# CENTER FOR DRUG EVALUATION AND RESEARCH

***APPLICATION NUMBER:***

# 022225Orig1s000

# PHARMACOLOGY REVIEW(S)

**Tertiary Pharmacology/Toxicology Review**

**From**: Timothy J. McGovern, Ph.D., ODE Associate Director for Pharmacology and Toxicology, OND IO
**NDA:** 22225
**Agency receipt date:** October 22, 2014
**Drug:** BRIDION (sugammadex sodium)
**Sponsor:** Organon USA (subsidiary of Merck)

**Indication:** Adult patients with shallow or profound neuromuscular blockade induced by rocuroniun or vecuronium

**Reviewing Division:** Division of Anesthesia, Analgesia, and Addiction Products

**Introductory Comments:** This is the third cycle review for NDA 22225. The pharmacology/toxicology reviewer and supervisor concluded in previous review cycles (2008 and 2013) that the nonclinical data support approval of sugammadex for the indication listed above. Dr. Paul Brown conducted an initial tertiary review of this NDA in 2008 and concurred with the Division conclusion that the nonclinical data support approval of this NDA for adults. He also agreed with the Division recommendations that the pregnancy labeling include a description of the neonatal mortality observed in the peri/postnatal study, the collection of additional information on bone effects prior to conducting multiple dose pediatric studies, and the request that the sponsor should provide additional information on the possible risk of carcinogenicity, particularly to bone. The sponsor submitted additional nonclinical studies to address the requirements of the original Not Approvable letter (2008) in their Complete Response of 2012. These studies adequately addressed the concerns identified in the 2008 Not Approvable letter to support pediatric dosing and labeling. The primary focus of the pharmacology/toxicology review for this cycle is on the nonclinical sections of the proposed label.

Drs. Z Alex Xu and R. Daniel Mellon from DAAAP evaluated the sponsor's proposed label and recommended modifications. The primary recommended changes include revision of the Pregnancy section to align with the final Pregnancy and Lactation Labeling Rule and inclusion of text regarding increased pup mortality in the pre- and postnatal development study, inclusion of text under Pediatric Use that discusses findings in a juvenile rat study, revision of text in section 13.1 and a description of bone toxicity studies in rats in section 13.2. I have discussed the proposed revisions with the Division and am in agreement with the recommendations. Additional changes may be made following discussion of the proposed revisions with the sponsor leading up to approval of the drug product.

The sponsor proposed "selective relaxant binding agent" as an established pharmacologic class (EPC) for sugammadex. The Division and the Pharmacology/Toxicology Associate Directors have been considering the appropriateness of this EPC in addition to others across the review cycles and has concluded at this time that none of the considered EPC



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terms are ideal, could be misleading to practitioners, or are not clinically meaningful. Therefore, the EPC will be intentionally omitted.

**Conclusion:**
I agree with the Division pharmacology/toxicology conclusion that sugamaddex can be approved from the pharmacology/toxicology perspective. I have reviewed the proposed labeling and agree with the recommendations made by the Division regarding the relevant nonclinical sections.



Reference ID: 3736753

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/s/

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TIMOTHY J MCGOVERN
04/21/2015

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH
DIVISION OF ANESTHESIA, ANALGESIA, AND ADDICTION PRODUCTS

# SUPERVISOR'S SECONDARY REVIEW
# PHARMACOLOGY/TOXICOLOGY REVIEW AND EVALUATION

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| NDA NUMBER: | 22225 |
| SERIAL NUMBER: | SDN 99 |
| DATE RECEIVED BY CENTER: | 10/22/2014 |
| PRODUCT: | **Bridion (sugammadex sodium)** |
| INTENDED CLINICAL POPULATION: | Adult patients |
| SPONSOR: | Organon USA (subsidiary of Merck) |
| REVIEW DIVISION: | DAAAP |
| PHARM/TOX SUPERVISOR: | R. Daniel Mellon, PhD |
| DIVISION DIRECTOR: | Sharon Hertz, MD |
| PROJECT MANAGER: | Diana Walker, PhD |
| Date review submitted to DARRTs: | April 20, 2015 |

## Executive Summary

# I. Recommendations

## A. Background and Regulatory History

This is the third review cycle for NDA 22225. Dr. Alex Xu completed the primary review for this and the two previous cycles. Dr. Adam Wasserman completed the secondary review for the two previous cycles. Dr. Paul Brown conducted a tertiary review for this new molecular entity (NME) during Cycle 1. At the completion of Cycle 2, Drs. Xu and Wasserman concluded that the NDA may be approved from a nonclinical pharmacology toxicology perspective without any post-marketing commitments or requirements. Labeling recommendations were not finalized during the first two review cycles.

As this is an NME, a tertiary review will also be completed this cycle by Dr. Timothy McGovern.

## B. Overview of Nonclinical Review Issues During this Cycle

There were no new nonclinical pharmacology or toxicology studies submitted during this review cycle. The nonclinical pharmacology toxicology review for this cycle focuses exclusively on the drug product labeling, including a continued discussion of an appropriate FDA Established Pharmacological Class (EPC) for the product. A discussion regarding the specific labeling recommendations provided by Dr. Xu and discussed in the previous secondary reviews by Dr. Wasserman is provided in the labeling recommendations table and subsequent text.

## C. Recommendations on labeling

The following labeling recommendations have been discussed with the Cycle 3 nonclinical review team of Drs. Xu and Chang. They have not yet been discussed with the rest of the review team or the Applicant. See the approval letter for final drug product labeling. Dr. Xu's recommended deletions are in strike-through red text and his additions are in blue text. Following further discussion of the labeling, further refinement of the labeling has been discussed with Drs. Xu and Chang. These edits are presented below in purple text.

| Label proposed by the Sponsor in the Cycle 3 submission | Recommended revision | Rationale |
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Reference ID: 3735657

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| **Highlights**<br>**Indications and Usage**<br>[Tradename] is (b) (4) indicated for the reversal of (b) (4) neuromuscular blockade induced by rocuronium or vecuronium. | **Highlights**<br>**Indications and Usage**<br>[Tradename] is (b) (4) indicated for the reversal of (b) (4) neuromuscular blockade induced by rocuronium or vecuronium. | Dr. Xu recommends (b) (4) I concur. There have been extensive discussions over the last two cycles regarding options for (b) (4) The Applicant's proposal of (b) (4) is not acceptable. See below for further discussion. |
| **8.1 Pregnancy**<br>(b) (4)<br><br>(b) (4) | **8.1 Pregnancy**<br>(b) (4)<br>*Risk Summary*<br>(b) (4)<br><br>There are no data on BRIDION use in pregnant women to inform any drug-associated risks.<br><br>In animal reproduction and development studies, there was no evidence of ~~embryofetal toxicity~~ teratogenicity when sugammadex was administered intravenously to rats and rabbits during organogenesis at exposures up to 6 and 8 times, respectively, the maximum recommended human dose (MRHD) of 16 mg/kg. However, there was an increase in the incidence of incomplete ossification of the sternebra and reduced fetal body weights in the rabbit study. In a pre- and | This section was revised per the FINAL Pregnancy and Lactation Labeling Rule (PLLR) published on 12/04/2014. This is optional at this time, but the Agency is willing to grant a waiver of the current CFR requirements in order to comply with the Final PLLR. The text in green was provided by the maternal health team. The reader is referred to their review for rationale behind those recommendations.<br><br>(b) (4) (b) (4) was removed and narrative summary of the risks was added per PLLR. Greater details of the studies are provided in the Animal Data section below.<br><br>Drs. Xu and Wasserman dismissed reduced ossification and reduced fetal body weights since they correlated with evidence of maternal toxicity. Dr. Xu notes that the effects in sternebra are outside of the |

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| | postnatal development study, sugammadex treatment resulted in an apparent increase in pup mortality which correlated with pup cannibalism by the Dam. An effect of sugammadex on steroid hormone and/or pheromone levels cannot be ruled out as contributing to this behavior *[see Data]*. The background risk of major birth defects and miscarriage for the indicated population are unknown. However, the background risk in the U.S. general population of major birth defects is 2-4% and of miscarriage is 15-20% of clinically recognized pregnancies. | historical control range. Although maternal toxicity is a possible explanation for these findings, the effect of the drug on bone suggests reasonable biological plausibility that the delayed ossification could be result of the drug. Further the reduced fetal body weights, in my opinion, regardless of the maternal toxicity, is still an adverse finding in this species. Therefore, I recommend including this finding in the labeling.<br><br>The effects of the drug in the PPD study appear to be real and although we do not believe that cannibalism of offspring is clinically relevant the underlying mechanism is unknown and therefore, I agree that these findings should be included in the labeling. |
| | *Animal Data*<br>In an embryofetal development study in rats, pregnant animals were administered sugammadex intravenously at 0, 20, 100, and 500 mg/kg (0.2, 1.2, and 6.2 times of MHRD, respectively, based on AUC comparison) during fetal organogenesis (Gestational Day 6 - 17). No treatment-related maternal and embryofetal changes were observed. | See exposure margin table for calculations and data source. |
| | In an embryofetal study in rabbits, pregnant New Zealand white rabbits were administered intravenous sugammadex at 0, 20, 65, 200 mg/kg (0.6, 2.4, and 8.2 times of MRHD by AUC, respectively) during organogenesis (Gestational Day 6-18). Fetal body weights were decreased (5, 10, and 14%, respectively) and reduced ossification of bone was observed in the offspring in the presence of maternal toxicity (decreased body weight and food consumption) ~~which was likely due to maternal body weight decrease associated with decreased food consumption in the drug-treated~~ | Clear maternal toxicity was noted in the MD and HD as evident by decreased body weights. Fetal body weights were also decreased suggesting embryotoxicity, possibly due to maternal toxicity. However, the incomplete ossification of sternebra was outside the historical control range and the data suggesting that sugammadex can have an impact on developing bone suggests biological plausibility that this could be due to a direct effect of the drug, even with little getting to the fetus. |

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| | ~~groups compared to the control~~. There was no evidence of teratogenicity at any dose. ~~The NOAEL for embryofetal toxicity in rabbits was 20 mg/kg, but the NOAEL for reproduction and development is considered to be 200 mg/kg.~~<br><br>In a prenatal and postnatal development study, pregnant rats (22/group) were administered sugammadex at 0, 30, 120, and 500 mg/kg (0.2, 1.2, and 6.2 times MHRD by AUC) from Gestational Day (GD) 6 to Postnatal Day (PND) 21 (corresponding to the beginning of organogenesis through parturition and subsequent pup weaning). Increased postnatal mortality at ≥ 120 mg/kg on PND 1-4 was observed, which correlates with an increase in pups cannibalized by the Dam. The reason for the cannibalization has not been identified, and the clinical relevance of the underlying mechanism for the cannibalization is not known; however, an effect of sugammadex on steroidal hormones and/or pheromones cannot be ruled out. In addition, there were no drug-related effects on parturition in rats during evaluations for prenatal or postnatal development. | In the pre- and postnatal study, there appears to be a treatment-related decrease in pup survival. This was largely driven by an apparent treatment-related increase in cannibalization which would obviously not be clinically relevant. However, the underlying mechanism causing this effect was still unknown. Dr. Xu speculates that in utero exposure to the drug substance may have resulted in a change in odor or taste of the pup resulting in cannibalization by the Dam. In Dr. Adam Wasserman's secondary review he indicated that the possibility of a direct effect on the fetus has not been ruled out as the missing pups were not able to be evaluated. He believed the data should not be entirely ignored in labeling and that further follow-up studies may address this concern. No further studies were required after the second cycle review. Dr. Xu also concludes that the underlying mechanism for cannibalization has not been determined, and that the finding may have human relevance particularly because of the possibility that sugammadex could sequester steroidal structures. In his tertiary review of Cycle 1, Dr. Paul Brown concurred that these findings should be included in the label. The potential for sugammadex to sequester compounds such as progesterone, estrogen, or other hormones and pheromones that may affect maternal behavior have not been examined or considered to date. I concur that the finding is perplexing and potentially of clinical significance given the very real |

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| | | possibility that the sugammadex may be altering circulating levels of steroidal-like compounds that have not yet tested and the fact that the standard studies we conduct may not provide an adequate assessment of the impact of diminished steroidal compounds on overall development. Including this in the labeling seems prudent, since this is not an obvious secondary effect of the drug and physicians would not likely associate an unexpected adverse effect with this treatment in the absence of this knowledge. |

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| (b) (4) | **8.2 Lactation**<br><br>*Risk Summary*<br>No data are available regarding the presence of sugammadex in human milk, the effects of sugammadex on the breast fed infant, or the effects of sugammadex on milk production. However, sugammadex is present in rat milk *[see Data]*. The developmental and health benefits of breast feeding should be considered along with the mother's clinical need for BRIDION and any potential adverse effects on the breastfed infant from BRIDION or from the underlying maternal condition.<br><br>*Data*<br>In a milk excretion study in rat dams following single intravenous dose of 20 mg/kg sugammadex on Postnatal Day 9, the maximum level was achieved at about 30 minutes after dosing with a ratio of milk to plasma level approximately 1:1. The oral exposure via milk did not induce effects on survival, body weight and physical or the behavioral developmental parameters monitored in rats in the prenatal and postnatal development studies *[see Use in Specific Populations (8.1)]*. | This section was revised per the PLLR. The input and revisions from the MHT have been included. The new **Lactation** replaces (b) (4) The text in green was provided by the MHT. See their review for final labeling language related to human data.<br><br>The data from the pre- and post-natal studies will be included (b) (4) and the data from the rat milk excretion study is described in more detail. |

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| **8.4 Pediatric Use**<br><br>The safety and efficacy of [TRADENAME] in pediatric patients have not been established. | **8.4 Pediatric Use**<br><br>The safety and efficacy of BRIDION in pediatric patients have not been established.<br><br>Sugammadex has been shown to be retained in bone and teeth with a significantly longer half-life than plasma. Bone and tooth deposition is significantly higher in juvenile than adult rats. Four-week daily sugammadex administration in juvenile rats caused slight but irreversible bone length decrease (approximately 5%) and reversible whitish discoloration with the No-observed-effect-level (NOEL) which is 0.1-fold ~~estimated local bone and teeth~~ the MRDH of 16 mg/kg based on adult plasma AUC. ~~concentration of a 4 mg/kg single dose in pediatrics of 4-year old. Weekly intermittent dosing (better reflected the likely clinical use)~~ Administration of sugammadex weekly for 8 weeks to juvenile rats did not produce similar changes in bone and teeth at a dose 1.2-times the MRHD of 16 mg/kg based on an adult AUC comparison ~~fold estimated local bone and teeth concentration of a 4 mg/kg single dose in pediatrics of 4-year old~~ *[see Nonclinical Toxicology (13.2)]*. | Juvenile animal studies required to be completed under either a Pediatric Study Plan (PSP) or a Pediatric Written Request (PWR) must be included in the label in Section 8.4 even if adverse changes are not observed. The juvenile animal studies for sugammadex were not required for either a PSP or PWR. However, the juvenile animal bone toxicity studies were required by the Agency due to the concern for bone toxicity especially in pediatrics because of the high deposition rate of sugammadex in juvenile rats compared to adults. Dr. Xu recommends that they should be included in labeling. I concur, specifically because retention of this drug in bone and teeth is not expected based on the intended pharmacology. As such, including the data with the safety margins should alert the prescribing official of a unique aspect of this drug which should improve the ability to associate an unexpected bone or tooth development adverse effect to the drug treatment based on this knowledge. |
| **11 DESCRIPTION**<br><br>[TRADENAME] (sugammadex) (b) (4) | **11 DESCRIPTION**<br><br>~~[TRADENAME] (sugammadex)~~ (b) (4) | (b) (4) . I concur with Dr. Xu's recommendation (b) (4) |

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| **12 Clinical Pharmacology**<br>**12.2 Pharmacodynamics**<br>[Tradename] may contain (b) (4) (b) (4) of the mono OH-derivative of sugammadex. In preclinical pharmacology studies (b) (4) was demonstrated (b) (4) nearly similar efficacy in reversing rocuronium- or vecuronium-induced blockade. | **12 Clinical Pharmacology**<br>**12.2 Pharmacodynamics**<br>BRIDION may contain up to 7% (b) (4) (b) (4) of the mono OH-derivative of sugammadex. In preclinical pharmacology studies, (b) (4) the mono OH-derivative was demonstrated (b) (4) to have ~50% of the affinity as sugammadex for rocuronium and vecuronium and that product with up to 7% of the mono OH-derivative has nearly similar efficacy in reversing rocuronium- or vecuronium-induced blockade. Although sugammadex has greatest affinity for aminosteroid neuromuscular blocking agents such as rocuronium and vecuronium, plasma levels of endogenous or exogenous compounds with a similar steroidal structure, such as some hormones, hormonal contraceptives, and pheromones may also be reduced following administration of sugammadex *[see 7.2 Interactions Potentially Affecting the Efficacy of Other Drugs, and 13.2 Animal Toxicology and/or Pharmacology].* | Dr. Xu notes that the binding data suggest that the mono OH-derivative has approximately 50% of the binding affinity for rocuronium and vecuronium and the addition of 10-20% of mono OH-derivative to sugammadex did not seem to decrease efficacy in a mouse model. He notes that the statement that suggests (b) (4) is not correct. Given the impact on hormonal contraceptives and the unusual findings in the pre- and post-natal development study, Dr. Xu recommends including some information regarding the fact that sugammadex can bind more than just rocuronium and vecuronium and cross reference the appropriate sections of the label.<br><br>I completely concur with this recommendation. |
| **12.3 Pharmacokinetics**<br>**Distribution** | **12.3 Pharmacokinetics**<br>**Distribution**<br>In nonclinical drug distribution studies, sugammadex is retained in sites of active mineralization, such as bone and teeth with a half-life of 172 and 8 days, respectively *[see 8.4 Pediatrics and 13.2 Animal Toxicology and/or Pharmacology].* | Dr. Xu recommends that given the unexpected distribution of this compound to these tissues, the information should be included in 12.3 and there should be a cross references to the information in 8.4 and 13.2.<br><br>I completely concur. |
| **Metabolism**<br>In (b) (4) clinical studies, no metabolites of sugammadex have been observed and only renal excretion of the unchanged product was observed as the route of elimination. | **Metabolism**<br>In (b) (4) clinical studies, no metabolites of sugammadex have been observed and only renal excretion of the unchanged product was observed as the route of elimination. | Dr. Xu notes that the text is accurate, although reference to (b) (4) is not necessary. I concur. The (b) (4) statement may be removed (b) (4) . |

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| **13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility**<br>(b) (4) | **13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility**<br><br>*Carcinogenesis*<br>(b) (4) Long-term animal studies to evaluate the carcinogenic potential of sugammadex (b) (4) -have not been conducted. | I concur with Dr. Xu's proposed labeling changes. |
| (b) (4) | *Mutagenesis*<br>(b) (4)<br><br>Sugammadex and its mono OH-derivative tested negatively in in vitro bacterial reverse mutation assays (Ames test), in vitro chromosomal aberration assays in human peripheral blood lymphocytes, and in vivo micronucleus assays in mice and rats. | I concur with Dr. Xu's proposed labeling changes. |
| (b) (4) | *Impairment of Fertility*<br>(b) (4)<br><br>A fertility and early embryonic development study in Sprague-Dawley rats in which male rats were treated daily for 29 days prior to mating and through the mating period and female rats were treated daily for 14 days prior to mating to Day 5 post-coitum via intravenously administered with saline or sugammadex at 20, 100, and 500 mg/kg (0.2-, 1.2-, and 6.2-times of MHRD by AUC) did not show adverse effects on fertility. | I concur with Dr. Xu's proposed labeling changes. See below for documentation regarding the source data employed for the exposure margin calculations.<br><br>TK data were not obtained in this study. The AUC comparisons are based on AUC data in a 4-week general toxicity study in rats. |

| 13.2 Animal Toxicology and/or Pharmacology | 13.2 Animal Toxicology and/or Pharmacology | |
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| (b) (4) | (b) (4) | Dr. Xu notes that the proposed text is promotional and does not provide useful information to the prescriber.<br><br>I concur.<br><br>Description of (b) (4) should be deleted from this section.<br><br>Although the juvenile animal study results are briefly summarized in Section 8.4. The studies are described in this section in more detail. Dr. Xu recommends that both the adult and juvenile studies are described together in this section to clearly delineate the differences noted. I agree. |
| | Bone and teeth retention of sugammadex occurred in rats after intravenous injection, with mean half-lives of 172 and 8 days, respectively. Sugammadex bound to hydroxyapatite in an *in vitro* study and distributed in the bone formation area where hydroxyapatite is present for mineralization *in vivo*. | Given the unexpected prolonged retention in bone, including these data in the labeling is appropriate to inform physicians that unexpected adverse effects on these tissues may be related to the use of sugammadex based on biological plausibility. |
| | In an adult rat bone toxicity study, a single dose of sugammadex at 2000 mg/kg (approximately 24-times the maximum recommended human dose (MRHD) of 16 mg/kg by AUC comparison) ~~Studies in adult rats~~ caused a ~~have shown that single dose administration of 2000 mg/kg sugammadex~~ slight~~ly~~ increase~~d~~ in bone resorption, but had no effect on teeth color~~or appearance~~. No adverse bone effects were seen following a single dose of sugammadex at 500 mg/kg (4-times the MRHD dose of 16 mg/kg based on a plasma AUC | I do not agree that (b) (4) |

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| | comparison).<br><br>In a bone repair study, adult rats were treated with intravenous sugammadex weekly for 6 weeks at 0, 30, 120, or 500 mg/kg (approximately 0.4, 1.4, and 6.4-times the MRHD, respectively based on a plasma AUC comparison). High dose animals showed a statistically significant increase in callus formation and decrease in bone formation ~~following 6 weekly administrations of sugammadex at 500 mg/kg (107 times the estimated bone concentration of a 4 mg/kg single-dose in human adult) as compared to the control~~, suggesting a slight delay in the bone healing process.<br><br>In a bone deposition study, sugammadex concentrations were ~~in juvenile animals was~~ significantly higher in juvenile rats ~~as~~ compared to adult rats (13% vs. 3% of the administered dose, respectively) following a single intravenous dose of 30 mg/kg (0.3-times the MRDH based on adult AUC). ~~single dose intravenously administration due to the high growth rate of the bone during developmental period.~~<br><br>In a juvenile animal bone toxicity study, 7-day old rats were dosed once daily for 28 days with 0, 30, 120, and 500 mg/kg sugammadex (0.1, 0.6, and 2.5-times the MRDH based on adult AUC). Sugammadex at 120 and 500 mg/kg decreased ulna and femur bone length by approximately 5% which did not recover after an 8-week treatment-free period. Reversible whitish discoloration and disturbance of enamel formation were also observed in the incisors at these dose levels. In molars, this effect was only observed at 500 mg/kg. The no-adverse effect level (NOAEL) was 30 mg/kg ~~(approximately 12 times~~ | |

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| | ~~the MRDH based on AUC)~~.<br><br>In a second juvenile toxicity study, 7-day old rats were dosed once weekly for 8 weeks with 0, 7.5, 30, or 120 mg/kg sugammadex (up to 1.2-times the MRHD based on adult AUC). No adverse effects on bone or teeth were noted. ~~Similar bone and teeth effects were not observed in a subsequent follow-up intermittent juvenile rat study with 8 weekly dosing of sugammadex at doses up to 120 mg/kg (approximately 33 times the estimated bone concentration of a 4 mg/kg single dose in pediatrics of 4 year old.~~ | |

## Established Pharmacological Class

The Established Pharmacologic Class (EPC) in drug product labeling is intended to provide the health care professional information about a drug and how it relates to other therapeutic options. As per the FDA guidance, the EPC is defined on the basis of any of the following three attributes of the drug and must also be clinically meaningful:

| Basis of EPC Options | | Options for EPC |
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| Mechanism of Action (MOA) | Reversal Agent for vecuronium or rocuronium neuromuscular junction blockade | Vecuronium and Rocuronium Reversal Agent<br>Vecuronium and Rocuronium Sequestrant |
| Physiologic effect (PE) | Reversal of neuromuscular blockade | Neuromuscular Blocking Agent Reversal Agent<br>Neuromuscular Blocker Reversal Agent<br>Aminosteroid Neuromuscular Blocking Agent Reversal Agent<br>Aminosteroid Neuromuscular Blocking Agent Sequestrant |
| Chemical Structure (CS) | Modified gamma-cyclodextrin | Cyclodextrin<br>Gamma-Cyclodextrin<br>Modified Gamma-Cyclodextrin |

(b) (4)

[redacted]

(b) (4)

## Basis of Exposure Margin Calculations

The exposure margins calculated in the above labeling recommendations are documented in the table below, as there are some differences in opinion between the Applicant and the review staff on the values that should be employed. The Applicant's proposed exposure margins are based on the range of recommended human doses of 2 mg/kg to 16 mg/kg rather than the maximum recommended human dose of 16 mg/kg.

**Table 1: Exposure Margins Based on AUC Pivotal Toxicology Studies**

| Study | Doses tested (mg/kg) | Nonclinical $AUC_{(0\text{-inf})}$ (mcg•h/mL) | Human $AUC_{(0\text{-inf})}$ (mcg•h/mL)[6] | Exposure Margin |
|---|---|---|---|---|
| 4-Week IV Rat Toxicology Study (Study 020117) | 30<br>120<br>500 | 48.5<br>215.5<br>1051.5 | 170 | 0.3x<br>1.3x<br>6.2x |
| Rat Fertility and Early Embryonic Development | 20<br>100<br>500 | 41.7[1]<br>208.5[1]<br>1051.5[1] | 170 | 0.2x<br>1.2x<br>6.2x |
| Rat Embryofetal Development | 20<br>100<br>500 | 41.7[1]<br>208.5[1]<br>1051.5[1] | 170 | 0.2x<br>1.2x<br>6.2x |
| Rabbit Embryofetal Development | 20<br>65<br>200 | 100.5[2]<br>403.7<br>1400.8 | 170 | 0.6x<br>2.4x<br>8.2x |
| Rat Pre- and Post-Natal Development | 30<br>120<br>500 | 48.5[1]<br>215.5<br>1051.5 | 170 | 0.3x<br>1.3x<br>6.2x |
| Single Dose Bone | 2000 | 4140[3] | 170 | 24x |

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| Study Adult Rat (Study 050396) | | | | |
| Single Dose Bone Study Adult Rat (Study 060338) | 30<br>120<br>500 | 63.2[4]<br>165.5[3]<br>704.5[3] | 170 | 0.4x<br>1.2x<br>4x |
| Adult Rat Bone Fracture Study (6-weekly doses) (Study 090319) | 30<br>120<br>500 | 63.2[4]<br>229.5<br>1096.0 | 170 | 0.4x<br>1.4x<br>6.4x |
| | | | | |
| 28-Day Daily Juvenile Bone Study (PND 7) (Study 060086) | 30<br>120<br>500 | 22.3[5]<br>97.5<br>432 | 170[8] | 0.1x<br>0.6x<br>2.5x |
| 8-Week Weekly Juvenile Rat Bone Study (PND 7) (Study 080229) | 7.5<br>30<br>120 | Unknown<br>43.8[7]<br>206.5 | 170[8] | <0.3<br>0.3<br>1.2x |

[1]There are no TK data for the rat reproductive and developmental toxicology studies. The AUC data are derived from the 4-week IV toxicology study (Study 020117). As the human AUC is based on $AUC_{(0\text{-inf})}$ ideally the animal data would also employ $AUC_{(0\text{-inf})}$ data; however, as these data were not calculated for females in this study; therefore, we have to employed the mean values from $AUC_{(0\text{-}3)}$. This is a reasonable substitute value, as the $AUC_{(0\text{-}3)}$ is virtually identical to the $AUC_{(0\text{-inf})}$ in males. Further, we used the data from the multiple doses of the drug, as this would more closely represent the exposures in the repeat-dose toxicology studies. The Sponsor employed a value of 1050 mcg•h/mL however it is not clear from the submission where this value came from (however, we note that 1050 is the $AUC_{(0\text{-inf})}$ in males in the 4-week IV tox study). Exposures for 20 and 100 mg/kg doses were extrapolated via linear regression from the 4-week general toxicology study (Study 020117) using GraphPad Prism.
[2]TK data are based on data actually obtained in the rabbit embryo-fetal development study.
[3]Plasma AUC data are from a single-dose IV TK study in rats (Study 060080).
[4]Plasma $AUC_{(0\text{-}3)}$ from single-dose TK data the 4-week rat IV toxicology study (Study 020117). The $AUC_{(0\text{-}3)}$ was used instead of the $AUC_{(0\text{-inf})}$ as there were no adequate female data for $AUC_{(0\text{-inf})}$. Values do appear comparable.
[5]TK data were obtained in Study 060086. Values represent them mean of male and female means after multiple doses, but only in males treated with 30 mg/kg.
[6]Human data was obtained from Study 19.4.102 which was a single dose study comparing exposure in Japanese and Caucasian subjects. According to this report, a single dose of 16 mg/kg sugammadex produced a mean $AUC_{(0\text{-inf})}$ of 9670 mcg•min/mL in Japanese subjects and 10185 mcg•min/mL (170 mcg•h/mL) in Caucasian subjects. For the purposes of labeling, we used the values from the Caucasian subjects, as this is likely to more closely resemble the typical U.S. body build across the diverse races represented in the U.S. population and is the more conservative approach. The Applicant's annotated labeling indicates that they based their exposure margins on a mean human AUC at 16 mg/kg of 160 mcg•h/mL. The submission does not indicate the source of this value.
[7]There were no PK data from Study 080229, although the juvenile animals were dosed weekly in this study, the only AUC data available is based on single doses. As such AUC reported here for exposure margins are from Study 060338.
[8]PK data are not available for pediatric patients dosed with 16 mg/kg of sugammadex. This value is the adult AUC, which, we recognize may not be accurate for pediatrics.

Estimations of sugammadex bone and teeth concentrations for multiple-dose studies were made by both the Sponsor and Dr. Gemma Kuijpers from the Division of Bone, Reproduction, and Urologic Products (DBRUP). These estimates suggested a

greater safety margin for bone effects than the plasma levels, which was used by the previous review team members to justify the recommendation that the drug product may be approved and clinical studies in pediatrics may be allowed to proceed.

Bone deposition estimates were obtained in Study 090310 and Study 060353. In **Study 090310**, Wistar rats of different ages (7 days, 6 weeks, and 12 weeks of age) were dosed once weekly with sugammadex as per the following study design:

| Group | Rat strain, age at start dosing | Gender/ No. of animals | Dose ($mg \cdot kg^{-1}$; $MBq \cdot kg^{-1}$) | Dosing regimen | Sacrifice time[d] |
|---|---|---|---|---|---|
| 1<br>2<br>3 | Wistar rats (Hsd:CpB, WU), 7 days old[a] | 3M +3F<br>3M +3F<br>3M +3F | 8.9 ; 0.75<br>30 ; 3<br>120 ; 12 | Once weekly for 7 weeks: day 7, 14, 21, 28, 35, 42 and 49 of age (7 dosages in total) | Day 56 (7 days after last dose) |
| 4<br>5<br>6 | Wistar rat (RccHan: WIST), ± 6 weeks old[b] | 3M +3F<br>3M +3F<br>3M +3F | 30 ; 3<br>120 ; 12<br>500 ; 50 | Once weekly in week 6, 7, 8, 9, 10 and 11 of age (6 dosages in total) | 12 weeks (7 days after last dose, coinciding with day of fracture) |
| 7<br>8<br>9 | Wistar rat (RccHan: WIST), ± 12 weeks old[c] | 3M +3F<br>3M +3F<br>3M +3F | 30 ; 3<br>120 ; 12<br>500 ; 50 | Once weekly in week 12, 13, 14, 15, 16 and 17 of age (6 dosages in total) | 18 weeks (7 days after last dose, coinciding with 6 weeks after fracture |
| 10[e] | Wistar rat (RccHan: WIST), ± 6 weeks old | 1M +1F | 8; 6 | Single dose | 24 hours after dose |

M =male and F = female

[a] corresponds to intermittent dosing in juvenile rat study (Study 080229).

[b] corresponds to Part 1 (pre-fracture exposure) of fracture repair study (Study 090319).

[c] corresponds to Part 2 (post fracture exposure) of fracture repair study(Study 090319).

[d] From animals of group 1-3: femurs, tibias, vertebrae L4-L6, incisors and molars were collected. For animals of group 4-6 femurs, tibias, incisors and molars and for the animals of group 7-10 femurs and tibias were collected.

[e] Group 10 generated femurs and tibias for the microautoradiography study[1]. The microautoradiography study is performed under study number 090071 and is reported separately.

Sugammadex concentrations in bone were evaluated for the PND7 rats in the left and right femur and tibia as well as the L4-L6 vertebra in both males and females. The values in the table below represent the mean of the males and females for the mean of all bones tested, as per the Sponsors tables 8.1-27 and 8.1-29 in **Study Report 090310**.

**Study 060353** compared single-dose administration of 30, 120, 400 mg/kg radiolabeled sugammadex to young adult rats, multiple-dose administration of 30 mg/kg in young adult rats, and single-dose administration of 30 mg/kg in aged and juvenile rats. The study report does not present the data in a manner that would allow a mcg sugammadex/g bone comparison to be readily determined as the data are primarily expressed as a percent of the radiolabel in bone and the data are not

pooled across the bones sampled. As such the single-dose values in the table below are the means of male and female femurs from Tables 9.1-9, 9.1-10. Scapula generally contained the highest concentration of radiolabel compared to the other bones assessed. Femur was used for Table 2 below as there was evidence of decreased ulna and femur length in the 28-day juvenile animal bone study (Study 060086). The estimate of bone concentration in Group 4 was obtained from Figure 9.2-11 as values were not reported in the study report. Missing entries are present in the table when it was unclear from the report what value could be estimated for those treatment groups.

Table 2: Bone Concentration Summary from Study 060353

| Group | Age | Treatment (mg/kg) single or multiple dose | Single or Multi Dose | Route | % Radiolabel retained in bone (mean M&F) | Bone (Femur) concentration (mcg/g bone) | Teeth (molars) concentration (mcg/g teeth) |
|---|---|---|---|---|---|---|---|
| 1 | Young Adult | 30 | SD | IV | 2.8% | 73.5 | 18.0 |
| 2 | Young Adult | 120 | SD | IV | 2.8% | 186.5 | 32.5 |
| 3 | Young Adult | 400 | SD | IV | 2.8% | 273.5 | 50.0 |
| 4 | Young Adult | 30 | MD (daily) | IV | | 3800? | 850 |
| 5 | Aged | 30 | SD | IV | 0.45% | | |
| 6 | Young Adult | 30 + 4 Rocuronium | SD | IV | | | |
| 7 | Juvenile | 30 | SD | SC | 13.3% | | |

The challenge with the use of these data to extrapolate bone concentration-based exposure margins is that the concentrations are not uniform across bones sampled or even across different regions of the same bone. Further, there are no mass-balance data for pediatric patients. Therefore, the same worst-case scenario approach for bone deposition in adults cannot be directly applied here (i.e., 15% not eliminated in urine assumed to be in bone). However, based on the table above in the rat, it can be estimated that bone deposition in the juvenile rat is 4.75 times higher than in the young adult rat (13.3% / 2.8% = 4.75x). Therefore, we could estimate the pediatric worst-case scenario to be approximately 18 x 4.75 = 85.5 mcg/g bone.

Table 3: Estimated Exposure Margins Based on Bone Deposition Data

| **Study** | **Doses tested (mg/kg)** | **Single-Dose Nonclinical Bone Concentration (mcg/g bone)** | **Multiple-Dose Nonclinical Bone Concentration Estimate (mcg/g bone)** | **Estimated Worst-Case Human Bone Concentration after 16 mg/kg** | **Exposure Margin for 16 mg/kg Dose** |
|---|---|---|---|---|---|
| Applicant's Position Paper on | 8<br>30<br>400 | 24<br>65<br>250 | | 18 mcg/g[5] | 1.3x<br>3.6x<br>13.9x |

| | | | | | |
|---|---|---|---|---|---|
| Bone Effects (source of values reported are not clear from NDA) | 500<br>2000 | 313<br>500 | | | 17.4x<br>27.8x |
| Single Dose Bone Toxicity Study in Adult Rats (Study 050396) | 2000 | 330[1] | | 18 mcg/g[5] | 18x |
| Single Dose Bone Toxicity Study in Adult Rats (Study 060338) | 30<br>120<br>500 | 73.5[2]<br>186.5<br>286.5 | | 18 mcg/g[5] | 4x<br>10x<br>16x |
| Bone Retention Study (Study 050322) | 8.6 | 1.33 | | 18 mcg/g[5] | 0.07x |
| Second Bone Adult PK Study (Study 060353) | 30<br>120<br>400 | 73.5[3]<br>186.5<br>273.5 | | 18 mcg/g[5] | 4.1x<br>10.4x<br>15.2x |
| Second Bone Adult PK Study (Study 060353) | 30 | | 3800[7] | 18 mcg/g[5] | 211x |
| Multiple-dose Weekly bone PK study in **PND7** rats (Study 090310) | 8.9<br>30<br>120 | | 52.6<br>170.2<br>409.3 | 85.5 mcg/g[6] | 0.6x<br>2.0x<br>4.8x |
| Multiple-dose Weekly bone PK study in **12 week old rats** (Study 090310) | 30<br>120<br>500 | | 107.7<br>234.7<br>398.0 | 18 mcg/g[5] | 6x<br>13x<br>22x |
| **Adult** Weekly Rat Bone Fracture Study (Study 090319) | 30<br>120<br>500 | | 107.7[9]<br>234.7<br>398.0 | 18 mcg/g[5] | 6x<br>13x<br>22x |
| 28-Day Daily Juvenile Bone Study (**PND 7**) (Study 060086) | 30<br>120<br>500 | | 3800[7]<br>Unknown<br>Unknown | 85.5 mcg/g[6] | 44x<br>>44x<br>>44x |
| 8-Week Weekly Juvenile Rat Bone Study | 7.5 (8.9)<br>30<br>120 | 50[4]<br>160<br>400 | 52.6[8]<br>170.2<br>409.3 | 85.5 mcg/g[6] | 0.6x<br>2.0x<br>4.8x |

| | | | | | |
|---|---|---|---|---|---|
| (**PND 7**) (Study 080229) | | | | | |

[1]Study 050396 did not include an assessment of bone concentration. The study does not specify the age of the animals, but refers to the animals as young adults and provides body weights for the Wistar rats tested. Based on other studies, "young adult" rats have been 12-weeks of age. The value of 330 mcg/g was based on a nonlinear regression analysis of the data from Study 060353 via GraphPad Prism using one site saturation curve.
[2]There were no bone concentration assessments completed for Study 060338. Bone concentration estimates for 30 and 120 mg/kg were from Study 060353. The estimate for 500 mg/kg was from the nonlinear regression analysis of the data from Study 060353 via GraphPad Prism using one site saturation curve.
[3]Data are from Document ID INT00033724 Study (060353). "Young adult" animals were dosed with a single-dose of 30, 120, or 400 mg/kg IV. The data were compared to 30 mg/kg dosed daily for 4 weeks. In addition, single-doses of 30 mg/kg were assessed in "aged" rats (~1 year old) and "juvenile" rats (7 day old). The study results indicate that ~13.3% of the dose administered was in the bones of "juvenile rats" vs. 2.8% in "young adult" and 0.4% in "aged." Therefore, one could say that juvenile bone deposition should be 4.75x that of young adult (13.3/2.8 = 4.75).
[4]Bone concentrations were not obtained in Study 080229. The data are from Study 090310.
[5]Human adult bone and teeth concentrations were estimated from worst-case scenarios, assuming all of the drug not accounted for in the clinical single-dose mass balance study (≤15%) was deposited in bone and teeth. This is based on an assumption of even distribution over tissues containing hydroxyapatite (which is 10 kg total weight of bone and teeth). There are flaws in this assumption, since the drug appears to bind at greater levels to active bone formation and remodeling and the rat data suggest that deposition in teeth (molars) is approximately 25 to 35% of bone. There are no multiple-dose clinical mass balance data.
[6]Estimated as 4.75 times the adult deposition based on the juvenile to young adult bone deposition ratio in rats (18 x 4.75 = 85.5 mcg/g).
[7]There were no bone concentrations determined for Study 060086. The value for 30 mg/kg multiple dosing was from Study 060353 estimated from the femur, since decreased femur length was reported in this study. The value was estimated as a mean of the male and female data from Figures 9.2-11 and 9.2-12 as it was not reported in the study report.
[8]Bone concentrations were not obtained in this study. Data are from Study 090310.
[9]There are no bone concentration data for Study 090319. Values are from Study 090310.

Although one can estimate bone concentrations based on the assumptions outlined in the table above, given the limitations of the bone data and uncertainty over the bone and teeth exposure margin predictions, I recommend that the drug product labeling only reflect the plasma exposure levels.

## D. Overall Recommendation

From a nonclinical pharmacology toxicology perspective, I concur with Drs. Xu and Wasserman that NDA 22225 may be approved with the labeling recommendations provided in this review.

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/s/

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RICHARD D MELLON
04/20/2015

**DEPARTMENT OF HEALTH AND HUMAN SERVICES**
**PUBLIC HEALTH SERVICE**
**FOOD AND DRUG ADMINISTRATION**
**CENTER FOR DRUG EVALUATION AND RESEARCH**

# PHARMACOLOGY/TOXICOLOGY NDA REVIEW AND EVALUATION

| | |
|---|---|
| Application number: | 22225 |
| Supporting document/s: | 99 |
| Applicant's letter date: | 10/22/2014 |
| CDER stamp date: | 10/22/2014 |
| Product: | BRIDION® (Sugammadex sodium)/Org 25969 |
| Indication: | Adult patients with shallow or profound neuromuscular blockade induced by rocuronium or vecuronium |
| Applicant: | Organon USA Inc., a subsidiary of Merck & Co. |
| Review Division: | Division of Anesthesia, Analgesia, and Addiction Products (HFD-170) |
| Reviewer: | Z. Alex Xu, PhD, DABT |
| Team Leader | Jay H. Chang, PhD |
| Supervisor: | R. Daniel Mellon, PhD |
| Division Director: | Sharon Hertz, MD |
| Project Manager: | Diana Walker, PhD |

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Reference ID: 3721694

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# 1 Executive Summary

## 1.1 Introduction

NDA 22225 was originally submitted on 10/31/2007 to seek marketing approval for Bridion (sugammadex sodium) for reversal of moderate or deep neuromuscular blockade induced by rocuronium or vecuronium. This is the third review cycle. As per previous nonclinical reviews, from the nonclinical pharmacology toxicology perspective, the NDA was recommended to be approved for the proposed adult use. However, the NDA was ultimately not approved due to lack of adequate characterization of potential adverse hypersensitivity/anaphylaxis reactions and coagulation events. Although I recommended that the NDA may be approved after the first review cycle, as noted in the action letter dated 2008, I recommended juvenile animal studies be conducted to support the safety of multiple-dose pediatric studies based on data that indicated the drug bound to bone. These studies were submitted and reviewed during the second review cycle, and the Applicant updated the label to include the new nonclinical information. However, since the NDA was not approved in the second cycle, a labeling review was not completed during the second review cycle.

In the third cycle review for NDA 22225, no new nonclinical data were submitted and there are no outstanding nonclinical issues. Therefore, from the nonclinical perspective, the NDA may still be approved. This review focuses on changes to the nonclinical sections of the proposed label and the conversion of the label to be in accordance to the Pregnancy and Lactation Labeling Rule (PLLR). Although PLLR labeling format is not required at this time, the Agency is willing to grant a waiver of the standard CFR labeling requirements to allow for voluntary implementation of PLLR labeling format. It should be noted that the recommended changes to the label described in this review are not final. Subsequent revision is expected after discussion within the Division and negotiation with the Applicant.

## 1.2 Brief Discussion of Nonclinical Findings

No nonclinical studies were included in this submission

## 1.3 Recommendations

### 1.3.1 Approvability

From the nonclinical standpoint, the application for use of sugammadex in the adult population may be approved.

### 1.3.2 Additional Non Clinical Recommendations

None

### 1.3.3 Labeling



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The table below shows a comparison of the Applicant's proposed label language, this reviewer's recommended changes, and the rationale for the recommended changes. It should be noted that these recommended changes are not final. Subsequent revision is expected after discussion within the Division and negotiation with the Applicant.



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| Label proposed by the Sponsor in this submission | Recommended revision | *Rationale* |
|---|---|---|
| **Highlights**<br>**Indications and Usage**<br>[Tradename] is (b) (4) indicated for the reversal of moderate or deep neuromuscular blockade induced by rocuronium or vecuronium. | **Highlights**<br>**Indications and Usage**<br>[Tradename] is (b) (4) -indicated for the reversal of moderate or deep neuromuscular blockade induced by rocuronium or vecuronium. | (b) (4) . |
| **8.1 Pregnancy**<br>(b) (4)<br><br>(b) (4) | **8.1 Pregnancy**<br>(b) (4)<br>*Risk Summary*<br>(b) (4)<br><br>There are no data on BRIDION use in pregnant women to inform any drug-associated risks.<br><br>In animal reproduction and development studies, in the absence of maternal toxicity, there was no evidence of embryofetal toxicity when sugammadex was administered intravenously to rats and rabbits during organogenesis at dose exposures up to 6 and 8 times, respectively, the maximum recommended human dose (MRHD) of 16 mg/kg. In a pre- and postnatal development study, sugammadex treatment resulted in an apparent increase in pup | This section was revised per the Pregnancy and Lactation Labeling Rule (PLLR) published on 12/04/2014. The input and revisions from the maternal health team (MHT) have been included.<br><br>The previous (b) (4) . The pregnancy category was removed and narrative summary of the risks was added per PLLR. The human statements were provided by the MHT (green text).<br><br>Information of studies included in the Animal Data subsection per PLLR requirement<br>• Types of studies<br>• Animal species<br>• doses or exposures in terms of human dose or exposure equivalents<br>• Duration and timing Study findings<br>• Maternal toxicity<br>• Limitations of the data<br>Proprietary name is used since the formulation of the drug product used in these studies was the same as the approved drug product.<br><br>Prenatal and postnatal study: Although it is clear that the observed treatment-related cannibalization is not directly clinically relevant, the underlying mechanism causing this effect was still questionable. In my 1st cycle primary review (dated 06/20/08 in DARRTS), I concluded that in utero exposure to the drug substance may have resulted in a |

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| | mortality which correlated with pup cannibalism by the Dam. An effect of sugammadex on steroid hormone and/or pheromone levels cannot be ruled out as contributing to this behavior [see Data]. The background risk of major birth defects and miscarriage for the indicated population are unknown. However, the background risk in the U.S. general population of major birth defects is 2-4% and of miscarriage is 15-20% of clinically recognized pregnancies.<br><br>*Animal Data*<br>In an embryofetal development study in rats, pregnant animals were administered intravenous sugammadex at 0, 20, 100, and 500 mg/kg (0.2, 1.0, 5.6 times of MHRD by AUC, respectively) during fetal organogenesis (Gestational Day 6 - 17). No treatment-related maternal and embryofetal changes were observed.<br><br>In an embryofetal study in rabbits, pregnant New Zealand white rabbits were administered intravenous sugammadex at 0, 20, 65, 200 mg/kg (0.6, 2.4, and 8.2 times of MRHD by AUC, respectively) during organogenesis (Gestational Day 6-18) by IV injection. A minor body weight decrease (5-14%) was observed in the offspring which was likely due to maternal body weight decrease associated with decreased food consumption in the drug-treated groups compared to the control. The NOAEL for embryofetal toxicity in rabbits was 20 mg/kg, but the NOAEL for reproduction and development is considered to be 200 mg/kg.<br><br>In a prenatal and postnatal development study, pregnant rats (22/group) were administered sugammadex at 0, 30, 120, and 500 mg/kg (0.3, 1.3, 5.6 times MHRD by AUC) from Gestational Day (GD) 6 to Postnatal Day | change in odor or taste resulting in cannibalization, which is not likely to be clinically relevant. This was not concurred by Dr. Adam Wasserman (nonclinical supervisor at that time) in his secondary review (Dated 06/16/08 in DARRTS). He indicated that the possibility of a direct effect on the fetus could occur has not been ruled out. He believed the data should not be entirely ignored in labeling and that further follow-up studies may address this concern. Upon further consideration during this review cycle, I am inclined to believe that the underlying mechanism for cannibalization has not been determined, and that the finding may have human relevance particularly because of the pharmacological effects of sugammadex to sequester steroidal structures. The potential for sugammadex to sequester compounds such as progesterone, estrogen, or other hormones and pheromones that may affect maternal behavior have not been examined or considered to date. |



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| | (PND) 21 (corresponding to the beginning of organogenesis through parturition and subsequent pup weaning). Increased postnatal loss at ≥ 120 mg/kg on PND 1-4 was observed, which correlates with an increase in pups cannibalized by the Dam. The reason for the cannibalization has not been identified, and the clinical relevance of the underlying mechanism for the cannibalization is not known; however, an effect of sugammadex on steroidal hormones and/or pheromones cannot be ruled out. In addition, there were no drug-related effects on parturition in rats during evaluations for prenatal or postnatal development. | |



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| (b) (4) | **8.2 Lactation**<br><br>*Risk Summary*<br>No data are available regarding the presence of sugammadex in human milk, the effects of sugammadex on the breast fed infant, or the effects of sugammadex on milk production. However, sugammadex is present in rat milk [see Data]. The developmental and health benefits of breast feeding should be considered along with the mother's clinical need for BRIDION and any potential adverse effects on the breastfed infant from BRIDION or from the underlying maternal condition.<br><br>*Data*<br>In a milk excretion study in rat dams following single intravenous dose of 20 mg/kg sugammadex on Postnatal Day 9, the maximum level reached at about 30 minutes after dosing with a ratio of milk to plasma level approximately 1:1. The oral exposure via milk did not induce effects on survival, body weight and physical or the behavioral developmental parameters monitored in rats in the perinatal and postnatal development studies [see Use in Specific Populations (8.1)]. | This section was revised per the PLLR. The input and revisions from the MHT have been included. The new **Lactation** replaces (b) (4) The text in green was provided by the MHT. See their review for final labeling language related to human data.<br><br>The data from the pre- and post-natal studies will be included in 8.1 and the data from the rat milk excretion study will be described in more detail. |

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| **8.4 Pediatric Use**<br><br>The safety and efficacy of [TRADENAME] in pediatric patients have not been established. | **8.4 Pediatric Use**<br><br>The safety and efficacy of BRIDION in pediatric patients have not been established.<br><br>Sugammadex has been shown to be retained in bone and teeth with a significantly longer half-life than plasma. Bone and tooth deposition is significantly higher in juvenile animals than adults in rats. Four-week daily sugammadex administration in juvenile rats caused slight but irreversible bone length decrease (approximately 5%) and reversible whitish discoloration with the No-observed-effect-level (NOEL) which is 12-fold estimated local bone and teeth concentration of a 4 mg/kg single-dose in pediatrics of 4-year old. Weekly intermittent dosing (better reflected the likely clinical use) administration for 8 weeks in juvenile rats did not produce similar change in bone and teeth at a dose achieving greater than 33-fold estimated local bone and teeth concentration of a 4 mg/kg single-dose in pediatrics of 4-year old [see Nonclinical Toxicology (13.2)]. | Juvenile animal studies required to be completed under either a Pediatric Study Plan (PSP) or a Pediatric Written Request (PWR) must be included in the label in Section 8.4 even if adverse changes are not observed. Although these studies were not required for either a PSP or PWR, we recommend that they be included in labeling.<br><br>The Final PPLR and FDA draft guidance for industry: Pregnancy, Lactation, and Reproductive Potential:<br>Labeling for Human Prescription Drug and Biological Products — Content and Format were not published when this NDA was originally submitted. However, the bone toxicity studies were required by the Agency due to the concern for bone toxicity especially in pediatrics because of the high deposition rate of sugammadex in juvenile rats compared to adults. Therefore, I believe these juvenile studies should be included in the label.<br><br>Sugammadex is a compound that structurally belongs to the molecular family of γ-cyclodextrins. Cyclodextrins were reported to cause renal changes in adult rats. Given the lack of full maturity of the kidneys in pediatric patients aged 0-2 years old, a concern was raised whether sugammadex may be associated with more severe adverse effects to kidneys when it is administered to pediatric patients in this age range in this review cycle. The Division did not require the Applicant to conduct juvenile toxicity studies to evaluate the effects of sugammadex on immature kidney functions previously, but the Applicant voluntarily conducted this evaluation.<br><br>Nonclinical data indicated that sugammadex caused reversible minimal to moderate renal tubular vacuolation in single-dose and 4-week repeat-dose studies in both adult and juvenile rats at doses ≥ |

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|---|---|---|
| | | 500 mg/kg and ≥ 120 mg/kg, respectively, with similar incidence and severity. In comparison to adults, juvenile rats dosed from 7-days old did not show a significant difference in the severity or the incidence of the kidney change. In addition, the sugammadex exposure level in 7-day old rats, the age when the kidneys are still considered to be developing, was similar to that in adults with equivalent dose administration. The NOEL for the kidney change in the 4-week juvenile rat study was 120 mg/kg which provides an adequate safety margin (1.6-fold) for MRHD on AUC comparison. Overall, there is no significant safety concern for kidney adverse effects in a pediatric population aged 0-2 years old for multiple-dose sugammadex treatment. Therefore, it is not necessary to include this information in the label. |
| **11 DESCRIPTION**<br>[TRADENAME] (sugammadex) (b) (4) | **11 DESCRIPTION**<br>~~[TRADENAME] (sugammadex)~~ (b) (4) | (b) (4) |

| | | (b) (4) |
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| **12 Clinical Pharmacology**<br>**12.2 Pharmacodynamics**<br>[Tradename] may contain (b) (4) [redacted] of the mono OH-derivative of sugammadex. In preclinical pharmacology studies (b) (4) was demonstrated [redacted] (b) (4) nearly similar efficacy in reversing rocuronium- or vecuronium-induced blockade. | **12 Clinical Pharmacology**<br>**12.2 Pharmacodynamics**<br>[Tradename] may contain up to 7% [redacted] (b) (4) of the mono OH-derivative of sugammadex. In preclinical pharmacology studies, ~~it~~ the mono OH-derivative was demonstrated [redacted] (b) (4) to have ~50% of the affinity as sugammadex for rocuronium and vecuronium and that product with up to 7% of the mono OH-derivative has nearly similar efficacy in reversing rocuronium- or vecuronium-induced blockade. Although sugammadex has greatest affinity for aminosteroid neuromuscular blocking agents such as rocuronium and vecuronium, plasma levels of endogenous or exogenous compounds with a similar steroidal structure, such as some hormones, hormonal contraceptives, and pheromones may also be reduced following administration of sugammadex *[see 7.2 Interactions Potentially Affecting the Efficacy of Other Drugs, and 13.2 Animal Toxicology and/or Pharmacology].* | Binding data suggest approximately 50% of the binding affinity for rocuronium and vecuronium and the addition of 10-20% of mono OH-derivative to sugammadex did not seem to decrease efficacy in a mouse model. The statement that [redacted] (b) (4) [redacted] is not correct. Given the impact on hormonal contraceptives and the unusual findings in the pre- and post-natal development study, we recommend including some information regarding the fact that sugammadex can bind more than just rocuronium and vecuronium and cross reference the appropriate sections of the label. |
| **12.3 Pharmacokinetics**<br>**Distribution** | **12.3 Pharmacokinetics**<br>**Distribution**<br>In nonclinical drug distribution studies, sugammadex is retained in sites of active mineralization, such as bone and teeth with a half-life of 172 and 8 days, respectively *[see 8.4 Pediatrics and 13.2 Animal Toxicology and/or Pharmacology].* | Given the unexpected distribution of this compound to these tissues, we recommend including these data in 12.3 and cross referencing the information in 8.4 and 13.2. |
| **Metabolism**<br>In [redacted] (b) (4) clinical studies, no metabolites of sugammadex have been observed and only renal excretion of the unchanged product was observed as the route of elimination. | **Metabolism**<br>In [redacted] (b) (4) clinical studies, no metabolites of sugammadex have been observed and only renal excretion of the unchanged product was observed as the route of elimination. | Text is acceptable, although [redacted] (b) (4) is not necessary and may be removed. |

| 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility | 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility | |
|---|---|---|
| (b) (4) | *Carcinogenesis*<br>(b) (4) Long-term animal studies to evaluate the carcinogenic potential of sugammadex (b) (4) have not been conducted. | Subtitles were added to assist the reader. The proposed text was modified to reflect standard CFR language and to remove explanatory statements that could be deemed promotional rather than simply stating the findings. A single-dose use does not make it sufficient to waive carcinogenicity studies. For this product, long-term bone retention occurred with single-dose treatment, which raised concern for potential bone carcinogenicity. The Applicant provided appropriate justification to resolve this issue in the 1st resubmission. Therefore, I recommend we just state that carcinogenicity studies have not been conducted. |
| (b) (4) | *Mutagenesis*<br>(b) (4)<br>Sugammadex and its mono OH-derivative tested negative in in vitro bacterial reverse mutation assays (Ames test), in vitro chromosomal aberration assays in human peripheral blood lymphocytes, and in vivo micronucleus assay in mice and rats. | The proposed language is not clear regarding the studies completed and has therefore, been reworded. Results for both sugammadex and its mono OH-derivative are specified because these compounds were tested separately and described in the product labeling. |
| (b) (4) | *Impairment of Fertility*<br>(b) (4)<br>A fertility and early embryonic development study in Sprague-Dawley rats in which male rats were treated daily for 29 days prior to mating and through the mating period and female rats were treated daily for 14 days prior to mating to Day 5 post-coitum via intravenously administered with | The *impairment of fertility* section was reworded to remove unnecessary information and include more information on the study design to assist interpretation. Only the fertility and early embryofetal toxicity study should be described in this section. (b) (4)<br>Although TK data were not obtained in this study, the AUC comparisons |

| | | |
|---|---|---|
| | saline or sugammadex at 20, 100, and 500 mg/kg (0.2, 1.0, and 6.2 times of MHRD by AUC) did not show adverse effects on fertility. | are based on AUC data in a 4-week general toxicity study in rats taken from other studies.<br><br>Sponsor's values: Clinical AUC at 16 mg/kg = 160 mcg.h/mL<br>Male and Female rat AUC at 500 mg/kg = 985 mcg.h/mL.<br>1050/160=6.6 |
| **13.2 Animal Toxicology and/or Pharmacology**<br><br>(b) (4) | **13.2 Animal Toxicology and/or Pharmacology**<br><br>(b) (4) | The proposed text is promotional and does not provide useful information to the prescriber.<br><br>The proposed text is also considered promotional. Although the juvenile animal study results are summarized in Section 8.4, the studies are described in this section in more detail. |
| | Bone and teeth retention of sugammadex occurred in rats after intravenous injection, with mean half-lives of 172 and 8 days, respectively. Sugammadex bound to hydroxyapatite in an *in vitro* study and distributed in the bone formation area where hydroxyapatite is present for mineralization *in vivo*. Studies in adult rats have shown that single dose administration of 2000 mg/kg sugammadex slightly increased bone resorption, but had no effect | (b) (4) It is believed that the teeth concentration is significantly lower than bone concentration when sugammadex is |

| | | |
|---|---|---|
| | on teeth color and appearance.<br><br>A bone repair study in adult rats showed a statistically significant increase in callus formation and decrease in bone formation following 6 weekly administrations of sugammadex at 500 mg/kg (107 times the estimated bone concentration of a 4 mg/kg single-dose in human adult) as compared to the control, suggesting a slight delay in the bone healing process. The NOAEL was identified to be 120 mg/kg (52 times the estimated bone concentration of a 4 mg/kg single-dose in human adult).<br><br>Bone deposition in juvenile animals was significantly higher as compared to adult in rats (13% vs. 3%) after 30 mg/kg single-dose intravenously administration due to the high growth rate of the bone during developmental period. In juvenile rats dosing from Postnatal Day 7, 28-day daily administration of sugammadex at 120 and 500 mg/kg caused slight bone length decrease (approximately 5%) which did not recover within an 8-week treatment-free period. Reversible whitish discoloration and disturbance of enamel formation were also observed in the incisors at these dose levels. In molars, this effect was only observed at 500 mg/kg. The no-observed-effect-level (NOEL) was 30 mg/kg (approximately 12-times the estimated bone concentration of a 4 mg/kg single-dose in pediatrics of 4 year-old). Similar bone and teeth effects were not observed in a subsequent follow-up intermittent juvenile rat study with 8 weekly dosing of sugammadex at doses up to 120 mg/kg (approximately 33 times the estimated bone concentration of a 4 mg/kg single-dose in pediatrics of 4 year-old). | administered based on distribution data. (b) (4) [redacted]<br><br>Description of (b) (4) [redacted] should be deleted from this section. |

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**This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.**

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/s/

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ZENGJUN XU
03/25/2015

JAY H CHANG
03/25/2015

RICHARD D MELLON
03/25/2015
I concur.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

# Supervisory Pharmacologist Memorandum

| | |
|---|---|
| NDA NUMBER: | **22-225** |
| SDN: | **51** |
| DATE RECEIVED BY CENTER: | **12/20/2012** |
| PRODUCT: | |
| **(Proposed) Trade Name:** | (b) (4) |
| **Established Name:** | **Sugammadex** |
| INDICATION (paraphrased): | (1) Routine reversal of moderate or deep neuromuscular blockade induced by rocuronium or vecuronium in adult patients<br>(2) Immediate reversal of neuromuscular blockade induced by rocuronium or vecuronium in adult patients |
| INTENDED CLINICAL POPULATION: | **Adults undergoing surgical procedures requiring neuromuscular blockade with rocuronium or vecuronium** |
| SPONSOR: | **Organon USA Inc.** |
| REVIEW DIVISION: | **Division of Anesthesia, Analgesia and Addiction Products (HFD-170)** |
| PHARM/TOX REVIEWER: | **Zengjun Xu, Ph.D, DABT** |
| PHARM/TOX SUPERVISOR: | **Adam Wasserman, Ph.D.** |
| DIVISION DIRECTOR: | **Bob Rappaport, M.D.** |
| PROJECT MANAGER: | **Diana Walker, Ph.D.** |

## I. BACKGROUND

Sugammadex sodium (Org 25969, previously referred to as (b) (4) in regulatory documents, now with proposed tradename (b) (4)) is a modified gamma cyclodextrin developed by Organon which is designed to capture, and therefore functionally inactivate amonosteroidal neuromuscular blocking drugs rocuronium and vecuronium (both of which are also manufactured by Organon). Sugammadex sodium injection was originally submitted as NDA 22-225 by Organon USA on October 30, 2007 for the routine reversal of shallow or profound neuromuscular blockade induced by rocuronium or vecuronium as well as immediate reversal of neuromuscular blockade at 3 minutes after administration of rocuronium. Both uses were limited to the adult population. Primary nonclinical review was conducted by Zengjun Xu, Ph.D., DABT. As part of the nonclinical review, consults were obtained from Gemma Kuijpers, Ph.D. of the Division of Metabolic and Endocrine Products (7/21/2008) and from Fred Hyman, D.D.S. of the Division of Dermatologic and Dental Products (4/11/2008) to provide a safety evaluation related to deposition of sugammadex in bone and teeth, respectively, in nonclinical models. From the nonclinical perspective the data supported approval and this recommendation was confirmed through primary, secondary and tertiary reviews conducted by Dr. Xu (6/20/2008), myself (6/16/2008), and Paul Brown, Ph.D., Associate Director for Pharmacology/Toxicology for the Office of Drug Evaluation II (7/18/2008).

Although approval was recommended from the nonclinical perspective for adult indications and usage, concerns were raised in reviews, consults and at the Advisory Committee meeting held on March 11, 2008, about use of sugammadex in pediatric patients. (b) (4) The principal issue involved the apparent deposition and long residence time ($t_{1/2}$ ~ 170 days in rats) of sugammadex in mineralizing tissues, particularly in bone and to a lesser extent teeth. Not surprisingly, deposition was significantly greater in the bones of juvenile rats when compared to young adult or aged rats. A juvenile animal toxicity study in which sugammadex was administered daily to rats for 4 weeks[1] revealed a reduced ulna and femur length when assessed in vivo and ex-vivo, respectively, as well as other changes in several bone parameters (including bone mineral density and bone thickness). A NOAEL was identified in the study; however, the safety margin compared to clinical AUC plasma exposure in humans at 4 mg/kg was low (0.5X), although by bone concentration estimate comparison – which clinically was of uncertain accuracy – the margin was substantially higher (33X). (b) (4) Additionally, as sugammadex was thought to bind to growing bone there was also concern regarding an effect of sugammadex on healing bones subsequent to trauma or surgical interventions. Though the level of concern in adults was low and was not considered a deficiency for the first cycle, it was thought by review staff and the Advisory Committee to be important to evaluate this potential effect.

[1] Org 25969: toxicity study in the neonatal/juvenile rat by SC and IV administration (Study Report Number INT00038389)



Reference ID: 3364115

The NDA ultimately received a Not Approvable determination in a letter of July 31, 2008, due to clinical deficiencies principally involving the lack of information characterizing hypersensitivity and the risk of anaphylaxis as well as the absence of information on coagulation parameters with an observation of increased events of hemorrhagic adverse events compared to placebo.

As part of the Not Approvable letter the Applicant was advised that, although not considered deficiencies for the original NDA submission, additional nonclinical data would be required prior to multiple-dose pediatric trials, approval of a pediatric indication, or inclusion of pediatric data in the label. Broadly, these evaluations centered on providing evidence to support the safety of sugammadex use in growing or repairing bone and consisted of a new juvenile rodent study with intermittent dosing and long follow-up with extensive evaluation of bone integrity, dimensions, and structure; re-analysis of findings observed in the 4-week juvenile animal study described above, and a study of sugammadex in a nonclinical fracture healing model. Additional studies included further characterization of the sugammadex binding site in bone and clarification of bone distribution. Finally, due to the long residence time and the more extensive deposition of sugammadex in bone expected in the pediatric population, the Applicant was required to provide data along with a persuasive argument that sugammadex was unlikely to cause tumors in children receiving the drug.

The applicant, now a subsidiary of Merck & Co, Inc., submitted a Complete Response to the Not Approval determination on December 20, 2012 (SDN51), to obtain the adult indications originally sought. (b) (4) Pediatric study protocols were not submitted in the Complete Response; however, the Applicant provided nonclinical data conducted to address the requirements listed in the Not Approvable letter. Nonclinical review was performed by Dr. Xu with consultative review again performed by Dr. Kuijpers (now in the Division of Bone, Reproductive and Urologic Products).

## II. SUBMITTED NON-CLINICAL DATA

The Applicant submitted several studies[2,3] which provided data to support the contention made in the original NDA submission that sugammadex binds to hydroxyapatite in bone at sites of active mineralization, including a reanalysis of a previously submitted microautoradiography study (Study 060217). The in vitro assessment of hydroxyapatite (HA) binding involved incubation of radiolabeled Org 25969 with HA in suspension with or without competition from rocuronium bromide, the bisphosphonates alendronate or

[2] In vitro binding of [14C]-Org 25969 (Org 25969-2) to hydroxyapatite (Study Number 090085/Study Report Number INT00127799)
[3] Microautoradiography in bone after a single intravenous dose of [14C]-Org 25969 to Wistar rats (Study Number 090071/Study Report Number INT00142585)

pamidronate, tetracycline or sodium citrate. Org 25969 avidly bound to HA in a saturable manner (maximum predicted to be 19.5 nmol/mg HA) and only bisphosphonates effectively competed for the binding. Little to no loss of radiolabel binding occurred with high concentrations of tetracycline or sodium citrate, indicating that sugammadex and bisphosphonates share a specific binding site. Interestingly, rocuronium bromide at ten-fold molar excess failed to reduce binding which is at odds with in vivo data in which rocuronium significantly reduces bone deposition of sugammadex. This, as noted by Dr. Kuijpers, is likely more related to an alteration in distribution of the bound complex, which seems possible or perhaps due to the conditions of the in vitro assay in which incubation was allowed to occur for 24 hr, a much greater period of time for Org 25969 to attach to HA than would normally be allowed in circulation based on plasma half-life.

A microautoradiographic study of bone deposition of [$^{14}$C]-Org 25969[3] in femur and tibia samples obtained from several studies[4,5,6] with similar study parameters allowed comparison of Org 25969 distribution at 24 hr, 21 days, and 84 days post dose administration. As described by Dr. Xu in his review, Org 25969 did not appear to be present in the epiphyseal growth plate or other non-mineralized regions (articular surface, bone marrow) but rather was distributed to areas of active bone growth and mineralization, consistent with the in vitro work described above. In particular, radiolabel was observed in the proximal metaphyseal region adjacent to the growth plate including calcified cartilage and newly formed trabecular bone, as well as in epiphyseal trabecular bone and diaphyseal periosteal and endosteal cortical bone. An apparent change in radiolabel distribution occurred between the acute 24 hr and the two delayed observation times. While initially radiolabel was present in the metaphysis directly adjacent to the growth plate and only at trabecular and cortical bone surfaces, label was located more distally in the diaphysis and more centrally inside the cortical and trabecular bone compartments at the later 3- and 12-week observation times. Dr. Xu states that the shift of labeled compound is due to new bone formation. Dr. Kuijpers in her consult additionally considered that the change in distribution potentially reflects redistribution from sites of bone resorption to adjacent sites of bone mineralization. It would seem possible that both processes are involved and it is perhaps notable that, as pointed out by Dr. Kuijpers, despite the apparent similarity between the binding sites of Org 25969 and bisphosphonates, the distribution of these two types of compounds over time is dissimilar due to the latter drug's inhibition of bone resorption. I am in agreement with Dr. Xu that the redistribution or movement of the compound to hydroxyapatite sites elsewhere in the bone does not represent a safety concern.

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[4] Study to quantitatively assess binding of [14C]-Org 25969 (Org 25969-2) and Org 25969 (sugammadex) in bone and teeth upon intermittent dosing in Wistar rats (Study Number 090310/Study Report Number INT00141634)

[5] Investigational single dose toxicity study on bone quality, structure and turnover in rats (Study Number 060338/Study Report Number INT00033963)

[6] Bone retention kinetics in rat after single dose and multiple dose (Study Number 050322/Study Report Number INT00010275)

The applicant conducted a single-dose study of the distribution kinetics of Org 25969 in PND7 (juvenile) rats in comparison to young adult (6-week old) rats[7] to which radiolabeled Org 25969 was administered. Many relevant tissues were obtained (plasma, site of administration, long bones and vertebrae, incisors, and molars). Animals (1 animal/timepoint) were sacrificed beginning 3 hours post-administration and ending 47-weeks later (juvenile start) or 35-weeks later (young adult start). As noted by Dr. Xu in his review, plasma levels were higher in juvenile animals – ascribed to immature renal function. A biphasic elimination from bones appeared to occur with significant reduction in bone concentrations within the first 1-5 weeks with slow elimination from the bone compartment for the subsequent 30-42 weeks. The maximal % of the dose residing in bone in juveniles appeared to be slightly less than 7% while in young adult rats this was closer to 3.5%. Juveniles appeared to have a much greater level of bone uptake initially and therefore the loss in label was much greater. However, these rats are growing bone at high rates and therefore radiolabel becomes diluted when considered as a % of dose/gram pooled bones but when considered as a % of dose in all the pooled bones the level of apparent elimination is much reduced. Absorption in teeth was more limited being ~0.6% of the total dose in juveniles and ~0.1% in young adults. Multiple, intermittent dosing of radiolabeled Org 25969 was explored in a subsequent study[4] in which drug was administered to juvenile rats (beginning postnatal week 1; 7 weekly doses), young adult rats (beginning postnatal week 6; 6 weekly doses), and adult rats (beginning postnatal week 12; 6 weekly doses) in order to determine the extent of bone absorption in the intermittent dose juvenile rat toxicity study and the bone fracture study (described below). Retained levels in both bone and teeth were slightly higher when equivalent dosing was started in the juvenile period when compared to the young adult period and in the latter levels were slightly greater than those in adult rats. This is not unexpected as Dr. Xu notes due to the different degree of mineralization and greater growth curve earlier in development. He also notes the general similarity between the level of absorbed radiolabel in the juvenile and young adult rats, but identifies the difficulty in comparing the absorption data from this study to those from a previous single-dose study comparing juvenile to adult rodents since different data analysis techniques were used (non-pooled bones were used in the present study). I also add that with repeated intermittent administration over 6 or 7 weeks the differences in developmental age become less prominent.

To address the observations of slightly reduced bone length from the 4-week daily dosing toxicity study in juvenile rats[1] conducted for the original NDA submission, the Applicant re-analyzed the femur length data[8] to determine the impact of reduced body weight. In addition, they conducted a new study[9] for this resubmission in which juvenile rats were administered Org 25969 intermittently – once per week – over 8 weeks, an administration

[7] Bone retention kinetics in juvenile and young adult Wistar rats after a single subcutaneous injection of [14C]-Org 25969 (Org 25969-2) and Org 25969 (sugammadex)(Study Number 100045; Study Report Number INT00150298)

[8] Statistical evaluation of the effect of Org 25969 on ulna length and femur length correcting for effects on body weight in juvenile rats of study 060086 (Study Report Number INT00122920)

[9] An 8-week intermittent dosing toxicity study with Org 25969 to assess effects on bone quality, structure, turnover, strength and growth and development in juvenile Wistar rats (Study Report Number INT00150243)



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regimen which was proposed to represent a more realistic maximal usage with which to judge effects on bone. As described by both Dr. Xu and Dr. Kuijpers in their most recent reviews, the re-analysis using animal body weight as a covariate failed to eliminate the significant reduction in bone length. It therefore reaffirmed that the reduced bone length in the original study could not be explained by general body weight changes and was therefore an independent effect of Org 25969, occurring at a dose of ≥ 120 mg/kg/day administered over 28 days. In the present juvenile animal toxicity study submitted with the NDA, post-natal day 7 (PND7) rats were administered Org 25969 at doses of 0, 7.5, 30, or 120 mg/kg weekly for 8 weeks (SC route the first two weeks, IV thereafter). The Sponsor stated the high dose was chosen to provide for a sufficiently high exposure without affecting body weight or animal condition. The study incorporated significant investigation of bone quality and integrity including biochemical markers of bone turnover, in vivo measurements of ulna length, urinalysis, gross and histopathologic assessment of tissue, specific assessments of growth plate thickness in humerus and tibia, femoral cross-section, femur and ulna length and weight, μCT of femur and vertebrae, and measures of bone strength. Administration of Org 25969 up to 120 mg/kg/week for 8 weeks did not affect animal condition or body weight. A significant decrease in osteocalcin, a marker for osteoblast activity, was observed at 120 mg/kg/week only at the recovery sacrifice of unclear significance. Drs. Xu and Kuijpers noted a significantly reduced growth plate thickness of the humerus (8% below control) in males of the Org 25969 120 mg/kg/week group which was not observed in females and was not present at recovery in either gender. Notably, no change was observed in any other structural aspect of bone and there was no consistent dose- or treatment-related effect on ulna length, bone mass, bone density, or bone strength or stiffness at the end of 8 weeks of dosing or at the conclusion of an 8 week recovery period. Pamindronate, a bisphosphonate used as a positive control, produced significant alterations in serum and urinary biomarkers, changed bone structure and affected bone biomechanical properties. Dr. Kuijpers considers the reduced growth plate thickness to potentially reflect a direct effect on chondrocytes in the growth plate even in the absence of drug binding, or alternatively an indirect effect through binding of Org 25969 to mineralized bone or calcified cartilage producing enhanced chondrocyte apoptosis or reduced proliferation.

Dr. Kuijpers notes a published study[10] in which a β-cyclodextrin-conjugated alendronate produced a local anabolic bone effect when injected into bone. This effect was hypothesized by the investigators to represent sequestration of a bone anabolic factor(s) by the cyclodextrin which remains in contact with bone due to linkage of the cyclodextrin to the bisphosphonate. While this isolated publication is intriguing and the investigators did demonstrate that a bone anabolic effect could occur through intentional prior complexation of this molecule with prostaglandin E1 (PGE1), I agree with Dr. Kuijpers that an analogous theory to explain the present observation of reduced growth plate thickness should be considered speculative. Cyclodextrins are used to solubilize numerous small molecule compounds and cannot complex with proteins as even the larger gamma-cyclodextrins, to which Org 25969 belongs, are too small for proteins (Bone Morphogenetic Protein BMP-3 is for example 50 KDa compared to rocuronium

[10] Liu XM, Wiswall AT, Rutledge, JE, Akhter MP, Cullen DM, Reinhardt RA, and Wang D. Osteotropic β-cyclodextrin for local bone regeneration. Biomaterials 29 (2008) 1686-1692.



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500 Da). Org 25969 is unlikely to retain any compound without the structural characteristics of a steroid (and aminosteroid), such as prostaglandins, lipids, etc. as the inclusion site has been specifically designed to capture these types of molecules. However, there are steroid-related compounds with notable effects on bone such as steroid hormones and vitamin D which may have some degree of affinity with Org 25969, although this seems like a remote probability as the finding does not appear similar to the one reported by Liu and colleagues and there was no evidence of a similar effect in the tibia. We therefore have no evidence for a direct or indirect effect on chondrocyte proliferation at this time. The absence of this evidence does not imply that it is not a possibility; and the reduced growth plate thickness is consistent with the small changes in bone length in the 4-week juvenile toxicity study. However, I agree with Drs. Xu and Kuijpers that the growth plate data itself are of questionable toxicologic importance and almost certainly of limited clinical relevance. The effect on growth plate was only observed in one bone type, one gender, at one timepoint, and did not translate into changes in bone density, structure or strength. Importantly, this also was not observed in the recovery period – which implies any effect on the growth plate would be transient – and the no effect level affords a significant safety margin based on AUC plasma exposure comparison (6.4X) and even more so when using estimated bone concentrations in children as a comparison (≥33X).

The study which has the most relevance to the present clinical sugammadex usage in adult surgical cases is a fracture healing study[11] conducted in the young adult rat. In order to determine if sugammadex could impact or impair the bone healing response the Applicant utilized a well-established closed femur fracture model in the rat (for details see the reviews by Drs. Xu and Kuijpers). Briefly, a specific and controlled femoral diaphyseal fracture was created at post-natal week 12 with animals either receiving placebo, or Org 25969 30, 120 or 500 mg/kg once weekly for 6 weeks either post-fracture or prior to fracture, with sacrifice for evaluation at post-natal week 24. A number of bone-specific evaluations were conducted to determine if sugammadex affects the bone healing response including: semi-quantitative histopathology; peripheral quantitative CT (pQCT) to assess bone mineral density (BMD), bone mineral content (BMC) and bone area (BA); μCT to assess callus volume (TV) and bone volume (BV); and several biomechanical tests to assess bone strength.

With Org 25969 treatment prior to fracture there was no consistent effect observed on any endpoint and though increased bone bridging was noted in males in all dosed groups but not in females this may be due to the large difference in control values between the genders. However, post-fracture treatment with Org 25969 at 500 mg/kg over 6 weeks resulted in a change in the histologic profile of the fracture site that was consistent in both treated male and female groups, which Drs. Xu and Kuijpers consider suggestive of a possible delay in bone healing. In particular, the study reported that an earlier less mature type of bone (greater degree of cartilage/fibrocartilage compared to woven bone) was present at the fracture site, that there was reduced bridging (in fact this appeared to be absent in males) of the fractured bone ends, and that some increased fibrosis was noted in both male and female rats. Dr. Xu noted that for the purpose of increasing concurrent

[11] Effects of Org 25969 on fracture repair in the Wistar rat (Report number 090319; Study AA86536)



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control group size to better understand the level of variability, pre-fracture and post-fracture treated animals receiving saline placebo can reasonably be combined. This resulted in a slightly broader range of values for the concurrent control group, which appeared to diminish the effect size in males with little consistent change in females.

**Histologic assessment of fracture site with separate and combined control group values compared to Org 25969 500 mg/kg**

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | Post-Fx control | Combined control† | Post-Fx 500 mg/kg | Post-Fx control | Combined control† | Post-Fx 500 mg/kg |
| Callus extent | 1.89 ± 0.60 | 2.06 ± 0.63 | 2.50 ± 0.48* | 2.00 ± 0.77 | 1.94 ± 0.80 | 2.10 ± 0.57 |
| Type bone | 1.33 ± 0.50 | 1.28 ± 0.47 | 1.00 ± 0.00 | 2.09 ± 0.83 | 1.99 ± 0.74 | 1.40 ± 0.52* |
| Bridging site | 0.78 ± 1.39 | 0.50 ± 1.0 | 0.00 ± 0.00 | 2.45 ± 1.44 | 2.54 ± 1.32 | 1.00 ± 0.94* |
| Fibrosis | 1.00 ± 0.71 | 1.22 ± 0.72 | 1.80 ± 0.42* | 0.64 ± 0.81 | 0.65 ± 0.68 | 1.30 ± 0.67 |

†, Control rats from the pre- and post-fx saline treatment regimens are combined (note no statistical reanalysis)
*, $p<0.05$ vs. Post-Fx control only

Histopathology Evaluation Scoring Criteria

| Characteristic | Grading | Score |
|---|---|---|
| Extent of Fracture Callous Formation | Marked | 3 |
| | Moderate | 2 |
| | Mild | 1 |
| | None | 0 |
| Type of new bone formed | Mostly lamellar | 4 |
| | Even mix of woven and lamellar bone | 3 |
| | Mostly woven bone | 2 |
| | Mostly cartilage /fibrocartilage | 1 |
| | No new bone formed | 0 |
| Bridging of the fracture site by new bone | Complete | 4 |
| | Mostly complete with small gaps | 3 |
| | Approximately 50% complete | 2 |
| | Occasional/very little | 1 |
| | None | 0 |

There was no evidence for an effect of Org 25969 with either administration paradigm on the mechanical strength of the healing fracture site, which included the parameters maximum load, energy and stiffness and derived parameters reflecting intrinsic bone properties. There was also no effect of Org 25969 on pQCT densitometry parameters including BMC, BMD or bone area. Thus, the data were not suggestive of less mineralized or weakened bone. Consequently, no correlation was noted between the histologic observations in the post-fracture high-dose Org 25969-treated group described above and the pQCT or biomechanical test results, although the latter were highly variable and the method would lack sensitivity to detect small effects. This lack of correlation limits the toxicological relevance of the histologic findings. Both Drs. Xu and Kuijpers consider the study NOAEL for Org 25969 effects in post-fracture setting to be

120 mg/kg/week with 500 mg/kg/week to be the NOAEL for Org 25969 exposure in the pre-fracture setting. I concur with this evaluation and agree that the large safety margins with pre-fracture administration (28X using AUC; ≥ 40X using estimated bone concentration) and post-fracture administration (5.8X using AUC; ≥ 20X using estimated bone concentration) suggested very limited clinical relevance with normal usage in adult (b) (4) patients.

Both Drs. Xu and Kuijpers have considered the Applicant's rationale for a low level of concern for an enhanced risk of bone tumors while acknowledging the kinetics of sugammadex in this tissue. The Applicant has defined the binding site of sugammadex as hydroxyapatite and there is no apparent proliferative effect on bone cells. The compound is of size and charge that would not be expected to enter into cells to directly interact with DNA or transcriptional machinery and genotoxicity studies were negative. I concur that there is no apparent risk for drug-related tumor development with use of sugammadex.

## III. Discussion

The first cycle review of NDA 22-225 in 2008 noted the significant distribution and long residence time of sugammadex in bone of nonclinical animal models, and in particular juvenile animals, which created concern for patients in which there is significant bone development (i.e. pediatric patients) and to a lesser extent for patients in which bone healing is taking place. The Applicant has provided a number of studies for the present cycle submission to address concerns related to these observations which, in agreement with primary and consultative review, I find reassuring. For the present cycle which proposes an indication for reversal of neuromuscular block in adults only, the study of fracture repair in young, growing rats indicates that the risk of impaired bone healing with use of sugammadex is remote. The histologic changes observed within a variable background of normal healing profiles in the absence of biomechanical or other structural changes does not elicit great toxicologic concern and the no effect level provides a ≥6X AUC safety margin (≥ 20X using the less conservative estimated bone concentration). It is worth noting that the study utilized weekly dosing over 6 weeks – a level of clinical usage that would be considered uncommon.

(b) (4)

While studies in the original NDA submission identified a potential effect of sugammadex on long bone length when administered daily for 28 days to the juvenile rat, the data provided in this resubmission did not substantiate the finding with use of a once weekly dosing regimen. Reduction of growth plate thickness at the highest dose used in the new study is not considered to be toxicologically significant as the observation was transient, seen only in one gender, was noted in only one bone (humerus) only, and did not coincide with changes in bone density, mineral composition, structure or strength. The finding is likely of little clinical significance as well due to the significant margins expected with clinical usage as the no observed adverse level affords a ≥6X plasma exposure margin over the expected pediatric exposure and a ≥33X margin over the estimated bone concentration in this population.



Reference ID: 3364115

(b) (4)

## IV. CONCLUSION AND RECOMMENDATIONS

### A. Recommendation on approvability

From the nonclinical standpoint, the application for use of sugammadex in the adult population may be approved.

### B. Recommendation for nonclinical studies

None. The Applicant has appropriately addressed the nonclinical issues raised in the Not Approvable letter (b) (4).

### C. Recommendations on labeling

The label will be finalized in a subsequent cycle due to deficiencies identified by other disciplines. I will withhold specific labeling language until that time. (b) (4) The revision of the proposed label in order to be in conformance with the Pregnancy and Lactation Labeling Rule will be negotiated with members of the Pediatric and Maternal Health Staff as well. Finally, the extent to which nonclinical data will be described in the Animal Toxicology section remains to be decided.

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/s/

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ADAM M WASSERMAN
08/27/2013
Recommend approval from the nonclinical perspective

**DEPARTMENT OF HEALTH AND HUMAN SERVICES**
**PUBLIC HEALTH SERVICE**
**FOOD AND DRUG ADMINISTRATION**
**CENTER FOR DRUG EVALUATION AND RESEARCH**

# PHARMACOLOGY/TOXICOLOGY NDA/BLA REVIEW AND EVALUATION

| | |
|---|---|
| Application number: | 22-225 |
| Supporting document/s: | 51/Resubmission |
| Applicant's letter date: | 12/19/2012 |
| CDER stamp date: | 12/20/2012 |
| Product: | (b) (4)® (Sugammadex sodium injection) |
| Indication: | Routine reversal of moderate or deep neuromuscular blockade induced by rocuronium or vecuronium in adult patients |
| Applicant: | Organon USA Inc., Whitehouse Station, NJ |
| Review Division: | Division of Anesthesia, Analgesia, and Addiction Products (HFD-170) |
| Reviewer: | Z. Alex Xu, PhD, DABT |
| Supervisor/Team Leader: | Adam Wasserman, PhD |
| Division Director: | Bob Rappaport, MD |
| Project Manager: | Diana Walker PhD |

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# 1 Executive Summary

## 1.1 Introduction

Sugammadex (Org 25969) is a compound structurally belonging to the molecular family of γ-cyclodextrins (CDs). It is developed to bind to the hydrophobic region of the steroidal neuromuscular blocking (NMB) drugs rocuronium and vecuronium with high affinity resulting in reversal of neuromuscular blockade. The original NDA was submitted seeking approval of sugammadex for 1) shallow or profound neuromuscular blockade induced by rocuronium or vecuronium (recommended dose: 4 mg/kg), and 2) immediate reversal of neuromuscular blockade at 3 minutes after administration of rocuronium in adult patients (recommended dose: 16 mg/kg) on 10/31/07. From the nonclinical perspective, sugammadex was recommended to be approved for the proposed adult use. However, due to the concerns regarding long-term bone deposition of Org 25969 and observed slightly reduced bone length in a 4-week juvenile rat study, additional bone studies were needed before any multiple-dose clinical trial in pediatric patients and/or for approval of the drug to be used in pediatrics based on evaluation of nonclinical data by this reviewer and Dr. Gemma Kuijpers from the Division of Metabolism and Endocrinology products (DMEP), the consultation reviewer for the bone effect of Org 25969. The original NDA was not-approvable due to particular concerns of inadequate anaphylaxis and coagulation evaluation. In the Not-Approvable letter issued on 07/31/2008, the following nonclinical comments/requests were conveyed to the Sponsor.



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Nonclinical evaluations will be necessary prior to any multiple-dose pediatric trials, approval of a pediatric indication, or inclusion of pediatric data in the label:

1. Provide an evaluation of sugammadex in a nonclinical model of bone fracture to examine potential effects of the drug on bone healing. You are encouraged to submit protocols of such studies to the Agency for comment prior to their conduct.

2. Provide data on growth plate morphology to help understand the longitudinal growth reduction observed in the 28-day juvenile rat study.

3. Provide a repeat-dose study of sugammadex in the juvenile rat with an extended period of recovery, such that bone length, material properties, and integrity at full skeletal maturity may be evaluated in order to clarify the observation of slight but lasting decreased bone length and body weight observed in the 28-day juvenile toxicity study. Within this study, micro-computed tomography (μCT), bone turnover markers, and bone strength assessment should be obtained and evaluated. Also, the study should incorporate positive control arms to verify assay sensitivity. Lastly, though not required, the inclusion of vertebral evaluation would be helpful to interpret bone effects since vertebrae have a more homogenous trabecular structure than long bones such as femur. You are encouraged to consider an alternative, intermittent dosing paradigm in order to minimize effects of sugammadex on body weight while allowing for significant drug accumulation in the skeleton.

4. Provide definitive information on the binding site of sugammadex in bone as well as a reevaluation of bone localization of sugammadex as presentation of the data on bone localization was not clear and errors in descriptions were noted in the submitted materials.

5. Provide data, which may be derived from published literature, in vitro studies, and/or in vivo studies, along with a persuasive, well-supported rationale, to show that the risk of administration and long-term retention of sugammadex in the bones of pediatric patients will not confer a risk for bone tumor development in this population. Otherwise, an evaluation of the carcinogenic potential of sugammadex may be required. Although plasma levels of sugammadex rapidly decline with acute administration, the long retention of sugammadex in skeletal bone may be considered to be chronic exposure (i.e., greater than six months) and raises concern regarding the potential for development of tumors of this tissue, especially in the pediatric population, which is known to develop primary bone tumors at rates that exceed those in the adult population.

This is the complete response submission of this NDA for the same indication in adults. In this submission, the Applicant included additional studies to investigate the bone effects of sugammadex. The adequacy and sufficiency of these studies to address the nonclinical requirements outlined in the Not-Approvable letter were evaluated by this reviewer. The Division also requested consultation review of these studies from Dr. Kuijpers who is currently in the Division of Reproductive and Urologic Products (DRUP).

## 1.2 Brief Discussion of Nonclinical Findings

Nonclinical studies included in this submission were in response to the nonclinical comments/requests listed in the Non-Approvable letter for the original NDA submission. These requests were aimed to address the issues that were identified based on the review of nonclinical data included in the original NDA submission. These included 1) the location and binding site of Org 25969 in the bone were not clear after absorption and there was no sufficient evidence to indicate that Org 25969 does not deposit in the



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growth plate of bone; 2) no sufficient evidence to support the safety of multiple-dose clinical trial in pediatrics; 3) the effect of Org 25969 on bone healing was not evaluated; 4) potential concern of bone tumor development due to the long-term retention of Org 25969 in the bone after administration.

*1) The location and binding site of Org 25969 in the bone.*

The Applicant predicted that Org 25969 binds to extracellular hydroxyapatite in the bone based on the structure of the molecule, but no data was included to support this in the original NDA submission. The *in vitro* [$^{14}$C]-Org 25969 binding study (study number 090085) included in this submission was to investigate whether Org 25969 binds to hydroxyapatite. Results of this study showed that [$^{14}$C]-Org 25969 bound to hydroxyapatite *in vitro* in a less than concentration-proportional manner. The binding of Org 25969 to hydroxyapatite was not affected by sodium citrate or tetracycline, but significantly decreased in the presence of bisphosphonate compound alendronate in a concentration-dependent manner, indicating that the binding site of Org 25969 to hydroxyapatite at the same site of the bisphosphonates (P-C-P group – 2 phosphonate groups sharing a common carbon atom). This finding suggested that Org 25969 may be confined in the bone area that is undergoing bone mineralization, which was confirmed by the microautoradiography study in rats included in this submission (study 090071).

A previously conducted microautoradiography study in rats (study 060217) included in the original NDA revealed that labeled Org 25969 in active bone formation areas such as periosteal and endosteal aspects of cortical bone and endosteal aspects of trabecular bone. Although the study report stated that Org 25969 did not bind to growth plates, growth plates were not identifiable in most of the examined bone tissues. In addition, poor micrograph quality and errors in the micrograph descriptions diminished the reliability of the data in this study. In the newly submitted microautoradiography study (study 090071), growth plates were clearly identified in the examined bone sections. Results of the study showed that no radioactivity is present in epiphyseal growth plate and the articular surface, rather in the subarticular region (metaphysis) 24 hours after administration of [$^{14}$C]-Org 25969. The subarticular region is the place where new bone formation occurs with active bone mineralization following cartilaginous tissue growth. In addition, 24 hours after administration, radiolabeled Org 25969 was also found to be present in the outer trabecular, endosteal, periosteal and inner articular aspect surfaces, areas of bone mineralization during bone remodeling. With time lapse after [$^{14}$C]-Org 25969 administration (21- and 81-day after dosing), labeled Org 25969 was seen distally in the diaphysis but not in the area of new bone formation. These data indicated that Org 25969 does not deposit in growth plate of the bone and thus is not likely to directly interfere with bone elongation and growth. Although Org 25969 binds with hydroxyapatite at the same site with bisphosphonates, it does not appear to produce similar anti-resorptive effect as bisphosphonates based on the available nonclinical data because the anti-resorptive effect of bisphosphonates is due to the drug's ability to block the enzyme farnesyl diphosphate synthase, but not through binding with hydroxyapatite. Overall, both the *in vitro* binding study and *in vivo* microautoradiography study indicated that Org 25969 binds with hydroxyapatite and



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locates in the new bone formation area where mineralization occurs after absorption in the bone. These studies fulfill the nonclinical request 4 listed in the Non-Approvable letter.

*2) Nonclinical data to support the safety of multiple-dose clinical trial in pediatrics*

In the previously submitted 28-day juvenile rat toxicity study in the original NDA (study 063592), minor decrease in ulna length (≤5%), femur length (≤3.5%) and weight (≤6.5%) were observed at 120 and 500 mg/kg Org 25969. The Applicant indicated that the minor change was due to smaller size of the animals in Org 25969 treated groups likely due to decreased food consumption, and thus did not represent bone effect of Org 25969. The Division did not agree with this conclusion because the bone length decrease was not coincident with the body weight decrease and thus requested more information to clarify this finding. The NOAEL was identified to be 30 mg/kg which corresponds to approximately 22 μg·hr/ml in plasma AUC and 150 μg/g bone Org 25969. By plasma AUC comparison, exposure at NOAEL in juvenile rats (22 μg·hr/ml) did not cover human exposure at 4 mg/kg (40 μg·hr/ml). Although estimated bone concentration provided sufficient coverage, it is uncertain whether AUC level or local bone concentration level shall be used for safety margin calculation since the bone effect in juvenile rats has not been well characterized from the nonclinical data in the original NDA submission.

In this submission, data of the 4-week juvenile rat study were re-analyzed using ANOVA analysis with body weight as the covariate. The results showed that the minor bone length decrease was still statistically significant, further suggesting that the decreased bone length following Org 25969 administration in juvenile rats might not be associated with body weight change.

The 8-week intermittent dosing toxicity in juvenile rats (study 080229) was conducted to further clarify the observation in the 28-day juvenile toxicity study. In this study, juvenile rats were dosed weekly from postnatal day (PND) 7 for 8 doses at 0, 7.5, 30, and 120 mg/kg Org 25969, and the animals were sacrificed the second day after last dosing and at the end of 8-week recovery. In addition, an additional dosing group (10 rats/sex/group) was treated with 250 μg/kg Pamidronate as positive control on bone parameters; results of pamidronate treatment group indicated the rat is a sensitive model for bone change. The protocol outline of this study was submitted to the division for advice prior to the study initiation. The Agency agreed that weekly dosing appeared to be better mimicking clinical dosing situation as compared to daily dosing but recommended a higher top dose in order to maximize the chance of detecting a bone effect in an advice letter to the Applicant. However, the Applicant believed that a dose that has no impact on growth but achieves significant systemic and local (bone and teeth) exposure multiples of Org 25969 clinical exposure should be chosen because this study should differentiate between a direct effect on bone growth and a secondary effect related to a general lower growth rate. Therefore, the study was conducted with the high dose at 120 mg/kg. We did not agree with this rationale because it is possible that there is no relationship between the bone effect and body weight change but



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happen to occur at the same dosing level due to different mechanism. However, if a NOAEL can be identified in this study and provide sufficient safety margin to cover the dosage that will be used in future pediatric trials, the issue of the safety concern on the bone effect of sugammadex in pediatrics may be considered to be appropriately addressed.

In the newly conducted juvenile rat study, bone effects of Org 25969 were evaluated by in vivo ulna length measurement, blood concentration analysis of bone turnover markers including osteocalcin and C-telopeptide crosslinks (CTx), histopathology exanimation of the bone, postmortem measurement of femur and terminal ulna length, µCT evaluation, and bone strength tests. These tests/analyses used are considered to be adequate and sufficient to characterize adverse changes in the bone. The results of the study did not show significant changes in the parameters tested except a statistically significant decrease (15%) of osteocalcin at the end of 8-week recovery period but not at the end of dosing. The significance of this finding was unclear since there were no effects on bone density or strength. The NOAEL in this study was identified to be 120 mg/kg. Of note, the results of this study can not override the results of the previously submitted 4-week daily dosing toxicity study. Although data showed that Org 25969 did not bind in growth plate in the bone, binding of Org 25969 with extracellular hydroxyapatite may indirectly affect bone growth as Dr. Kuijpers indicated in her consultation review. The absence of bone effects at 120 mg/kg in this 8-week intermittent study may be due to the lower bone concentration as compared to the estimated bone concentration at the 120 mg/kg daily dose in the 4-week study.

In order to determine the sufficiency of the 8-week weekly dosing juvenile rat study to support the safety of future clinical trial in pediatric patients, safety margins were calculated based on AUC and estimated bone concentration comparison between animals and human. Data of bone concentration in human adults and pediatrics are not available. The bone concentration of Org 25969 was estimated to be maximally 4.5 µg/g and 18 µg/g bone at 4 mg/kg and 16 mg/kg, respectively, based on a mass balance study in human adult subjects. In pediatric subjects, bone retention rate is estimated to be 2-3 fold higher than that in adults (i.e. 12 µg/g in 4 yrs old and 9 µg/g in 10 yrs old vs. 4.5 µg/g in adult with single dose of 4 mg/kg) by Dr. Gemma Kuijpers based on extrapolation from rat data and comparison on bone weight/body weight ratio between human adult and pediatrics (refer to her consultation review for more details). Of note, this bone concentration estimation was based on single-dose administration. With multiple dose administration such as weekly dosing, an accumulation factor of 2 should be applied based on rat data.

The safety margins for the 4 mg/kg human dose were shown below based plasma AUC, and bone concentration estimates.

Safety Margin for sugammadex multiple-dose treatment at 4 mg/kg in pediatric subjects

| | | Rat | Human | Safety Margin |
|---|---|---|---|---|
| | | | | |
| Juvenile rat | Dose | 120 NOAEL | 4 | |
| | AUC (ug x h/mL) | 254 | 40 | 6.4x |



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| | | | | |
|---|---|---|---|---|
| | Bone conc (ug/g) | 400 | 18 (child 10 yrs) | 22x |
| | | | 24 (child 4 yrs) | 16.5x |

As shown above, the 8-week intermittent bone study in juvenile provide 6.4-fold and ≥ 16.5-fold safety margin (b) (4) based on AUC and bone concentration comparison.

(b) (4)

Although the Applicant did not have a plan to conduct a clinical study with 16 mg/kg dose, it is possible this dose will be tested in pediatric patients for immediate reversal of neuromuscular blockade as proposed in adult patients. Assuming a linear increase of plasma exposure (AUC) and bone concentration, which is the worst scenario for bone concentration estimation because bone deposition of Org 25969 was found to be less than dose proportional, the 8-week toxicity study in juvenile provides sufficient margin for (b) (4) clinical study in pediatric patients (b) (4)

Overall, the submitted 8-week intermittent toxicity study in juvenile rats provides sufficient evidence to support the (b) (4) pediatric study with (b) (4) Of note, in real clinical situation, 8 doses with weekly frequency in an individual patient will be very rare. Therefore, the 8-week intermittent toxicity study in juvenile is considered to be an exaggerated evaluation for the safety of sugammadex bone effects in the real clinical situation. This juvenile rat study successfully fulfills the nonclinical request 3 list in the Non-Approvable letter. In addition, this study also fulfills the request 2 because growth plate morphology was included in this study.

Possibility of sugammadex multiple daily dosing is even lower in clinical situation. However, a short term (i.e. < one week) daily dosing may occur in rare occasions. The 8-week intermittent toxicity study in juvenile rats is not considered to be appropriate to support daily dosing clinical use of sugammadex because of the difference in dosing interval (weekly dosing in animal study vs. daily dosing in human). However, the previous conducted 4-week daily dosing toxicity study provided information on safety of potential multiple daily dose clinical study. As indicated above, although exposure (22 µg·hr/ml) at NOAEL (30 mg/kg) in this study did not cover human exposure at 4 mg/kg (40 µg·hr/ml) by AUC comparison, the bone concentration at NOAEL provided

approximately 8-fold and 2-fold coverage to the estimated bone concentration in pediatric patients with daily dosing at 4 mg/kg and 16 mg/kg, respectively.

The question has been raised whether plasma AUC or bone concentration is more relevant in safety evaluation regarding bone effect. Bone concentration may be more relevant because bone effect was due to the deposition and binding of Org 25969 to hydroxyapatite in the bone which depends on the plasma exposure as well as the bone growth rate. The caveat for using bone concentration in the safety evaluation is that the exact bone concentration in pediatric subjects is not known but based on estimation from mass balance study in human adults. However, this may represent a conservative estimation because the assumption was made that all unrecoverable Org 25969 was deposited in the bone which is the worst case scenario. In addition, in vivo rat study indicated that presence of rocuronium decreases Org 25969 binding in the bone up to 30% when the molar ratio of rocuronium to Org 25969 was 1:2. Since Org 25969 will be given to patients with rocuronium present in the blood in the pediatric clinical trial or clinical use if approved for this indication, the 8-week intermittent toxicity study in juvenile without rocuronium treatment provides additional safety margins for Org 25969 uses in patients. Therefore, a short-term (i.e. < one week) daily pediatric use up to 16 mg/kg is considered to be safe based on the 4-week daily dose toxicity study.

*3) Effects of Org 25969 on bone repair*

The effect of Org 25969 on bone repair was evaluated in adult animals (study number: 090319). The bone fracture was produced by surgery at 12 weeks of age and animals were administered Org 25969 for 6 weeks by weekly dosing prior to or after fracture surgery at 0, 30, 120, and 500 mg/kg. The purpose of the study was to determine whether pre- and post-fracture treatment of Org 25969 may adversely affect the bone healing process. The results of the data did not show evidence of adverse change on bone healing in animals with pre-fracture treatment in any Org 25969 dosing group as compared to control as assessed by pQCT scan, µCT scan, bone strength test, and histopathology examination. With post-fracture treatment, statistically significant changes were observed at 500 mg/kg groups as compared to the concurrent control. Histopathology examination revealed slight increase in callus formation, slight decrease in bone formation and the extent of bridging at the fracture site by new bone at 500 mg/kg. All these suggest a slight delay in bone healing process. It is uncertain whether the changes in the histologic scores had clinically relevant consequences because significant changes were not observed BMD and BMC as well as bone strength test. In addition, the values of the parameters that were shown to be statistically significant were within the range of control values if male and female data are combined. However, the observed changes which suggest slight delay in bone healing process appear to be consistent with the observed bone growth reduction in the 4-week toxicity study in juvenile rats because the bone healing process and bone growth are generally similar. The observed findings in this study should not be dismissed and the NOAEL in post-fracture treated animals is identified to be 120 mg/kg, while in pre-fracture treated animals the NOAEL is 500 mg/kg. The safety margins are calculated based on the AUC and bone concentration comparison between animals in this bone repair study and



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human. The bone concentration was based on same estimation as shown in the safety margin calculation for the 8-week juvenile rat study. The safety margins of the NOAELs in the bone repair study to support adult and pediatric multiple-dose administration at 4 mg/kg are shown in the following table.

Safety margin for multiple-dose administration at 4 mg/kg in adult and pediatric patients with bone fracture

| | | Rat | Human | Safety margin |
|---|---|---|---|---|
| | | | | |
| Young adult rat | Dose | 120 NOAEL | 4 | |
| (Fx healing study) | AUC (ugxh/mL) | 230 | 40 | **5.8x** |
| | Bone conc (ug/g) | 235 | 9 (adult) | **26x** |
| | | | 18 (child 10 yrs) | **13x** |
| | | | 24 (child 4 yrs) | **10x** |
| | | | | |
| | Dose | 500 NOAEL | 4 | |
| | AUC (ugxh/mL) | 1101 | 40 | **28x** |
| | Bone conc (ug/g) | 480 | 9 (adult) | **53x** |
| | | | 18 (child 10 yrs) | **27x** |
| | | | 24 (child 4 yrs) | **20x** |

The bone repair study in adult rats provides sufficient safety margin to support Org 25969 treatment in adults with the proposed dosage. Significant adverse effects are not expected on the bone healing process in patients with bone fractures following 4 mg/kg or 16 mg/kg weekly treatment for 6 weeks. (b) (4) This bone repair study in young adult rats is considered to successfully fulfill the nonclinical request 1 in the Non-approvable letter

*4) Potential concern for bone tumor development*

The Division raised the concern of bone carcinogenicity because of the extended duration of Org 25969 bone retention as demonstrated in rats. In a meeting with the Applicant on December 1st, 2008, the Agency indicated demonstration of binding to hydroxyapatite as well as submission of bone metabolism and histopathology data from the requested juvenile rat study with extended duration of follow-up would address the carcinogenicity concern. In this submission, the Sponsor submitted an in vitro study clearly demonstrated that Org 25969 binds with hydroxyapatite, a component of bone extracellular matrix, at the same site for bisphosphonate binding. In addition, a microautoradiography study indicated that Org 25969 does not deposit in the growth plate, the area responsible for bone growth and elongation. In addition, histopathology examination did not show signs of carcinogenicity such as hyperplasia or hypertrophy of bone cells after 16-week Org 25969 exposure in the bone. Furthermore, bisphosphonates which bind the same site at hydroxyapatite do not cause bone-related cancer after long-term exposure based on a FDA published paper (Whitaker et al., 2012). This suggests that Org 25969 may not be associated with significant risk for

bone tumor development. Therefore, the request 5 in the Non-Approvable letter is considered to be sufficiently fulfilled.

Overall, the newly submitted nonclinical studies sufficiently addressed the issued identified in the original NDA submission, and the requests in the Non-approvable letter are fulfilled.

## 1.3 Recommendations

### 1.3.1 Approvability

The NDA may be approved

### 1.3.2 Additional Non Clinical Recommendations

There are no additional nonclinical recommendations.

### 1.3.3 Labeling

Labeling will be finished in the next cycle. Topics that will be addressed from nonclinical perspective include 1) creating an established pharmacologic class (EPC), 2) Converting the pregnancy (8.1), Labor and Delivery (8.2), and Nursing mother (8.3) section into PLLR format, and 3) Adding nonclinical information regarding the bone effect of sugammadex in the animal Toxicity and/or Pharmacology section (13.2).

# 2 Drug Information

## 2.1 Drug

CAS Registry Number (Optional) N/A

Generic Name Sugammadex

Code Name Org 25969

Pharmacologic Class To be determined

## 2.2 Relevant INDs, NDAs, BLAs and DMFs

IND 68,029

## 2.3 Drug Formulation

The same as the formulation in the original NDA submission

# 3 Studies Submitted

## 3.1 Studies Reviewed

| Title | Report Designation | Location |
|---|---|---|



Reference ID: 3362422

| ***Pharmacokinetics Study Reports*** | | |
|---|---|---|
| [$^{14}$C-Org 25969]: The distribution of radioactivity in bone after a single intravenous dose of [$^{14}$C]-Org 25969 to Wistar rats | INT00122920 (Study 060217) | eCTD |
| Microautoradiography in bone after a single intravenous dose of [14C]-Org 25969 to Wistar rats | INT00142585 (Study 090071) | eCTD |
| Study of quantitatively assess binding of [$^{14}$C]-Org 25969 (Org 25969-2) and Org 25969 (Sugammadex) in bone and teeth upon intermittent dosing in Wistar rats | INT00141634 (study 090310) | eCTD |
| Bone retention kinetics in juvenile and young adult Wistar rats after a single subcutaneous injection of [$^{14}$C]-Org 25969 (Org 25969-2) and Org 25969 (Sugammadex) | INT00150298 (study 100045) | eCTD |
| In vitro binding of [$^{14}$C]-Org 25969 (Org 25969-2) to hydroxyapatite | INT00127799 (090085) | eCTD |
| ***Toxicology Study Reports*** | | |
| Statistical evaluation of the effect of Org 25969 on ulna length and femur length correcting for effects on body weight in juvenile rats of study 060086 | INT00122920 (Study 060086) | eCTD |
| An 8-week intermittent dosing toxicity with Org 25969 to assess effects on bone quality, structure, turnover, strength and growth and development in juvenile Wistar rats | INT00150243 (Study 080229) | eCTD |
| Effects of Org 25969 on fracture repair in the Wistar rat | 090319 (study AA86536) | eCTD |

## 3.2 Studies Not Reviewed

None

## 3.3 Previous Reviews Referenced

Primary review of the original NDA submission by this reviewer (DARRTS on 06/20/2008)
Supervisory review of the original NDA submitted by Dr. Adam Wasserman (DARRTS on 06/16/2008)
Consultation review for the bone effect of Org 25969 for the original NDA submission by Dr. Gemma Kuijpers (DARRTS on 07/21/2008)
Consultation review for the bone effect of Org 25969 for this submission by Dr. Gemma Kuijpers (DARRTS on 08/15/2013)



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# 4 Pharmacokinetics/ADME/Toxicokinetics

**Study title:** [$^{14}$C-Org 25969]: The distribution of radioactivity in bone after a single intravenous dose of [$^{14}$C]-Org 25969 to Wistar rats

| | |
|---|---|
| Study Number: | 060217 |
| Study report Number: | INT00122920 |
| Conducting laboratory and location: | (b) (4) |
| Date of study initiation: | 08/14/2006 |
| GLP compliance: | No |

This study report is a replacement of study report INT00029187 with more detailed description of the results. The study report INT00029187 was included in the original NDA submission and numbered as INT00029186 by the Applicant which was also cited in my review (the study report was summarized in my review of the original NDA submission). In comparison, the current study report updated the result section (shown below as extracted from the study reports) but no change in the conclusion. The applicant stated that the results of the study showed that the deposition of silver granules ([$^{14}$C]-Org 25969) was at or adjacent to sites of active bone formation when the dose of [$^{14}$C]-Org 25969 was administered. Bone formation subsequent to this showed no silver deposition. It appeared that bone formation had continued normally. As I pointed out in my review of the original NDA review, among the 3 animals which were dosed with [$^{14}$C]-Org 25969, growth plates could only be identified in 2 scapulas and one femur of examined tissues. Therefore, additional evidence was needed in support of the conclusion of Org 25969 absence in growth plate. However, the updated study report in this submission did not include this information to address this issue as shown below (extracted from the study report).



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Comparison of the result section between current study report (INT00122920) and INT00029187

| INT00029187 result section | INT00122920 result section |
|---|---|
| The distribution of radioactivity in sections of femur and scapula of rats killed at either 1 day, 21 days or 84 days following a single intravenous dose of [$^{14}$C]-Org 25969 (8.6 mg/kg; 5.6 MBq/kg) and from control rats was assessed qualitatively by microautoradiography. Photomicrographs of the resulting sections are presented in Figures 1 - 9.<br><br>In all three animals that received a single intravenous dose of [$^{14}$C]-Org 25969 (8.6 mg/kg; 5.6 MBq/kg), there was a band of deposition of silver granules at or close to the periosteal and endosteal aspect of the cortex, and endosteal aspect of trabeculae, in both the scapula and the femur. No silver deposition was seen in growth plates. No differences in intensity of silver granule deposition between animals were detectable.<br><br>**Animal 124 (control; Figures 1 and 2)**<br><br>Scapula No silver granules present. (Growth plate identifiable.)<br><br>Femur No silver granules present. (Growth plate identifiable.)<br><br>**Animal 1001 (killed at 1 day after dosing; Figures 3 and 4)**<br><br>Scapula Patchy band of deposition of silver granules at periosteal and endosteal aspect of cortex, and endosteal aspect of trabeculae. (Growth plate not identifiable.)<br><br>Femur Band of deposition of silver granules at periosteal and endosteal aspect of cortex, and endosteal aspect of trabeculae. (Growth plate not identifiable.)<br><br>**Animal 3001 (killed at 21 days after dosing; Figures 5, 6 and 7)**<br><br>Scapula Band of deposition of silver granules at or close to periosteal and endosteal aspect of cortex, and endosteal aspect of trabeculae. (Growth plate identifiable.)<br><br>Femur Band of deposition of silver granules at or close to periosteal and endosteal aspect of cortex, and endosteal aspect of trabeculae. No discernible differences in extent and location of binding between cortical and trabecular bone. No silver deposition was seen in the growth plate, or in the most recently formed trabeculae immediately adjacent to it. (Growth plate identifiable.)<br><br>**Animal 5001 (killed at 84 days after dosing; Figures 8 and 9)**<br><br>Scapula Band of deposition of silver granules at or close to periosteal and endosteal aspect of cortex, and endosteal aspect of trabeculae. (Growth plate identifiable.)<br><br>Femur Band of deposition of silver granules close to periosteal and endosteal aspect of cortex, and endosteal aspect of trabeculae. (Growth plate not identifiable.) | The distribution of radioactivity in sections of femur and scapula of rats killed at either 1 day, 21 days or 84 days following a single intravenous dose of [$^{14}$C]-Org 25969 (8.6 mg/kg; 5.6 MBq/kg) and from control rats was assessed qualitatively by microautoradiography. Photomicrographs of the resulting sections are presented in Figures 1 - 9.<br><br>In all three animals that received a single intravenous dose of [$^{14}$C]-Org 25969 (8.6 mg/kg; 5.6 MBq/kg), there was a band of deposition of silver granules at or close to the periosteal and endosteal aspect of the cortex, and endosteal aspect of trabeculae, in both the scapula and the femur. No silver deposition was seen in growth plates. No differences in intensity of silver granule deposition between animals were detectable.<br><br>**Animal 124: control; Figures 1 (scapula) and 2 (femur)**<br><br>Scapula Very slight, random background staining in the form of black debris present over cortical bone, bone marrow, tissue-cavities and outside the section. Given the even distribution, partial localization above tissue-cavities and outside the section and the low intensity of staining this is considered to be a procedural artifact.<br><br>Femur No silver granules present. The growth plate was identifiable in this slide, but is not presented in areas selected for photography.<br><br>**Animal 1001: killed at 1 day after dosing; Figures 3 (scapula) and 4 (femur)**<br><br>Scapula Patchy band of deposition of silver granules at periosteal (presented in selected micrographs) and endosteal aspect of cortex, and endosteal aspect of trabeculae. The endosteal aspect of the cortex and trabeculae were present in the slide, but are not presented in the micrographs.<br><br>Femur Band of deposition of silver granules at periosteal and endosteal aspects of cortex (neither are presented in the micrographs selected), and endosteal aspect of trabeculae (presented in the selected micrographs). The growth plate was not identifiable in this slide.<br><br>**Animal 3001: killed at 21 days after dosing; Figures 5 (scapula), 6 (femur) and 7 (femur)**<br><br>Scapula Band of deposition of silver granules at or close to periosteal and endosteal aspects of cortex, and endosteal aspect of trabeculae (upper left corner in selected low magnification micrograph). In the high magnification micrographs only the deposition at the endosteal aspect is presented.<br>Femur Band of deposition of silver granules at or close to periosteal and endosteal aspects of cortex, and endosteal aspect of trabeculae (Figure 7, trabeculae not presented on areas selected for the micrographs). No discernible differences in extent and location of binding between cortical and trabecular bone (comparison of Figures 6 and 7). No silver deposition was seen in the growth plate, or in the most recently formed trabeculae immediately adjacent to it (Figure 6). Bone formation and mineralization has occurred on periosteum and endosteum since the dosing of the radiolabel. The growth plate was identifiable and is presented in Figure 6, low magnification.<br><br>**Animal 5001: killed at 84 days after dosing; Figures 8 (scapula) and 9 (femur)**<br><br>Scapula Band of deposition of silver granules at or close to periosteal and endosteal aspects of cortex, and endosteal aspect of trabeculae. Bone formation and mineralization has occurred on periosteum and endosteum since the dosing of the radiolabel.<br><br>Femur Band of deposition of silver granules close to periosteal and endosteal aspects of cortex, and endosteal aspect of trabeculae. Bone formation and mineralization has occurred on periosteum and endosteum since the dosing of the radiolabel. The growth plate was not identifiable in this slide. |

**Study title:** *In vitro* binding of [14C]-Org 25969 (Org 25969-2) to hydroxyapatite

| | |
|---|---|
| Study Number: | 090085 |
| Study report Number: | INT00127799 |
| Conducting laboratory and location: | Schering-Plough Research Institute, Schaijk, the Netherlands |
| GLP compliance: | No |
| Drug, lot #, and % purity: | CoA of $^{14}$C-Org 25969 and Org 9426 (rocuronium bromide) were included |

The objectives of the study were 1) to determine the extent and characteristic of binding of [$^{14}$C]-Org 25969 to hydroxyapatite *in vitro*; 2) to determine whether the in vitro binding of [$^{14}$C]-Org 25969 to hydroxyapatite is affected by Org 9426 (rocuronium bromide); 3) to determine the competitive effect of sodium citrate, tetracycline, alendronate and pamidronate on the binding of Org 25969 to hydroxyapatite *in vitro.*

Hydroxyapatite suspension (1.5 mg/ml in TBS) was incubated in duplicate with [$^{14}$C]-Org 25969 in the presence and absence of Org 9426, tetracycline, sodium citrate, alendronate or pamidronate for 24 hours at 37°C. The incubation samples were centrifuged, supernatant samples were separated and pellets were washed with TBS. The amount of radioactivity was determined in the supernatant, wash and pellet samples. The extent of binding of [$^{14}$C]-Org 25969 to 1.5 mg/ml hydroxyapatite is shown in the table below as extracted from the study report.

% Org 25969 binding to hydroxyapatite

| [$^{14}$C]-Org 25969[a] (nmol·mg$^{-1}$) | Binding to hydroxyapatite %[b] | Binding to hydroxyapatite (nmol·mg$^{-1}$ hydroxyapatite) |
|---|---|---|
| 0.017 | 89 | 0.015 |
| 0.17 | 91 | 0.151 |
| 1.7 | 83 | 1.38 |
| 17 | 76 | 12.6 |
| 167 | 11 | 18.0 |

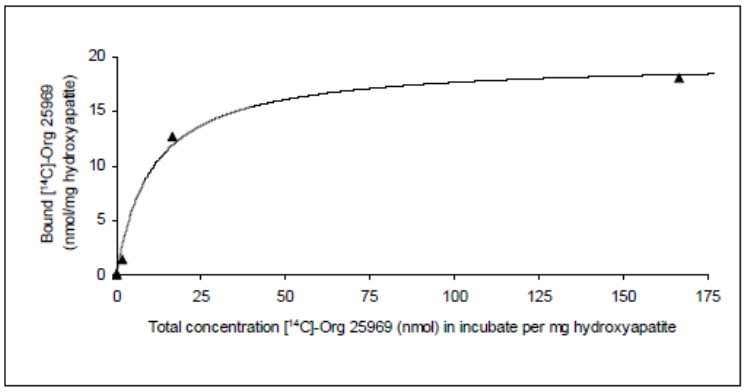
[a] Total concentration [$^{14}$C]-Org 25969 (nmol) in incubate per mg hydroxyapatite
[b] % of the total concentration [$^{14}$C]-Org 25969 bound to hydroxyapatite

Fitting these data with a simple $E_{max}$ model ($E = (E_{max} \times \text{Concentration})/(\text{Concentration}+EC_{50})$, model 101 in WinNonlin Professional version 5.0.1) showed that the Org 25969 binding to hydroxyapatite increased non-linearly to the concentration Org 25969 as shown in the figure below (extracted from the study report). The estimated $E_{max}$ was 19.5 nmol [$^{14}$C]-Org 25969/mg hydroxyapatite, and the estimated $EC_{50}$ of this binding was 10.6 nmol [$^{14}$C]-Org 25969/mg hydroxyapatite.



Reference ID: 3362422

Figure 9-1 Fitted curve of the extent of binding of [$^{14}$C]-Org 25969 to hydroxyapatite




At 1.7 nmol [$^{14}$C]-Org 25969 per mg hydroxyapatite, Org 9426 (rocuronium HCl) was added to test the effect of Org 9426 on Org 25969 binding with hydroxyapatite. The results showed that hydroxyapatite binding of [$^{14}$C]-Org 25969 was not affected by Org 9426 in a range of 0.17 to 17 nmol per mg hydroxyapatite (data not shown). In addition, the extent of hydroxyapatite binding of [$^{14}$C]-Org 25969 (1.7 nmol/mg) were not affected by sodium citrate and tetracycline (1.7 – 167 nmol/mg). However, presence of bisphosphonates (alendronate and pamidronate) significantly decreased [$^{14}$C]-Org 25969 (1.7 nmol/mg) binding to hydroxyapatite in a concentration-dependent manner at 17 nmol/mg as shown below (extracted from the study report).

% Org 25969 binding to hydroxyapatite in the presence of bisphosphonates

| Competitor[a] | Alendronate | | Pamidronate | |
|---|---|---|---|---|
| | Org 25969 binding to hydroxyapatite | | | |
| (nmol·mg$^{-1}$) | % | (nmol·mg$^{-1}$) | % | (nmol·mg$^{-1}$) |
| 0[b] | 83 | 1.36 | 83 | 1.36 |
| 1.7 | 87 | 1.44 | 88 | 1.46 |
| 7 | 86 | 1.43 | 87 | 1.44 |
| 17 | 86 | 1.44 | 88 | 1.46 |
| 67 | 58 | 0.96 | 63 | 1.06 |
| 167 | 3 | 0.05 | 1 | 0.01 |

[a] concentrations alendronate and pamidronate (nmol) in incubate per mg hydroxyapatite

[b] Data for [$^{14}$C]-Org 25969 binding in the absence of bisphosphonate were obtained in a separate experiment

The bisphosphonate binding moiety is a P-C-P group (2 phosophonate groups sharing a common carbon atom) and the tetracycline binding moiety is the tricarbonylmethane group in the A-ring in calcium-hydroxyapatite. These results suggested that Org 25969 bind to the same binding site as bisphosphonate compounds. This study did not provide information whether Org 25969 bind to other components in the bone.

**Study title:** Study to quantitatively assess binding of [$^{14}$C-Org 25969] (Org 25969-2) and Org 25969 (sugammadex) in bone and teeth upon intermittent dosing in Wistar rats

| | |
|---|---|
| Study Number: | 090310 |
| Study report Number: | INT00141634 |
| Conducting laboratory and location: | Merck Research Laboratories, West Point, PA |
| GLP compliance: | No |

## Methods and materials

Male and female rats of 1-week (juvenile), 6-week (young adults), and 12-week old (adults) were SC and IV injected with Org 25969 including [$^{14}$C]-Org 25969 according to the following scheme (extracted from the study report).

Table of study design

| Group | Rat strain, age at start dosing | Gender/ No. of animals | Dose (mg·kg$^{-1}$; MBq·kg$^{-1}$) | Dosing regimen | Sacrifice time[d] |
|---|---|---|---|---|---|
| 1 | Wistar rats (Hsd:CpB, WU), 7 days old[a] | 3M +3F | 8.9 ; 0.75 | Once weekly for 7 weeks: day 7, 14, 21, 28, 35, 42 and 49 of age (7 dosages in total) | Day 56 (7 days after last dose) |
| 2 | | 3M +3F | 30 ; 3 | | |
| 3 | | 3M +3F | 120 ; 12 | | |
| 4 | Wistar rat (RccHan: WIST), ± 6 weeks old[b] | 3M +3F | 30 ; 3 | Once weekly in week 6, 7, 8, 9, 10 and 11 of age (6 dosages in total) | 12 weeks (7 days after last dose, coinciding with day of fracture) |
| 5 | | 3M +3F | 120 ; 12 | | |
| 6 | | 3M +3F | 500 ; 50 | | |
| 7 | Wistar rat (RccHan: WIST), ± 12 weeks old[c] | 3M +3F | 30 ; 3 | Once weekly in week 12, 13, 14, 15, 16 and 17 of age (6 dosages in total) | 18 weeks (7 days after last dose, coinciding with 6 weeks after fracture |
| 8 | | 3M +3F | 120 ; 12 | | |
| 9 | | 3M +3F | 500 ; 50 | | |
| 10[e] | Wistar rat (RccHan: WIST), ± 6 weeks old | 1M +1F | 8; 6 | Single dose | 24 hours after dose |

M =male and F = female
[a] corresponds to intermittent dosing in juvenile rat study (Study 080229).
[b] corresponds to Part 1 (pre-fracture exposure) of fracture repair study (Study 090319).
[c] corresponds to Part 2 (post fracture exposure) of fracture repair study(Study 090319).
[d] From animals of group 1-3: femurs, tibias, vertebrae L4-L6, incisors and molars were collected. For animals of group 4-6 femurs, tibias, incisors and molars and for the animals of group 7-10 femurs and tibias were collected.
[e] Group 10 generated femurs and tibias for the microautoradiography study[1]. The microautoradiography study is performed under study number 090071 and is reported separately.

This study was to obtain quantitative information on absorption of Org 25969 in rat bone and teeth under the conditions used in the newly submitted intermittent dose toxicity study in the juvenile rat (study number 080229) and the fracture repair study in rats (study number 090319). In addition, bone samples generated from group 10 were used for microautoradiography study (study number 090071). Animals of ≤ 14 days old were weekly SC injected to avoid high level drop-out due to technical complications induced by IV at this age range; IV injection was used for animals ≥ 21 days. Kinetic data in juvenile rats of previous studies showed that Org 25969 is well absorbed when dosed



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subcutaneously and the bioavailability was close to 100%. It should be noted that animals were sacrificed 7 days after the last dose of Org 25969 in this study. Org 25969 was quickly distributed to the bone and the concentration reached peak within 24 hours and started to decline thereafter based on the results of previous distribution studies. Therefore, data of bone concentration obtained in this study likely to be lower than the peak concentration of Org 25969 after administration, which provides a more conservative evaluation when these values are used for safety margin calculation.

All collected bone (femur, tibia, vertebrae L4 – L6 for juvenile only) and teeth (incisor and molar) samples were trimmed and weighed. The excised bones of group 1-9 and molars of group 1-6 were dissolved and subsequently assessed for radioactivity by liquid scintillation counting (LSC); % of cumulative dose (i.e. total doses administered)/bone or teeth, % of cumulative dose/gram bone or teeth were calculated (% of cumulative dose/bone or teeth/weight of bone or teeth). The absolute Org 25969 amount (mg equivalent/gram bone or teeth) was calculated based on the equation (mg equivalent/gram bone or teeth = % of radioactive cumulative dose/gram bone or teeth × cumulative nonradioactive dose (mg)).

The % of cumulative radioactive dose/whole bone tissue or teeth vs. % of cumulative radioactive dose/g bone or teeth tissue are shown in the table below. Data of vertebrae in the juvenile dosing groups were similar to that of the tibia (not shown). A less than dose-proportional deposition was observed in juvenile, young adult, and adult groups. This suggests the Org 25969 deposition in the bone is saturable. In addition, [$^{14}$C]-Org 25969 deposition was decreased with the increase of animal age, which is consistent with the previous observation. This appeared to be because of the decrease in bone growth rate with the age increase in rats, which further suggests that Org 25969 incorporates into the bone during bone formation following administration. However, these data showed that % of cumulative dose in juvenile animals after 7-weekly doses was only slightly higher than that in young adult and adult animals after 6-weekly dosing (e.g. 0.38% in juvenile vs. 0.32% in young adult vs. 0.16% in adult male femur bone at 30 mg/kg including 3 MBg/kg [$^{14}$C]-Org 25969). In comparison, in a previously conducted bone retention study in rats (INT00033724), bone deposition in juvenile was significantly higher than that in young adult (7-8 weeks of age) after single-dose of 30 mg Org 25969 with 3 MBg/kg (young adult) or 15 MBg/kg (juvenile) [$^{14}$C]-Org 25969 (e.g. 13.6% in juvenile vs. 2.4% per pooled bone in young adult). This difference may be because 1) in the study INT00033724, the % of dose was derived from pooled bone tissue including (femur, scapular, skull bones, tibia, humerus, ossicles, and vertebrae (L4, 5, 6) while the % of dose was calculated in individual bones such as femur and tibia; 2) bone growth diluted the deposited Org 25969 in the bone during the dosing period.

As shown below, % cumulated dose/g bone or teeth was slightly higher in females as compared to males while there was no evident difference when the absorption was expressed as % cumulated dose/whole bone or teeth. This was likely because males have larger bone mass or density than females



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Mean % of cumulative dose/whole bone or teeth vs. mean % of cumulative dose/g bone

| Dosing group | Sex | Juvenile (7 days of age) | | | | Young adult (6-week) | | | | Adult(12-week) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Femur | Tibia | Incisor | Molar | Femur | Tibia | Incisor | Molar | Femur | Tibia |
| 8.9 mg/kg | M | 0.46/ 0.76 | 0.38/ 0.77 | 0.10/ 0.43 | 0.16/ 0.88 | | | | | | |
| | F | 0.48/ 0.97 | 0.40/ 1.04 | 0.11/ 0.48 | 0.17/ 1.07 | | | | | | |
| 30 mg/kg | M | 0.38/ 0.60 | 0.32/ 0.63 | 0.09/ 0.31 | 0.13/ 0.60 | 0.32/ 0.40 | 0.23/ 0.37 | 0.07/ 0.21 | 0.05/ 0.21 | 0.16/ 0.15 | 0.13/ 0.17 |
| | F | 0.40/ 0.83 | 0.35/ 0.90 | 0.09/ 0.37 | 0.17/ 0.81 | 0.35/ 0.57 | 0.25/ 0.53 | 0.08/ 0.25 | 0.08/ 0.28 | 0.13/ 0.19 | 0.11/ 0.22 |
| 120 mg/kg | M | 0.29/ 0.41 | 0.22/ 0.44 | 0.05/ 0.21 | 0.10/ 0.44 | 0.22/ 0.27 | 0.16/ 0.26 | 0.03/ 0.08 | 0.04/ 0.13 | 0.08/ 0.08 | 0.06/ 0.09 |
| | F | 0.29/ 0.53 | 0.23/ 0.54 | 0.06/ 0.23 | 0.10/ 0.47 | 0.19/ 0.35 | 0.14/ 0.33 | 0.04/ 0.15 | 0.04/ 0.16 | 0.07/ 0.11 | 0.07/ 0.14 |
| 500 mg/kg) | M | | | | | 0.10/ 0.12 | 0.07/ 0.11 | 0.02/ 0.05 | 0.01/ 0.16 | 0.03/ 0.03 | 0.03/ 0.04 |
| | F | | | | | 0.08/ 0.15 | 0.06/ 0.14 | 0.02/ 0.07 | 0.01/ 0.06 | 0.03/ 0.05 | 0.03/ 0.06 |

It should be noted that the absorption in the molar was higher than that in the incisor of juvenile animal (40-100%). This was not seen in young adult animals. In rats, incisors are open-rooted and grow throughout life. After eruption, the rate of growth is generally consistent (about 40-50 days for new tooth generated at the base to reach the tip). This may explain why the absorption amount of Org 25969 in incisors was generally similar between juvenile and young adult groups. Rats have three sets of molars. By 6 weeks of age, all molars are erupted in rats, and by 125 days molar growth slows greatly. After this the molars continue to grow and wear away, but at such a slow rate as to be almost indiscernible. Higher Org 25969 tooth deposition during juvenile period was likely due to relatively more active tooth morphogenesis and amelogenesis during this period. Rat incisors erupt out of the gum 8-10 days after birth. Rodent incisors consist of two halves. The labial half is covered with enamel and resembles a crown, while the lingual half is covered with dentin without enamal coverage, which may be associated with the relatively low level of Org 25969 in incisors as compared to molar during the juvenile administration.

Based on absorbed activity levels, the absolute Org 245969 amounts absorbed were calculated and shown below.

Mean mg equivalent Org 25969/gram bone or teeth

| Dosing group | Sex | Juvenile (7 days of age) | | | | Young adult (6-week) | | | | Adult(12-week) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Femur | Tibia | Incisor | Molar | Femur | Tibia | Incisor | Molar | Femur | Tibia |
| 8.9 mg/kg | M | 0.052 | 0.053 | 0.030 | 0.061 | | | | | | |
| | F | 0.055 | 0.059 | 0.027 | 0.060 | | | | | | |
| 30 mg/kg | M | 0.17 | 0.17 | 0.09 | 0.17 | 0.19 | 0.18 | 0.10 | 0.10 | 0.10 | 0.13 |
| | F | 0.18 | 0.19 | 0.08 | 0.17 | 0.18 | 0.17 | 0.078 | 0.088 | 0.084 | 0.10 |
| 120 mg/kg | M | 0.40 | 0.43 | 0.20 | 0.43 | 0.51 | 0.48 | 0.15 | 0.24 | 0.24 | 0.26 |
| | F | 0.42 | 0.44 | 0.18 | 0.38 | 0.44 | 0.41 | 0.18 | 0.20 | 0.20 | 0.24 |



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| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Group 3 (500 mg/kg) | M | | | | | 0.87 | 0.84 | 0.38 | 0.38 | 0.43 | 0.48 |
| | F | | | | | 0.74 | 0.73 | 0.34 | 0.32 | 0.31 | 0.38 |

**Study title:** Microautoradiography in bone after a single intravenous dose of [$^{14}$C]-Org 25969 to Wistar rats

Study Number: 090071
Study report Number: INT00142585
Conducting laboratory and location: Merck Research Laboratories, West Point, PA
GLP compliance: No

The animals of group 10 (one male and one female; 6 wk old) in study 090310 (study report INT00141634) were sacrificed 24 hours after single dosing of 6 MBq/kg [14C]-Org 25969 and 8 mg/kg Org 25969. Femurs and tibias were collected; the right femur and tibia samples were used for dissolving and radioactivity counting and the left femur and tibia samples for microautoradiography. Control femur and tibia samples were obtained from rats dosed with Org 25969 (no radiolabel) in study 060338 (**Study Title:** Second investigational single dose toxicity study to assess the effects of Org 25969 on bone quality, structure and turnover in male and female Wistar rats, INT00033963) which was submitted in the original submission. In addition, femur and tibia samples of animals sacrificed 21 and 84 days after single-dose 5.6 MBq/kg [14C]-Org 25969 and 8.6 mg/kg Org 25969 from study 050322 (**Study title:** Bone retention kinetics in rat with Org 25969 after a single and multiple intravenous injection, conducted in 2006, INT00010275) were also collected from animal carcasses of rats for microautoradiography.

Radioactivity assay revealed that % of the dose retrieved per gram bone was 1.4% in both femur and tibia 24 hours after single-dose administration; % of the dose retrieved per bone was 0.75% in femur and 0.59% in tibia. The total mg equivalent of Org 25969 retrieved per gram bone was approximately 0.02 mg/g in both femur and tibia after single-dose administration.

Following a single intravenous dose of [$^{14}$C]-Org 25969, binding to rat femoral bone predominantly occurs on the outer trabecular, endosteal, periosteal and inner articular surfaces, and at the interface of metaphysis and epiphyseal growth plate where active mineralization occurs (i.e. calcified cartilage) after 24 hour administration. No incorporation occurs into non-calcified cartilaginous tissues such as the epiphyseal growth plate and the articular surface, and bone marrow as shown in the photograph included in the study report.



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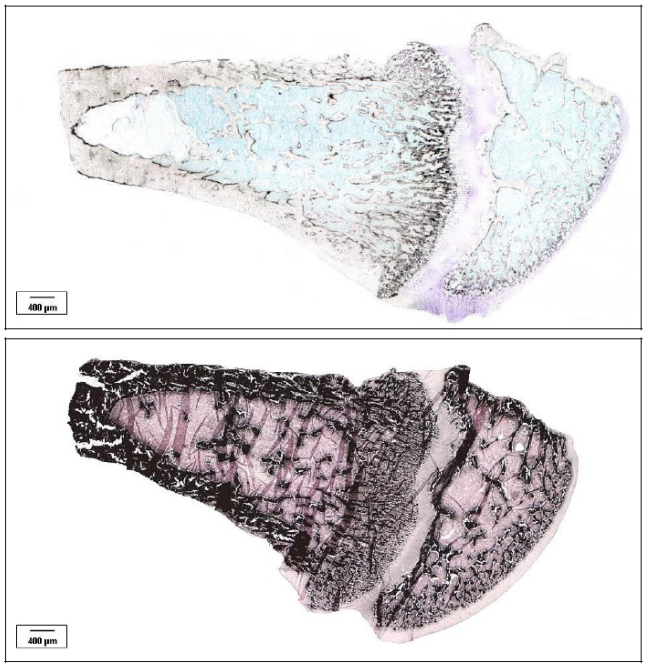
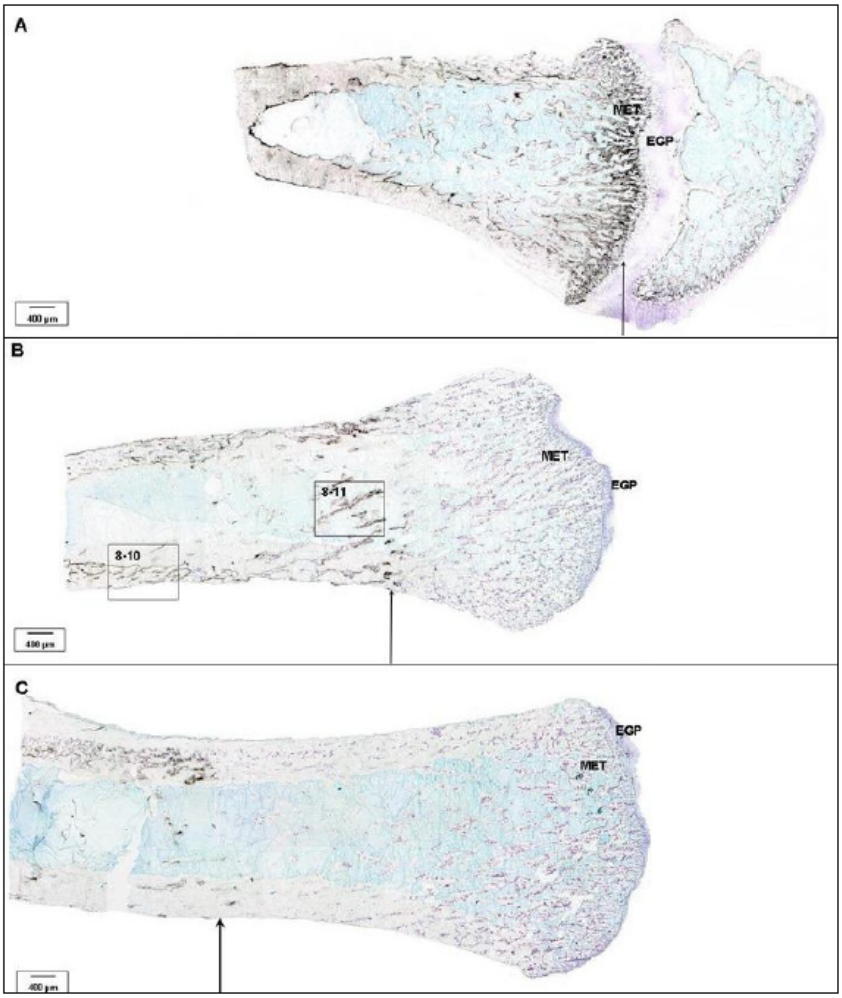
Figure 8–8 Comparative overviews of the rat femur by microautoradiography (upper panel) and the von Kossa staining for mineralization (lower panel)




Femur growth and modeling resulted in [$^{14}$C]-Org 25969 incorporated at the epiphyseal side of the metaphysis directly after dosing, and gradually and passively being carried along in metaphysis and diaphysis due to longitudinal growth of the femur. In the cortical bone the [$^{14}$C]-Org 25969 gradually became located more central as a result of new bone formation from endost and periost. There were no morphologic changes of the tissues involved.

**Figure 8–9 Microautoradiography overviews of [$^{14}$C]-Org 25969 distribution in the femur of rats, killed 24 h (A: 1007), 21 days (B: 3003) and 84 days (C: 5003) after single dose of [$^{14}$C]-Org 25969 (A: 8 mg·kg$^{-1}$; 6 MBq·kg$^{-1}$, B and C: 8.6 mg·kg$^{-1}$; 5.6 MBq·kg$^{-1}$)[1]**




EGP: Epiphyseal Growth Plate, MET: Metaphysis.
The arrow indicates the site of incorporation of [$^{14}$C]-Org 25969 at t=0.
Counterstaining: Toluidin blue

**Study title:** Bone retention kinetics in juvenile and young adult Wistar rats after a single subcutaneous injection of [$^{14}$C]-Org 25969 (Org 25969-2) and Org 25969 (sugammadex)

| | |
|---|---|
| Study Number: | 100045 |
| Study report Number: | INT00150298 |
| Conducting laboratory and location: | Merck Research Laboratories, West Point, PA |
| GLP compliance: | No |
| Drug, lot #, and % purity: | none |

The objective of this study was to investigate the plasma and tissue kinetics in bone and teeth of Org 25969 (sugammadex) in the juvenile and young adult Wistar rats following a single administration of [14C]-Org 25969 (Org 25969-2) and Org 25969. Male and female juvenile (1 week old at start dosing) and young adult (6 weeks old at starting dosing) were administered subcutaneously 4 MBq/kg [$^{14}$C]-Org 25969 and 30 mg/kg Org 25969. Animals were sacrificed after dosing at the time-points as shown below (extracted from the Applicant's study report). Plasma, tissue of the dosing site, femur, ulna, tibia, humerus, vertebrae (L4, 5, and 6), incisors, and molars were collected for radioactivity analysis by liquid scintillation counting (LSC). Of note, there were only one male and female at each time-point.

Table of study design

| Group | Life stage at dosing | Rats[1] no. of animals (weight range[2]) | Sacrifice time[3] |
|---|---|---|---|
| 1 | Juvenile (7 days) | 21M (14-18 g) + 21F (10-18 g) | 3, 24 and 72 hours and 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 18, 21, 25, 30, 35, 41, 47[4] weeks) |
| 2 | Young adult | 9M (207-223 g) + 9F (157-170 g) | 3, 24 and 72 hours and 1, 3, 5, 10, 21 and 35 weeks |

[1] M =male and F = female;
[2] weight range at start dosing
[3] From all animals in the study, plasma and the following tissues were collected (one male and one female per time point): The dosing site, femur, ulna, tibia, humerus, vertebrae (L4, 5, 6)), incisors and molars.
[4] : 2 males and 1 female were sacrificed after 47 weeks as the last sampling time and no animals at 53 weeks. One juvenile female (1042) was already missing at 3 days after dosing.

Low retention at the dosing site after subcutaneous dosing (<0.1% of dose at 24 h after dosing was noted with no difference between the two age groups.

Plasma radioactivity concentration (ng equivalent/gram plasma) versus time obtained in male and female juvenile and young adult Wistar rats after a single dose of [$^{14}$C]-Org 25969 and Org 25969. The plasma radioactivity levels after dosing are higher in juveniles and the decline was slower as compared to young adults, which was likely due to incomplete renal function at the age of 7 days in rats. Five weeks after dosing, plasma radioactivity levels are all below 0.2 ng eq·gram-1. The $AUC_{(0\text{-last})}$ were (assuming the 1 gram plasma equals 1 ml) 0.41 µg eq days/ml and 0.36 µg eq days/ml for juvenile males and females respectively and 0.11 and 0.12 µg eq days/ml for young adult males and females respectively. The AUC level in this study was significantly lower as compared to those in other studies such as the previously conducted 4-week repeat dose after 30 mg/kg administration ($AUC_{0\text{-inf}}$ = ~9.1 µg·hr/ml vs. ~43 µg·hr/ml after single dose administration of 30 mg/kg to rats of 7 days old). This was because the first blood sample was collected at 3-hour after dosing while the first blood sample was collected at 0.5 hour after dosing in the previous 4-week rat study. As previous studies revealed, $t_{max}$ was generally reached within 0.5 hours after dosing and the $t_{1/2}$ was 0.4-0.9 hours in rats.

A biphasic wash out of the radioactivity was noted in pooled bone as shown below (% of the dose/gm pooled bone). It should be noted that the value of each time-point was derived from only one animal and therefore individual variation may exist. The data showed slow elimination of Org 25969 in the bones. Significantly high amount of Org 25969 was absorbed in juvenile animals as compared to the young adults. The data from % of dose/gram may be misleading because it suggested a great decline of radioactivity, but if take the data of %/total pooled bones, these data may indicate that the absorbed radioactivity stayed within the bone for at least a week without elimination. These data also suggested that between 5-47 weeks, there was very little amount of elimination based on the data of %/total pooled bones, indicating slow elimination of Org 25969 from the bone. It should be noted that there was only one animal/sex/time point. The values shown in the following table represented data from one animal. Therefore, individual difference might exist.

% of the [$^{14}$C]-Org 25969 dose per gram pooled bone and per whole pooled bone

| time of sacrifice | % of the dose/gram pooled bones | | | |
|---|---|---|---|---|
| | juvenile males | juvenile females | YA males | YA females |
| 3 h | 31.24 | 28.87 | 1.08 | 1.37 |
| 24 h | 48.47 | 29.11 | 1.10 | 1.18 |
| 72 h | 13.30 | 18.21 | 0.56 | 0.90 |
| 1 wk | 9.28 | 8.80 | 0.83 | 1.31 |
| 3 wk | 1.74 | 2.30 | 0.50 | 0.77 |
| 5 wk | 0.81 | 1.20 | 0.30 | 0.55 |
| 10 wk | 0.49 | 0.58 | 0.29 | 0.62 |
| 21 wk | 0.37 | 0.70 | 0.22 | 0.38 |
| 35 wk | 0.32 | 0.55 | 0.18 | 0.39 |
| 47 wk | 0.24* | 0.42 | | |

| time of sacrifice | % of the dose/ pooled bones | | | |
|---|---|---|---|---|
| | juvenile males | juvenile females | YA males | YA females |
| 3h | 6.94 | 6.24 | 3.27 | 3.58 |
| 24h | 6.93 | 7.63 | 3.62 | 2.96 |
| 72h | 6.30 | 6.41 | 2.31 | 2.57 |
| 1 wk | 5.47 | 6.07 | 2.43 | 2.91 |
| 3 wk | 3.36 | 3.95 | 2.18 | 2.65 |
| 5 wk | 2.48 | 3.01 | 1.77 | 1.95 |
| 10 wk | 2.75 | 2.38 | 1.68 | 2.11 |
| 21 wk | 2.58 | 3.01 | 1.39 | 1.50 |
| 35 wk | 2.13 | 2.59 | 1.34 | 1.64 |
| 47 wk | 1.84* | 1.86 | | |

In teeth, a mono-phase was observed. Significantly higher amount absorbed in the molar as compared to the incisor. From wk 1 to week 35, there was small amount of Org 25969 eliminated. From week 10, Org 25969 was not detectable in the incisors.

% of the [$^{14}$C]-Org 25969 dose per gram pooled teeth and per whole pooled teeth

| time of sacrifice | % of the dose/gram molar sample | | | | % of the dose/gram incisor sample | | | |
|---|---|---|---|---|---|---|---|---|
| | juvenile males | juvenile females | YA males | YA females | juvenile males | juvenile females | YA males | YA females |
| 3 h | 19.78 | 26.05 | 0.46 | 0.43 | 40.49 | 44.16 | 0.39 | 0.61 |
| 24 h | 27.67 | 19.68 | 0.27 | 0.43 | 56.73 | 37.85 | 0.44 | 0.57 |
| 72 h | 12.64 | 15.34 | 0.25 | 0.33 | 19.93 | 21.84 | 0.24 | 0.49 |
| 1 wk | 11.00 | 8.47 | 0.37 | 0.49 | 16.20 | 17.92 | 0.41 | 0.66 |
| 3 wk | 3.81 | 3.77 | 0.34 | 0.26 | 1.70 | 1.74 | 0.34 | 0.47 |
| 5 wk | 3.42 | 3.20 | 0.21 | 0.22 | 0.17 | 0.20 | 0.16 | 0.18 |
| 10 wk | 2.52 | 1.76 | 0.16 | 0.18 | 0.01 | 0.01 | 0.00 | 0.01 |
| 21 wk | 2.75 | 3.24 | 0.21 | 0.18 | 0.00 | 0.01 | 0.00 | 0.00 |
| 35 wk | 2.88 | 2.50 | 0.18 | 0.20 | 0.00 | 0.00 | 0.00 | 0.00 |
| 47 wk | 1.39* | 1.55 | | | 0.00* | 0.00 | | |

| time of sacrifice | % of the dose/ molar sample | | | | % of the dose/ incisor sample | | | |
|---|---|---|---|---|---|---|---|---|
| | juvenile males | juvenile females | YA males | YA females | juvenile males | juvenile females | YA males | YA females |
| 3 h | 0.53 | 0.58 | 0.07 | 0.07 | 0.60 | 0.41 | 0.08 | 0.14 |
| 24 h | 0.50 | 0.38 | 0.07 | 0.07 | 0.51 | 0.31 | 0.09 | 0.13 |
| 72 h | 0.52 | 0.54 | 0.05 | 0.06 | 0.51 | 0.52 | 0.05 | 0.10 |
| 1 wk | 0.52 | 0.53 | 0.06 | 0.08 | 0.51 | 0.59 | 0.09 | 0.14 |
| 3 wk | 0.53 | 0.55 | 0.08 | 0.04 | 0.22 | 0.20 | 0.10 | 0.12 |
| 5 wk | 0.57 | 0.59 | 0.05 | 0.04 | 0.04 | 0.03 | 0.05 | 0.06 |
| 10 wk | 0.43 | 0.35 | 0.02 | 0.03 | 0.01 | 0.00 | 0.00 | 0.00 |
| 21 wk | 0.45 | 0.47 | 0.03 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 |
| 35 wk | 0.40 | 0.36 | 0.02 | 0.03 | 0.00 | 0.00 | 0.00 | 0.00 |
| 47 wk | 0.25* | 0.21 | | | 0.00* | 0.00 | | |

# 5 Toxicology Studies

**Study title:** An 8-week intermittent dosing toxicity study with Org 25969 to assess effects on bone quality, structure, turnover, strength and growth and development in juvenile Wistar rats

| | |
|---|---|
| Study no.: | 080229 |
| Study report location: | eCTD |
| Conducting laboratory and location: | Department of Toxicology and Drug Disposition (TDD), MSD, Schaijk, The Netherlands |
| Date of study initiation: | 2009 |
| GLP compliance: | Yes |
| QA statement: | Yes |
| Drug, lot #, and % purity: | Org 25969, L00027962, 95.4% |

**Key Study Findings**

- Juvenile rats from age of 7 days were treated with Org 25969 weekly for 8 doses at 0, 7.5, 30, and 120 mg/kg with a positive control group included (250 µg/kg pamidronate). Recovery groups were included for 8-week treatment-free observation.



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- Animals were sacrificed one day after the last dosing or at the end of 8-week recovery.
- The results of the positive control group indicated that the rat is a sensitive model for bone change.
- Org 25969 treatment-related changes in body weight were not observed.
- Evaluations of bone effects of org 25969 included in vivo ulna length measurement, postmortem femur and ulna length measurement, µCT examination, and bone strength and architecture assessment tests. In addition, histopathology examination of bone and teeth was conducted with measurement on epiphyseal disc from the epiphyseal growth plate of humerus and tibia.
- No significant changes in bone turnover markers C-telopeptide crosslinks (CTx) in the Org 25969 treated groups as compared to the control. Statistically significant decrease (15%) in osteocalcin concentration in the blood was observed at 120 mg/kg Org 25969 recovery group. This effect was not seen at the end Org 25969 dosing. The significance of this finding was unclear since there were no effects on bone density or strength. In comparison, in the positive control group (pamidronate), significant decrease (approximately 20%) was observed in osteocalcin and CTx concentration at the end of dosing. The change of osteocalcin was not reversible after 8-week recovery.
- Significant Org 25969 treatment effects were not seen in the bone and teeth. NOAEL is identified to be 120 mg/kg.

**Methods**

| | |
|---|---|
| Doses: | 0, 7.5, 30, 120 mg/kg Org 25969 |
| Frequency of dosing: | Weekly from PND 7 for 8 doses in total |
| Route of administration: | SC from PND 7-14; IV for PND 21-58 |
| Dose volume: | 5 ml/kg |
| Formulation/Vehicle: | 0.9% NaCl solution |
| Species/Strain: | Wistar rats (Hsd: CpB WU) |
| Number/Sex/Group: | 20 rats/sex/group (10/sex/group for recovery) |
| Age: | 7 days at dosing |
| Weight: | 14 g in average at dosing |
| Satellite groups: | TK groups not included |
| Unique study design: | An additional dosing group (10 rats/sex/group) was treated with 250 µg/kg Pamidronate as positive control on bone parameters*. Recovery group (10 sex/group) of each treatment was included (8-week recovery period) as shown below |
| Deviation from study protocol: | Deviations do not appear to affect study quality |

* In a study from Pataki et al (1997), dosing of Pamidronate to young adult rats at dose levels of 3.7, 370 µg·kg-1 for ten consecutive days resulted in significant dose-related effects on bone parameters (trabecular bone volume, trabecular width and trabecular number) and metaphyseal microscopic anatomy).

Treatment regimen

| Grp | Dose Org 25969 ($mg \cdot kg^{-1}$) | Dosing regimen | Gender/Animal No. Main part (sacrifice at day 57 of age) | Gender/Animal No. post dosing part (sacrifice at day 112 of age) |
|---|---|---|---|---|
| 1 | 0 | Once weekly for 7 weeks: postnatal day 7, 14, 21, 28, …, 49, 56 (8 weekly dosings) | 10 M + 10 F | 10 M + 10 F |
| 2 | 7.5 | | 10 M + 10 F | 10 M + 10 F |
| 3 | 30 | | 10 M + 10 F | 10 M + 10 F |
| 4 | 120 | | 10 M + 10 F | 10 M + 10 F |
| 5 | 250 $\mu g \cdot kg^{-1}$ Pamidronate | | 10 M + 10 F | 10 M + 10 F |

M = male, F= female

Study Background

The 8-week intermittent dosing toxicity in rats was conducted to clarify the observation in the 28-day juvenile toxicity study included in the originally NDA submission. In the 28-day juvenile toxicity study, animals (7 days of age at dosing start) were treated with Org 25969 daily for 28 days at 0, 30, 120, and 500 mg/kg Org 25969. There was no body weight change during the treatment period, but minor decrease in body weight gain of both males (5-8%) and females (<5%) at 120 and 500 mg/kg Org 25969 were observed as compared to the control during the 8-week treatment free period. At the end of 8-week recovery period, the body weight of animals treated with Org 25969 at 120 and 500 mg/kg were still slightly lower than that of control in both males (4%) and females (3%). In addition, minor decreased ulna length (≤5%), femur length (≤3.5%) and weight (≤6.5%) were observed with the most severe effects at the end of 8-week recovery period at 120 and 500 mg/kg Org 25969 dose levels. The protocol outline of this study was submitted to the division for advice prior to study initiation in 2009. The Agency recommended that a higher top dose should be selected in order to maximize the chance of detecting a bone effect. For both males and females a high dose in the range of 300-500 mg/kg was suggested. However, the Applicant did not take the Division's recommendation into consideration and decided to use the originally planned dosage with justification of dose selection in this study report (as copied below).



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**3.2 JUSTIFICATION OF DOSE SELECTION**

This study should unequivocally differentiate between a direct effect on bone growth and a secondary effect related to a general lower growth rate. It was expected that this could be achieved by choosing a dose and dose regimen that has no impact on rat pup growth, but nevertheless achieves significant systemic and local (bone and teeth) exposure multiples of Org 25969. Intermittent dosing was considered to be appropriate as it was expected to achieve that goal and mimics frequent, intermittent use in man as could occur in case of repeated anesthesia with induction of neuromuscular blockade.

In Wistar rat pups dosed at day 7 or 14 of age a single dose of 120 or 500 $mg \cdot kg^{-1}$ via the s.c. route did not result in decreased growth as assessed by body weight[4]. However, in rats at day 21 a single i.v. dose of 500 $mg \cdot kg^{-1}$ results in slightly lower body weight increase in the 24 h following the injection (weight gain day was 71% and 82% in males and females, respectively, Figure 1 top panels). At day 26 (6 days after dosing) the body weight of males dosed with 500 $mg \cdot kg^{-1}$ was comparable to control, however the weight gain in female pups had only recovered to 93% of control (Figure 3-1).[4] Although statistical significance was not always present, it was concluded that absence of a risk of general toxicity affecting body weight gain was present at the dose level of 120 $mg \cdot kg^{-1}$, noted that repeated weekly dosing was going to be applied.

Therefore, 120 $mg \cdot kg^{-1}$ was chosen as the top dose level that should result in significant exposure to Org 25969 in bone and teeth, without a significant effect on body weight gain.

I do not agree with the Sponsor on the dose selection rationale. To demonstrate the association between the observed decreased bone length and decreased body weight in the previously conducted 28-day study, all possibilities must be taken into consideration. With the selected dosage, the Sponsor attempted to exhibit that there will be no bone length decrease if there is no body weight decrease, thus indicate the previously observed bone length was due to the effect on body weight in the 28-day study. However, I do not think it is logically correct. Bone effect may be parallel and not associated with the effect on body weight when a dose level is reached (i.e. 500 mg/kg). At a lower dose (i.e. the proposed dose in this study), it is possible both the body weight and bone effect are not evident. However, if a NOAEL can be identified in this study which provide sufficient safety margin coverage for the dosage that will be used in future pediatric trials, the issue of the safety concern on the bone effect of sugammadex in pediatric subjects may be considered to be appropriately addressed.

## Observations and Results

Experimental parameters included mortality and morbidity, clinical observations including assessment of incisor and molar color and morphology, body weight, in vivo ulna length, clinical pathology (hematology, clotting, blood biochemistry and urinalysis), bone related biochemistry and urinalysis, histopathology of target organs and incisors and molars, femur and ulna weight and length, Micro Computer Tomography (µCT) on femur and vertebral body and bone strength assessment of femur and vertebrae.

## Clinical Signs and Mortality



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All animals survived to the end of dosing.
The incisors and molars were observed twice per week for assessment of color and morphology (eruption, signs of incisor overgrowth, enamel defects etc.) during the dosing period. During the recovery period incisors and molars were observed once weekly based on the study report. Mottled discoloration of incisors was observed starting from SD35 in all groups including control as shown below; the severity of this change was not graded by the Sponsor.

Incidence of discolored incisor

| | Control | 7.5 mg/kg | 30 mg/kg | 120 mg/kg | Positive control |
|---|---|---|---|---|---|
| Male (n=20) | 3 | 5 | 3 | 3 | 13 |
| Female (n=20) | 5 | 2 | 5 | 5 | 11 |
| Total (n=40) | 8 | 7 | 8 | 8 | 24 |
| distribution | Upper only | Upper and/or lower | Upper and/or lower | Upper and/or lower | Upper and/or lower |

The individual data of the teeth observation was not included in the report. It is not known whether the severity of discoloration and number of incisors with discoloration may be different between the control and treated groups. The Sponsor indicated that "The frequency of mottled appearance of the incisors was higher in the pamidronate treated group and is considered to be related to pamidronate treatment. Incidence and distribution of the incisor discoloration of Org 25969 treated animals is considered not to be indicative of a drug-treatment relation." The individual data may not be critical with the histopathology data available.

**Body Weights**

Body weight was examined twice weekly prior to and during dosing period, and once weekly during recovery period. The mean body weight of males and females at the day of dosing (7 days old), end of dosing (56 days old), and last week of recovery (104 days old) is shown below. Dose-dependent decrease of body weight as observed in the previously conducted 4-week juvenile rat study was not seen in this study after 8 weekly dosing of Org 25969. In the positive control group, there was a slight body weight decrease in males (3-5%) but not in females at the end of dosing and end of recovery period.

Mean body weight (g)

| Body weight (g) | Control | | 7.5 mg/kg | | 30 mg/kg | | 120 mg/kg | | Positive control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | M | F | M | F | M | F | M | F |
| 1st day dosing | 33 | 32 | 32 | 32 | 33 | 31 | 33 | 32 | 32 | 31 |
| End of dosing | 329 | 218 | 316 | 223 | 331 | 215 | 334 | 213 | 312 | 214 |
| End of recovery | 449 | 259 | 431 | 268 | 454 | 266 | 451 | 262 | 436 | 263 |



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**Feed Consumption**

Not examined

**In vivo ulna length:** Ulna length was determined every two weeks using sliding calipers. Ulna length was determined for all animals on day 6, day 20, 34, 48, 62, 76, 90 and 112 of age. The mean value prior to the dosing (7 days old), end of dosing (62 days old), and last week of recovery (112 days old) is shown below. Decrease of ulna length as observed in the previously conducted 28-day daily dosing juvenile rat toxicity study was not observed in this study after 8 weekly doses of Org 25969. In addition, ulna length was not changed in the positive control group as compared to vehicle control.

Mean ulna length (mm)

| Ulna length (mm) | Control | | 7.5 mg/kg | | 30 mg/kg | | 120 mg/kg | | Positive control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | M | F | M | F | M | F | M | F |
| One day prior to dosing | 14.67 | 14.54 | 14.42 | 14.65 | 14.90 | 14.50 | 14.71 | 14.45 | 14.63 | 14.51 |
| End of dosing | 38.13 | 36.29 | 38.28 | 35.61 | 39.50 | 36.32 | 38.66 | 36.57 | 38.33 | 36.16 |
| End of recovery | 41.96 | 38.51 | 42.05 | 37.76 | 41.91 | 38.55 | 42.60 | 38.76 | 41.35 | 38.24 |

**Hematology:** Significant changes were not seen in any of the treated groups as compared to control at the end of dosing and recovery period.

**Clinical Chemistry:** In addition to the parameters that are usually analyzed, bone turnover parameters including osteocalcin and C-telopeptide crosslinks (CTx) were examined at the end of dosing and recovery. In the positive control group (pamidronate), a statistically significant decrease (approximately 20%) was observed in osteocalcin and CTx concentration at the end of dosing. Decrease of osteocalcin did not appear to be reversible after 8-week recovery while the CTx concentration had no difference as compared to control. Similar change was not seen in Org 25969 treated groups except that a statistically significant decrease of osteocalcin concentration (15%) were observed in both males and females at the end of recovery. Both $Ca^{2+}$ and phosphorus (PHOS) was decreased after 8-week pamidronate treatment. These changes were statistically significant as compared to control, but the magnitude of Ca decrease in both male and females and PHOS decrease in females was <5% which is not considered to be biologically significant. The PHOS was decreased at 18% in males at the end of dosing. All these changes were reversible after 8-week recovery. Similar changes were not seen in Org 25969 treated groups as compared to control. There were no other changes that are considered to be treatment-related. Dr. Kuijpers indicated that “Osteocalcin is a marker of osteoblast activity and is related to formation of osteoid rather than to formation of mineralized bone. The significance of this finding was unclear since there were no effects on bone density (‘mass’) or strength.”



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**Urinalysis:** the following parameters were examined

| | |
|---|---|
| Volume (Quantity) | (Graduated flask) |
| Creatinine (CREA) | (Quantitative, PAP method at the Modular) |
| Total calcium (Ca) | (O-cresolphtalein method) |
| Inorganic phosphorus (PHOS) | (Molybdate UV method) |
| N-acetyl-beta-D-glucosaminidase (NAG) | (Quantitative, 3-cresolsulphtalinein method at the Modular® ) |
| Deoxypyridinolines (DPD) | Chemiluminescence immunoassay, Immulite 2000) |

In addition, NAG, Ca, PHOS and DPD were evaluated as calculated creatinine ratios (normalized values) and creatinine excretion was calculated.

Significant DPD decrease was seen in both male (35%) and females (42%) after 8-week treatment of pamidronate as compared to control. At the end of recovery, DPD was decreased in both males (22%) and females (38%), likely suggesting a partial reversibility. This was confirmed by decreased DPD/CREA ratios. A similar change was not seen in Org 25969 treated groups.

**Gross Pathology:** No treatment-related changes observed

**Organ Weights**

Organs weights were measured including kidney, liver, and lung. The results did not show any evidence of change in the weight of the tested organs/tissues in Org 25969 and pamidronate treated groups as compared to control.

In addition, femur (left and right), ulna (left and right) weights were also recorded. The results did not indicate treatment-related change in Org 25969 and pamidronate treated groups as compared to control.

**Histopathology**

Stained sections of the organs and tissues were microscopically examined including kidney, liver, lung, urinary bladder, tibia, humerus, femur, ulna, vertebral bodies L1-L3, vertebral body L4 + L5, mandible incisors and molars, and treatment sites in animals of vehicle control, 120 mg/kg Org 25969, and pamidronate treatment group at the end of dosing and end of the recovery. In addition to regular examination, special attention was paid to the bones and epiphyseal disc including photographs from the epiphyseal growth plate of humerus and tibia of 3 animals/sex/group. Thickness of the growth plate was measured on digital images captured with a 20x lens using a digital camera. Treatment-related changes were not detected.

*Bone*

Slightly increased trabecular bone density was observed in humerus (11/20) and tibia (20/20) of animals in pamidronate treated group. This effect was more pronounced after 8-week treatment-free period. At the end of recovery, increased trabecular density was observed in humerus of 20/20 animals (18 slight and 2 moderate) and tibia of 17/20



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animals (10 slight and 7 moderate). Trabecular bone density change was not observed in 120 mg/kg Org 25969 treatment group.

Measurement of the epiphyseal discs of humerus and tibia did not reveal evidence of change in animals of 120 mg/kg Org 25969 and pamidronate treated groups as compared to the vehicle control.

*Teeth*
Focal ameloblast disorganization of the incisor was observed in 120 mg/kg Org 25969 group (3/20, minimal) and pamidronate group (4/20, 3 minimal and 1 slight). This change was also seen in one animal of the vehicle control group (1/20, minimal). In addition, vacuolation was also seen in 1/20 animals at the incisor ameloblast layer and surrounding tissue of 120 mg/kg Org 25969 group; this change, however, was considered to be an artifact by the pathologist. At the end of recovery, Minimal incisor focal ameloblast disorganization was observed in vehicle control (3/20), 120 mg/kg Org 25969 (1/20), and pamidronate treatment group (2/20). Thus this change may not be considered treatment related and/or adverse. Minimal incisor focal vacuolation at ameloblast layer was observed in one animal of Org 25969 treatment group. In the pamidronate, 2/20 animals were reported to be with vacuolation at ameloblast layer of the incisor; one case was indicated to be minimal in severity but the other one did not have information of severity. The association of this change to the treatment can not be excluded. However, these changes may not be associated with significant adverse effect due to the minimal severity and low incidence.

There was no change in molar teeth in any treatment group.

Changes such as vacuolation and foamy macrophage were noted in the tissues tested in 120 mg/kg Org 25969 and pamidronate treatment group. These changes are consistent with the findings in previous Org 25969 toxicity studies which were not considered to be adverse.

**Special Evaluation**

1) Postmortem measurement of femur and terminal ulna length and: The length of each ulna and each femur was measured by means of a sliding vernier caliper. The results did not show any evidence of treatment-related changes in all Org 25969 treatment groups as compared to control. In pamidronate treatment groups, a small but statistically significant decrease of femur length in males was observed at the end of dosing as compared to the control. Femur length in males in pamidronate group was also smaller than that of the control (↓1.8%) with no statistical significance.

Length (mm) and weight (g) of femur and ulna

| Ulna | | Control | | 7.5 mg/kg | | 30 mg/kg | | 120 mg/kg | | Positive control | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M | F | M | F | M | F | M | F | M | F |
| End of | Length | 28.9 | 27.4 | 28.7 | 27.7 | 29.0 | 26.9 | 28.9 | 27.5 | 28.7 | 26.9 |



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| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| dosing | Weight | 0.29 | 0.23 | 0.28 | 0.21 | 0.28 | 0.21 | 0.28 | 0.23 | 0.28 | 0.23 |
| End of recovery | Length | 33.7 | 29.8 | 33.2 | 30.2 | 33.2 | 30.5 | 33.8 | 30.4 | 32.9 | 30.1 |
| | Weight | 0.37 | 0.25 | 0.36 | 0.27 | 0.37 | 0.26 | 0.38 | 0.26 | 0.37 | 0.27 |
| Femur | | Control | | 7.5 mg/kg | | 30 mg/kg | | 120 mg/kg | | Positive control | |
| | | M | F | M | F | M | F | M | F | M | F |
| End of dosing | Length | 31.9 | 29.7 | 31.7 | 29.7 | 32.1 | 29.5 | 31.8 | 29.7 | 31.1* | 29.2 |
| | Weight | 0.80 | 0.61 | 0.79 | 0.61 | 0.78 | 0.60 | 0.76 | 0.61 | 0.80 | 0.65 |
| End of recovery | Length | 37.7 | 32.8 | 37.7 | 33.2 | 37.5 | 33.5 | 38.0 | 33.1 | 37.0 | 32.9 |
| | Weight | 1.10 | 0.73 | 1.09 | 0.76 | 1.09 | 0.76 | 1.12 | 0.72 | 1.11 | 0.78 |

* Statistical significance, $p < 0.05$, ↓ 2.5% from the control

2) Femur cross section dimensions: The dimensions of the cross section of the right femur were measured by micro computed tomography (µCT) as stated in the study report. Likely, the µCT parameter "cortical cross section bone area diaphysis femur" as shown in the section 3 below was the parameter that it referred to. The data of femur diaphysis cortical cross section bone area are summarized in the following table. Significant changes were not seen in Org 25969 treated groups and positive control group as compared to the vehicle control.

Femur diaphysis cortical cross section bone area ($mm^2$)

| Cross section bone area (mm2) | End of dosing | | | | | End of recovery | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | VC* | 7.5 | 30 | 120 | PC* | VC | 7.5 | 30 | 120 | PC |
| Males | 4.52 | 4.49 | 4.68 | 4.55 | 4.33 | 6.57 | 6.47 | 6.68 | 6.58 | 6.25 |
| Females | 3.89 | 3.92 | 3.88 | 3.99 | 3.79 | 4.78 | 4.95 | 5.01 | 4.80 | 4.96 |

*VC = vehicle control; PC = positive control

3) Bone mineral density, bone strength and architecture assessment:

The following cortical and trabecular bone parameters were determined by µCT

- Cortical cross section bone area diaphysis femur ($mm^2$)
- Trabecular BMD ($mg \cdot cm^3$)
- Cortical BMD diaphysis femur ($mg \cdot cm^3$)
- Cortical thickness diaphysis femur (mm)
- Cortical bone volume diaphysis femur ($mm^3$)
- Cortical closed porosity diaphysis femur (%)
- Cortical endosteal circumference diaphysis femur (mm)
- Cortical mean eccentricity diaphysis femur
- Cortical periosteal circumference diaphysis femur (mm)
- Trabecular bone volume ($mm^3$)
- Trabecular tissue volume ($mm^3$)
- Trabecular bone volume over tissue volume (%)
- Trabecular pattern factor ($mm^{-1}$)
- Trabecular number ($mm^{-1}$)
- Trabecular thickness (mm)
- Trabecular separation (mm)
- Degree of anisotrophy (measure of trabecular orientation)
- Structure model index (plate/rod characterisation of trabecular bone)
- Cortical mean polar moment of inertia diaphysis femur ($mm^4$)

Bone strength parameters were determined after performing the following tests

1. *indentation test of the distal femur*
2. *compression test of lumbar vertebral body L5*
3. *three-point bending test of femoral shaft*
4. *cantilever bending test of femoral neck*

The following parameters were found to be different with statistical significance in the pamidronate group as compared to the vehicle control group, indicating that this animal model is sensitive to bone change induced by bisphosphonate compounds.

- Cortical bone mineral density diaphysis femur (lower compared to placebo),
- Cortical bone volume diaphysis femur (lower compared to placebo),
- Cortical endosteal circumference diaphysis femur (higher compared to placebo),
- Cortical thickness diaphysis femur (lower compared to placebo),
- Trabecular bone mineral density distal femur (higher compared to placebo),
- Trabecular bone volume distal femur (higher compared to placebo),
- Trabecular bone volume/tissue volume ratio distal femur (higher compared to placebo),
- Trabecular structure model index distal femur (lower compared to placebo),
- Trabecular number distal femur (higher compared to placebo),
- Trabecular pattern factor distal femur (lower compared to placebo),
- Trabecular separation distal femur (lower compared to placebo),
- Trabecular thickness distal femur (higher compared to placebo),
- Trabecular tissue volume distal femur (lower compared to placebo),
- Trabecular bone mineral density vertebra L5 (higher compared to placebo),
- Trabecular bone volume vertebra L5 (higher compared to placebo),
- Trabecular structure model index vertebra L5 (lower compared to placebo),
- Trabecular number vertebra L5 (higher compared to placebo),
- Trabecular pattern factor vertebra L5 (lower compared to placebo),
- Trabecular separation vertebra L5 (lower compared to placebo),
- Femur cantilever energy total (lower compared to placebo),
- Femur indentation energy total (higher compared to placebo),
- Femur indentation end of displacement (higher compared to placebo),
- Femur indentation maximum force (higher compared to placebo),
- Femur indentation stiffness (higher compared to placebo),
- Vertebra compression maximum force (higher compared to placebo), and
- Vertebra compression stiffness (higher compared to placebo).

The follow graph showed the effect of pamidronate treatment on trabecular bone density as an example.

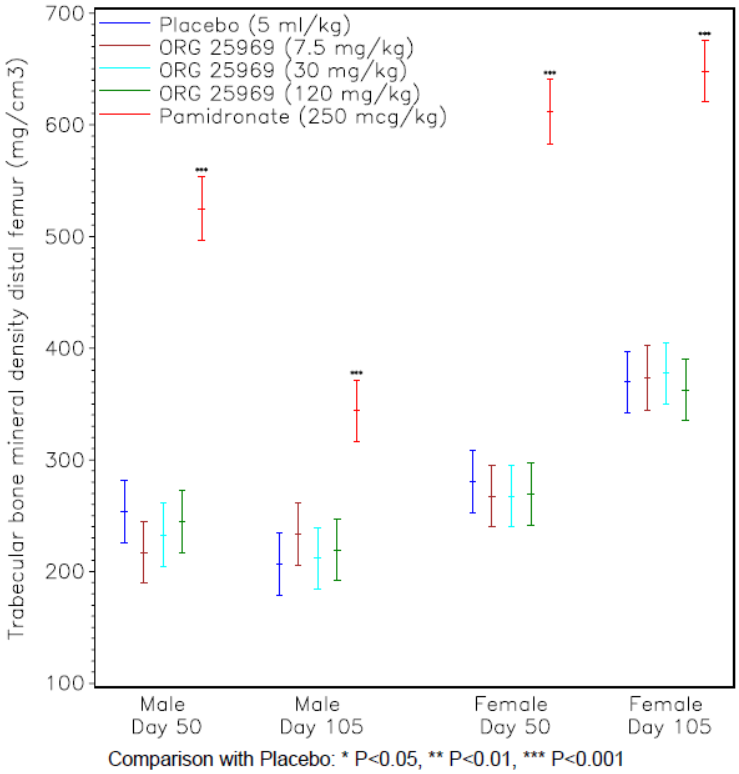


**Figure 1 Estimated mean and 95% confidence interval of Trabecular bone mineral density distal femur**

For Org 25969 treatment, statistically significant change was observed in several parameters including cortical mean eccentricity diaphysis femur, trabecular thickness vertebra L5, vetebra compressed end of displacement, trabecular bone volume/tissue volume ratio vertebra L5, trabecular degree of anisotrophy vertebra L5, and demur cantilever end of displacement. However, the significance was seen mostly in one gender (male or female) and one group (occasionally in two groups but never in all 3 groups). In addition, dose-dependent manner was not observed. An example was shown below. Overall, Org 25969 did not appear to significantly affect the bone structure, density and strength in juvenile rats after 8-week intermittent weekly administration at up to 120 mg/kg.

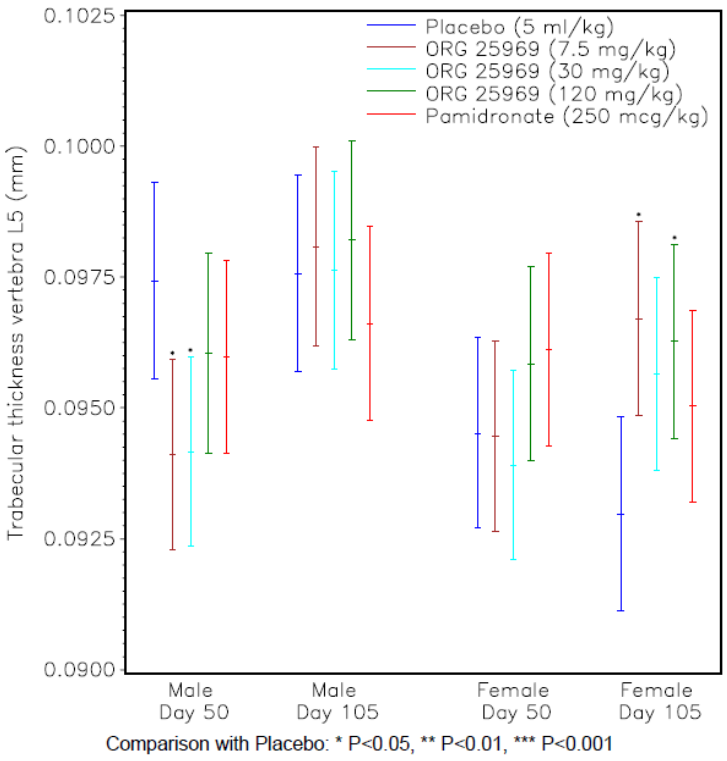


**Figure 5 Estimated median (geometric mean) and 95% confidence interval of Trabecular thickness vertebra L5**

### Toxicokinetics

Not conducted in this study

### Dosing Solution Analysis

Dosing concentration and homogeneity appeared to be acceptable.

**Study title:** Effects of Org 25969 on fracture repair in the Wistar rat

| | |
|---|---|
| Study no.: | 090319 |
| Study report location: | eCTD |
| Conducting laboratory and location: | (b) (4) |
| Date of study initiation: | 2009 |
| GLP compliance: | Yes* |
| QA statement: | Yes |
| Drug, lot #, and % purity: | Org 25969, L00027962, 95.4% |

* µCT assay was not GLP compliant

### Key Study Findings

- This study was designed to investigate whether fractured bone repair may be affected by Org 25969 in adult rats with pre-fracture treatment or post-fracture treatment for 6 weekly doses at 0, 30, 120, and 500 mg/kg
- Femur fracture was produced in animals at 12-weeks of age; and bone repair was evaluated 12 weeks after fracture.
- Evaluations included peripheral quantitative computed tomography (pQCT) for densitometry µCT scan for bone structure including callus volume and bone volume in the callus, mechanical testing for bone strength, and histopathology examination for callus and bone formation as well as evaluation of bridging of the fracture site by new bone.
- Large variability was seen animals with all endpoints evaluated
- pQCT scan did not find significant adverse changes in bone mineral content, area and density in animals of all Org 25969 groups as compared to control.
- Statistically significant changes were not seen in callus volume (TV) and bone volume in the callus (BV) derived from µCT scan. However, data showed a trend of slight increase in TV, slight decrease in BV, and increase in BV/TV ratio at 500 mg/kg in both males and females after post-fracture administration of Org 25969.
- Strength testing did not detect significant changes in bone strength in Org 25969 treated groups as compared to control
- Statistically significant changes were observed at 500 mg/kg post fracture in the extent of callus (↑) and fibrosis (↑) of males and bone formation (↓) and extent of bridging formation at the fracture site (↓) of females as compared to the concurrent control. These changes are consistent with the observations of µCT scan and possibly suggest a slight delay in bone healing as compared to the concurrent control. However, the extent of these changes appeared to be within the range of all control values if the pre-fractured treatment control data were taken into consideration with male and female data combined.
- The NOAEL for pre-fracture treatment = 500 mg/kg; the NOAEL for post-fracture treatment = 120 mg/kg



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**Methods**

| | |
|---|---|
| Doses: | 0, 30, 120, and 500 mg/kg Org 25969 |
| Frequency of dosing: | Weekly for 6 weeks |
| Route of administration: | Intravenously, 1 ml/min |
| Dose volume: | 5 ml/kg |
| Formulation/Vehicle: | Org 25969 solution in 0.9% saline |
| Species/Strain: | Wistar (RccHan: WIST) rats |
| Number/Sex/Group: | 20 animals/sex/group |
| Age: | 4-5 weeks for pre-fracture treatment and 10-11 weeks for post-fracture treatment |
| Weight: | 99-220 g for pre-fracture treatment and 238-425 g for post-fracture treatment |
| Satellite groups: | none |
| Unique study design: | This study is specifically designed to investigate the effects of Org 25969 on bone repair. Animals were administered Org 25969 for 6 weeks prior to (part 1) or after (part 2) femur fracture produced by surgery. All animals were sacrificed 12 weeks after fracture as seen in the table below. Among the 20 animal/sex/group, half (subgroup A, 10/sex/group) were used for µCT, pQCT and bone strength assessment while the other half (subgroup B) were used for histopathology examination of the fracture repair. |
| Deviation from study protocol: | Deviations do not appear to significantly affect study quality |

Experiment design:

Table 1: Treatment Groups

| Group | Dosing paradigm | Dose Org 25969 (mg/kg/dose) | Dosing regimen | Fracture at | Follow-up/time of terminal evaluation | Gender /Animal #[a]. |
|---|---|---|---|---|---|---|
| 1 | Pre-fracture exposure (Part I) | 0 | Once weekly at 6, 7, 8, 9, 10 and 11 weeks of age | 12 weeks of age (± 3 days) | 12 weeks after day of fracture | 20 M + 20 F |
| 2 | | 30 | | | | 20 M + 20 F |
| 3 | | 120 | | | | 20 M + 20 F |
| 4 | | 500 | | | | 20 M + 20 F |
| 5 | Post-fracture exposure (Part II) | 0 | Once weekly for 6 weeks starting on the day after fracture[b] | 12 weeks of age (± 3 days) | 6 weeks after last day of dosing = 12 weeks after day of fracture | 20 M + 20 F |
| 6 | | 30 | | | | 20 M + 20 F |
| 7 | | 120 | | | | 20 M + 20 F |
| 8 | | 500 | | | | 20 M + 20 F |

[a] Each experimental group was split into subgroups of 10 M + 10 F which were intended to be used for either microCT, pQCT and strength assessments (subgroups A) or histopathological assessment of fracture repair (subgroups B) except as noted in the deviations section. See result section for study inclusion

[b] 6 weeks of treatment covered all critical stages of fracture repair.

*Surgery for closed fracture of femur*
On the day of surgery, animals were sedated and the patellar groove of the right femur was exposed. A penetrating hole was made into the patellar groove surface of the femur. A needle was inserted into the medullary canal, rotated, and then removed to create a pilot hole for pin placement. A steel pin, appropriately sized for animal age/weight was placed into the pilot hole and into the medullary canal, with the length being cut to fit approximately from the distal condyle of the femur to the lesser trochanter. After the patella was replaced over the patellar groove to its normal position, and the muscle and skin was closed, the animal was placed in the fracture device. The fracture blade was positioned over the midshaft of the right femur and the anvil was raised until the fracture blade contacted the cutaneous surface overlying the midshaft. The anvil was then tightened until an appropriate preload was achieved. The weight was then dropped from a height specified for the particular size animal to create a midshaft fracture. A post-operative radiograph was taken to confirm the fracture and check for proper alignment of the fractured ends after the surgery. In addition, radiographs were taken approximately two weeks post surgery to verify the pin had not migrated outside the medullary space. Animals with inappropriate fractures/no fracture or pin migration were removed from the study.

## Observations and Results

### In-life observation
Animal death (3 cases) was reported after the first dose which were likely due to the uncontrolled rate of IV injection. This has not been reported in previous rat studies. Spare animals were added to replace the animal loss. According to the study report, an amendment was thereafter issued to reduce the rate of IV (1 ml/min). After the installment of the amendment to closely monitor dosing rate by noting start and stop time of dosing, 2 deaths occurred during the study period. In total, 6 animals died during the dosing phase in group 2 (1 animal), 3 (1), 4 (3), and 8 (1). No animal death was observed in control groups, suggesting that Org 25969 treatment cannot be excluded.

Significant treatment-related clinical observations and body weight change was not observed in Org 25969 treated animals as compared to the control during the study.

### Postmortem examinations
At 12-week post surgery, animals were euthanized and the fractured (right) and contralateral (left) femurs were collected for histopathology, mechanical testing, and densitometry. Tissues selected for histopathology were stored in 10%NBF, and tissues selected for densitometry and mechanical testing were wrapped in saline soaked gauze and stored frozen until the time of testing.

*pQCT scan for densitometry*

Peripheral computed tomography (pQCT) was performed on the excised unfractured left and fractured right femurs using a Stratec XCT-RM. pQCT scan was performed at 50% of the total femoral length from the distal end of the femur. The positions were verified using scout views and one 0.5-mm slice perpendicular to the long axis of the femoral



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shaft were acquired. Bone mineral content (BMC, mg/mm), bone area (BA, $mm^2$) and bone density (BMD, $mg/cm^3$) were analyzed. Femurs at fracture side tended to have higher bone mineral content and area and lower bone mineral density than non-fractured side (data are not shown). This was expected as fractured side contained more calluses and thus higher bone mineral content and area than non-fractured side

There appeared to be trend of decrease on the tested parameters in Org 25969 treated groups as compared to control in the fractured femur as shown below. However, the magnitude of the change was minimal (generally <10%) and did not show statistically significant difference as compared to control, which may not indicate toxicity.

% change of pQCT parameters in the fractured bone from the vehicle control

| densitometry | | Pre-fractured treatment | | | | Post-fractured treatment | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | 30 mg/k | 120 mg/kg | 500 mg/kg | Control | 30 mg/k | 120 mg/kg | 500 mg/kg |
| M | BMC | 24.1 | ↓2.5 | ↓1.1 | ↓5.6 | 25.0 | ↓1.6 | ↑10.8 | ↓2.2 |
| | BMD | 817.9 | ↓4.0 | ↓8.8 | ↓7.1 | 804.3 | ↓2.1 | ↑0.9 | ↓2.1 |
| | BA | 29.5 | ↓1.9 | ↑7.6 | ↑1.9 | 31.1 | ↑2.6 | ↑8.5 | ↑1.2 |
| F | BMC | 18.3 | ↓3.0 | ↓5.8 | ↓9.6 | 22.05 | ↓5.5 | ↓15.3 | ↓8.5 |
| | BMD | 822.6 | ↑1.5 | ↓0.8 | ↓2.6 | 902.1 | ↓4.7 | ↓7.1 | ↓7.2 |
| | BA | 22.35 | ↓5.2 | ↓3.8 | ↓7.4 | 24.45 | ↓1.1 | ↓4.1 | ↓1.6 |

*µCT scan for bone structure*

The fractured right femur was scanned using a Skyscan 1076 machine. The samples were scanned at 18 µm resolution. A single scan length of 18mm was performed on the fracture site. The region of interest (ROI) or volume of interest (VOI) of the analysis was focused only on the callus tissue at the fracture area. The parameters determined by µCT were callus volume (TV), bone volume in the callus (BV) and bone volume over callus volume ratio (TV/CV). The results were summarized in the table below. Statistically significant differences were not seen between treated groups and control in all 3 tested endpoints as shown in the table below.

% change of µCT parameters from the vehicle control

| μCT | | Pre-fractured treatment | | | | Post-fractured treatment | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | 30 mg/k | 120 mg/kg | 500 mg/kg | Control | 30 mg/k | 120 mg/kg | 500 mg/kg |
| M | TV ($\mu M^3$) | 535.6 ± 104.6 | ↑14 | ↑25 | ↑17 | 904.6 ± 526.5 | ↓24 | ↓13 | ↑26 |
| | BV ($\mu M^3$) | 94.6 ± 14.4 | ↑7.8 | ↑23 | ↑23 | 149.5 ± 22.89 | ↓7.4 | ↑16 | ↓22 |
| | BV/CV % | 17.9 ± 2.22 | ↓6.1 | ↓1.1 | ↑4.3 | 19.3 ± 5.93 | ↑9.8 | ↑9.8 | ↓23 |
| F | TV ($\mu M^3$) | 538.6 ± 403.6 | ↓29 | ↓25 | ↓23 | 503.2 ± 109.7 | ↓1.2 | ↑82 | ↑37 |
| | BV ($\mu M^3$) | 82.6 ± 17.01 | ↓9.2 | ↓6.1 | ↓23 | 90.4 ± 16.97 | ↑1.1 | ↓4.3 | ↓4.6 |
| | BV/CV % | 18.8 ± 5.42 | ↑5.3 | ↑4.3 | ↑3.7 | 18.5 ± 4.49 | ↑0.9 | ↓24 | ↓25 |

Dr. Kuijper indicated that decreased BV/CV in the post-fractured treatment at high dose groups in both male and females may suggest delay in bone formation.
However, caution is needed to interpret the data as treatment related bone formation delay if the decreased BV/CV was likely mainly due to callus volume increase. It should be noted that the values of bone volume (BV) in both males and females at 500 mg/kg were within the range of control animals with the mean in males at 500 mg/kg slightly lower as compared to control. For callus volume, extreme values (> 3 fold of the mean and > 2 fold of the up-limit of others in the group) existed in male control (1/9), male 120 mg/kg (1/10), male 500 mg/kg (3/9), female 120 mg/kg (3/9), and female 500 mg (1/10) of post-fracture groups. In addition, one animal (1/10) in control group of pre-fracture had an extreme value in callus volume. In these animals with high callus volume, bone volume was not increased concomitantly. Since the incidence of these extreme values were not dose-dependent, it is not clear whether this represented Org 25969 treatment related change.

*Mechanical testing*

Half of fractured femurs, from animals either pre-treated before fracture or post-treated after fracture, were subject to three point bending test at the femoral shaft.

The anterior to posterior diameter at the midpoint of the femoral shaft was taken with an electronic caliper and recorded. The fractured femur was placed on the lower supports of a three point bending fixture with the anterior side facing downward in an Instron Mechanical Testing Instrument. The upper loading device was aligned to the center of the femoral shaft. The load was applied at a constant displacement rate of 6 mm/min until the femur broke or failed. The locations of maximum load, stiffness and energy absorbed were selected manually from the load and displacement curve and values calculated by instrument software. The intrinsic properties, ultimate strength, elastic modulus and toughness, were calculated from maximum load, stiffness, energy absorbed, anterior-posterior diameter and moment of inertia. The results of the mechanical testing did not show dose-dependent changes in any of these parameters



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and statistically significant difference was not seen between treated groups and control as shown in the table below (extracted from the study report). In addition, data showed large individual variability. Overall, mechanical testing did not reveal treatment related changes after Org 25969 administration.

Table 11: Three Point Bending Test Summary Results, Male

| Limb | Group | | Maximum Load N | Stiffness N/mm | Energy mJ | Ultimate Strength N/mm$^2$ | Elastic Modulus MPa | Toughness MJ/m$^3$ |
|---|---|---|---|---|---|---|---|---|
| Right - Fractured limb (Pre-fracture dosing) | Group 1 - Vehicle | mean | 163.05 | 338.34 | 67.20 | 49.68 | 901.95 | 2.45 |
| | | Std | 72.15 | 194.06 | 36.27 | 26.78 | 569.94 | 1.63 |
| | | SEM | 22.82 | 61.37 | 11.47 | 8.47 | 180.23 | 0.51 |
| | | n | 10 | 10 | 10 | 10 | 10 | 10 |
| | | stat | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| | Group 2 - Org 25969 (30mg/kg) | mean | 137.07 | 343.11 | 53.81 | 41.76 | 906.55 | 2.13 |
| | | Std | 66.14 | 184.64 | 29.79 | 18.63 | 577.12 | 1.59 |
| | | SEM | 19.94 | 55.67 | 8.98 | 5.62 | 174.01 | 0.48 |
| | | n | 11 | 11 | 11 | 11 | 11 | 11 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | Group 3 - Org 25969 (120mg/kg) | mean | 152.04 | 377.43 | 62.17 | 46.77 | 1063.76 | 2.00 |
| | | Std | 64.07 | 221.11 | 36.74 | 26.59 | 768.92 | 1.00 |
| | | SEM | 18.49 | 63.83 | 10.61 | 7.68 | 221.97 | 0.29 |
| | | n | 12 | 12 | 12 | 12 | 12 | 12 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | Group 4 - Org 25969 (500mg/kg) | mean | 130.97 | 436.68 | 35.12 | 43.57 | 1232.20 | 1.49 |
| | | Std | 45.22 | 264.23 | 13.36 | 17.59 | 853.13 | 0.97 |
| | | SEM | 13.63 | 79.67 | 4.03 | 5.30 | 257.23 | 0.29 |
| | | n | 11 | 11 | 11 | 11 | 11 | 11 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| Right - Fractured limb (Post-fracture dosing) | Group 5 - Vehicle | mean | 72.18 | 104.22 | 48.61 | 23.72 | 265.85 | 2.03 |
| | | Std | 25.67 | 61.01 | 26.98 | 12.18 | 188.01 | 1.18 |
| | | SEM | 8.56 | 20.34 | 8.99 | 4.06 | 62.67 | 0.39 |
| | | n | 9 | 9 | 9 | 9 | 9 | 9 |
| | | stat | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| | Group 6 - Org 25969 (30mg/kg) | mean | 122.76 | 173.82 | 86.23 | 29.44 | 271.00 | 3.69 |
| | | Std | 71.26 | 145.81 | 59.54 | 16.56 | 151.81 | 4.86 |
| | | SEM | 23.75 | 48.60 | 19.85 | 5.52 | 50.60 | 1.62 |
| | | n | 9 | 9 | 9 | 9 | 9 | 9 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | Group 7 - Org 25969 (120mg/kg) | mean | 107.71 | 161.53 | 80.47 | 31.68 | 451.66 | 2.86 |
| | | Std | 48.31 | 119.78 | 65.11 | 16.23 | 555.93 | 1.88 |
| | | SEM | 15.28 | 37.88 | 20.59 | 5.13 | 175.80 | 0.59 |
| | | n | 10 | 10 | 10 | 10 | 10 | 10 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | Group 8 - Org 25969 (500mg/kg) | mean | 85.96 | 184.71 | 50.27 | 27.17 | 437.70 | 2.18 |
| | | Std | 24.31 | 139.89 | 25.78 | 10.93 | 353.54 | 1.40 |
| | | SEM | 8.10 | 46.63 | 8.59 | 3.64 | 117.85 | 0.47 |
| | | n | 9 | 9 | 9 | 9 | 9 | 9 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

n.a. not applicable; n.s. not significantly different from vehicle group

Table 12: Three Point Bending Test Summary Results, Female

| Limb | Group | | Maximum Load N | Stiffness N/mm | Energy mJ | Ultimate Strength N/mm$^2$ | Elastic Modulus MPa | Toughness MJ/m$^3$ |
|---|---|---|---|---|---|---|---|---|
| Right - Fractured limb (Pre-fracture dosing) | Group 1 - Vehicle | mean | 147.43 | 420.33 | 41.48 | 59.05 | 1812.09 | 1.55 |
| | | Std | 59.38 | 105.52 | 25.39 | 22.34 | 560.19 | 0.78 |
| | | SEM | 19.79 | 35.17 | 8.46 | 7.45 | 186.73 | 0.26 |
| | | n | 9 | 9 | 9 | 9 | 9 | 9 |
| | | stat | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| | Group 2 - Org 25969 (30mg/kg) | mean | 130.43 | 360.29 | 43.61 | 58.40 | 1797.01 | 1.76 |
| | | Std | 67.03 | 134.39 | 27.47 | 28.79 | 730.55 | 1.02 |
| | | SEM | 20.21 | 40.52 | 8.28 | 8.68 | 220.27 | 0.31 |
| | | n | 11 | 11 | 11 | 11 | 11 | 11 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | Group 3 - Org 25969 (120mg/kg) | mean | 129.79 | 405.33 | 35.36 | 57.61 | 1901.04 | 1.55 |
| | | Std | 73.62 | 258.50 | 17.71 | 29.18 | 1218.45 | 0.70 |
| | | SEM | 22.20 | 77.94 | 5.34 | 8.80 | 367.38 | 0.21 |
| | | n | 11 | 11 | 11 | 11 | 11 | 11 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | Group 4 - Org 25969 (500mg/kg) | mean | 172.82 | 420.46 | 54.60 | 83.36 | 2254.04 | 2.47 |
| | | Std | 75.85 | 186.01 | 36.45 | 38.00 | 1205.67 | 1.92 |
| | | SEM | 22.87 | 56.08 | 10.99 | 11.46 | 363.52 | 0.58 |
| | | n | 11 | 11 | 11 | 11 | 11 | 11 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| Right - Fractured limb (Post-fracture dosing) | Group 5 - Vehicle | mean | 127.23 | 341.63 | 48.33 | 45.64 | 1142.94 | 2.07 |
| | | Std | 91.52 | 225.92 | 54.10 | 38.96 | 763.78 | 3.23 |
| | | SEM | 28.94 | 71.44 | 17.11 | 12.32 | 241.53 | 1.02 |
| | | n | 10 | 10 | 10 | 10 | 10 | 10 |
| | | stat | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| | Group 6 - Org 25969 (30mg/kg) | mean | 117.42 | 354.43 | 30.54 | 46.81 | 1430.49 | 1.19 |
| | | Std | 53.28 | 153.90 | 15.23 | 25.51 | 771.48 | 0.67 |
| | | SEM | 16.85 | 48.67 | 4.82 | 8.07 | 243.96 | 0.21 |
| | | n | 10 | 10 | 10 | 10 | 10 | 10 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | Group 7 - Org 25969 (120mg/kg) | mean | 110.68 | 356.08 | 33.32 | 47.47 | 1502.23 | 1.64 |
| | | Std | 54.68 | 232.26 | 18.00 | 23.67 | 1157.96 | 1.11 |
| | | SEM | 18.23 | 77.42 | 6.00 | 7.89 | 385.99 | 0.37 |
| | | n | 9 | 9 | 9 | 9 | 9 | 9 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | Group 8 - Org 25969 (500mg/kg) | mean | 109.55 | 301.89 | 38.39 | 41.05 | 1206.73 | 1.47 |
| | | Std | 70.57 | 249.27 | 30.24 | 23.25 | 1029.67 | 1.09 |
| | | SEM | 22.32 | 78.83 | 9.56 | 7.35 | 325.61 | 0.34 |
| | | n | 10 | 10 | 10 | 10 | 10 | 10 |
| | | stat | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

n.a. not applicable; n.s. not significantly different from vehicle group

*Histopathology*

A coronal section was cut at approximately mid-point of the femoral shaft at 4-6 µm/ thick, (1 section/femur). The tissue specimen included the site of the fracture plus at least 1 cm of each end from the fracture site. Slides were stained with hematoxylin and eosin (H&E). Histopathology evaluation included extent of fracture callous formation, type of new bone formed, and bridging of the fracture site by new bone. The scoring criteria were shown below as extracted from the study report.

Table 2: Histopathology Evaluation Scoring Criteria

| Characteristic | Grading | Score |
|---|---|---|
| Extent of Fracture Callous Formation | Marked | 3 |
| | Moderate | 2 |
| | Mild | 1 |
| | None | 0 |
| Type of new bone formed | Mostly lamellar | 4 |
| | Even mix of woven and lamellar bone | 3 |
| | Mostly woven bone | 2 |
| | Mostly cartilage /fibrocartilage | 1 |
| | No new bone formed | 0 |
| Bridging of the fracture site by new bone | Complete | 4 |
| | Mostly complete with small gaps | 3 |
| | Approximately 50% complete | 2 |
| | Occasional/very little | 1 |
| | None | 0 |

In addition, inflammation, fibrosis and necrosis were graded (none to severe: 0 - 3). As stated in the study report, there were no significant treatment related effects upon extent of fracture callus, type of bone formed at the fracture site, or extent of bridging by new bone at the fracture site in male and female rats of Org 25969 treated as compared to control after pre-fracture or post fracture treatment. In addition, there were no significant treatment-related effects on the extent of inflammation, fibrosis, or necrosis at the fracture site in male or female rats of Org 25969 treatment as compared to control.

The features at the fracture site varied among both control rats and treated rats, as the amount of fracture callus ranged from mild to marked, while the type of new bone formed within the fracture callus usually was composed primarily of woven bone or primarily of cartilage or fibrocartilage. Bridging of the fracture site by new bone varied from none to complete.

Dr. Gemma Kuijpers indicated in her consultation review that “Some histologic characteristics of the fractures were altered by 500 mg/kg Org 25969’, and concluded that “Org 25969 delayed fracture healing in animals treated with 500 mg/kg <u>after</u> fracture. Thus, the NOAEL for that group is 120 mg/kg/week. The NOAEL for the pre-fracture treated animals is 500 mg/kg.” These histologic parameters included the following as verbatimly cited from Dr. Kuijpers review (ss=statistically significant; ns = not statistically significant):

- High dose: Increased extent of callus (M, ss, moderate effect), increased fibrosis (M, ss; F, ns), decreased “type of bone” (i.e. more cartilage and less woven/lamellar bone, or delayed bone formation) (M, ns; F, ss) and decreased bridging (M, ns; F, ss, marked effect).
- Mid dose: Increased fibrosis, decreased bridging and decreased bone type (ns) in F but not M

I have noted the observations that Dr. Kuijpers indicated. The observed changes may suggest a slight increase callus formation but a slight decrease in bone formation in the post-fracture Org 25969 treated groups as compared to the concurrent control. Toxicological significance of these changes is not clear. Since the control groups of pre-fracture treatment were only dosed with saline and these animals were fractured and sacrificed at the same time as the animals for post-fracture treatment. Therefore, these pre-fracture control groups may be considered a secondary control of the post-fracture treatment groups. In comparison to the values of both control groups, the observed changes were within the range of control values.

Mean of the parameters of histopathology examination in control and Org 25969 treated groups

| Histopathology (mean ± SD) | | Pre-fractured treatment | | | | Post-fractured treatment | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | 30 mg/kg | 120 mg/kg | 500 mg/kg | Control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| M | Extent of callus | 2.22 ± 0.67 | 2.09 ± 0.54 | 2.00 ± 0.60 | 2.18 ± 0.75 | 1.89 ± 0.60 | 2.30 ± 0.82 | 2.30 ± 0.48 | 2.50* ± 0.48 |
| | Type of bone | 1.22 ± 0.44 | 1.36 ± 0.50 | 2.08 ± 1.00 | 1.64 ± 0.67 | 1.33 ± 0.50 | 1.20 ± 0.42 | 1.30 ± 0.48 | 1.00 ± 0.00 |
| | Bridging Fx site | 0.22 ± 0.67 | 1.45* ± 1.51 | 2.00* ± 1.41 | 1.91* ± 1.64 | 0.78 ± 1.39 | 0.70 ± 1.49 | 0.70 ± 1.34 | 0.00 ± 0.00 |
| | Fibrosis | 1.44 ± 0.73 | 1.00 ± 0.89 | 0.50 ± 0.67 | 0.82 ± 0.75 | 1.00 ± 0.71 | 1.00 ± 0.67 | 1.10 ± 0.88 | 1.80* ± 0.42 |
| | Necrosis | 2.11 ± 1.27 | 2.82 ± 0.40 | 2.25 ± 0.87 | 2.45 ± 0.52 | 1.67 ± 0.87 | 2.00 ± 0.67 | 1.90 ± 0.32 | 2.30 ± 0.48 |
| F | Extent of callus | 1.88 ± 0.83 | 1.55 ± 0.69 | 1.55 ± 0.52 | 1.73 ± 0.65 | 2.00 ±0.77 | 2.27 ± 0.79 | 2.22 ± 0.83 | 2.10 ± 0.57 |
| | Type of bone | 1.88 ± 0.64 | 2.27 ± 0.47 | 1.82 ± 0.40 | 2.00 ± 0.89 | 2.09 ± 0.83 | 1.82 ± 0.40 | 1.67 ± 0.87 | 1.40* ± 0.52 |
| | Bridging Fx site | 2.63 ± 1.19 | 3.55 ± 0.52 | 2.73 ± 1.62 | 2.25 ± 1.51 | 2.45 ± 1.44 | 2.18 ± 1.33 | 1.33 ± 1.66 | 1.00* ± 0.94 |
| | Fibrosis | 0.38 ± 0.52 | 0.00* ± 0.00 | 0.36 ± 0.81 | 0.55 ± 0.82 | 0.64 ± 0.81 | 0.91 ± 0.83 | 1.33 ± 0.87 | 1.30 ± 0.67 |
| | Necrosis | 2.88 ± 0.35 | 2.64 ± 0.50 | 2.64 ± 0.50 | 2.73 ± 0.47 | 2.73 ± 0.47 | 2.64 ± 0.50 | 2.78 ± 0.44 | 2.80 ± 0.63 |

# 6 Carcinogenicity

The Division raised the concern of bone carcinogenicity because of the extended duration of Org 25969 bone retention as demonstrated in rats. In a meeting with the Applicant on December 1st, 2008, the Agency indicated demonstration of binding to hydroxyapatite as well as submission of bone metabolism and histopathology data from the requested juvenile rat study with extended duration of follow-up would address the carcinogenicity concern. In this submission, the Sponsor submitted an in vitro study clearly demonstrated that Org 25969 binds with hydroxyapatite at the same site for bisphosphonate binding, a component of bone extracellular matrix. In addition, a



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microautoradiography study indicated that Org 25969 does not deposit in the growth plate, the area responsible for bone growth and elongation. The newly conducted Org 25969 toxicity study had a maximum observation period of 16 weeks (8-week dosing + 8 week recovery observation) and signs of carcinogenicity such as hyperplasia were not observed in histopathology examination. Therefore, this reviewer believes Org 25969 does not impose significant risk for bone tumor development. Firstly, Org 25969 mainly binds to extracellular matrix which is involved in mineralization in the bone, which is not likely associated with cell proliferation or differentiation. Secondly, due to the large size and the hydrophilic property of the outside surface, Org 25969 molecules do not appear to easily cross cell membrane of bone cells directly, which may modulate cell proliferation or differentiation. It should be noted that, bisphosphonate which binds the same site at hydroxyapatite, did not cause bone-related cancer after long-term exposure based on a FDA published paper (Whitaker et al., 2012). Dr. Kuijpers concurred on this conclusion and stated in her consult review that “is unlikely that the presence of Org 25969 in bone would be tumorigenic to bone cells (osteoclasts or osteoblasts) or other cells”.

# 7 Special evaluation

**Title:** Statistical evaluation of the effect of Org 25969 on ulna length and femur length correcting for effects on body weight in juvenile rats of study 060086 (INT00122920)

In the 4-week toxicity study in the juvenile rat submitted in the original NDA (Study 060086), animals (7 days of age at dosing start) were treated with Org 25969 daily for 28 days at 0, 30, 120, and 500 mg/kg Org 25969. Minor but statistically significant decrease in ulna length (≤5%), femur length (≤3.5%) and weight (≤6.5%) were observed at the end of treatment with the most severe effects at the end of 8-week recovery period at 120 and 500 mg/kg Org 25969 dose levels as shown in the tables below (femur only as an example) (extracted from my review for original NDA submission).

Bone length and weight at the end of Org 25969 dosing

| femur | C | 30 mg/kg | 120 mg/kg | 500 mg/kg | C | 30 mg/kg | 120 mg/kg | 500 mg/kg |
|---|---|---|---|---|---|---|---|---|
| Sex | M | M | M | M | F | F | F | F |
| Length (mm) | 23.4 | 22.8* | 23.0* | 22.9* | 22.7 | 22.8 | 22.6 | 22.2 |
| Weight (mg) | 0.424 | 0.408 | 0.426 | 0.420 | 0.386 | 0.373 | 0.395 | 0.371 |
| Weight/BW (%) | 0.414 | 0.418 | 0.409 | 0.412 | 0.425 | 0.417 | 0.443 | 0.419 |

Bone length and weight at the end of recovery

| femur | C | 30 mg/kg | 120 mg/kg | 500 mg/kg | C | 30 mg/kg | 120 mg/kg | 500 mg/kg |
|---|---|---|---|---|---|---|---|---|
| Sex | M | M | M | M | F | F | F | F |
| Length (mm) | 34.1 | 33.9 | 33.4 | 32.9* | 31.1 | 30.8 | 30.3* | 30.2* |
| Weight (g) | 1.006 | 1.038 | 1.000 | 0.956 | 0.780 | 0.754 | 0.727* | 0.729* |
| Weight/BW (%) | 0.302 | 0.303 | 0.315 | 0.299 | 0.377 | 0.372 | 0.363 | 0.363 |

* Statistically significant, $p < 0.05$ or $p < 0.01$

The Applicant indicated that the minor bone length decrease was due to the body weight decreased in Org 25969 treated groups as compared to control. Minor decrease in mid (120 mg/kg) and high dose (500 mg/kg) (5-8%) in males was observed during recovery period; this change was statistically significant during Day 35-70. Changes in females were smaller than males (≤5%). The Division did not agree with the Applicant because the change in bone length was not coincident with the body weight decrease. The Applicant re-analyzed the data from the 4-week juvenile study using ANCOVA analysis with body weight as the covariate (INT00122920) to examine whether the bone length change was associated with the body weight change in a statistical perspective. The analysis was performed with male and female data combined. The following graph shows the ANCOVA model for analyses of the femur length at PND35 (at the end of Org 25969 dosing) as an example.

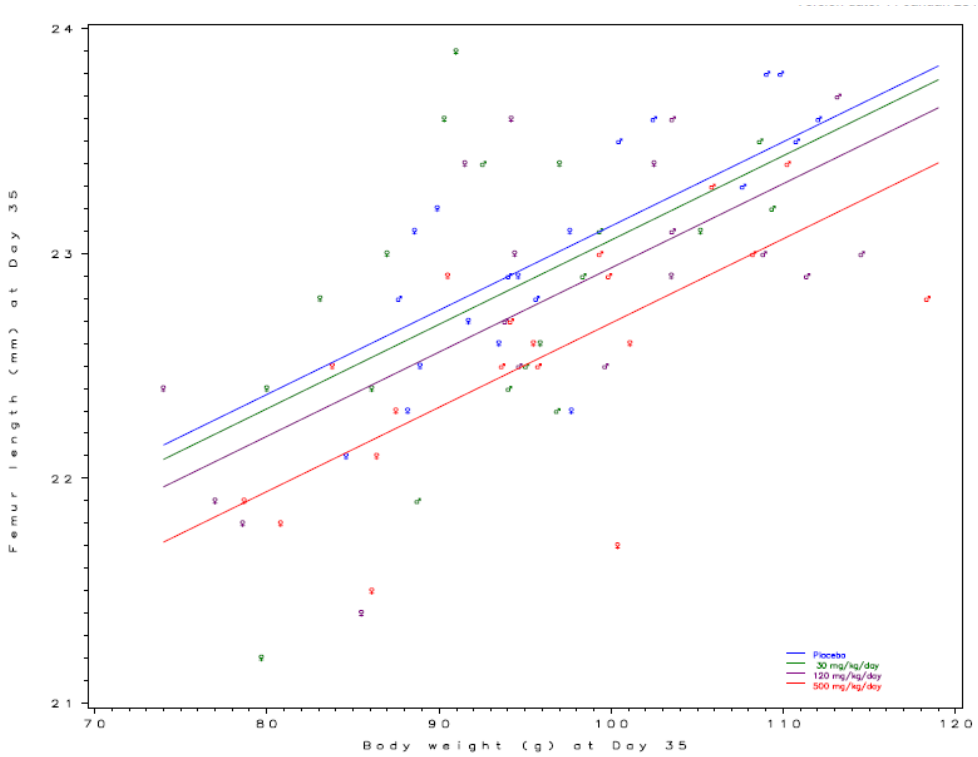


**Figure 2 Femur length versus bodyweight at postnatal day 35. The lines represent the ANCOVA model.**

After body weight correction, the ulna length is still statistically significantly smaller in the 500 mg/kg/day dose group compared to control at the end of recovery. Furthermore, a statistically significantly smaller femur length was found at 500 mg/kg/day dose group at the end of dosing and at the end of recovery. For the 120 mg/kg/day dose group, a statistically significantly smaller femur length compared to control was found at the end of recovery. No statistically significant difference was observed at 30 mg/kg/day and therefore is identified as NOAEL. These results further indicated that the observed bone length decrease in Org 25969 treated groups at 120 and 500 mg/kg in the previously submitted 4-week juvenile toxicity study was not due to the body weight decrease.

# 8 Integrated Summary and Safety Evaluation

*Summary of the nonclinical data associated with bone evaluation in the original NDA submission*

*In vivo* studies in the rat indicated that Org 25969 is rapidly distributed to the bone and is retained in these tissues for extended periods of time with mean $t_{1/2}$ of 172 days (range from 67 – 210 days), which raised the concern of bone toxicity due to long-term retention. A micro-autoradiography study in young male adult rats showed labeled Org 25969 in active bone formation areas such as periosteal and endosteal aspects of cortical bone and endosteal aspects of trabecular bone in femur and scapula. The

Applicant stated that Org 25969 did not bind to growth plates based on the results of the study. However, the nonclinical review team concluded that additional evidence is needed in support of the conclusion of Org 25969 absence in growth plate because growth plates were not identifiable in most of the examined bone tissues, and poor micrograph quality and errors in the micrograph descriptions diminished the reliability. Nonclinical data in the original NDA did not characterize the binding site of Org 25969 in the bone tissue completely.

The bone effects of Org 25969 were evaluated in adult rats. Single-dose 2000 mg/kg administration caused a statistically significant change in bone parameters via μCT assay and bone turnover marker assay, suggesting a minor bone resorptive effect with greater severity in females than males. The mechanism for bone change after single-dose Org 25969 administration has not been characterized. The Applicant proposed a possible hormonal explanation for the reduced trabecular strength and volume because a PTH surge was observed in both rats and dogs upon Org 25969 administration in the original NDA. However, this immediate (appeared 3 minutes after dosing) and transient (lasted <30 min) PTH surge is probably not relevant in the reduction of trabecular mass and strength since bone changes persisted through 3-6 weeks following single dose administration. The NOAEL of Org 25969 on bone effect was identified to be 500 mg/kg after single dose administration, corresponding to approximately 6-fold or 17-fold safety margin for human administration at 16 mg/kg based on AUC or bone concentration estimation, which considered to be sufficient to support the safety of Org 25969 for human use under the proposed dosage. However, the effect of Org 25969 on healing of bone fracture in non-clinical models and human has not been studied and therefore is not known. This concern was raised because higher level of Org 25969 may deposit in the bone during bone healing process.

Bone effects of Org 25969 were examined in juvenile rat studies to support pediatric trials because of the high amount of bone absorption in juvenile animals as observed in the distribution studies. In a single-dose treatment study in juvenile rats, significant changes in bone were not observed at up to 500 mg/kg Org 25969 administration. Assuming that plasma AUC of Org 25969 in pediatrics is similar to that in human adult, single-dose pediatric exposure with proposed dosage in adults does not appear to impose significant safety concern.

A 28-day repeat-dose toxicity study was conducted to further investigate the bone effect of Org 25969 in rats. The results revealed minor decreased ulna length, femur length and weight after 4-week daily treatment with more severe effect apparent on Day 91 (end of recovery period) at 120 and 500 mg/kg. These changes were not coincident with the slight body decrease occurred during the early stage of recovery period (Day 35-70). In addition, decrease in polar moment of inertia, a parameter indicative of the ability of bone to resist torsion, was observed on Day 91 in all groups of males and 500 mg/kg group of female. All these may suggest a prolonged effect on bone growth. It is not known if this effect was primarily due to systemic or local bone exposure of Org 25969. Bone strength assessment and bone turnover markers were not conducted in this study. The NOAEL was identified to be 30 mg/kg which corresponds to approximately 22



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μg·hr/ml in plasma AUC and 150 μg/g bone Org 25969. By plasma AUC comparison, exposure at NOAEL in juvenile rats (22 μg·hr/ml) did not cover human exposure at 4 mg/kg (40 μg·hr/ml). By bone concentration estimates comparison, exposure at NOAEL provided approximately 33 × and 8 × coverage to the human exposure at 4 mg/kg and 16 mg/kg, respectively. However, since the bone effect in juvenile rats has not been well characterized, it was uncertain during the original NDA review cycle whether AUC level or local bone concentration level shall be used for safety margin calculation. It should be noted that both human plasma and bone exposure values were derived from data of adults. Additional safety factors may need to be implemented when they are extrapolated to pediatrics. Therefore, additional non-clinical studies are needed to further characterize the bone effect of Org 25969 especially the mechanism of the lasting bone length decrease in the juvenile rat model to determine safety for pediatric use.

Overall, the bone distribution and effects of Org 25969 were not well-characterized by the nonclinical studies included in the original NDA submission. These included 1) the binding site of Org 25969 was not clear, 2) there was no sufficient evidence to indicate that Org 25969 doe not deposit in the growth plate of bone; 3) the effect of Org 25969 on bone healing was not evaluated, 4) While Org 25969 studies in rats appeared to support the safety of single dose pediatric clinical study up to 16 mg/kg, additional nonclinical studies were needed to demonstrate the safety of proposed clinical dose (likely to be similar to adult human dose according to medical team) prior to the initiation of multiple-dose clinical trial in pediatrics, as well as to support the approval of pediatric indication or inclusion of pediatric data in the label. The comments/requirements included in the non-approvable letter reflected the Division's thoughts to address these issues as well as the concern of bone tumor development raised due to the long-term retention of Org 25969 in the bone.

*Summary and discussion of the Nonclinical Studies included in this NDA re-submission*

The in vitro [$^{14}$C]-Org 25969 binding study (study number 090085) was conducted to investigate whether Org 25969 binds to hydroxyapatite in the bone as proposed in the original NDA submission. Results of this study showed that [$^{14}$C]-Org 25969 bound to hydroxyapatite *in vitro* in a less than dose-proportional manner. The estimated $E_{max}$ for [$^{14}$C]-Org 25969 binding to hydroxyapatite *in vitro* was 19.5 nmol [$^{14}$C]-Org 25969/mg hydroxyapatite, and the estimated $EC_{50}$ of this binding was 10.6 nmol [$^{14}$C]-Org 25969/mg hydroxyapatite. The binding of Org 25969 to hydroxyapatite was not affected by sodium citrate or tetracycline, however, binding was significantly decreased in the presence of bisphosphonate compound alendronate in a dose-dependent manner, indicating that the binding site of Org 25969 to hydroxyapatite at the same site of the bisphosphonates (P-C-P group – 2 phosphonate groups sharing a common carbon atom). In addition, data of this study showed that binding of Org 25969 (1.7 nmol [$^{14}$C]-Org 25969/mg hydroxyapatite) to hydroxyapatite was not affected in the presence of rocuronium. This finding was not consistent with the previous finding in an *in vivo* rat study submitted with the original NDA package. In this previously submitted *in vivo* study (INT00033724), a single IV injection of 4 mg/kg rocuronium (half molar dose of 30



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mg/kg Org 25969) immediately after a single intravenous injection of 30 mg/kg Org 25969 containing 3 MBq/kg [$^{14}$C]-Org 25969 to young adult Wistar rats resulted in a decrease in the percentages of the dose bound to bones and molar (about 36% and 67% for males and 31% and 41% for females, respectively), indicating the presence of the neuromuscular blocker may prevent binding of [$^{14}$C]-Org 25969 to bone. The mechanism for this difference is not known. Dr. Kuijpers indicated "the decrease in the in vivo study may have been a secondary effect due to an effect on the systemic PK of Org 25969." Nevertheless, the results in the *in vivo* rat study indicated that intact animals without rocuronium treatment is a more conservative model to evaluate the safety of org 25969 in patients with neuromuscular blockade induced by rocuronium or vecuronium regarding the bone effects.

To further investigate the location and binding of Org 25969 in the bone, the Applicant submitted an amended study report (Study number: INT00122920) for the microautoradiography study included in the original NDA submission (study number: 060217; study report: INT00029187). However, no information was included in the amended study report to address the issues identified in this study. In the newly conducted microautoradiography study in rats included (study 090071; Study report: INT00142585), growth plates were clearly identified in the examined bone sections of animals (one animal/time point) sacrificed on SD1, SD21, and SD84 following a single intravenous dose of [$^{14}$C]-Org 25969. The results of the study showed that no radioactivity is present in the non-calcified cartilaginous tissues such as the epiphyseal growth plate and the articular surface, rather in the subarticular region (metaphysis) where mineralization occurs 24 hours after administration. Growth plate (epiphyseal plate) contains cartilage cells which undergo mitosis, pushing the epiphysis away from the diaphysis resulting in bone elongation in the long bones such as femur and tibia. This suggested that the effect of Org 25969 on bone growth may not be significant since it does not appear to be involved in the bone elongation process. The subarticular region is the place where new bone formation occurs following cartilaginous tissue growth. During bone formation, bone matrix is secreted within the fibrous membrane and hydroxyapatite is present for calcification. It is reasonable to conclude that org 25969 bound to hydroxyapatite and deposited in the new formed bone area after administration. In addition, 24 hours after administration, Org 25969 was also found to be present in the outer trabecular, endosteal, periosteal and inner articular aspect surfaces, areas which are sites of bone mineralization during bone remodeling. After 21 and 84 days of administration, labeled Org 25969 was not seen in the area of new bone formation but distally in the diaphysis. Radioactivity was observed in the trabecular and cortical bone at day 21, but only cortical bone at day 84. The Sponsor indicated that "The change of location is associated with growth of the femur and not with redistribution of the radiolabel as black granular silver staining is still distinct". However, Dr. Kuijpers indicated that redistribution was possible by comparing autoradiography data obtained in a study with [$^{14}$C]-clodronate, a bisphosphonate compound (Osterman et al., 1997). In this clodronate study, radiolabel was situated further away from the growth plate and under the cortical bone surface after up to 79-days after dosing, due to longitudinal and appositional bone growth, respectively. Time lapse did not change the continuous pattern of the distribution, as opposed to what was seen with Org 25969, as



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Dr. Kuijpers indicated. However, the potential for redistribution is not necessarily a safety concern since Org 25969 still located in the hydroxyapatite mineralization area as shown in the microautoradiography study. Overall, both the in vitro binding study and microautoradiography study provided sufficient information to fulfill the nonclinical requirement 4 in the non-approvable letter which is to "Provide definitive information on the binding site of sugammadex in bone as well as a reevaluation of bone localization of sugammadex as presentation of the data on bone localization was not clear and errors in descriptions were noted in the submitted materials".

In the previously submitted 28-day toxicity study in rats in the original NDA, animals (7 days of age at dosing start) were treated with Org 25969 daily for 28 days at 0, 30, 120, and 500 mg/kg Org 25969. Minor decrease in ulna length (≤5%), femur length (≤3.5%) and weight (≤6.5%) were observed with the most severe effects at the end of 8-week recovery period at 120 and 500 mg/kg Org 25969 dose levels. The Applicant indicated that the minor bone length decrease was due to smaller size of the animals in Org 25969 treated groups. The Division did not agree with the Applicant after review of the 28-day daily dosing study because the change in bone length was not coincident with the body weight decrease. In this submission, data of this study were re-analyzed using ANOVA analysis with body weight as the covariate (INT00122920). The results showed that the minor bone length decrease was still statistically significant, further suggesting that the decreased bone length following Org 25969 administration in juvenile rats might not be associated with body weight change.

The 8-week intermittent dosing toxicity in rats (study 080229) was conducted to further clarify the observation in the 28-day juvenile toxicity study in order to fulfill the nonclinical requirement 3 in the non-approvable letter. In this 8-week intermittent dosing study, juvenile rats were dosed weekly from PND7 for 8 doses at 0, 7.5, 30, or 120 mg/kg Org 25969, and the animals were sacrificed the second day after last dosing. The protocol outline of this study was submitted to the division for advice prior to the study initiation. The Agency agreed that weekly dosing appeared to be better mimicking the clinical dosing situation as compared to daily dosing but recommended the Applicant to pick a higher top dose in order to maximize the chance of detecting a bone effect. However, the Applicant believed that a dose that has no impact on growth but achieves significant systemic and local (bone and teeth) exposure multiples of Org 25969 clinical exposure should be chosen because this study should differentiate between a direct effect on bone growth and a secondary effect related to a general lower growth rate. In my opinion, the Applicant's rationale for dosing selection was not logically correct because it is possible that there is no relationship between the bone effect and body weight change but happen to occur at the same dosing level due to different mechanism. However, if a NOAEL can be identified in this study and provide sufficient safety margin to cover the dosage that will be used in pediatric patients, the issue of the safety concern of the bone effect of sugammadex in pediatrics may be considered to be appropriately addressed. In this study, an additional dosing group (10 rats/sex/group) was treated with 250 µg/kg Pamidronate as positive control on bone parameters; results of pamidronate treatment group indicated the rat is a sensitive model for bone change.



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Significant difference between the Org 25969 administered groups and control were not found using assays including *in vivo* ulna bone length measurement, postmortem ulna and femur length measurement, µCT parameters including bone mass, volume and thickness, and parameters of bone strength tests. Dr. Kuijpers noted that "A small (8%) decrease in humeral but not tibial growth plate thickness was noted in males, but not females, at the 120 mg/kg high dose, on Day 50 but not Day 105". She further indicated in her review that "This decrease may indicate some negative effect on chondrocyte proliferation or enhanced apoptosis of the hypertrophic chondrocyte and may have been accompanied by a reduction in humerus bone length" (humerus length was not measured in the study). However, she considered the high dose (120 mg/kg) as the NOAEL in this study with the results of all other parameters taken into consideration. I agree with this NOAEL identification. The observed change in humerus epiphysical disc thickness may not represent a treatment-related effect because this change was only observed in one gender and similar changes were not seen in tibia (tibia length was not changed wither). In addition, the overall change was small (30 mm in control vs. 28 mm at 120 mg/kg), which may not suggest a significant biological effect. Overall, I agree with Dr. Kuijpers that significant adverse changes were not seen in Org 25969 treated groups after 8 weekly dosing as compared to control and 120 mg/kg is considered to be the NOAEL in this study. This study fulfilled the nonclinical requirement 3 in the non-approvable letter on providing a repeat-dose study of sugammadex in the juvenile rat to clarify the observed bone length change in the 28-day juvenile toxicity study. Of note, the results of this study can not override the results of the previously submitted 4-week daily dosing toxicity study. Although data showed that Org 25969 did not bind in growth plate in the bone, binding of Org 25969 with extracellular hydroxyapatite may indirectly affect bone growth as Dr. Kuijpers indicated. The absence of bone effects at 120 mg/kg in this 8-week intermittent study may be due to the lower bone concentration as compared to that at the 120 mg/kg daily dose in the 4-week study because of the long $t_{1/2}$ of Org 25969 in the bone. As to the plasma exposure of Org 25969, it is not expected to be significantly different between the two dosing regimens because of the short $t_{1/2}$ of Org 25969 in the blood.

The effect of Org 25969 on bone repair was evaluated in adult animals (study number: 090319). The bone fracture was produced by surgery at 12 weeks of age and animals were administered Org 25969 for 6 weeks by weekly dosing prior to or after fracture surgery at 0, 30, 120, and 500 mg/kg. The purpose of the study was to determine whether pre- and post-fracture treatment of Org 25969 may adversely affect the bone healing process. The results of the data did not show evidence of adverse change on bone healing in animals with pre-fracture treatment in any Org 25969 dosing group as compared to control as assessed by pQCT scan, µCT scan, bone strength test, and histopathology examination. With post-fracture treatment, statistically significant changes were observed at 500 mg/kg groups as compared to the concurrent control. µCT scanning and histopathology examination revealed slight increase in callus formation and slight decrease in bone formation at 500 mg/kg. In addition, the extent of bridging at the fracture site by new bone also appeared to be slightly decreased at this dosing level by histopathology examination. All these suggest a slight delay in bone healing process. Dr. Kuijpers dismissed the slight change in the µCT parameters



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because of lack of statistical significance, but not the histopathological findings. I agree with her on this evaluation and also agree with her that "it is uncertain whether the changes in the histologic scores had clinically relevant consequences" because significant changes were not observed BMD and BMC as well as bone strength test. I think the toxicologic significance of these changes may be questionable. Since the control groups of pre-fracture treatment were only dosed with saline and these animals were fractured and sacrificed at the same time as the animals for post-fracture treatment. The pre-fracture control groups may be considered a secondary control of the post-fracture Org 25969 treatment groups. In comparison to the values of both control groups, the values of the parameters that were shown to be statistically significant were within the range of control values if male and female data are combined. However, I also noted that the observed changes which suggest slight delay in bone healing process appear to be consistent with the observed bone growth reduction in the 4-week toxicity study in juvenile rats. Thus, I agree with Dr. Kuijpers to not dismiss the observed findings in this study and conclude that the NOAEL in post-fracture treated animals is 120 mg/kg, while in pre-fracture treated animals is 500 mg/kg. This bone repair study in adult rats fulfilled the nonclinical requirement 1 to "provide an evaluation of sugammadex in a nonclinical model of bone fracture to examine potential effects of the drug on bone healing".

Requirement 2 in the non-approvable letter is also considered to be fulfilled because data of growth plate morphology were included in the microautoradiography study and the 8-week intermittent toxicity study.

Studies included in this submission are considered to be sufficient to suggest long-term retention of sugammadex in the bones of pediatric patients may not confer a risk for bone tumor development. These studies demonstrated that Org 25969 binds to extracellular matrix and likely not associated with bone cell proliferation and differentiation. In addition, histopathology examination did not show signs of carcinogenicity such as hyperplasia or hypertrophy of bone cells after 16-week Org 25969 exposure in the bone.

*Safety Margin Calculation*

There is no pediatric clinical study protocols included in this submission. Based on a plan for pediatric trials submitted previously, the dosage will likely be a single treatment of 2 mg/kg and 4 mg/kg in future clinical studies. The Applicant did not indicate whether a multiple dose clinical study in pediatrics will be conducted or not in the pediatric study plan. To determine if the 8-week intermittent toxicity study in juvenile rats may support the safety of future clinical trials in pediatrics, safety margins for pediatric dosage are calculated. In addition, safety margins for human dose were also calculated based on the bone repair study to determine the safety of sugammadex in human subject with fracture.



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The plasma $AUC_{0-24hr}$ of Org 25969 was approximately 40 µg·hr/ml and 160 µg·hr/ml at 4 mg/kg and 16 mg/kg in human, respectively. The bone concentration of Org 25969 in adult human was estimated to be maximally 4.5 µg/g and 18 µg/g bone at 4 mg/kg and 16 mg/kg, respectively, based on a mass balance study in human adult subjects. From this human mass balance study and other single dose recovery clinical studies, it was estimated that the recovery of Org 25969 in urine is at minimum 85% (worst-case estimate). Assuming the 15% of the unrecovered Org 25969 were entirely distributed into the bone and teeth (worst case scenario estimation), the amount of Org 25969 in the bone was calculated with bone and teeth weight being approximately 13.3% of human body weight in adult. A mass balance study has not been conducted in pediatrics. Because of the higher growth rate, bone concentration in pediatrics would be higher than adults after Org 25969 administration. Dr. Kuijpers estimated that bone retention in pediatric patients may be 2-3 fold higher than that in adults (i.e. 12 µg/g in 4 yrs old and 9 µg/g in 10 yrs old vs. 4.5 µg/g in adult) based on rat data and comparison on bone weight/body weight ratio between human adult and pediatrics. The animal data Dr. Kuijpers used for this extrapolation was from a bone retention study that was submitted in the original NDA (INT00033724) with the ratio of bone and teeth weight vs. body weight taken into consideration. The results in this study showed that the extent of Org 25969 deposition per pooled bones was approximately 13%, 3%, and 0.5% in juvenile (7 days of age), young adult (6 weeks of age), and aged animals (1 year of age), respectively, 24 hours after single-dose administration of 30 mg/kg Org 25969 including 3 MBq/Kg [$^{14}$C]-Org 25969 (young adult and aged) or 15 MBq/kg [$^{14}$C]-Org 25969 (juvenile). An later conducted rat bone retention kinetics study included in this submission showed that the % of the dose per pooled bones was approximately 7.3% and 3.3% in juvenile (7 days of age) and young adults (6 weeks of age), respectively, 24 hours after single dose of 30 mg/kg Org 25969 including 4 MBq/Kg [$^{14}$C]-Org 25969 (INT00150298). These data suggested that Dr. Kuijpers' estimation of pediatric bone concentration be reasonable (details of this estimation can be found in Dr. Kuijpers consultation review).

Plasma exposure and bone concentration was not examined in the 8-week juvenile rat weekly toxicity study and the adult rat bone repair study. Bone concentrations of Org 25969 after weekly dosing were examined in a separate rat study (INT00141634). In this study, Org 2596 was administered weekly for 7 weeks beginning in juvenile at 7 days old to mimic the animal age and dosing regimen of the 8-week intermittent toxicity study, 6 weeks in young adult (6 weeks of age) to mimic animal age and the pre-fracture dosing regimen in the bone repair study, and 6 weeks in adult (12 weeks of age) to mimic the animal age and post-fracture dosing regimen in the bone repair study. It should be noted that animals were sacrificed 7days after end of dosing in this study; therefore, the values of bone concentrations calculated in this study may be slight lower than the peak Org 25969 concentration in these animals. The AUC value was derived from the single-dose Org 25969 PK studies in adult rats since the animals were all at the adult age at the end of dosing in both of these studies. Of note, this value was similar to the AUC in juvenile at 7 days of age after single-dose sugammadex administration.



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The safety margins for the 4 mg/kg human dose were calculated by Dr. Kuijpers and the table is shown below rats based on dosage, plasma AUC, and bone concentration comparison. Of note, the usefulness of dosing level comparison is limited with the AUC data available, and thus will not be discussed here.

Safety Margin for sugammadex single-dose treatment at 4 mg/kg in adult and pediatric subjects

| | | Rat | Human | Safety Margin |
|---|---|---|---|---|
| | | | | |
| Juvenile rat | Dose | 120 NOAEL | 4 | 30x |
| | AUC (ugxh/mL) | 254 | 40 | 6.4x |
| | Bone conc (ug/g) | 400 | 4.5 (adult) | 89x |
| | | | 9 (child 10 yrs) | 44x |
| | | | 12 (child 4 yrs) | 33x |
| | | | | |
| | | Rat | Human | Multiple |
| | | | | |
| Young adult rat | Dose | 120 NOAEL | 4 | 30x |
| (Fx healing study) | AUC (ugxh/mL) | 230 | 40 | **5.8x** |
| | Bone conc (ug/g) | 235 | 4.5 (adult) | **52x** |
| | | | 9 (child 10 yrs) | **26x** |
| | | | 12 (child 4 yrs) | **20x** |
| | | | | |
| | Dose | 500 NOAEL | 4 | 125x |
| | AUC (ugxh/mL) | 1101 | 40 | **28x** |
| | Bone conc (ug/g) | 480 | 4.5 (adult) | **107x** |
| | | | 9 (child 10 yrs) | **53x** |
| | | | 12 (child 4 yrs) | **40x** |

I agree with this evaluation. As shown above, the submitted 8-week intermittent weekly dosing toxicity in juvenile provide sufficient safety margins to support the safety of 4 mg/kg human dose in adult and pediatrics regarding bone effect based on both plasma AUC and estimated bone concentration comparison. Because the safety of sugammadex 4 mg/kg and 16 mg/kg in adults and single-dose clinical study in pediatrics were determined to be supported by data submitted in the original NDA submission, this review will focus on the adequacy and sufficiency of the 8-week study to support the safety of future multiple dose pediatric clinical study. It should be noted that the AUC values and bone concentration estimation in human was based on single-dose administration. For AUC, due to the short $t_{1/2}$ of sugammadex in vivo (<1 hour), the AUC level of sugammadex after weekly administration was generally equivalent to that of single-dose. Therefore, the 8-weekly intermittent toxicity study in juvenile rats technically will support the safety of a multiple-dose pediatric clinical study of same duration (up to 8 weeks) with 4 mg/kg daily weekly dosing based on AUC comparison.

For bone concentration, binding of sugammadex to the bone was not easily saturated and bone concentration increased in a less than dose-proportional manner based on the original NDA submission. Data from in the original NDA submission showed that the bone concentration was about 200 µg/g bone at 120 mg/kg after single dose administration in young adult rats (6 weeks of age). In comparison, PK data in this submission showed that the bone concentration was approximately 0.46 mg/g bone at



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120 mg/kg after 6 weekly dosing starting from young adult rats (6 weeks of age). In addition, 7 weekly dosing at 120 mg/kg starting from juvenile rats (7 days of age) and ending at 8 weeks of age resulted in approximately 0.43 mg Org 25969 per gram bone. These data indicated that weekly dosing up to 7 doses from juvenile or young adult rats may cause about 2-fold accumulation as compared to single-dose administration. As the above safety margin table showed, the 8-week intermittent bone study in juvenile provided ≥ 33-fold safety margin for pediatrics at 4 mg/kg with single-dose administration based bone concentration comparison. Therefore, this study will likely provide ≥ 16.5-fold safety margin for pediatric at 4 mg/kg with weekly administration up to the same duration (8 weekly dosing) based on bone concentration comparison if similar bone accumulation is assumed in human as that in rats. The question has been raised that whether plasma AUC or bone concentration is more relevant in safety evaluation regarding bone effect. Bone concentration may be more relevant because bone effect was due to the deposition and binding of Org 25969 in the bone which depends on the plasma exposure as well as the growth rate of bone. The caveat for using bone concentration in safety evaluation is that the exact bone concentration in pediatrics is not known and but based on estimation from mass balance study in human adults. However, this estimation as indicated above may be a conservative estimation because assumption was made that all unrecoverable Org 25969 was absorbed in the bone which is the worst scenario. In addition, in vivo rat study indicated that presence of rocuronium decreases Org 25969 binding in the bone up to 30% when the molar ratio of rocuronium to Org 25969 was 1:2. Since Org 25969 will be given to patients with rocuronium present in the blood in the pediatric clinical trial or clinical use if approval, the 8-week intermittent toxicity study in juvenile without rocuronium treatment provides additional safety margins for Org 25969 use in patients.

The Applicant plans to conduct clinical trials in pediatrics from age of 18 yrs old down to 2 month old based on the submitted pediatric trial plan for single dose clinical studies. This raised the question whether the currently available data support the safety of sugammadex treatment in pediatrics below 2 years old because renal functions are not considered to be mature. Published data demonstrated that the renal function continue to develop during postnatal period up to 4-6 weeks in rats (Solomon, 1977; Zoetis and Hurtt, 2003). With the large safety margin to pediatric patients at ≥ 4 yr old as shown in the table above, the 8-week intermittent toxicity study initiated in PND7 rat pups is considered to be adequate to support the safety of sugammadex treatment in pediatric patients under 2 years old.

Although the Applicant did not have a plan to conduct a clinical study with 16 mg/kg dose, it is possible this dose will be tested in pediatrics for immediate reversal of neuromuscular blockade as proposed in adult patients. Assuming a linear increase of AUC and bone concentration which is the worst scenario because bone deposition of Org 25969 was found to be less than dose proportional, the 8-week toxicity study in juvenile provides sufficient margin for 8-week weekly dosing clinical study in pediatrics based on plasma AUC (6.4/4 = 1.6-fold) and bone concentration (≥ 16.5/4 =4-fold).



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Of note, in real clinical situation, 8 doses with weekly frequency in an individual patient will be very rare. Therefore, the 8-week intermittent toxicity study in juvenile is considered to be an exaggerated evaluation of the safety of sugammadex bone effects in real clinical situation. Possibility of sugammadex multiple daily dosing is even lower in clinical situation. However, a short term (i.e. < one week) daily dosing may occur in rare occasions. The 8-week intermittent toxicity study in juvenile rats is not considered to be appropriate to support daily dosing clinical use of sugammadex because of the difference in dosing regimen. However, the previous conducted 4-week daily dosing toxicity study provided information on safety of potential multiple daily dose clinical study or clinical use after approval. As indicated above, although exposure (22 µg·hr/ml) at NOAEL (30 mg/kg) in this did not cover human exposure at 4 mg/kg (40 µg·hr/ml) by AUC comparison, it provided approximately 33-fold and 8-fold coverage to the estimated adult human exposure at 4 mg/kg and 16 mg/kg, respectively, by bone concentration comparison. Again, the estimated bone concentration in human was based on single-dose mass balance human study which is a conservative estimation. With the application of similar accumulation factor (2-fold) and estimated difference between the adult and pediatrics in bone deposition after sugammadex administration (2-fold), the exposure at the NOAEL in the 4-week toxicity study will provide 8-fold and 2-fold coverage to the estimated bone concentration in pediatrics with daily dosing at 4 mg/kg and 16 mg/kg, respectively. Therefore, a short-term (i.e. < one week) daily dose pediatric study up to 16 mg/kg is considered to be safe based on the 4-week daily dose toxicity study.

Similarly, the bone repair study in adult rats provide sufficient safety margin to support Org 25969 treatment in adults with the proposed dosage. Significant adverse effects were not expected on the bone healing process in patients with bone fractures following 4 mg/kg or 16 mg/kg weekly treatment for 6 weeks. The plasma AUC and bone concentration in the adult rat bone healing study also covered the pediatric use of sugammadex at 4 mg/kg and 16 mg/kg. The adequacy of this bone repair study in adult rats to support the safety of sugammadex treatment to pediatric patients with bone fracture was discussed with Dr. Kuijpers. Since bone growth is continuous in rats during adulthood, this model is considered to be appropriate to mimic the bone healing process in pediatrics with bone fracture. Therefore, sugammadex treatment in pediatric patients with bone fracture is also considered to be safe with the proposed dosage.

Overall, nonclinical studies included in this submission successfully fulfilled the requirements outlined in the non-approvable letter for the original NDA submission. In addition, the 8-week intermittent toxicity study provided sufficient evidence to support the initiation of multiple-dose clinical trials in pediatric patients. It should be noted that it was determined that nonclinical data in the original NDA submission were sufficient to support the safety of pediatric study regarding teeth effect and additional nonclinical studies were not likely to be required based on the consult review and AC recommendation (see details in Dr. Wasserman's secondary review for the original NDA submission). In addition, significant changes were not observed in the teeth in the 8-week intermittent toxicity study. Therefore, the effects of sugammadex in teeth were not



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discussed in this review. There are no additional nonclinical recommendations from this reviewer.

Reference List

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Zoetis T, Hurtt ME (Species comparison of anatomical and functional renal development. Birth Defects Res B Dev Reprod Toxicol 68:111-120.2003).

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/s/

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ZENGJUN XU
08/23/2013

ADAM M WASSERMAN
08/23/2013
I concur with Dr. Xu that the application may be approved from the nonclinical perspective. Please also see my Supervisory memo.

# PHARMACOLOGY/TOXICOLOGY CONSULT REVIEW

**Nonclinical Pharmacology/Toxicology**
**DAAAP Consult**

**For Internal Use Within FDA Only**

| | |
|---|---|
| **IND/NDA#:** | **NDA #22-225** |
| Sponsor: | Merck |
| Drug: | Org 25969, sugammadex sodium injection ( (b) (4)) |
| Indication: | Reversal of neuromuscular blockade induced by rocuronium or vecuronium |
| Clinical dose: | Routine reversal: 4 mg/kg; Immediate reversal: 16 mg/kg |
| Route: | IV bolus injection |
| Submission: | December 20, 2012 |
| | |
| Reviewer: | Gemma Kuijpers, CDER/DRUP, Pharmacology/Toxicology |
| Consult request date: | March 4, 2013 |
| Review date: | August 19, 2013 |
| | |
| To: | Diane Walker, ODE2/DAAAP |
| Through: | Lynnda Reid, Ph.D., CDER/DRUP, Supervisory Pharmacologist |
| Tracking No.: | 406 |

## BACKGROUND

Sponsor has developed a modified gamma-cyclodextrin derivative (Org 25969; sugammadex) for reversal of neuromuscular blockade (NMB) by rocuronium or vecuronium. The mechanism of action consists of complex formation and removal of the NMB agent from the neuromuscular junction.

## NDA submission

The Sponsor applied for approval for the use of sugammadex in the adult population in their October 30, 2007, submission of NDA 22-225 (b) (4) The NDA was given a Not Approvable (NA) letter on July 31, 2008, based on insufficient data to fully assess safety, particularly related to the risk of anaphylaxis. Also included in the NA letter were recommendations for additional nonclinical studies needed to support pediatric studies or indications (APPENDIX 1). DMEP was consulted at the time of the original NDA submission since sugammadex binds to bone tissue and data from a juvenile rat study provided a signal that the interaction may have negative consequences for bone growth in children.

## NDA resubmission

Sponsorship of the NDA was transferred to Merck. The new Sponsor resubmitted the NDA on December 20, 2012. The Sponsor is seeking approval to market sugammadex for routine reversal of moderate or deep NMB induced by rocuronium or vecuronium,



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and immediate reversal of NMB at 3 minutes after administration of rocuronium. Specifically, Merck is proposing the use of sugammadex in three situations:

For routine reversal:
1. *A dose of **4 mg/kg** sugammadex is recommended if recovery has reached 1-2 post-tetanic counts (PTC), train-of-four (TOF)-count 0 (deep blockade) following administration of rocuronium- or vecuronium-induced blockade.*
2. *A dose of **2 mg/kg** sugammadex is only recommended if spontaneous recovery has reached the reappearance of T2 (moderate blockade) following rocuronium- or vecuronium-induced blockade.*

For immediate reversal:
3. *If there is a clinical need for immediate reversal at 3 minutes following administration of rocuronium, a dose of **16 mg/kg** sugammadex is recommended.*

In the resubmission, the Sponsor provided the reports of several new nonclinical studies to address the pediatric development issue raised in the NA letter. The Agency provided feedback on the protocols for 4 of these nonclinical studies in 2009.

**NONCLINICAL INFORMATION IN ORIGINAL NDA**

Sugammadex binds to the skeleton and teeth (in rats) and is retained in these hard tissues for extended periods of time ($T_{1/2}$ = 67-210 days), while it is excreted much faster from the systemic circulation ($T_{1/2}$ = 1-2h). Specific binding to bone matrix hydroxyapatite was postulated based on the presence of multiple anionic carboxylic acid residues i.e. thiopropinate groups in the substituted cyclodextrin molecule, but in vitro binding data were not included.

The binding of sugammadex to bone was characterized in *in vivo* distribution and microautoradiography studies. Retention in rat bones was 13% (juvenile), 3% (young adult), and 0.5% (aged rats) of the administered dose per pooled bones, at 24h post dose. The increased bone retention in juvenile rats was thought to be due to rapid growth of bone containing multiple sites of bone resorption, formation and mineralization. A similar finding of increased bone retention in young vs. older rats has been reported for alendronate. A microautoradiography study in young rats showed radiolabel on cortical and trabecular bone surfaces. There was no binding to the femoral growth plate or calcified cartilaginous zone. The Sponsor concluded that sugammadex binds to or near sites of 'active bone formation'.

Treatment of juvenile rats for 4 weeks with an 8-week recovery period (doses 0, 30, 120, 500 mg/kg/day) resulted in a slight, dose-dependent, persistent reduction in bone length (NOAEL 30 mg/kg/day). Body weight was slightly reduced particularly upon recovery in males. Slight, reversible changes in trabecular and cortical bone structure were also observed. It was unclear how the effects on bone length and body weight were related to each other since they did not always coincide. Bone resorption or formation markers and bone strength were not measured. Quantitative data on growth plate dimensions were not obtained. The safety margin for this effect was small (1x) based on AUC, but much larger



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(≥33x) based on bone concentration comparison. Sponsor dismissed the bone effects because they were small and considered to be associated with the slight general growth retardation and therefore nonspecific and not adverse.

The DAAAP Nonclinical Reviewers (Dr. Zengjun Xu, Dr. Adam Wasserman) recommended that the original NDA could be approved for the adult population without further nonclinical evaluation. Nonclinical studies were also not required for initiation of single-dose clinical trials in pediatric patients. However, nonclinical evaluations were deemed necessary prior to multiple-dose pediatric trials, approval of a pediatric indication, or inclusion of pediatric data in the label (NA letter, APPENDIX 1). Feedback from DAAAP to Sponsor regarding the additional nonclinical evaluations was provided in the Type A meeting of December 1, 2008. In addition, comments were provided by DAAAP to the submission of July 23, 2009 containing outlines of four planned nonclinical studies.

## NONCLINICAL STUDY REPORTS IN NDA RESUBMISSION

Eight (8) nonclinical study reports were included in the resubmission. DBRUP was consulted by DAAAP to review the submitted nonclinical information, specifically to decide whether the new (bone) data support pediatric studies to be conducted in the future. Specific pediatric study protocols have not yet been submitted.

| Nr. | Report | Title |
|---|---|---|
| | | |
| #1 | INT00122920 (Study 060217) | [$^{14}$C-Org 25969]: The distribution of radioactivity in bone after a single intravenous dose of [$^{14}$C]-Org 25969 to Wistar rats |
| #2 | INT00145198 (INT0038389) (Study 060086) | Statistical evaluation of the effect of Org 25969 on ulna length and femur length correcting for effects on body weight in juvenile rats of study 060086 |
| #3 | INT00127799 (Study 090085) | In vitro binding of [$^{14}$C]-Org 25969 (Org 25969-2) to hydroxyapatite |
| #4 | INT00142585 (Study 090071) | Microautoradiography in bone after a single intravenous dose of [$^{14}$C]-Org 25969 to Wistar rats |
| #5 | INT00150298 (study 100045) | Bone retention kinetics in juvenile and young adult Wistar rats after a single subcutaneous injection of [$^{14}$C]-Org 25969 (Org 25969-2) and Org 25969 (Sugammadex) |
| #6 | INT00141634 (study 090310) | Study of quantitatively assess binding of [$^{14}$C]-Org 25969 (Org 25969-2) and Org 25969 (Sugammadex) in bone and teeth upon intermittent dosing in Wistar rats |
| #7 | INT00150243 (Study 080229) | An 8-week intermittent dosing toxicity with Org 25969 to assess effects on bone quality, structure, turnover, strength and growth and development in juvenile Wistar rats |
| #8 | 090319 (study AA86536) | Effects of Org 25969 on fracture repair in the Wistar rat |

**Study #7** was conducted in juvenile rats to determine whether the body and/or bone growth effect observed in the original 28-day daily dosing study could be reproduced with a more clinically relevant weekly dosing regimen. **Study #8** was a new fracture healing study in young adult rats. The accompanying **Study #6** was done to provide data



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on bone retention (parameter: [ug Org 25969/gram bone]) following the intermittent weekly dosing regimen employed in Studies #7 and #8.

**Study #1** was the amended report of previous microautoradiography study (Study 060217). **Study #2** was a re-analysis of data from the previous 28-day juvenile rat study regarding the bone length effect. **Study #3** was a new in vitro study on the binding of Org 25969 to HA. **Study #4** was a repeat microautoradiography to evaluate the localization of Org 25969 in bone tissue of young growing animals. **Study # 5** was done in juvenile and young adult rats in order to compare retention (parameters: [%dose/gram bone] and [%dose/pooled bones] of a single dose of Org 25969 in juvenile vs. young adult rats - and to extend bone retention data to later times. AUC data were also obtained in this study.

**DAAAP questions:**

The consulting Division has the following questions in order to determine whether the nonclinical studies included in this NDA resubmission addressed the identified issues in the Not-Approvable-Letter:

1. Do you think that the potential effects of sugammadex on bone healing have been sufficiently evaluated and the data indicate no safety concern of sugammadex treatment on healing of fractured bone?
2. Do you think that the data in the microautoradiography study (INT00142585) provided sufficient morphological information on growth plate to help understand the effect of sugammadex on bone growth and development?
3. Are the dosing selection rationale and study design employed in the 8-week intermittent dosing toxicity study in juvenile rats (INT00150243) adequate and did the study appropriately clarify the observations in the 28-day juvenile toxicity study included in the original NDA?
4. Based on the data of the microautoradiography study (INT00142585) and the 8-week intermittent dosing toxicity study in juvenile rats (INT00150243), do you agree that sugammadex is not likely to adversely affect bone growth and development?
5. Do you think that the nonclinical studies included in this submission provided definitive information on the binding site and localization of sugammadex in bone? Did the data provide sufficient evidence to demonstrate that Org 25969 binds to hydroxyapatite in the bone and distributes in the area of bone mineralization only?
6. Do you think the nonclinical studies included in this submission addressed the concern regarding the potential for bone tumor development as indicated in the Not Approvable Letter?
7. Do you think that the nonclinical studies included in this submission provided sufficient evidence to support the initiation of a multiple dose clinical studies in pediatric patients?

***Question 1:***



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*Do you think that the potential effects of sugammadex on bone healing have been sufficiently evaluated and the data indicate no safety concern of sugammadex treatment on healing of fractured bone?*

Fracture healing study

"Effects of Org 25969 on fracture repair in the Wistar rat" (b) (4) Reference AA86536, Study 090319, Study #8)

This study was conducted to evaluate the effect of sugammadex on fracture repair when given before or after occurrence of a fracture. Young Wistar rats were given weekly IV doses of Org 25969 (0, 30, 120, 500 mg/kg/week) (N=20/s/g) for 6 weeks, before or after fracture. A mid-diaphyseal closed femoral fracture (modified Einhorn model), was created using a fracture device with a fracture blade and a weight dropped from a pre-specified height. Before the fracture was made, a steel pin was inserted in the femoral medullary canal for fracture stabilization. The fracture was created one week after the last dose in pre-Fx treated animals. Dosing was started 1 day after fracture in post-Fx treated rats. All animals were sacrificed 12 weeks after the day of fracture, at 24 weeks of age. The dosing regimen was aimed at achieving significant systemic and bone exposure in the absence of general toxicity.

Table 1: Treatment Groups

| Group | Dosing paradigm | Dose Org 25969 (mg/kg/dose) | Dosing regimen | Fracture at | Follow-up/time of terminal evaluation | Gender /Animal #[a]. |
|---|---|---|---|---|---|---|
| 1 | Pre-fracture exposure (Part I) | 0 | Once weekly at 6, 7, 8, 9, 10 and 11 weeks of age | 12 weeks of age (± 3 days) | 12 weeks after day of fracture | 20 M + 20 F |
| 2 | | 30 | | | | 20 M + 20 F |
| 3 | | 120 | | | | 20 M + 20 F |
| 4 | | 500 | | | | 20 M + 20 F |
| 5 | Post-fracture exposure (Part II) | 0 | Once weekly for 6 weeks starting on the day after fracture[b] | 12 weeks of age (± 3 days) | 6 weeks after last day of dosing = 12 weeks after day of fracture | 20 M + 20 F |
| 6 | | 30 | | | | 20 M + 20 F |
| 7 | | 120 | | | | 20 M + 20 F |
| 8 | | 500 | | | | 20 M + 20 F |

[a] Each experimental group was split into subgroups of 10 M + 10 F which were intended to be used for either microCT, pQCT and strength assessments (subgroups A) or histopathological assessment of fracture repair (subgroups B) except as noted in the deviations section. See result section for study inclusion

[b] 6 weeks of treatment covered all critical stages of fracture repair.

Assessments (in-life) included: body weight (weekly), clinical observations, incisor examination, radiographs 2 weeks post-surgery to verify fracture. Fracture assessment after necropsy at 12 weeks post-fracture included (N=10/s/g were used for histopathology and the other N=10/s/g were used for pQCT, uCt and mechanical testing):

- pQCT of mid shaft area of both femurs (BMD, BMC, bone area; measured in 0.5 mm slice perpendicular to long axis of shaft at 50% of total femur length from distal end) in N=10/s/g
- Micro-CT of fracture callus tissue (BV, TV; data from 18-mm scan of fracture site and reconstructed 3D model) in N=10/s/g



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- Mechanical 3-point bending test of fractured femoral mid-shaft (extrinsic parameters: load, stiffness, energy; and extrinsic parameters: ultimate strength, elastic modulus and toughness) in N=10/s/g;
- Semi-quantitative histopathology of fractured femurs in N=10/s/g (mid-coronal sections scored for callus extent, type of new bone formed, bridging of Fx site, inflammation, fibrosis, necrosis). All sections contained the Fx site and ≥1 cm of bone on either side of the Fx site.
- There was no TK assessment. TK data are available from other studies.

As concluded in the DRUP Consult Review of Nov 17, 2009 the study design was adequate.

*Results*

- No effects on body weight, clinical signs and incisor color
- Radiographs were not analyzed
- pQCT densitometry (BMD, BMC, BA):
  - Parameters were not significantly affected in pre- or post-Fx treated
  - As expected, the fractured/healing femur had larger BMC and BA, but slightly lower BMD than the non-fractured contralateral limb.
- Micro-CT (TV, BV):
  - Pre-Fx treated: In males, TV (callus volume) and BV (bone volume in callus) were slightly but not significantly increased in all treated groups, although the effect on TV was significant in the mid dose group; in females, BV and TV were slightly but not significantly decreased in all treated groups.
  - Post-Fx treated: TV (callus volume) was increased and therefore BV/TV was decreased in high dose males and in mid and high dose females; however, variability was large and the effects were not statistically significant in either gender.
- Biomechanical strength (extrinsic and intrinsic):
  - There was large variability in these parameters, in both control and treated groups.
  - Parameters were not statistically significantly affected, in males or females.
  - Strength of the non-fractured site was not assessed.
- Histopathology (scores for various paramters):
  - Generally, callus extent varied from mild to marked (score 1-3); type of new bone usually was mainly (fibro)cartilage or mainly woven bone (score 1 or 2); and bridging varied from none to complete (score 0-4)
  - There were some effects on histopathology parameters in M (males) and/or F (females) (ss=statistically significant; ns = not statistically significant) in post-Fx treated:
    - High dose:
      - Increased extent of callus (Moderate ss effect in M, but <u>no effect</u> in F)



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        - Decreased "type of bone" (i.e. more cartilage and less woven/lamellar bone (M, ns; F, ss); decreased bridging (M, ns; F, ss, marked effect); increased fibrosis (M, ss; F, ns).
    - Mid dose: Decreased bone type, decreased bridging and increased fibrosis, in F (ns) but not M.
  - No effect on inflammation or necrosis at any dose in any group.
  - Effects on bone type, bridging and fibrosis in both sexes suggests possible interference with bone formation/healing in post-Fx treated animals.

It was concluded in the histopathology report:

- For pre-fracture treated animals: *"There were <u>no consistent effects</u> of treatment upon extent of fracture callus, type of bone formed at the fracture site, extent of bridging by new bone at the fracture site; or on extent of inflammation, fibrosis, or necrosis in either male or female rats treated with ORG 25969.*
- For post-fracture treated animals: *"There were <u>no consistent effects</u> of treatment upon extent of fracture callus, type of bone formed at the fracture site, extent of bridging by new bone at the fracture site; or on extent of inflammation, fibrosis, or necrosis in either male or female rats treated with ORG 25969 following fracture induction, <u>in spite of slightly different mean scores (some with statistical significance) for high dose male or high dose female rats, as compared to corresponding control groups</u>."*

*<u>Sponsor's conclusions:</u>*
Sponsor concluded that there were no relevant changes compared to vehicle-treated animals in pQCT, micro-CT, and bone strength parameters, and there were no effects on fracture healing based upon outcomes of the morphological (histopathology) evaluations. The statistically significant changes in morphological scores in the high dose groups were dismissed because they were considered "not consistent or meaningful" and because they showed no apparent correlation with differences in pQCT, uCT or biomechanical strength parameters. Hence, the Sponsor decided that the NOAEL was 500 mg/kg in both pre- and post-Fx treated rats.

*<u>Reviewer's comments and conclusions:</u>*
Histopathology data (type of bone, bridging, fibrosis) suggested that treatment with 500 mg/kg/wk Org 25959 after fracture for 6 weeks interfered with fracture healing. However, the histologic changes were not clearly correlated with changes in pQCT or uCT parameters. For example, the decrease in bone type in high dose females was not accompanied by a decrease in BMC and/or BMD, or a decrease in bone volume (by uCT). In addition, the histologic effects were not clearly accompanied by negative callus/bone strength changes: effects on biomechanical strength parameters in high dose males and females were opposite (increase in males vs. decrease in females), not statistically significant and inconsistently related to histologic effects.

It should be noted that the histopathology data were from other animals than the densitometry/mechanical testing data and this may explain part of the apparent



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discrepancy. Also, the pQCT data were from a very thin (0.5 mm) bone slice that may not have been representative of the whole fracture region.

Biomechanical strength data are important since they provide information on the most clinically relevant mechanical function of (healing) bone. However, as is usually observed in bone biomechanical testing, there was a large variability in the mechanical strength data and the study was unlikely to have sufficient power to resolve small effects. This Reviewer concludes that it is uncertain whether the changes in the histologic scores had clinically relevant consequences and believes that the histologic findings should not be dismissed. Additional animal study/ies with higher or more frequent doses given after fracture would be needed to confirm the histologic effect and determined whether this is accompanied by changes in mineral density and/or biomechanical integrity of the fracture region.

Bone regeneration in fractured bone is a process analogous to bone formation in growing bone. Thus, the decrease in the histologic score for "type of bone" in post-Fx treated animals may be correlated to the decrease in bone length that was previously observed in the 4-week repeat dose juvenile rat study. However, it is unlikely that the negative effects of Org 25969 are due to an inhibition of bone mineralization since this would be expected to be accompanied by increased osteoid (unmineralized bone matrix) in the metaphysis of growing bone and increased growth plate thickness (similar to rickets), which was not observed in the previous 28-day or the new 8-week juvenile rat study (see below). In order to unequivocally identify effects on bone mineralization, data from double fluorescent label histomorphometry studies would be needed.

In conclusion, treatment with 500 mg/kg Org25969 after fracture caused changes in histological scores in the fracture gap, potentially indicating interference of Org 25969 with bone healing and new bone formation. Correlations between changes in histologic parameters vs. densitometric/ CT/ strength parameters could be identified, but - as Sponsor pointed out - were not consistent. This would indicate that the histologic changes had no clear, biologically significant consequence. However, as discussed above, this Reviewer is not prepared to dismiss the histologic changes and concludes that the NOAEL in post-Fx treated animals (males and females) is **120 mg/kg,** while the NOAEL in pre-fracture treated animals (males and females) is **500 mg/kg**.

*AUC and bone concentrations*

The plasma AUC of Org 25969 in rats treated according to the current dose regimen was not determined. However, we can assume that the AUC in these animals is similar to the AUC on Day 1 in previous Study NL0043521, in which (12-week old?) young adult rats were given repeat doses of 30, 120, 500 mg/kg (AUC = 48, 230, 1101 ugxh/mL on Day 1).

Bone concentrations of rats dosed similarly as in the fracture healing study were evaluated in Study #6. Sacrifice was at 12 weeks or 18 weeks of age, 7 days after the last of six weekly doses. Thus, the time of sacrifice coincided with the time of fracture in the



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pre-fracture dosing group, and 6 weeks after fracture (1 week after last dose) in the post-dosing fracture group. Bone concentration data were given for femur, tibia and vertebrae. The data from Study #6 showed that in the 12-week old rats, the bone concentration at 500 mg/kg was ca. 800 ug/gr. In the 18-week old rats, one week after 6 weekly 120 mg/kg doses (NOAEL), the bone concentration was appr. 235 ug/gram (APPENDIX 2).

For the 18-week old (post-Fx treated) rats, the bone concentration had probably decreased since the last dose (by ca. 50%, data from Study #5). However, the concentration would also have increased over the 6 weeks of dosing since $T_{1/2}$ is long and Org 25969 would have accumulated in bone with the weekly dosing regimen. Thus, the average bone concentration over the entire dosing period (12-18 wks) can be assumed to be appr. 235 ug/gr.

For the pre-Fx treated rats, the bone concentration at the NOAEL of 500 mg/kg (800 ug/gr) is expected to decrease during the fracture healing period (to 60% of the Wk1 value in Wk3, based on data from Study #5). Thus, the bone concentration averaged over the first 6 weeks of healing can be assumed to be 480 ug/gr.

AUC data were not available from Study #5 or #6.

Bone concentration data from Study #6

| Study | Species | Dose regimen | Dose mg/kg | microgram/gr bone, 7 days after last dose |
|---|---|---|---|---|
| Study #6 | YA rat | 6 weekly doses (Age Wk6-wk12)<br>SAC at Wk12 (≈ pre-Fx treated) | 30 | 175 (femur & tibia) |
| | | | 120 | 460 |
| | | | 500 | **800 NOAEL (480*)** |
| | | | | |
| | YA rat | 6 weekly doses (Age Wk12- Wk18)<br>SAC at Wk18 (≈ post-Fx treated) | 30 | 100 |
| | | | 120 | **235 NOAEL (235*)** |
| | | | 500 | 390 |

YA=young adult
* Calculated average value over 6-week Fx healing period

*NOAEL and exposure multiples:*

Exposure multiples (rat:human) are shown below. The calculation is based on the assumption that AUC in children is similar as in adults (40 ugxh/mL) and bone concentration in children is dependent on age and is ca. 12, 9 and 4.5 ug/gr in 4-year olds, 10-year olds, and adults, respectively (APPENDIX 2). Rat AUC data were derived from the 1st 4-week toxicity study in young adult rats (APPENDIX 2). The multiples suggest that there are no significant safety concerns for bone fracture healing in patients treated with Org 25969 during surgery, before or after fracture.

Exposure multiples achieved in fracture healing study in young adult rats

| | | Rat | Human | Multiple (rat:human) |
|---|---|---|---|---|
| | | | | |
| Dose (mg/kg) | NOAEL, post-Fx treatment | 120 | 4 | 30x |
| AUC (ugxh/mL) | | 230 | 40 | 5.8x |



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| | | | | |
|---|---|---|---|---|
| Bone conc (ug/g) | | 235 | 4.5 (adult)* | 52x |
| | | | 9 (child 10 yrs) | 26x |
| | | | 12 (child 4 yrs) | 20x |
| | | | | |
| | | | | |
| Dose (mg/kg) | NOAEL, pre-Fx treatment<br>LOAEL, post-Fx treatment | 500 | 4 | 125x |
| AUC (ugxh/mL) | | 1101 | 40 | 28x |
| Bone conc (ug/g) | | 480 | 4.5 (adult)* | 107x |
| | | | 9 (child 10 yrs) | 53x |
| | | | 12 (child 4 yrs) | 40x |

*Human adult bone concentration (4.5 ug/g) was based on data from mass balance study with 15% dose retention assuming 10 kg bone in 75 kg person. Pediatric values were calculated based on assumptions of increased bone retention in young individuals of up to 30% in 4-yr olds, based on rat data (APPENDIX 2).

*Question 1:*
*Do you think that the potential effects of sugammadex on bone healing have been sufficiently evaluated and the data indicate no safety concern of sugammadex treatment on healing of fractured bone?*

***Response to Question 1:***
The fracture repair study included a comprehensive evaluation of Org 25969 on the healing of a diaphyseal bone fracture in young rats. PQCT, micro-CT, biomechanical and quantitative histology data suggested that in pre- or post-fracture treated animals there was no effect of Org 25969 at 6 weekly doses of 30 or 120 mg/kg on fracture healing. There was also no effect of Org25969 at 500 mg/kg/week on bone healing in pre-fracture treated animals. However, in post-Fx treated animals, the high dose of 500 mg/kg given weekly for 6 weeks after fracture, had negative effects on a number of histologic scores in males and females, which suggested a possible interference with the process of fracture healing. The histopathology effect was not obviously or consistently correlated to changes in uCT and/or mechanical strength parameters of the bone fracture site, suggesting the interference did not have any clinically significant functional consequences. However, this Reviewer is not prepared to dismiss the histologic findings. Studies with higher or more frequent doses and evaluations and evaluations at multiple time points would be needed to reproduce the histologic findings and confirm the absence of bone mineral density or strength effects. Based on the data from the current study, this Reviewer concludes that the NOAEL for the post-fracture treatment modality is **120 mg/kg/week**. Org25969 at 500 mg/kg/week for 6 weeks did not affect bone healing in pre-fracture treated animals and the NOAEL for this treatment modality is **500 mg/kg/wk**.

The exposure multiple (rat:human) at the 120 mg/kg NOAEL in post-Fx treated animals is 6x based on AUC. Exposure multiples are 20x and 52x based on bone concentration in children and adults, respectively. Reviewer believes that AUC multiples have more clinical relevance, since the concentration of Org 25969 in plasma or tissue fluid (not the concentrations in bone at steady state) would probably determine the effects of Org25969 in metabolically active fracture callus. The exposure multiple (rat:human) at the 500 mg/kg NOAEL in pre-Fx treated animals is 28x on AUC basis. Exposure multiples are 40x or 107x based on bone concentration in children or adults, respectively.



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In this Reviewer's opinion, the AUC multiples suggest that it is very unlikely that the fracture healing process will be affected in humans (adults or children) who suffered a fracture before they are undergoing surgery with NMB reversal using 2 or 4 mg/kg Org 25969. Conversely, the AUC multiples suggest that it is extremely unlikely that fracture healing will be affected in humans if they suffer a fracture after having previously undergone NMB reversal using Org 25969 at a 2 or 4 mg/kg dose. In evaluating the safety of human use based on these AUC multiples, it was kept in mind that the rat studies were conducted with multiples (6 weekly) doses whereas human treatment is more likely to occur with "single" doses.

***Question 2:***
*Do you think that the data in the microautoradiography study (INT00142585) provided sufficient morphological information on growth plate to help understand the effect of sugammadex on bone growth and development?*

Microautoradiography study
"Microautoradiography in bone after a single intravenous dose of [$^{14}$C]-Org 25969 to Wistar rats" (INT00142585, Study 090071, Study #4)

A second microautoradiography (INT00142585, Study 090071) was conducted using young adult rats since the data from the first study (INT00029186, Study 060217, entitled "[$^{14}$C-Org 25969]: The distribution of radioactivity in bone after a single intravenous dose of [$^{14}$C]-Org 25969 to Wistar rats") were not very valuable. The Sponsor also made an effort to clarify the data from this first study by submitting an amended study report (INT00122920, Study 060217, Study #1).

The data from the first study (Study 060217) were described more correctly in the results section. However there were still errors, for example in the description of Figures 3 and 5 (not shown in this review). Rats used for that study had been given a single 8.6 mg/kg $^{14}$C-radiolabelled dose of Org 25969 (in Study 050322) and were sacrificed at 24h, 21 days, or 84 days post dosing. Scapula and femur sections from undecalcified methylmethacrylate embedded tissue were displayed. In the 24-hour sample, silver granules were present on the periosteal and endosteal surfaces, including both cortical and trabecular aspects of bone. In the 21- and 84-day samples, substantial label was still present but appeared further away from the (cortical) bone surface indicating deposition of new bone on top of labeled surfaces. There was no label in the growth plate at any time point. At 21 days after dosing, there was no label in the trabeculae adjacent to the growth plate. Sponsor concluded that bone formation/growth and turnover were unaffected. However, that conclusion is not justified based on histological images alone.

The data from the second microautoradiography study (INT00142585) were of much better quality although images from only one animal per time point were provided. Rats used in this study were from three different studies and had been given single doses of $^{14}$C-labelled Org 25969 (Study 090310, 8 mg/kg $^{14}$C-Org 25969; Study 050322, 8.6 mg/kg $^{14}$C-Org 25969) or unlabeled Org 25969 (Study 060338, 30 mg/kg). Rats had been sacrificed at 24h, 21 days or 84 days after dosing and femurs and tibias were prepared for



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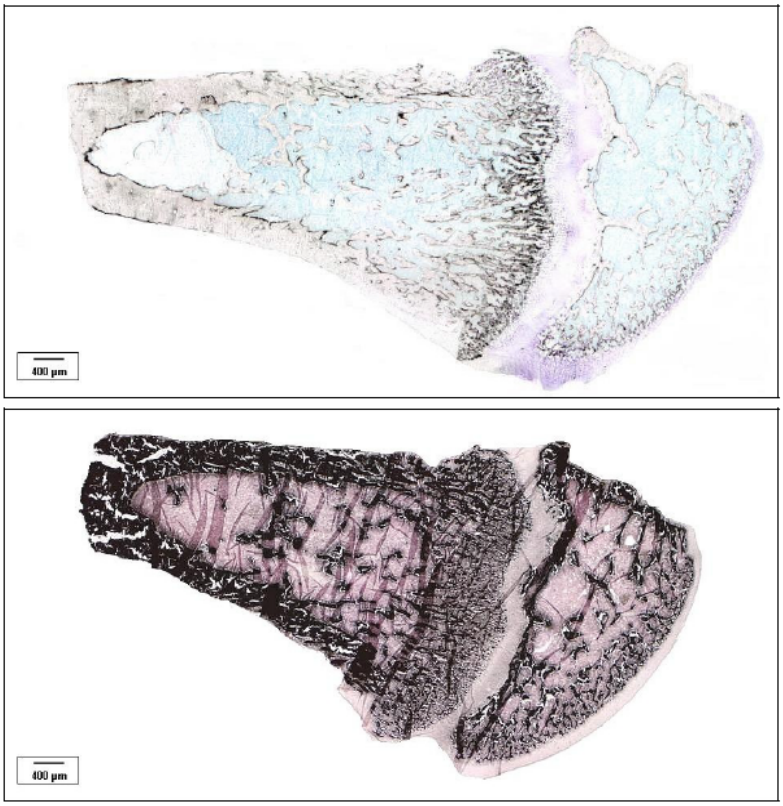
microautoradiography. Sections were cut from fixed undecalcified bone tissue embedded in methylmethacrylate. In the 24-hour sample from rat #M1007 (dosed in Study 090310), clear labeling of trabecular bone surfaces in the metaphysis, epiphysis and subarticular regions and of cortical bone surfaces in the diaphysis (periosteal, endosteal, and intracortical pores) was observed (Fig. 8-8, upper panel). Label was not observed in the growth plate or articular cartilage. Von Kossa stain for mineral (phosphate) suggested that only the surfaces of mineralized tissues were labeled (Fig. 8-8, upper and lower panel). Magnification showed that label was very prominent in the zone of calcifying cartilage, which is the distal part of the hypertrophic chondrocyte zone; and in the primary spongiosa (mixed layer of calcified cartilage and woven bone) just below the zone of calcifying cartilage (Fig. 8-2; not shown in this review). Secondary spongiosa (layer of woven and lamellar bone, with bone remodeling) was also labeled but to lesser extent.

**Figure 8–8 Comparative overviews of the rat femur by microautoradiography (upper panel) and the von Kossa staining for mineralization (lower panel)**




**Upper panel**: Distribution of radioactivity (black granular silver staining) in the femur of rat 1007, killed 24 h after a single dose of [$^{14}$C]-Org 25969 [8 mg·kg$^{-1}$; 6 MBq·kg$^{-1}$]. Counterstaining: Toluidin blue.

**Lower panel**: Serial slide taken from the same region and stained with von Kossa staining to visualize mineralization in the femur of the same rat. Counterstaining: Nuclear red.



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In the 21- and 84-day samples from two rats (#M3003 and #M5003) dosed in Study 050322 and also used in the first radiography study (#060217), the proximal metaphyseal region (calcified cartilage and primary spongiosa) did not contain any label (Fig. 8-9). Granules were only present in the metaphyseal trabeculae (secondary spongiosa) (at 21 days) and diaphyseal cortical bone (at 21 and 84days). The label was no longer confined to bone surfaces but appeared with a discontinuous pattern in the cortical and trabecular bone (Fig. 8-9 and 8-11). Sponsor argued that redistribution of Org 25969 was not apparent, but this Reviewer believes that redistribution may have occurred. Comparable autoradiography data have been obtained in a study in rats with $^{14}$C-clodronate, in which high uptake of clodronate was seen in the primary spongiosa just below the distal femoral growth plate (Osterman et al, 1997). Radiolabel was situated further away from the growth plate and under the cortical bone surface after up to 79-day follow-up, due to longitudinal and appositional bone growth, respectively. However, time lapse did not change the continuous pattern of the distribution, as opposed to what was seen with Org 25969.

There were no quantitative data on growth plate morphology (thickness, area).



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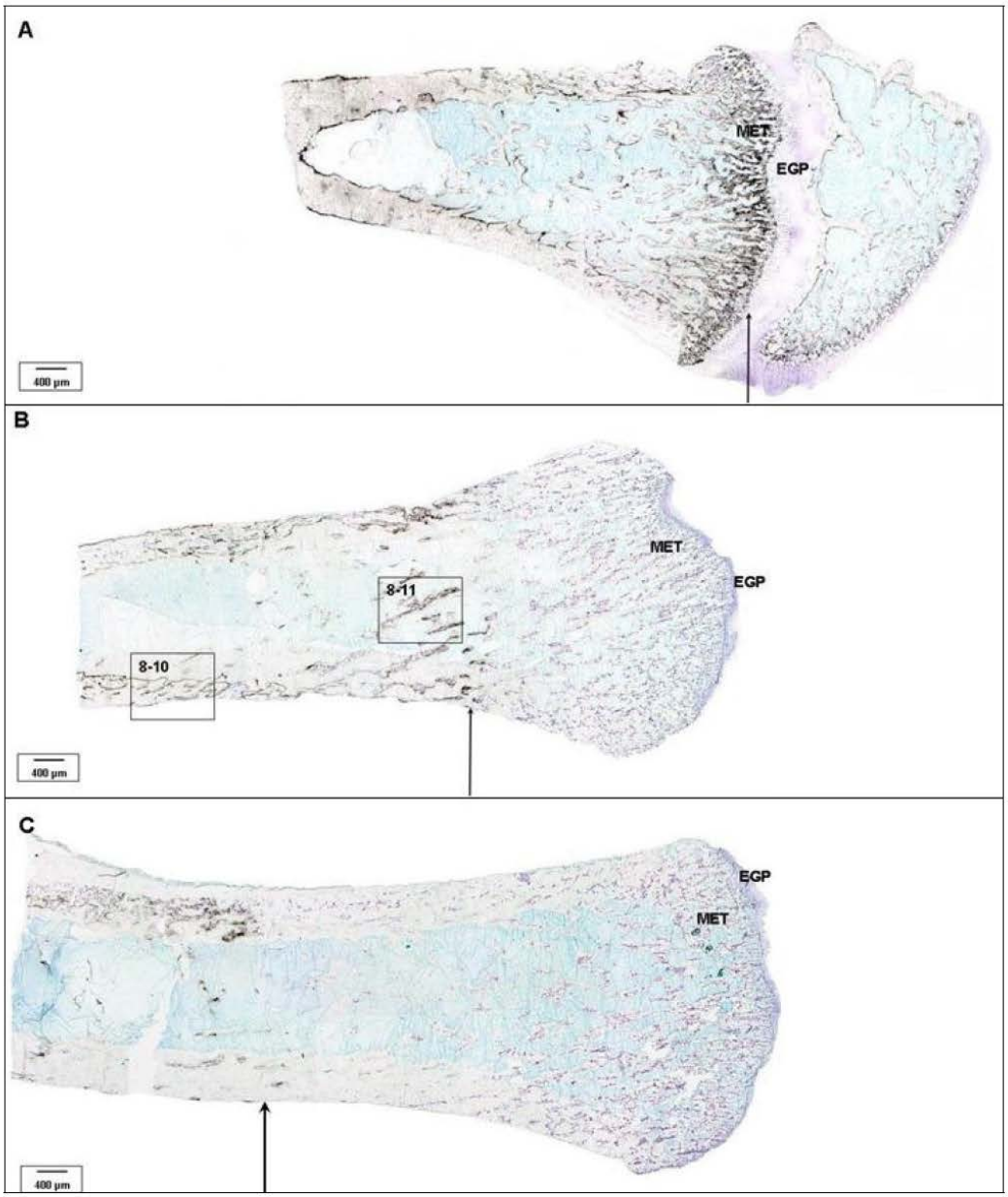
**Figure 8–9 Microautoradiography overviews of [$^{14}$C]-Org 25969 distribution in the femur of rats, killed 24 h (A: 1007), 21 days (B: 3003) and 84 days (C: 5003) after single dose of [$^{14}$C]-Org 25969 (A: 8 mg·kg$^{-1}$; 6 MBq·kg$^{-1}$, B and C: 8.6 mg·kg$^{-1}$; 5.6 MBq·kg$^{-1}$)[1]**




**EGP**: Epiphyseal Growth Plate, **MET**: Metaphysis.
The arrow indicates the site of incorporation of [$^{14}$C]-Org 25969 at t=0.
Counterstaining: Toluidin blue

[1] In the femurs shown in boxes B and C, the epiphysis and the epiphyseal growth plate is absent or partly present due to an accidental fracture following freezing during conservation and subsequent thawing and trimming.



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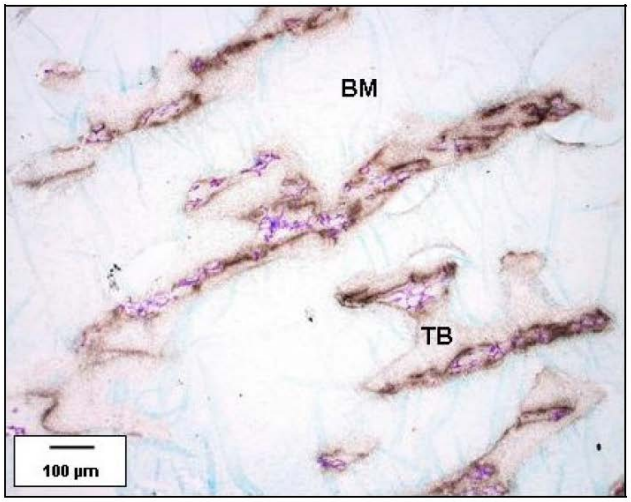
**Figure 8–11 Microautoradiography detail of [$^{14}$C]-Org 25969 distribution in the trabeculae of the femoral diaphysis of a rat sacrificed 21 days after dosing**




**TB**: Trabecular Bone, **BM**: Bone Marrow
This detail represents the corresponding box in Figure 8–9. Distribution of radioactivity (black granular silver staining) in the femur of rat 3005, killed 21 days after single dose of [$^{14}$C]-Org 25969 (8.6 mg·kg$^{-1}$; 5.6 MBq·kg$^{-1}$).

*Question 2:*
*Do you think that the data in the microautoradiography study (INT00142585) provided sufficient morphological information on growth plate to help understand the effect of sugammadex on bone growth and development?*

***Response to Question 2:***
The autoradiography study was adequate to clearly show in vivo binding of Org 25969 to bone surfaces. Study #3 (INT00127799, Study 090085) demonstrating high affinity in vitro binding of Org 25969 to hydroxyapatite (HA) is in agreement with these data. The data suggest that Org 25969 does not bind to other (extra)cellular compartments, e.g. cartilage or bone marrow. The binding to bone surfaces and not to the inner bone compartment is similar to findings with bisphosphonates and tetracycline. This is probably due to more extensive vascular and molecular access to HA binding sites on bone surfaces.

The data from this microautoradiography study do not specifically provide information on the longitudinal bone growth effect observed in the originally submitted 28-day juvenile rat study (Study INT00038039). Bone length or growth plate morphology was not evaluated in the animals used for this microautoradiography study. The autoradiography study does show that Org 25969 binds to the surfaces of calcified cartilage and bone tissue (trabecular and cortical) and that with passage of time the compound becomes located deeper in the bone (i.e. further away from the growth plate) and/or redistributes to other bone (resorption or formation) surfaces. The binding to mineralized tissue surfaces and not the growth plate itself suggests that the inhibitory effect of Org 25969 on bone growth that was previously observed in the juvenile rat



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study may have been a locally exerted indirect effect on growth plate chondrocyte activity (see Response to Question 3).

***Question 3:***
*Are the dosing selection rationale and study design employed in the 8-week intermittent dosing toxicity study in juvenile rats (INT00150243) adequate and did the study appropriately clarify the observations in the 28-day juvenile toxicity study included in the original NDA?*

Juvenile rat study (weekly dosing)
"An 8-week intermittent dosing toxicity with Org 25969 to assess effects on bone quality, structure, turnover, strength and growth and development in juvenile Wistar rats" (INT00150243, 080229, Study #7)

This second juvenile rat study was conducted to re-evaluate the effect of Org 25969 on longitudinal growth. Juvenile rats (N=20/s/g), 7 days old, were dosed weekly for 8 weeks with **0, 7.5, 30, or 120 mg/kg** (SC on postnatal days 7 and 14, IV thereafter). Pamidronate (0.25 mg/kg/wk, SC) served as positive control. A recovery period of 8 weeks followed the 8-week treatment period. The intermittent dosing regimen was aimed at achieving significant bone exposure in the absence of growth retardation due to general toxicity. The weekly dosing regimen mimics potential clinical use. It reflects an -approximately- yearly dose in humans between ages 2-10 years. In 2009, the Division (DAAAP) recommended that higher doses be used to maximize the likelihood of achieving a bone effect, but the Sponsor had already started the study when the advice was received.

Evaluations included body weight, in vivo ulna length, hematology, biochemistry, urinalysis, organ weights, gross anatomy, histopathology, thickness of growth plate in humerus and tibia, femur and ulna length and weight, bone structure and density (micro-CT) of femur and vertebrae, bone strength (distal femur indentation, vertebral compression, femur shaft 3-pt bending and femoral neck cantilever bending), femoral cross section. Evaluations were made on experimental **Day 50** (i.e. day after last dose given on postnatal day 56) and experimental **Day 105** (i.e. postnatal day 111, or last day of 8-week recovery period at end of study)

*Effects of Org 25969*
There was no effect on body weight, ulna in vivo length or incisor color.

Slight, but statistically significant decreases in serum osteocalcin levels were seen at 120 mg/kg on Day 105 in both males and females. Osteocalcin is a marker of osteoblast activity and is related to formation of osteoid rather than to formation of mineralized bone. The significance of this finding was unclear since there were no effects on bone density ('mass') or strength.

Bone histology of humerus, tibia and mandibula was normal. Growth plate morphology was unaltered and did not indicate an effect on endochondral bone formation.



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Growth plate thickness was defined as the distance "*from the first chondrocyte (in a nicely cut stack of chondrocytes) to the end of the calcified cartilage (just before the metaphysis)*". As expected, the thickness of the growth plate was decreased on Day 105 as compared to Day 50 (by ca. 50%) due to a decrease in chondrocyte proliferative activity and longitudinal growth rate with age. A small (8%) but statistically significant decrease in humeral but not tibial growth plate thickness was noted in males, but not females, at the 120 mg/kg high dose, on Day 50 but not Day 105 (Figure 1 below). It is not clear which growth plate zone (proliferative or hypertrophic chondrocyte zone, calcified cartilage?) was particularly affected. The decrease MAY have been accompanied by a decrease in humerus bone length. Humerus length was not measured in either the previous or the current juvenile rat study. Ulna and femur length were unchanged in the current study. Thus, the relation between the growth plate effect in the humerus and the previously observed effect on ulna or femur bone length is not clear.

The biology of the growth plate (GP) is complex and chondrocyte behavior is regulated by para- and autocrine factors enabling crosstalk between different cell types in the different GP regions, including the zone of calcifying cartilage. Study #4 showed no binding of Org25969 to chondrocytes or non-mineralized cartilage, only to mineralized cartilage and bone surfaces. The small effect on the thickness of the humerus growth plate may have been a <u>direct</u> effect of Org 25969 on chondrocyte proliferative activity or cartilage calcification, since retention of Org 25969 is not a prerequisite for an effect. Alternatively, Org 25969 binding to calcified cartilage and/or bone mineral may have affected GP chondrocyte proliferation and/or apoptosis in an <u>indirect</u> manner. Although this is speculative, the cyclodextrin moiety in the mineral tissue-bound Org 25969 molecule, by having affinity for a regulatory factor involved in GP biology, may have caused a negative effect on GP thickness (this study) and bone length (previous study). This theory was inspired by the results of a published study by Liu et al (Biomaterials 29, 2008), which showed that a bone-seeking alendronate-cyclodextrin conjugate caused a local bone anabolic reaction upon injection in the rat mandible. The authors hypothetically ascribed the finding to local sequestration of endogenous bone anabolic factors by the hydrophobic cyclodextrin annulus anchored to the bone by the alendronate part of the conjugate. Alendronate itself has been shown to <u>increase</u> GP thickness and <u>decrease</u> longitudinal bone growth due to inhibition of angiogenesis (resulting from hyperostosis in the metaphyseal area) which causes a delay in the apoptosis of hypertrophic chondrocytes. The latter mechanism is not relevant for Org 25969 since it is not an antiresorptive.

In a fracture healing study in young rats (Study 090319), changes in histologic parameters were identified which suggested a possible interference of Org 25969 with the bone regeneration/formation process. Fracture healing replicates the process of endochondral bone formation that takes place during bone growth in young animals/humans. Therefore, the histologic changes in the fracture healing study may be related to the bone length effect in the previous juvenile rat study and the growth plate effect in the current juvenile rat study. However, this is speculative since the underlying mechanisms for both of these findings are unclear.



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Histopathology of target organs (kidney, bladder, lungs and liver) did not show significant effects. The reversible presence of foamy epithelial cells in the urinary bladder in a few high dose 120 mg/kg males and females was considered to reflect retention of Org 25969 in this organ and not to be of toxicological significance.

There was no effect on bone length (absolute as well as normalized by body weight) (Fig 3.3-4 below) and no effect on weight of ulna and femur, neither on Day 50 or Day 105 after recovery. There was also no effect on femoral cross section. Thus, the significance of the small GP effect in the male humerus at 120 mg/kg is unclear.

There were no consistent dose-related effects on bone structural/densitometric parameters (uCT), and no effects on biomechanical properties of vertebrae and femur (See figures below).

The NOAEL for effects on bone length, structure and quality in this study is 120 mg/kg.

*Effects of pamidronate*

Pamidronate caused incisor discoloration, and decreases in serum Ca, P, osteocalcin and CTx and urinary DPD, consistent with inhibition of bone resorption and turnover. Most parameters recovered by Day 105.

Bone histology showed increased trabecular density in tibia and humerus. Growth plate morphology and thickness in humerus or tibia were not affected by pamidronate (Fig. 1).

There was no effect on bone length (absolute and/or normalized by body weight) (Fig 3.3-4) and weight of ulna and femur; and there was no effect on femoral cross section.

Increases in trabecular bone density were noted microscopically and by uCT. Imaging by uCT showed increases (up to 2-fold) in femoral and vertebral BMD, BV/TV, Tb.N. and $Tb.Sp^{-1}$ (Fig. 3.1-15). Biomechanical testing showed increased maximum load in the vertebra compression test (not the femur 3-pt bending test) and increased resistance (force, displacement, stiffness, energy) in the femur trabecular bone indentation test (Fig. 3.1-43), on both Days 50 and 105. However, femur cantilever (neck bending) strength parameters maximum force and energy were decreased in males and females on Day 50 and 105. The reason for the latter was unclear. Positive bone effects of pamidronate are expected particularly in cancellous bone based on pamidronate's anti-resorptive action.



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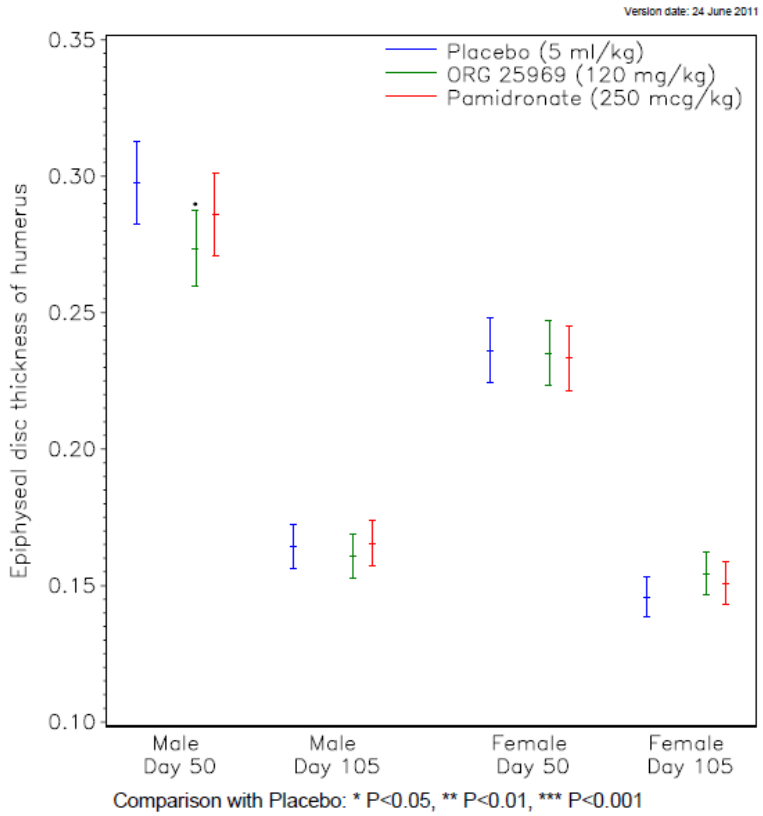


**Figure 1 Estimates and 95% confidence interval of Epiphysial disc thickness of humerus**

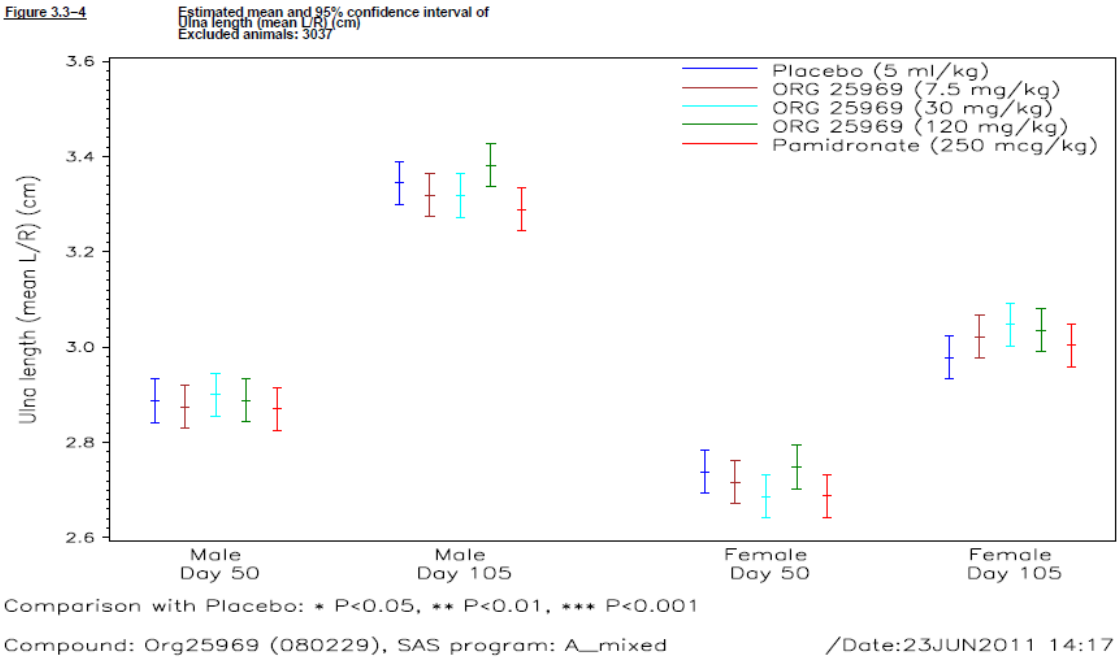




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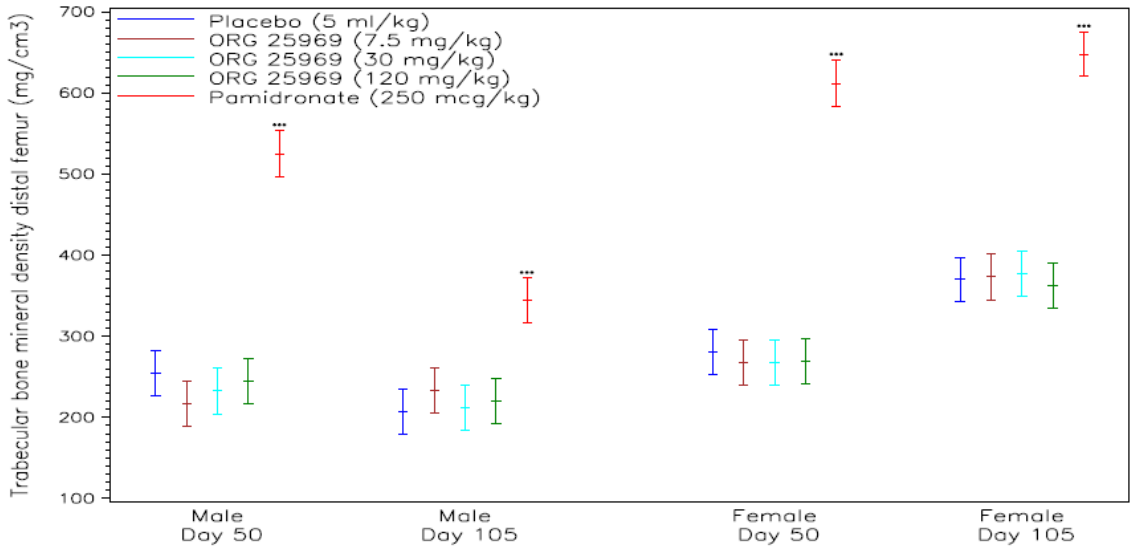
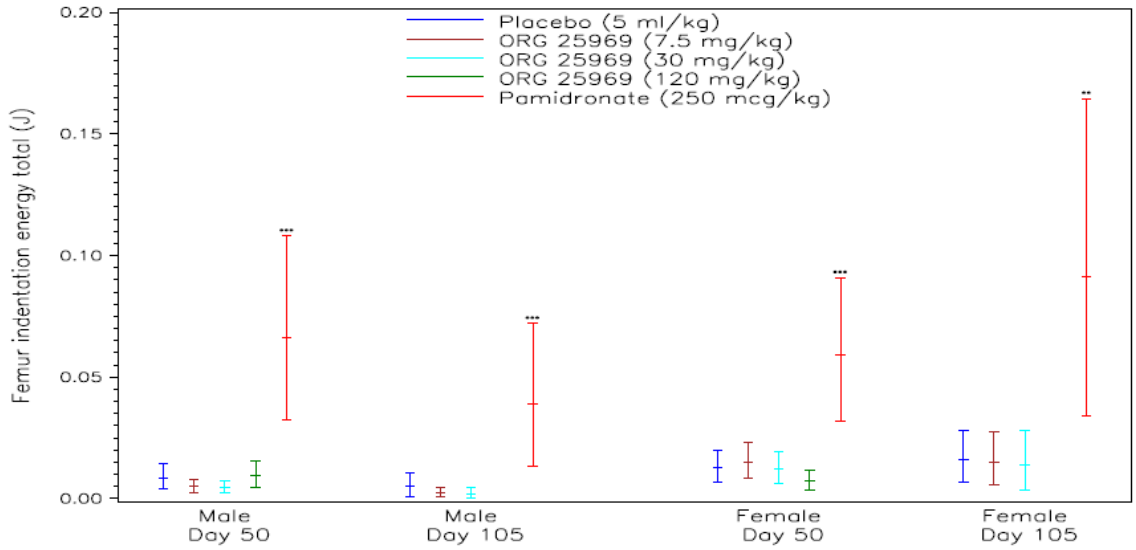
Figure 3.1–15 Estimated mean and 95% confidence interval of Trabecular bone mineral density distal femur (mg/cm3)




Comparison with Placebo: * P<0.05, ** P<0.01, *** P<0.001

Compound: Org25969 (080229), SAS program: A_mixed /Date:23JUN2011 14:12

Figure 3.1–43 Estimated median(geometric mean) and 95% confidence interval of Femur indentation energy total (J)




Comparison with Placebo: * P<0.05, ** P<0.01, *** P<0.001

Compound: Org25969 (080229), SAS program: A_mixed /Date:23JUN2011 14:16

Study #2 (INT00145198, or INT0038389; Study 060086)

The Sponsor also re-analysed the data on bone length and body weight from the original 28-day repeat dose study (INT0038389) in juvenile rats in Study #2 (INT00145198,



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Study 060086). In Study INT0038389 there was a small reduction in body weight and ulna and femur length at 120 and/or 500 mg/kg, in both males and females. The Sponsor had concluded that the effect on bone length was not a specific effect of Org 25969 but related to the body growth retardation and decided that the NOAEL was 500 mg/kg. However, the P/T Reviewer(s) in the NDA review concluded that the NAOEL was 30 mg/kg/day. A re-analysis with body weight as co-variable was performed by the Sponsor in order to investigate whether there is an additional effect of Org 25969 on ulna or femur length that cannot be explained by the effect of Org 25969 on bodyweight gain, and whether this direct effect on ulna an femur length is statistically significant. The re-analysis showed that there was body-weight independent significant reduction in ulna length at 500 mg/kg in M (males) and F (females) on Day 84, a reduction in femur length at 500 mg/kg in M and F at D35 and at 120 mg/kg and 500 mg/kg in M and F at Day 91. The latter effect was a 0.4mm and 0.8mm as compared to controls. It was concluded by the author of the statistical re-analysis that the NOAEL for the bone length reduction was 30 mg/kg. Despite this result, the Sponsor concluded again that, because the effect was small and likely influenced by imprecisions in bone length measurement, the NOAEL with respect to specific effects on bone growth and development was 500 mg/kg.

This Reviewer reiterates that the NOAEL for the bone length effect in the original 28-day repeat dose study in juvenile rats was 30 mg/kg/day.

*NOAEL and exposure multiples*

The NOAEL in the new 8-week intermittent dosing study was 120 mg/kg/week. TK analysis was not performed in this study, or in the accompanying Study#6. However, PK data can be derived from previous single dose AUC data from young adult rats. The AUC at 120 mg was 230 ugxh/mL on Day 1 in the 4-week young adult rat toxicity study. This AUC is selected as the most relevant AUC for evaluation of the current study results.

Bone concentrations in juvenile rats dosed once weekly for 7 (not 8) weeks (between 1 and 8 weeks of age) was determined in Study #6, after 7 doses of doses of 8.9, 30 and 120 mg/kg. The bone concentration at 120 mg/kg was 400 ug/gram.

In their study protocol submission, the Sponsor estimated that the cumulative bone concentration after 7 weekly doses of 120 mg/kg will be ca. 2000-2500 ug/g bone in the juvenile rats (500ug/g after 1st dose). The data from Study #6 showed much lower values. This was probably due, in part, to the fact that the bone content was measured one week after the last dose.

| | Species | Study/protocol | Dose mg/kg | microgram/gr bone |
|---|---|---|---|---|
| | | | | |
| Study #6 | Juvenile/ YA rat | 7 weekly doses (Day7-Day49) SAC at Day 56 (7 days after last dose) | 8.9 | 50 |
| | | | 30 | 160 |
| | | | 120 | **400 NOAEL** |

The NOAEL exposure multiple is 6.4-fold relative to the human dose of 4 mg/kg on AUC basis. NOAEL exposure multiples based on bone concentration vary for children of



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different ages, if we assume that higher percentages of a given dose are retained in the skeleton of younger individuals.

Exposure multiples

| | | Rat | Human | Multiple |
|---|---|---|---|---|
| | | | | |
| Juvenile rat | Dose | 120 | 4 | 30x |
| | AUC (ugxh/mL) | 254 | 40 (adult or child) | **6.4x** |
| | Bone conc (ug/g) | 400 | 4.5 (adult)* | **89x** |
| | | | 9 (child 10 yrs) | **44x** |
| | | | 12 (child 4 yrs) | **33x** |

*Human adult bone concentration (4.5 ug/g) was based on data from mass balance study with 15% dose retention assuming 10 kg bone in 75 kg person. Pediatric values were calculated based on assumptions of increased bone retention in young individuals of up to 30% in 4-yr olds based on rat data (see APPENDIX 2).

*Question 3:*
*Are the dosing selection rationale and study design employed in the 8-week intermittent dosing toxicity study in juvenile rats (INT00150243) adequate and did the study appropriately clarify the observations in the 28-day juvenile toxicity study included in the original NDA?*

***Response to Question 3:***
Study INT00150243 was appropriate as a re-evaluation of the effect of Org 25969 on longitudinal bone growth in juvenile rats. The study adequately evaluated the histology and quality (density and strength) of rat bone. Pamidronate was used as a positive control and confirmed the validity of the model. The data showed that at the doses employed there was no effect of Org 25969 on bone growth or quality in juvenile rats, despite the fact that the doses caused significant bone binding/accumulation. Pamidronate caused the expected increases in trabecular bone density and strength in vertebrae and distal femur but had no effect on bone length.

The cause of the small decrease in bone length by Org 25969 in the previous 28-day juvenile rat study (INT0038389) is not clear. Reanalysis of the data from the original juvenile rat study with body weight as covariable (Study #2, INT0038389) confirmed that there was an independent bone length effect. The new study showed a small decrease in the thickness of the humerus growth plate at the high dose in males only. Although the length of the humerus was not measured, this may have reflected a direct or indirect negative effect of Org 25969 on the activity of the growth plate and may be related to the small reduction in bone length in the previous 28-day juvenile rat study. However, this theory is speculative. Since the measurement of the growth plate thickness was subjective, and the decrease in thickness was very small and only seen in males, the effect was not considered reliable and substantial enough to set the NOAEL.

In this new study, a NOAEL of 120 mg/kg/week was defined with regard to effects on bone growth and bone quality. The previous juvenile rat study (INT0038389) yielded a NOAEL of 30 mg/kg/day. The new weekly NOAEL of 120 mg/kg is 6-fold the human/pediatric dose of 4 mg/kg based on AUC, and 33- to 89-fold the human dose based on bone concentration. The NOAEL and exposure multiples derived from this new



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study are relevant for the evaluation of pediatric studies to be conducted with single or intermittent doses of Org 25969.

***Question 4:***
*Based on the data of the microautoradiography study (INT00142585) and the 8-week intermittent dosing toxicity study in juvenile rats (INT00150243), do you agree that sugammadex is not likely to adversely affect bone growth and development?*

In this Reviewer's opinion, the previous observation of a small decrease in bone length in a multiple dose juvenile rat study (INT 0038389) warranted only minor clinical concern. The new data from the 8-week intermittent dosing toxicity study in juvenile rats (INT00150243) confirm that there is no significant clinical concern for a negative effect on bone growth or quality in pediatric studies with single or intermittent doses of 2-4 mg/kg. Reviewer believes that additional nonclinical studies are not needed. However, monitoring of height in children enrolled in pediatric studies may be considered.

***Question 5:***
*Do you think that the nonclinical studies included in this submission provided definitive information on the binding site and localization of sugammadex in bone? Did the data provide sufficient evidence to demonstrate that Org 25969 binds to hydroxyapatite in the bone and distributes in the area of bone mineralization only?*

Binding study
An in vitro study on binding (Study #3, INT00127799) was conducted to confirm affinity for hydroxyapatite (HA). The data clearly showed that Org 25969 binds to biological hydroxyapatite in a saturable manner (Fig. 9-1). Maximum capacity was 19.5 nmol Org 25969/mg HA). $EC_{50}$ was 10.6 nmol/mg (Fig. 9-1).

The extent of *in vitro* HA binding of **1.7 nmol/mg** [$^{14}$C]-Org 25969 was not affected by Org 9426 (rocuronium) (**0.17-17 nmol/mg**), sodium citrate and tetracycline (both 1.7- 167 nmol/mg ). In an earlier in vivo study (Study INT00033724) the binding to bone of Org 25969 given as single IV dose of 30 mg/kg (13.8 nmol/kg) (yielding an AUC of **20 pmolxh/gr**) was decreased (by ca. 35%) by a subsequent single IV dose of rocuronium of 4mg/kg (6.6nmol/kg) (yielding an AUC of **3.6 pmolxh/gr**). This apparently contradicts the binding data in Study #3. However, the decrease in the in vivo study may have been a secondary effect due to an effect on the systemic PK of Org 25969. Alendronate and pamidronate, but not tetracycline (or sodium citrate), when given at concentrations up to 167 nmol/mg, competed with Org 25969 for HA binding (Fig. 9-2), suggesting a shared binding site or region.



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**Figure 9-1 Fitted curve of the extent of binding of [$^{14}$C]-Org 25969 to hydroxyapatite**

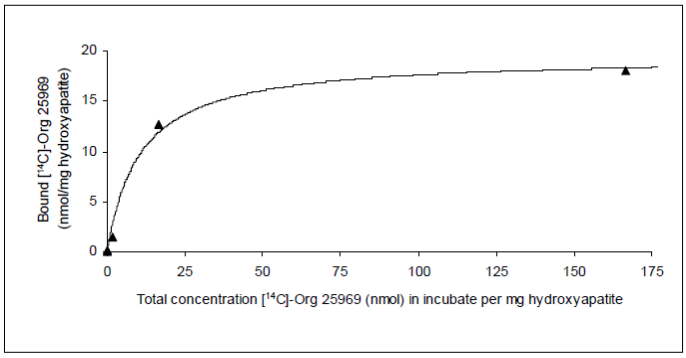


**Figure 9-2 Hydroxyapatite binding of 2.5 nmol·mL$^{-1}$ (1.7 nmol·mg$^{-1}$ hydroxyapatite) [$^{14}$C]-Org 25969 in the presence of sodium citrate, tetracycline, alendronate or pamidronate**

**Figure 9-2 Hydroxyapatite binding of 2.5 nmol·mL$^{-1}$ (1.7 nmol·mg$^{-1}$ hydroxyapatite) [$^{14}$C]-Org 25969 in the presence of sodium citrate, tetracycline, alendronate or pamidronate**

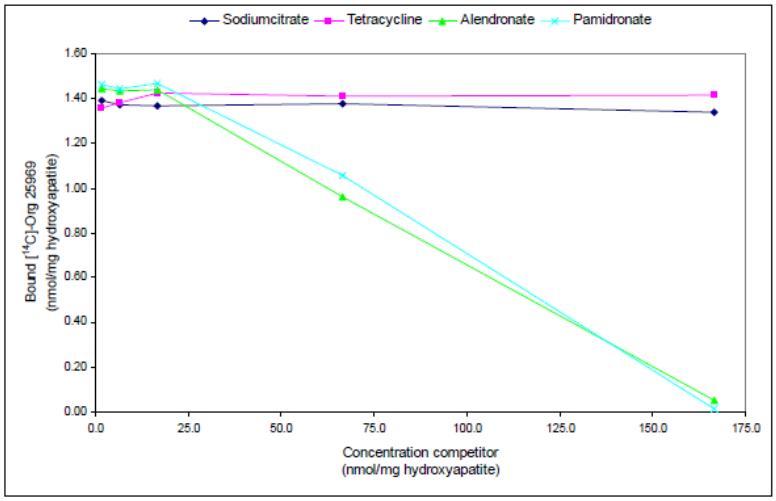




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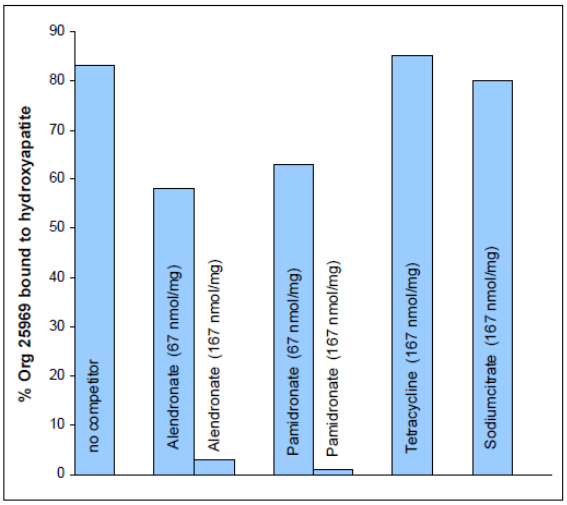
**Figure 9-3** **Comparison of the extent of binding of 2.5 nmol·mL$^{-1}$ (1.7 nmol·mg$^{-1}$ hydroxyapatite) [$^{14}$C]-Org 25969 to hydroxyapatite in the absence and presence of Org 9426, sodium citrate, tetracycline, alendronate and pamidronate**




The data show definite high affinity binding of Org 25969 to a binding site or region in HA that is close to the site or region which binds bisphosphonates (BPs). However, while BPs inhibit osteoclastic bone resorption, Org 25969 does not, at least not at the doses used in Sponsor's studies. In part, this may be explained by the fact that anti-resorptive effect of the nitrogen containing BP's (such as ALN and PAM) is due to their ability to block the enzyme farnesyl diphosphate synthase (FPPS). Org 25969 apparently has no toxic/inhibitory effects on osteoclast function. In fact, at a high dose (2000 mg/kg) it appeared to stimulate bone resorption.

Bisphosphonates are thought to bind to hydroxyapatite through interaction of the phosphate and amino groups with calcium ions in HA. Tetracycline binds to HA through interaction of the oxygen atoms on C10, C12 and C2 in tetracycline with calcium in HA (Perrin, Nature 1965). The lack of competition with tetracycline in the current study may be due to the selection of the concentration used. Alternatively, BP's and Org 25969 may bind to similar molecular binding sites in HA that are different from the site that tetracycline binds to.

*Question 5:*
*Do you think that the nonclinical studies included in this submission provided definitive information on the binding site and localization of sugammadex in bone? Did the data provide sufficient evidence to demonstrate that Org 25969 binds to hydroxyapatite in the bone and distributes in the area of bone mineralization only?*

Response to Question 5:
Yes. The in vitro data show definite binding of Org 25969 to hydroxyapatite, which is the main mineral component of bone tissue. The binding explains the in vivo distribution of Org 25969 to bone and its localization to bone surfaces. In vivo, Org 25969 binding may



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take place at resting sites or at resorptive or formative (i.e. mineralizing) surfaces. Bisphosphonates and tetracycline have been shown to bind to both types of surfaces and Org 25969 may do the same. This conclusion is supported by the fact that the localization of Org 25969 in the bone of young growing rats (in Study #4) is similar to the localization of bisphosphonates or tetracycline. The competition between bisphosphonate (alendronate) and Org 25969 and the lack of competition between tetracycline and Org 25969 observed in Study #3 does not provide any conclusive evidence about the nature of the Org 25969 HA binding site.

***Question 6:***

*Do you think the nonclinical studies included in this submission addressed the concern regarding the potential for bone tumor development as indicated in the Not Approvable Letter?*

Response to Question 6:

I don't believe there is a significant concern for bone tumor development. Org 25969 is not genotoxic and data from repeat dose toxicity studies with up to 8-week recovery periods have not shown a signal for carcinogenicity. Its binding to bone mineral and prolonged bone retention time appear to have no effect on bone metabolism (turnover) at clinically relevant doses. I believe it is unlikely that the presence of Org 25969 in bone would be tumorigenic to bone (osteoclasts or osteoblasts) or other cells.

***Question 7:***

*Do you think that the nonclinical studies included in this submission provided sufficient evidence to support the initiation of a multiple dose clinical studies in pediatric patients?*

Response to Question 7:

This reviewer believes that sufficient nonclinical juvenile animal studies have been conducted to support the initiation of a pediatric study program. Generally, the submitted data appear to support the initiation of clinical studies with single or intermittent/multiple doses of 2-4 mg/kg Org 25969 in pediatric patients. However, specific clinical protocols should be submitted and reviewed to decide whether the proposed studies are sufficiently safe. AUC comparisons as well as bone comparisons should be used to estimate their safety. This Reviewer believes that AUC comparisons are probably more relevant, partly because bone concentrations in children are uncertain, and partly because it is not clear whether average bone concentrations are relevant for the bone growth effect that was previously observed in juvenile animals.

ADDITIONAL QUESTION (from draft consult request, Febr 2013):

1. Based on the results of the bone retention kinetics study included in this submission, do you think that sugammadex may be completely eliminated from the bone in humans after a single dose administration?

Response:

The data from Study #5 suggest that the % dose per gram bone or per pooled bones decreases with time, although the terminal $T_{1/2}$ is very long. I expect



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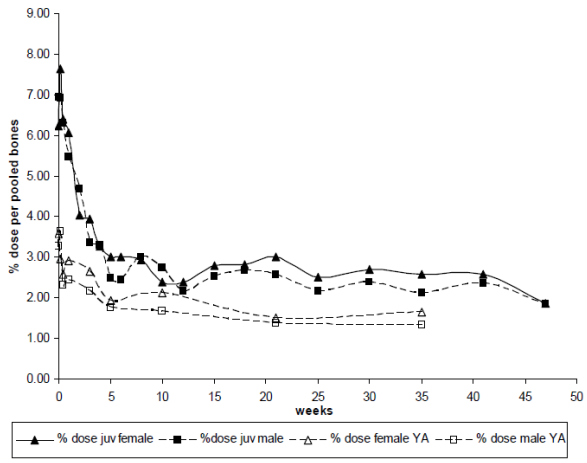
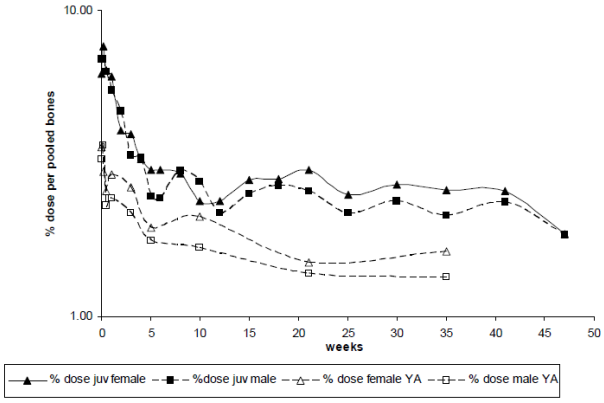
that eventually the bone content will be no longer measurable although this appears to take a long time (years). In humans, this is probably also the case. Since bone weight increases with the rat's age, the data could be converted to bone concentration (ug/gr, averaged over skeleton) to determine relevant $T_{1/2}$ values.

**Figure 8.2-4 Percentages of a dose retrieved per pooled bones versus time in male and female juvenile and young adult Wistar rats after a single dose of [$^{14}$C]-Org 25969 and Org 25969 (linear and log scale)**




Juv: juvenile (1 week at dosing), YA young adults (6 weeks at dosing)




Juv: juvenile (1 week at dosing), YA young adults (6 weeks at dosing)



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A biphasic wash out of the radioactivity was noted in pooled bones.
Levels expressed as % of the dose per gram pooled bones and per pooled bones were shown in the tables below. It should be noted that only one animal per time point was sacrificed. For juveniles, more time points were taken but only the time points as used for the young adults are given below.

| time of sacrifice | % of the dose/gram pooled bones | | | |
|---|---|---|---|---|
| | juvenile males | juvenile females | YA males | YA females |
| 3 h | 31.24 | 28.87 | 1.08 | 1.37 |
| 24 h | 48.47 | 29.11 | 1.10 | 1.18 |
| 72 h | 13.30 | 18.21 | 0.56 | 0.90 |
| 1 wk | 9.28 | 8.80 | 0.83 | 1.31 |
| 3 wk | 1.74 | 2.30 | 0.50 | 0.77 |
| 5 wk | 0.81 | 1.20 | 0.30 | 0.55 |
| 10 wk | 0.49 | 0.58 | 0.29 | 0.62 |
| 21 wk | 0.37 | 0.70 | 0.22 | 0.38 |
| 35 wk | 0.32 | 0.55 | 0.18 | 0.39 |
| 47 wk | 0.24* | 0.42 | | |

| time of sacrifice | % of the dose/ pooled bones | | | |
|---|---|---|---|---|
| | juvenile males | juvenile females | YA males | YA females |
| 3h | 6.94 | 6.24 | 3.27 | 3.58 |
| 24h | 6.93 | 7.63 | 3.62 | 2.96 |
| 72h | 6.30 | 6.41 | 2.31 | 2.57 |
| 1 wk | 5.47 | 6.07 | 2.43 | 2.91 |
| 3 wk | 3.36 | 3.95 | 2.18 | 2.65 |
| 5 wk | 2.48 | 3.01 | 1.77 | 1.95 |
| 10 wk | 2.75 | 2.38 | 1.68 | 2.11 |
| 21 wk | 2.58 | 3.01 | 1.39 | 1.50 |
| 35 wk | 2.13 | 2.59 | 1.34 | 1.64 |
| 47 wk | 1.84* | 1.86 | | |

*n=2

2. Was the dosing selection rational and study design employed in the 8-week intermittent dosing toxicity study in juvenile rats (INT00150243) appropriate?

Response:

Yes. The dose selection was appropriate since a NOAEL was defined.



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## APPENDIX 1

In the Not Approvable letter of July 31, 2008, the Division (DAAAP) indicated that "nonclinical evaluations will be necessary prior to any multiple-dose pediatric trials, approval of a pediatric indication, or inclusion of pediatric data in the label". The following recommendations were included in the letter:

1. Provide an evaluation of sugammadex in a nonclinical model of bone fracture to examine potential effects of the drug on bone healing. You are encouraged to submit protocols of such studies to the Agency for comment prior to their conduct.

2. Provide data on growth plate morphology to help understand the longitudinal growth reduction observed in the 28-day juvenile rat study.

3. Provide a repeat-dose study of sugammadex in the juvenile rat with an extended period of recovery, such that bone length, material properties, and integrity at full skeletal maturity may be evaluated in order to clarify the observation of slight but lasting decreased bone length and body weight observed in the 28-day juvenile toxicity study. Within this study, micro-computed tomography (μCT), bone turnover markers, and bone strength assessment should be obtained and evaluated. Also, the study should incorporate positive control arms to verify assay sensitivity. Lastly, though not required, the inclusion of vertebral evaluation would be helpful to interpret bone effects since vertebrae have a more homogenous trabecular structure than long bones such as femur. You are encouraged to consider an alternative, intermittent dosing paradigm in order to minimize effects of sugammadex on body weight while allowing for significant drug accumulation in the skeleton.

4. Provide definitive information on the binding site of sugammadex in bone as well as a reevaluation of bone localization of sugammadex as presentation of the data on bone localization was not clear and errors in descriptions were noted in the submitted materials.

5. Provide data, which may be derived from published literature, in vitro studies, and/or in vivo studies, along with a persuasive, well-supported rationale, to show that the risk of administration and long-term retention of sugammadex in the bones of pediatric patients will not confer a risk for bone tumor development in this population. Otherwise, an evaluation of the carcinogenic potential of sugammadex may be required. Although plasma levels of sugammadex rapidly decline with acute administration, the long retention of sugammadex in skeletal bone may be considered to be chronic exposure (i.e., greater than six months) and raises concern regarding the potential for development of tumors of this tissue, especially in the pediatric population, which is known to develop primary bone tumors at rates that exceed those in the adult population.



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## APPENDIX 2

AUC and bone concentrations in rats and humans

| Species | Body wt (gr) | Dose mg/kg | Actual dose | Bone weight | % dose in pooled bones (ug/pld B) | AUC (ugxh/mL) SD/28day-MD | microgram/gr bone (bone weight) |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| Juvenile rat (4-wk tox study) | 13gr | "4" | 0.05mg | 0.35gr (2.7% of bw) | (30%?) 15ugr | ? | 43 |
| | | **30SD** | 0.39mg | 0.35gr | (13.3%) =52ugr | 43/22 | 150 |
| | | 120 | 1.6mg | 0.35gr | (8.2%) =130ugr* | 254/98 | 371 |
| | | 500 | 6.5mg | 0.35gr | (3.4%) = 221ugr* | 1275/432 | 631 |
| | | | | | | | |
| Young adult rat (1st 4-wk tox study) | 250 gr (3mo old) | 30 | | 3gr (1.2% of bw) | 2.8% | 48/44 | 70* |
| | | 120 | | 3gr | ? | 230/207 | 175* |
| | | 500 | | 3gr | ? | 1101/1052 | 300*** |
| | | | | | | | |
| HUMAN | 60kg | 4 | 240mg | 8kg (wet weight) (8/60= 13%bw) | 15% =36mg (based on excretion data) | 40 | 4.5 |
| CHILD | 30kg (10 yrs) | 4 | 120mg | 3kg | 22.5% & | 40** | 9 |
| CHILD | 16 kg (4 yrs) | 4 | 64 mg | 1.6kg | 30% & | 40** | 12 |

* Values based on assumption that dose-dependence of bone concentration is similar as in Study #6
** Assuming AUC in children is same as in adults
***Based on data from Study INT00033724
& Hypothetical values for %dose in children's bone, modeled on age- and weight-dependence of this parameter in rats



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/s/

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GEMMA KUIJPERS
08/19/2013

LYNNDA L REID
08/19/2013

| | |
|---|---|
| NDA: | 22-225 |
| Sponsor: | Organon |
| Drug: | Sugammadex (Bridion) (Org 25969) |
| Class: | Selective relaxant binding agent |
| Indications: | Routine reversal of neuromuscular blockade induced by rocuronium or vecuronium in adults; Immediate reversal of NMB at 3 minutes after administration of rocuronium in adults. Not proposed for pediatric use at this time. |
| Clinical dose: | Routine reversal: 4 mg/kg (profound blockade: 2 mg/kg (shallow blockade)<br>Immediate reversal: 16 mg/kg |
| Route: | IV bolus injection |

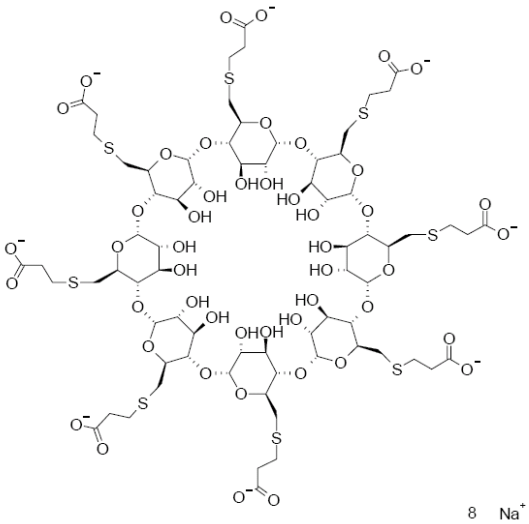
| | |
|---|---|
| Review: | June 6, 2008 |
| Reviewer: | Gemma Kuijpers |

**Background**

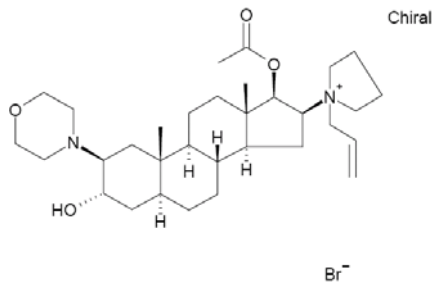
Sponsor has developed a modified gamma-cyclodextrin derivative (Org 25969; Sugammadex) for reversal of NMB by rocu- or vecuronium. Mechanism consists of complex formation and removal of the NMB agent from the neuromuscular junction. It is thought this provide more rapid relief of NMB than current pharmacological methods.

The cyclodextrin ring in gamma CD's consists of 8 glucose residues that form a cone-slice shaped molecule with cavity diameter of ca. 0.88nm. Cyclodextrin molecules bind nonpolar compounds and lipophilic drugs and are used in pharmaceutical, food, cosmetic and toiletry industries. The sugammadex molecule has very high affinity for amino steroid NMB agents such as rocuronium and when given after administration of the blocking agent (eg rocuronium 0.6-1.2 mg/kg) it is believed to carry the agent out of the circulation effectively reducing or reversing its blocking action. Sponsor contends the compound is not metabolized.

**Compound**




Sugammadex
Mw=2178 (Na form)




Org 9426
Rocuronium
Mw=610 (Br form)

**Bone physiology**
Bone is a complex mineralized tissue with a role in support and movement, systemic Ca and P metabolism, and possibly endocrine regulation of other tissues. Skeletal growth and homeostasis/maintenance is under control of systemic hormones (sex steroids, GH, thyroid, VitD) and multiple local tissue factors. Bone metabolism includes resorption and formation and is determined by actions of osteoclasts, osteoblasts and osteocytes. Osteoclasts resorb bone and release Ca, osteoblasts deposit bone matrix and aid in matrix mineralization, and osteocytes regulate bone cells. Resorption and formation take place in distinct (re)modeling units and are often coupled processes particularly during remodeling.

Adherence of compounds to bone can affect bone cell function resulting in changes in bone size or bone mineral content/density (BMC or BMD) or it can affect the intrinsic mechanical properties of the bone material. Well known bone seeking agents are bisphosphonates, tetracycline and fluoride. Of these, the first two are believed to adhere to hydroxyapatite, suppress osteoclastic bone resorption and increase bone mass without adversely affecting material properties. Fluoride may deteriorate bone quality and weaken bone.

The properties of bone tissue and its metabolism can be evaluated by qualitative or quantitative histology, evaluation of bone turnover markers, X-ray-based mass/density/structure measurements (DEXA, pQCT, uCT), Ca uptake/exchange and Ca homeostatic hormones (PTH, VitD).

**Secondary Pharmacodynamics**
Specificity of binding of sugammadex was tested by isothermal titration microcalorimetry. There was relatively high affinity for raloxifene, toremifene, tibolone, Steroid hormones also bound to sugammadex but with ≥10x less affinity than rocuronium.

**Distribution of sugammadex to bone and teeth**
Sugammadex is rapidly and widely distributed in all preclinical species and eliminated by the kidney with no appreciable role for metabolism in clearance. During development it was noted that sugammadex binds to the skeleton and teeth (in rats) and is retained in these hard tissues for extended periods of time (T1/2 in various bones = 67-210d).

Although no direct experimental evidence was provided it was hypothesized that sugammadex binds to bone and teeth due to a high affinity for hydroxyapatite (HA) i.e. the mineral phase of the bone and tooth matrix. This assumption was based on the presence of multiple anionic carboxylic acid residues i.e. thiopropinate groups in the substituted sugammadex molecule. Unmodified cyclodextrins are not known to bind to bone. Analogy to the high affinity of tetracycline, bisphosphonates and polycarboxylic EDTA acid for hydroxyapatite in bone and teeth was suggested. Although this hypothesis is plausible, data were not provided to support it.

Binding to bone was not easily saturated and bone concentration (mg/g) increased in less than dose-proportional manner at doses up to 500 mg/kg. Retention in teeth (incisors and molars) (mg/g, or % dose/g) was less than in bones and teeth lost radioactivity faster. Multiple daily doses lead to continued bone uptake with less than proportional increases in bone concentration. This is similar to the bone retention of

bisphosphonates such as alendronate. However, the alendronate concentration in young rat bone (ug/g) is several times higher than that of sugammadex at similar iv doses.

At a dose of 30 mg/kg retention in pooled bones was 13% (juvenile), 3% (young adult), and 0.5% (aged rats), at 24h post dose. The increased bone retention in juvenile rats is probably due to rapid growth of bone containing multiple sites of bone resorption, formation and mineralization. A similar finding of increased bone retention in young vs. older rats (2mo vs. 12mo) has been reported for alendronate. Sugammadex can cross the placenta and bind to fetal bone, but this binding has not been found to result in teratogenicity or abnormal fetal/pup (skeletal) development in rats or rabbits.

Microautoradiography in young male adult rats (INT00029186) showed label in periosteal and endosteal aspects of cortical bone in femur and scapula and on trabecular bone surfaces in femur. There was no binding to femoral growth plate or calcified cartilaginous zone. Bone formed subsequent to dose administration did not contain compound and 21d to 84d post dosing labeled compound appeared to be embedded in bone. Sponsor concluded sugammadex binds to or near sites of 'active bone formation', (possibly because the pattern was reminiscent to that of tetracycline labeling), however it was not clear whether there was binding to cells or to matrix only. The binding appeared different from that of a Tc-99 labeled bisphosphonate (and tetracycline) which localizes to areas of mineralization and resorption including calcified cartilage (Christensen and Krogsgaard 1981). The report of the microautoradiography study contained several errors and some of the micrographs were of poor quality.

Injection of rocuronium immediately after sugammadex injection at half molar dose (4 mg/kg rocu vs 30 mg/kg sugammadex, analogous to clinical dose ratio) diminished the extent of binding in bone and teeth by 33-50%. This suggests that residues involved in complex formation with the neuromuscular blocking agent are also important for binding to bone or that the inclusion of rocuronium in the molecule impairs bone interaction.

Sponsor conducted a number of preclinical disposition and toxicity studies in rats to characterize bone and teeth effects. A position paper was submitted to describe the bone tissue findings and evaluate their potential implications for clinical use. Based on experience with drugs targeting bone tissue DMEP was consulted to evaluate these studies.

**Animal bone studies**

In 4-week rat and dog (iv) toxicity studies there were no bone abnormalities at doses up to 500 and 250 mg/kg/day, based on standard toxicity study endpoints (histopathology incl. Von Kossa calcium stainnig).

Single dose studies in young adult rat

First study

A dedicated bone study was conducted in young adult rats (ca. 3 months of age, single iv doses of 0 and 2000 mg/kg, 6-week follow-up) to evaluate bone turnover and quality. There were no significant effects on body weight, femur length or diameter and teeth color. There was no clear effect on serum or urine Ca levels or excretion throughout evaluation period. Bone turnover marker data from D21 and D42 (data from D1 and D7 not provided) suggested increased bone resorption in females but not males at D21 (urine DPD and serum CTx increase) but no effect on bone formation.

Micro CT (femur, ex vivo) showed statistically significant effects on trabecular bone in metaphysis on D21 (decreases in Tb BMD and BV/TV due to decreased Tb.N and increased Tb.Sp but no effect on Tb.Th). Accordingly, mechanical testing of cancellous bone showed decreased indentation strength parameters maximum load, stiffness, energy, and stress. Effects were seen in both sexes but appeared larger in females. Effect size was minimal to moderate (5-25%). Some but not all changes in structural and/or strength parameters were recovered or reversed by D42, but there were inconsistencies in the data (eg Tb.BMD in males was still decreased on D42 but load and stiffness were increased). Cortical bone volume, thickness or BMD and femur diaphyseal bending strength (max load) were not affected although diaphyseal stiffness was slightly decreased on D21 and D42 in both sexes. Femoral neck strength was not affected. No teeth color effect observed. There was no vertebral structure or strength assessment. Inconsistencies in data prevent firm conclusions regarding effects and underlying mechanisms.

Second study

In a second study rats were dosed with single iv doses up to 500 mg/kg and sacrifice/evaluation at D1, 3, 7, 14, 21, 28, 35, 42. There were small nearly statistically significant effects in the 500 mg/kg group on Tb. BMD (decrease) and Tb.Sp (increase) similar to those in the first high dose study. Sponsor concluded there were no significant dose-dependent treatment effects on structural bone parameters (uCT, femur) at any dose (30, 120, 500 mg/kg). Strength and turnover markers were not assessed because there was no structural effect. Thus the NOAEL for the structural bone effects was 500 mg/kg.

The NOAEL dose of 500 mg/kg provides a safety margin of 26x based on AUC (human dose 4 mg/kg).

Comments:

- Small but significant effects of sugammadex on bone structure and strength of femoral bone were observed at dose of 2000 mg/kg only (first study).
- Cause of decrease in Tb parameters is not clear but it may have been increased resorption
- Decrease in cancellous bone strength was at least partly due to decreases in trabecular structural measures (TbN, Tb BV/TV, BMD) indicating a decrease in the amount of bone per unit volume. However there may have been a mass-independent component to this decrease in strength due to an effect on material properties. Analysis of the correlation between bone density (mg/cm3) and ultimate load in controls vs treated could provide clarification of this issue although variability in the data would probably prevent solid conclusions. Data on bone strength from the lower dose groups with no effect on structural/density parameters could also clarify this issue but are not available. However, absence of an effect on cortical and femoral neck maximal load suggested no deleterious effect on material properties.
- The decrease in stiffness in trabecular (D21) and cortical bone (D21, D42) suggested a change in the intrinsic mechanical (i.e. material) properties of the bone material. Decreased stiffness makes bone less resistant to bending. The relevance of the data on stiffness is unclear. Ultimate load (N) or energy (J) are usually believed to be the more important strength parameters predicting resistance to fracture and these were not affected in cortical bone or in the femoral neck.
- Apparent inconsistencies or anomalies in the data from the first study included: change in turnover markers in venous but not arterial blood; marked sex difference in cancellous and cortical density and strength (males << females), discordances in structure-strength data; inconsistencies in recovery data (D42).
- In a 1-month repeat dose toxicity study there was no histologic evidence of trabecular thinning at ≤500 mg/kg/day. However, the cumulative bone concentration in this study eg at 500 mg/kg/day probably exceeded the level attained at a single 2000 mg/kg dose. The apparent discrepancy may be due to different resolutions of the techniques. Bone microarchitecture and strength were not evaluated in the repeat dose toxicity study.
- Sponsor discussed the possibility that sugammadex may be a mild Ca chelator and induce a transient hypocalcemia and PTH release which might stimulate bone/Ca resorption. A PTH surge was observed in both rats and dogs upon sugammadex administration at >80 and >120 mg/kg. In the rat (30, 120, 500 mg/kg) this increase was maximal at 3 min post dose (4x at 500 mg/kg). PTH returned to baseline by 30 min. Serum Ca was increased for 30 min and urine P excretion was increased over 24h. In dogs the PTH surge lasted ca. 15 min. Sponsor concluded this transient effect is probably not relevant in the reduction of trabecular mass and strength since bone changes persisted through 3-6 weeks following single dose administration. I agree it is unlikely that the short term PTH surge was responsible for the bone quality effect.
- Transient stimulation of the osteoclast may have occurred due to binding of systemic or local factors (estrogen or cholesterol?, growth factors?) to sugammadex in bone. An increase in bone formation by an alendronate-B-cyclodextrin complex targeted to bone was ascribed to sequestration of regulatory factors in the B-cyclodextrin cavity (Liu et al, Biomaterials, 2008). However this is speculative.
- Large safety margin (26x at NOAEL of 500 mg/kg) indicates low likelihood of clinical relevance.

Juvenile rat studies

In a single dose study in rats (m+f) at ages 7, 14, 21 days (0, 120, 500 mg/kg) there were no effects on body weight, clinical signs, but slight toxicity in kidneys at 500 mg/kg in all age groups (tubule vacuolation) and disruption of teeth enamel epithelium in 1F @14d post dose. Bone length or structure was not evaluated. There were no histopathologic changes in femur.

In a study in 7-day old rats (M and F) dosed with 0, 30, 120, 500 mg/kg and sacrificed on D1, 3, 7, 14, 21, 28, 35, 42 post dose there was discoloration of incisors in all groups including control. No analysis of bone length or structure or turnover was performed.

In a pilot TK study in 7-day old rats animals were dosed for 2 weeks with 0, 30, 120, 500 mg/kg/day. There were histologic effects in lung, kidney and urinary bladder at 120 and 500 mkd.

28-day study

A 28-day multiple dose study was conducted in the 7-day old juvenile rat (dosing from D7-D35, recovery D35-D91) with doses of 0, 30, 120, 500 mg/kg/day (sc D7-21; iv D22-35). Study assessments included bone growth (length), structure (uCT), histopathology, TK. Bone resorption or formation markers and bone strength were not measured.

Results showed no significant effect on body weight during dosing. However, there was a small but significant decrease in BW gain in males after dose termination from D35-D70 at 500 mkd. Ulna length was minimally but significantly reduced (<5%) at 500 mkd in both males and females from D14-D84. Femur length was decreased on D35 (2-3%) and more so on D91 (3%). Femur diameter was slightly increased on D35 and decreased on D91. Thus there was a slight irreversible suppression of longitudinal bone growth with NOAEL 30 mg/kg/day. It is unclear how this effect was related to the body weight effect.

Body weight gain (g)

| | | M | | | | F | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mg/kg | | 0 | 30 | 120 | 500 | 0 | 30 | 120 | 500 |
| | | | | | | | | | |
| D7-D35 | | 93 | 93 | 93 | 91 (-2%) | 83 | 80 | 81 | 79 (-5%) |
| D35-D91 | | 222 | 225 | 207 | 209 (-6%) | 103 | 105 | 99 | 103 (nc) |

Ulna length (mm)

| | | M | | | | F | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mg/kg | | 0 | 30 | 120 | 500 | 0 | 30 | 120 | 500 |
| | | | | | | | | | |
| D28 | | 25 | 24.9 | 24.5 | 24.8 (-1%) | 24.7 | 24.4 | 24.3 | 24.1 (-2%) |
| D84 | | 35.7 | 35.8 | 35.3 | 34.8 (-3%) | 33.1 | 32.7 | 32.5 | 32.5 (-2%) |

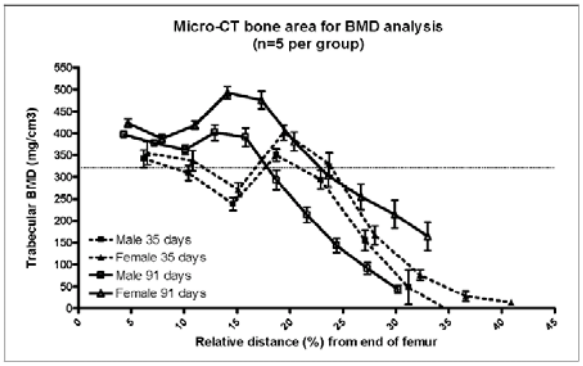
Femur length

| | | M | | | | F | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mg/kg/day | | 0 | 30 | 120 | 500 | 0 | 30 | 120 | 500 |
| | | | | | | | | | |
| Treatment (D35) | Necropsy | 23.4 | **22.8*** | **23*** | **22.9*** | 22.7 | 22.8 | 22.6 | 22.2 |
| | uCT | 23.9 | 23.3 | 23.8 | 23.4 (-2%) | 23.4 | 23.2 | 23.2 | 22.7 (-3%) |
| | | | | | | | | | |
| Recovery (D91) | Necropsy | 34.1 | 33.9 | 33.4 | **32.9*** | 31.1 | 30.8 | **30.3*** | **30.2*** |
| | uCT | 34.8 | 34.9 | **34.3*** | **33.9* (-3%)** | 31.8 | 31.6 | **31.1*** | **30.9* (-3%)** |

(% change as compared to controls)

Slight toxicity evidenced by minimal histopathologic changes in kidney, lung and urinary bladder was observed at 120 and 500 mkd. At 120 and 500 mkd disruption of enamel epithelial layer in incisors was observed. In the femur there was a low incidence of chondromucinous degeneration in the recovery 120 mkd (1/12F) and 500 mkd (1/12M) groups possibly associated with the longitudinal growth reduction. No other histologic bone changes (e.g. trabecular structure) were observed. Tooth color was affected at 500 mg/kg/day (whiter than control). Growth plate morphometry (area, height, diameter) was not performed.

Bone analysis was performed using uCT. For analysis of metaphyseal trabecular bone a slice was scanned in the area above the knee joint where trabecular BMD changes linearly (Figure below), and the location of the slice depended on age of animal (20-25% relative distance from end of femur i.e. 5mm-6mm for 35-day old, 17%-25% i.e. 6-7.5mm for 91-day old). Diaphyseal bone was scanned further away from the knee. Femur sections had to be re-oriented in treated animals due to reduction in femur length and diameter. This may have led to artifacts in trabecular measurements.




There were increases in femoral trabecular BMD, BV/TV, TbN, TbTh (up to 20%), with decreased SMI (ie more rod-like structure), decreased femur length (<5%) and increased diameter (≤5%) at doses of 120 and 500 mg/kg/day, significant mainly at the high dose. Cortical thickness was decreased (≤10%) while cortical BMD and BV/TV were unaffected. Thus bone was slightly shorter and wider with thinner cortex and increased trabecular density. Changes in trabeculae and cortex but not bone length were recovered or reversed by D91. NOAEL of structural bone effects was 30 mkd.

Increased polar moment of inertia (Ip) in females on D35 reflecting increased torsional/bending resistance was probably due to increased ML diameter and decreased Ip on D91 to decreased AP or ML diameter. This suggests that diaphyseal bone strength was probably not affected on D35 but may be slightly reduced on D91.

Sponsor concluded there were no specific effects on bone architecture, growth and development and NOAEL was 500 mg/kg/day. Effect on bone length (120, 500 mkd) was ascribed to growth ie body weight gain retardation due to limited toxicity observed at the 500 mkd high dose. Although Sponsor concluded that the NOAEL for bone structural changes (eg decreased Ct.Th) was 30 mg/kg/day, effects on bone structure were dismissed because they were small and considered to be associated with growth retardation and therefore nonspecific and not adverse.

Reviewer concludes longitudinal growth was retarded upon treatment and/or recovery and there were slight structural changes in trabecular and cortical bone. The NOAEL for these effects is 30 mg/kg/day. This NOAEL provides a safety margin of 1x based on AUC (human dose 4 mg/kg) and >33x based on estimated bone concentration.

Comments:

- The cause and significance of the small reduction in bone growth at the mid and high dose is unclear. Lack of reversibility suggests potential relationship to bone retention. Longitudinal bone growth is determined by growth plate activity but sugammadex does not appear to bind to this

region. Effect on bone length was not determined in single dose juvenile rat studies so single dose NOAEL is not known.

- The relation between the body weight and bone length/size effects is unclear. Effects on bone size appeared at doses/times/groups where there were no significant body weight effects which suggests an independent or specific bone growth effect. However, body weight suppression and slight toxicity in the mid and high dose groups confounds the data and it is not clear whether the study findings are causally or coincidentally related.
- Bone structural effects do not support that the bone length reduction is a result of general toxicity and growth retardation. Younger female rats (D35) have lesser and thinner trabeculae than older ones (D91), while Tb.N and Tb.Th were increased by treatment with 500 mg/kg/day.
- Relationship between changes in trabecular and cortical bone and reduced longitudinal growth is unclear. Possibly, sugammadex causes suppression of bone resorption analogous to bisphosphonates or tetracycline. Alendronate, a resorption inhibitor, can reduce bone length during an animal's growing phase. In the wild type and oim/oim mouse (animal model of osteogenesis imperfecta) treatment from 4 to 12 wks caused a reduction in bone length and an increase in growth plate height/area, perhaps secondary to an impairment in vascular-invasion induced hypertrophic cell apoptosis due to reduced resorption in the chondro-osseous junction (Evans et al, Bone 32, p.268, 2003).

Femur length in B6C3 mice treated with alendronate (2.5 mg/kg/wk, ip)

| | M (wt) | F (wt) | M+F (wt + oim) |
|---|---|---|---|
| | | | |
| Humerus length | -4% | -6% | |
| Ulna length | -0% | -2% | |
| Growth plate area | | | +30% |
| Hypertrophic zone height | | | +68% |

Data from : Evans et al, 2003

However, in another study in 6-wk old B6C3 mice, alendronate did not significantly reduce femur length or body weight (Camacho et al, 2001) but there was a clear effect on metaphyseal bone mineral density (1.7x) and reduced AP diameter. In rats, pigs and rabbits reductions in bone length due to bisphosphonate treatment have been demonstrated (Smith et al, 2005; Kim et al 2005, Pataki et al 1997). Observed effects on length were quite small (<5%) but statistically significant. In children an unequivocal effect on longitudinal bone growth of bisphosponates has not been demonstrated.

- Cortical thinning at end of treatment (D35) did not appear to affect bone strength (Ip) probably due to offsetting increase in bone diameter.
- There was a slight reduction in diaphyseal bone diameter and probably also in bone strength upon recovery in all dose groups (F, M). Significance is not clear.
- It is not known whether bone resorption or formation was affected in the juvenile study. Bone turnover markers or other measures of bone cell activity were not evaluated.
- The age range of the animal treated (D7-D35) is equivalent to a human age range of approximately 2-10 years.
- Safety margins are low when based on AUC (1x), higher when based on bone concentration (33x).
- Since the proposed human treatment is single dose administration, cumulative AUC may be better basis for comparison.
- The safety margin based on bone concentration is conservative since it is based on the bone concentration after a single dose of 30 mg/kg in rats.
- It is not clear which safety margin is most appropriate because the mechanisms underlying the bone effects are unknown.
- Clinical relevance of findings is not clear.

Safety margin calculation

Sponsor

**Table 2: Overview of safety margins for effects of Org 25969 on bone and teeth**

| Effect or no effect observed | Safety window based on: | Animal dose or exposure | Human dose or exposure | Safety margin |
|---|---|---|---|---|
| Trabecular microarchitecture and strength (SD), NOEL | Dose (mg·kg$^{-1}$) | 500 | 4 | 125 |
| | AUC (µg·h·mL$^{-1}$) | 1050 | 40 | 26.3 |
| | $C_0$ (µg·mL$^{-1}$) | 6000 | 50 | 120 |
| | Bone exposure (µg/g) | 313 [a)] | 4.5 | 70 |
| Rat femur histopath. (incl. epiphysis) (MD), NOAEL | Dose (mg·kg$^{-1}$) | ≥ 500 | 4 | ≥ 125 |
| | AUC (µg·h·mL$^{-1}$) | ≥ 1050 | 40 | ≥ 26.3 |
| | $C_0$ (µg·mL$^{-1}$) | ≥ 6000 | 50 | ≥ 120 |
| | Bone exposure (µg/g) | 5000 [b)] | 4.5 | ≥ 1111 |
| Juvenile bone dev't and micro-architecture (SD+MD), NOAEL | Dose (mg·kg$^{-1}$) | 500 [c)] | 4 | 125 |
| | AUC (µg·h·mL$^{-1}$) | 720 [c) g)] | 40 | 18 |
| | $C_{max}$ (µg·mL$^{-1}$) | 580 - 1800 [c)] | 50 | 11.6 – 36 |
| | Bone exposure (µg/g) | 150 [d)] | 4.5 | >33 [d)] |
| Rat fetal bone development, NOAEL | Dose (mg·kg$^{-1}$) | Not relevant [e)] | Not relevant [e)] | n.d. |
| | AUC | Not relevant [e)] | Not relevant [e)] | n.d. |
| | $C_0$ | Not relevant [e)] | Not relevant [e)] | n.d. |
| | Bone exposure | 450-600 | 4.5 | 100-133 |
| Juvenile rat incisor discoloration/disturbed amelogenesis in juvenile rat incisors and molars | Dose (mg·kg$^{-1}$, NOAEL) | 500 [f)] | 4 | 125 |
| | AUC (µg·h·mL$^{-1}$) | 720 [g)] | 40 | 18 |
| | $C_{max}$ (µg·mL$^{-1}$) | 580 - 1800 | 50 | 11.6 – 36 |
| | Teeth exposure (µg/g) | 220, 2200, 2500 [f)] | 4.5 | >48, 480, > 556 [f)] |

In most cases rounded values for systemic and bone exposure values are used for convenience.
n.d. = not determined; SD = single dose; MD = multiple dose, 4 weeks

[a)] In the study under reference 4 level of exposure extrapolated from the retention observed after a single dose of 400 mg/kg is 500/400 x 250 µg/g = 313 µg/g bone as Lowest Observed Effect Concentration (LOEC). After a single dose of 2000 mg/kg the bone exposure level is estimated to be approximately double that at 400 mg/kg, thus 2 x 250 µg/g = 500 µg/g.

[b)] The total exposure in rat bone at the dose of 500 mg·kg$^{-1}$·day$^{-1}$ in the 4-week toxicity study is difficult to estimate. Rough estimates indicate that exposure in bone highly likely exceeds the level of 5000 µg/g bone. A concentration of 2.5-5 mg Org 25969 per gram bone is already extrapolated for the dose of 30 mg·kg$^{-1}$·day$^{-1}$ from reference 4.

[c)] The NOAEL for an effect on bone micro-architecture has been estimated at the high level used in the 4-week juvenile rat toxicity study. The slight effects observed are attributed to the slight growth retardation caused by the mild general toxicity in this study. At 30 mg·kg$^{-1}$·day$^{-1}$ given for 4 weeks no effect at all is observed. If that is taken at the NOEL, the multiple dose design has to be taken into account representing a very worst case situation as compared to the single dose clinical use. Alternatively, the highest dose level of the single dose juvenile rat study (500 mg·kg$^{-1}$) can be taken as the NOAEL as no histopathological changes were observed in bone.

[d)] The extent of Org 25969 binding to bone in juvenile rats has been estimated in reference 4. At the dose level of 30 mg·kg$^{-1}$, approximately 150 µg Org 25969 per gram bone is reached. Thus, the minimal safety margin is 33, however most likely bone concentration has been significantly higher at 500 mg·kg$^{-1}$ and upon repeated dosing, thus the safety margin is much higher.

[e)] The maternal dose and exposure at which no effects occur in the embryo-fetal development study in rat and rabbit are considered of little relevance. Only a fraction of the maternal dose and exposure are transferred to the fetus due to the limited placental transfer of Org 25969. Thus, it would represent an overestimate of the safety margin. Fetal bone exposure is relevant as Org 25969 has been shown to accumulate with repeated dosing.

[f)] The single dose NOAEL for the discoloration of incisors and effects on amelogenesis in or molars and incisors of juvenile rat is 500 mg·kg$^{-1}$. The NOAEL expressed in mg·kg$^{-1}$ determined after 4 weeks of dosing (30 mg·kg$^{-1}$·day$^{-1}$) is not considered very relevant to man given the single dose application. Local exposure is the most relevant parameter to express the safety margin on. Data on retention in incisors and molars of juvenile rats at a single dose of 30 mg·kg$^{-1}$ Org 25969 levels of 220 µg per gram incisor are being reached. If in juvenile rats the concentration in incisors and molars increase approximately 10 to 15-fold upon dosing for 4 weeks, the exposure level at the end of the 4-week juvenile rat study is estimated to be around 2.2 mg/g incisor for the NOAEL dose level of 30 mg·kg$^{-1}$ at the end of the 4 week dosing period. The safety margin is then estimated to be around 480. In young adult rats no incisor or molar discoloration is observed at 30-500 mg·kg$^{-1}$·day$^{-1}$ and exposure levels in incisors and molars are 2-2.5 mg/g and 0.5-1 mg/g, respectively at the dose level of 30 mg·kg$^{-1}$. The extent of binding may not be linear to the dose, but this indicates that the safety margin based on incisor exposure is even (much) larger than factor 480. If exposure in human teeth is lower as compared to bone (as it is for rat) this can also be multiplied by this additional safety factor.

[g)] The AUC of 720 µg·h·mL$^{-1}$ at the dose of 500 mg·kg$^{-1}$ in the juvenile rat is estimated from the approximate average of juvenile rats of approx 2 to 3 weeks old and dosed s.c.. For 1 week old rats the AUC will be higher (±1200 µg·h·mL$^{-1}$), for 5 weeks old rats it will be slightly lower (±430 µg·h·mL$^{-1}$) as a consequence of higher clearance.

Safety margins bone effects

| Species | Study and Effect | Safety margin based on | Dose or exposure | Human dose or exposure | Safety margin |
|---|---|---|---|---|---|
| Young adult rat | Single dose study, Tb structure and strength decrease NOAEL | Dose (mg/kg) | 500 | 4 | 125x |
| | | Dose (mg/m2) | - | - | 20x |
| | | **AUC (ugxh/mL)** | **1050** | **40** | **26x** |
| | | Bone conc (ug/g) | 313 | 4.5* | 70x |
| | Multiple dose study, bone histopathology NOAEL | Dose (mg/kg) | ≥500 | 4 | ≥125x |
| | | Dose (mg/m2) | - | - | ≥20x |
| | | **AUC (ugxh/mL)** | **≥1050** | **40** | **≥26x** |
| | | Bone conc (ug/g) | ≥313 | 4.5* | ≥70x |
| Juvenile rat | Single dose study, bone histopathology NOAEL | Dose (mg/kg) | 500 | 4 | 125x |
| | | Dose (mg/m2) | - | - | 20x |
| | | **AUC (ugxh/mL)** | **720** | **40?** | **18x?** |
| | | Bone conc (ug/g) | 150 | 4.5?* | 33x? |
| | Multiple dose study, changes in bone structure and delayed growth/development NOAEL** | Dose (mg/kg) | 30 | 4 | 7.5x |
| | | Dose (mg/m2) | - | - | 1x |
| | | **AUC (ugxh/mL)** | **22** | **40?** | **<1x?** |
| | | Bone conc (ug/g) | 150 | 4.5?* | 33x? |

*Human bone concentration (4.5 ug/g) was based on data from mass balance study with 15% dose retention assuming 10 kg bone in 75 kg person and was not adjusted for pediatrics.
** NOAEL different from sponsor's

Comments:

- All NOAEL safety margins were large (≥18x), except the AUC-based safety margin derived from the 28-day multiple dose juvenile rat study (1x).
- Since mechanisms underlying observed effects are not known it is unclear which safety margins are more appropriate (AUC or bone ug/g basis).
- For single dose studies, AUC and bone concentration (ug/g) can be used as basis for safety margin calculations.
- For multiple dose studies, total cumulative bone concentration (ug/g) is not reliable as basis for safety margin calculation since it does not reflect the concentration of compound at the pharmacological site (and time) of action.
- Human bone exposure was calculated based on mass balance study data indicating a maximal bone retention of 15% of dose. Thus, safety margins based on bone concentration (ug/g) in adults are conservative.
- AUC and bone concentration in children is not known. Thus, safety margins for children are uncertain (?).

**Summary of bone findings**

- Bone retention of sugammadex was shown in disposition studies and decreased with animal age probably due to lower rate of bone metabolism (resorption, formation)
- Sugammadex was localized on cortical and trabecular bone surface but not in growth plate area.
- Histopathology evaluation of longitudinal bone sections did not identify clear bone effects in any study (young adult or juvenile rat, single or multiple dose).
- Single dose treatment of adult rat showed slight trabecular bone deterioration (decrease in mass and strength) at a single high dose (safety margin 26x, AUC basis). Effects were of unknown etiology but possibly due to resorption stimulation. Based on large safety margin clinical relevance of this finding is probably low.
- Treatment of juvenile rats for 4 weeks resulted in slight but persistent growth inhibition (safety margin 1x, AUC basis). This was accompanied by slight reversible changes in trabecular and cortical bone (safety margin 1x, AUC basis). Effects were dose dependent and likely treatment related. Association with slight body weight reduction was unclear. There were no data on bone turnover or biomechanical strength from the repeat dose juvenile study. However, trabecular data predict transiently increased metaphyseal bone strength while cortical data predict slightly reduced diaphyseal strength after treatment.

**Comments**

- Retention of sugammadex in bone is explained by likely affinity for HA. However, experimental evidence for this was not provided.
- There was no clear evidence of an effect of sugammadex on osteoclast or osteoblast cell function
- Mechanisms underlying effects in juvenile and young adult rats are unclear. Also, the apparent difference in bone effects in adult and juvenile rats is puzzling. There were insufficient data to clarify the inconsistency.
- The relation between the body weight and bone length/size effects in the juvenile rat study is unclear. Data suggest an independent or specific bone growth effect.
- Data on growth plate morphology are needed to provide insight in longitudinal growth reduction. Data may be obtained using samples from the completed 28-day juvenile rat study.
- Evaluation of vertebrae would be helpful to interpret bone effects since they have a more homogeneous trabecular structure.
- Presentation of data from bone localization study was poor
- Action of sugammadex appears to be different from that of bisphosphonates.
- Chelation of Ca and resulting PTH surge is unlikely to provide an explanation for the apparent resorptive effect in the single dose study
- Additional studies to address mechanism of action include in vitro bone resorption assay ($^{45}Ca$ release), assessment of bone turnover markers, hydroxyl-apatite crystal growth and dissolution assay, effect of in vitro bone decalcification on sugammadex retention.

**Concerns**

- Lack of data on binding properties to bone and its matrix components.
- Apparent contradiction in data between adult and juvenile rats (increase vs. decrease in Tb bone)
- Lack of data on bone cell function from multiple dose studies (resorption, formation)
- Potential effect of sugammadex on bone material properties upon repeat dose accumulation
- Unknown effect of sugammadex on healing of bone fractures

Questions from DAARP

***Methodologic questions***:

1. Are the technical methods (serum/plasma/urine biomarkers, µCT, evaluation time-points, strength measurements) used in the juvenile toxicity evaluation and dedicated bone studies in the young adult rat adequate and appropriate to evaluate risk to bone?

*Yes.*

*Biomarker evaluation (performed in young adult rat bone study) was adequate and appropriate.*

*Micro-CT evaluation (in juvenile and young adult rat bone studies) is adequate and provides high resolution 3D-data on macro- and microarchitecture of cortical and cancellous bone compartments.*

*Evaluation time-points generally appeared appropriate.*

*Biomechanical tests (indentation, bending, cantilever) and parameters measured (load, energy, stiffness) were adequate and provided information on extrinsic and intrinsic bone mechanical properties of cancellous and cortical bone.*

2. Has the microautoradiography study (INT00029186) in rat bone properly characterized the distribution of sugammadex within the bone? If not, would you recommend alternative assays for this evaluation?

*Microautoradiography employed in Study INT00029186 was an appropriate assay. However, characterization of bone localization was not optimal. Although radiolabel was clearly associated with cortical and trabecular bone surfaces, localization in the growth plate area was not well defined. Poor micrograph quality and errors in the micrograph descriptions diminished the reliability of the data.*

3. Has the Sponsor provided sufficient evidence or an acceptable scientific argument to explain the mechanism and binding site underlying sugammadex retention in bone?

*No. Additional studies would be needed to explain the basis for bone retention, e.g. hydroxyapatite crystal growth or dissolution assay or release of sugammadex from bone upon decalcification.*

4. Does the absence of positive controls (mimicking anabolic or catabolic states) in the dedicated bone studies affect your confidence level in the interpretation of the results?

*Yes. Data from compounds with known mechanism of action would have helped interpret the data in both young adult and juvenile rat studies.*

***Risk for adults***:

5. Do the findings in the bone in adult rats represent a risk for adult patients? Has the risk for adult patients been adequately addressed or characterized?

*The findings in the adult rat suggest a risk for thinning and weakening of trabecular bone. However, based on considerable safety margins for structural and mechanical effects (AUC or bone concentration basis) the risk for adverse bone effects in adult patients is not likely to be significant.*

***Risk for children***:

6. Are the bone findings described in the juvenile rat study significant or adverse?

*Findings in the repeat dose juvenile rat study included macro- and micro-structural bone changes. There was a slight irreversible suppression of longitudinal growth and there were slight changes in metaphyseal/trabecular and diaphyseal/cortical bone architecture. Effects on bone cell activity and growth plate characteristics were not evaluated. Based on AUC comparison the safety margin was very small, but based on bone concentration the margin was considerable. Safety margin calculations were based on assumptions and it is unclear which safety margin is most appropriate.*

*The mechanistic basis and significance of the findings is not clear. Longitudinal bone growth is determined by growth plate activity but sugammadex does not preferentially bind to this region. While a persistent longitudinal growth effect is undesirable, the positive effect on trabecular bone is unlikely to be adverse. The cortical bone changes are of questionable significance because of their nature, reversibility and/or inconsistent dose- and sex-dependence. The 28-day duration of the animal study mitigates the significance of the animal findings for single dose clinical treatment.*

a. Do you agree with the Sponsor that the altered bone parameters in the juvenile rat study likely represents a general and non-specific "growth retarding" effect and is not indicative of bone toxicity?

*No. The relation between the reduction in body weight and the changes in bone parameters (length, diameter, cortical thickness, trabecular density) in the juvenile rat study is unclear and a specific inhibitory effect on longitudinal bone growth can not be excluded.*

7. Do these findings represent a risk in the pediatric population?

*The findings are unlikely to represent a significant risk for longitudinal growth retardation or unfavorable bone effects upon single dose administration in children. However, they suggest a risk for slight growth retardation upon multiple dose administration. The findings may also predict alterations in bone structure and biomechanical behavior upon multiple dosing but it is unclear whether these translate into clinically significant adverse effects.*

a. If so, what is the risk?

*See response to Question 7.*

b. Are there further evaluations you would recommend to further address or characterize this (or any other) risk?

*Further evaluations can include (1) in vitro mechanistic studies, (2) quantitative evaluation of growth plate morphology (3) assessment of bone resorption/formation and bone strength upon multiple dosing of juvenile or young adult animals, and (4) single dose PK and skeletal retention studies in pediatric subjects to better define safety margins. Additional animal data could be obtained from samples retained in completed studies. Data from mechanistic studies may suggest potentially useful clinical biomarkers.*

*Because the effects observed in animals were small and safety margins were adequate for single or limited repeat dose administrations, I expect that additional in vivo animal studies will only provide a marginal improvement in the ability to project risk or safety in the pediatric population. I agree with sponsor that bone strength assessment is not practical for young animals of <2 months of age. The need for further evaluations depends on risk-benefit estimations and the likelihood of multiple dosing in the clinical pediatric setting.*

8. Are bone strength/integrity tests conducted in young adult rats combined with data obtained from the juvenile rat studies sufficient to evaluate risk in pediatric patients of all age groups? If not, would you recommend dedicated bone studies in the juvenile rat? Do you have

recommendations on study design (in particular single vs. multiple dose and appropriate recovery sacrifice time-points)?

*In the 4-week repeat dose juvenile rat study animals were treated during a period equivalent to a human age of approximately 2-10 years. This is a considerable period of time and the risk identified in the study is probably relevant for growing pediatric patients of all age groups. The 8-week recovery period in the rat represents only ≤1x the skeletal $T_{1/2}$ for sugammadex. It corresponds to approximately 5 years in humans from the perspective of musculoskeletal development. The single dose study in young adult rats does not provide much relevant information on pediatric risk.*

*For specific suggestions on additional studies see response to Question 7b.*

9. Has the potential for long-term risk of bone retention of sugammadex in the juvenile animal been sufficiently addressed or characterized?

*The potential for long-term risk of bone retention of sugammadex in juvenile animals has been reasonably well characterized. However, lack of data on mechanism of action and functional effects complicates risk assessment. Additional studies may help to better define the long-term risk for adverse bone effects if such studies include functional endpoints (e.g. bone resorption, bone strength), a positive control arm (e.g. bisphosphonate) and an extended recovery period (e.g. 4-6 months).*

*For specific suggestions on additional studies see response to Question 7b.*

***Risk of impaired bone-healing***:

10. Do you agree with the Sponsor that the data obtained by the Sponsor indicates the probable absence of risk for disruption of bone repair? If not, are there in vitro or in vivo nonclinical studies in nonclinical fracture models which are preferred to evaluate bone healing?

*No. A non-clinical fracture repair study is recommended to evaluate potential interference with bone healing.*

***General risk evaluation***:

11. Is evaluation of bone integrity principally in the young adult and juvenile rat sufficient to evaluate potential risk to adult and pediatric patients or is a second animal model (i.e. non-rodent) necessary for dedicated bone evaluations. If so, is there a preferred species for this evaluation?

*The young adult and juvenile rat models are adequate for evaluation of bone effects in adult and pediatric patients and a second animal model is not needed.*

12. Do you believe the approach the Sponsor used for safety margin calculations for bone concentration is appropriate and acceptable?

*Safety margins based on bone concentrations should be used with caution. The distribution in bone is likely to be inhomogeneous and the relevant bone concentration is the concentration at the site of pharmacologic action. Total bone concentration may not represent this concentration and thus the calculation may be inappropriate. Also, the approach is misleading if the estimated bone concentration after a single dose in humans is compared to the bone concentration at the end of a repeat dose period in animals. Finally, since the mechanisms underlying the observed bone effects are unclear (e.g. longitudinal growth reduction) it is not clear whether the bone concentrations are relevant. Thus it seems prudent to rely mainly on AUC-based safety margins with additional support from bone concentration-based margins.*

***Additional comment:***

*Labeling section 13.2 (Animal Toxicology and/or Pharmacology) describing bone effects needs to be revised.*

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Materials reviewed:

- Primary review (AXu), PeRC Consult.doc
- Sponsor's position paper
- NDA study reports
- Doc: Explanation for estimated local exposure level in rat bone

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/s/
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Karen Davis-Bruno
7/21/2008 10:54:44 AM
PHARMACOLOGIST

Tertiary Pharmacology Review

**By**: Paul C. Brown, Ph.D., ODE Associate Director for Pharmacology and Toxicology OND IO
**NDA:** 22-225
**Submission date:** October 31, 2007
**Drug:** sugammadex
**Sponsor:** Organon
**Indication:** routine reversal of shallow or profound neuromuscular blockade induced by rocuronium or vecuronium, and immediate reversal of neuromuscular blockade at 3 minutes after administration of rocuronium

**Reviewing Division:** Division of Anesthesia, Analgesia and Rheumatology Products

**Introductory Comments:**
The pharm/tox reviewer and supervisor found the nonclinical information submitted for sugammadex to be sufficient to support its use for the proposed indication. Additional information was considered necessary to support further development of the drug in pediatric patients.

**Reproductive and developmental toxicity:**
The sponsor has proposed (b) (4). The reviewer recommended a category of B but included description in the pregnancy section of some findings of neonatal mortality that were observed in a rat peri/postnatal study. The findings may be related to maternal cannibalism of the pups. The reason why cannibalism may be increased is not clear. The possibility that sugammadex causes some alteration of the fetus that results in an increase in cannibalization of the pups has not been eliminated. The supervisor recommended that the pregnancy category be C based on these findings. Category C would be appropriate if these findings are included in the labeling. I think these findings could be included in the labeling unless the sponsor provides additional information that the findings do not represent a risk to the neonate.

**Bone effects:**
Sugammadex binds to bone. Some effects on bone growth were noted in a juvenile rat study. The reviewer and supervisor recommended additional studies to better assess the effect of sugammadex on bone development and on bone response to fracture. The Anesthetic and Life Support Drugs Advisory Committee also recommended that such studies be undertaken. The pharm/tox supervisor recommended that these studies be conducted prior to multiple dose pediatric studies, approval of a pediatric indication or inclusion of pediatric data in the label. I agree.

**Carcinogenicity:**
The proposed use of sugammadex is acute. A carcinogenicity evaluation is seldom necessary for acute indications. However, sugammadex has a very long bone half-life ($t_{1/2ß}$ = 172 days). Therefore, the supervisor has recommended that the applicant be asked to provide a rationale as to why sugammadex will not confer a risk for bone tumor development. Otherwise, an evaluation of the carcinogenic potential of sugammadex may

be required. I agree that, in spite of the acute administration of the drug, the pharmacokinetics of sugammadex result in chronic exposure of at least some tissues. Consequently, I agree that the applicant should be asked to address this concern.

**Conclusions:**
I concur with the Division pharm/tox conclusion that the nonclinical data support approval of this NDA for adults.

I recommend that the pregnancy section of labeling include description of the neonatal mortality observed in the peri/postnatal study and that the pregnancy category be C.

Additional information on the bone effects should be collected before conducting multiple dose pediatric studies.

Additional information should be provided on the possible risk of carcinogenicity, particularly to bone.

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/s/
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Paul Brown
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PHARMACOLOGIST

**DEPARTMENT OF HEALTH AND HUMAN SERVICES**
**PUBLIC HEALTH SERVICE**
**FOOD AND DRUG ADMINISTRATION**
**CENTER FOR DRUG EVALUATION AND RESEARCH**

**Pharmacology and Toxicology Memorandum**

# ADDENDUM

| | |
|---|---|
| NDA NUMBER: | **22-225** |
| SERIAL NUMBER: | **000** |
| DATE RECEIVED BY CENTER: | **30-OCT-2007** |
| PRODUCT: | (b) (4) **Sugammadex sodium** |
| INTENDED CLINICAL POPULATION: | **routine reversal of shallow or profound neuromuscular blockade induced by rocuronium or vecuronium, and immediate reversal of neuromuscular blockade at 3 minutes after administration of rocuronium** |
| SPONSOR: | **Organon USA, a Division of Schering-Plough** |
| DOCUMENTS REVIEWED: | **N/A** |
| REVIEW DIVISION: | **Division of Anesthesia, Analgesia and Rheumatology Products (HFD-170)** |
| PHARM/TOX REVIEWER: | **Zengjun Xu, Ph.D., DABT** |
| PHARM/TOX SUPERVISOR: | **Adam Wasserman, Ph.D.** |
| DIVISION DIRECTOR: | **Bob Rappaport, M.D.** |
| PROJECT MANAGER: | **Allison Meyer** |

**Background:**
An informal consult request from Dr. Alan Schroeder, the CMC reviewer of this product, expressed concern about the safety of (b) (4) a compound formed during the (b) (4) of the drug product. According to Dr. Schroeder, (b) (4) is known to be formed as an impurity during (b) (4). The applicant has indicated that its level is below the reporting threshold ((b) (4)%, according to ICH), and it is therefore not specifically limited in the drug product. Evaluate the safety of this level of (b) (4), since it has (b) (4) This addendum is to specifically address the issue of the safety of (b) (4) Other issues raised by Dr. Schroeder in the informal consult have already been addressed in the primary review by this reviewer.

**Evaluation:**
(b) (4) The maximal proposed dosage of sugammadex is 16 mg/kg in human via IV injection, and the maximal exposure level of (b) (4) is ≤ (b) (4) mg/kg, 31-fold lower than the EPA reference dose via the oral route. It has been reported that (b) (4) was well absorbed with high bioavailability after oral administration ((b) (4)). (b) (4) is also reported to be metabolized and eliminated after administration. Following oral dosing of (b) (4), the dosed radioactivity was rapidly excreted with 50–75% of the dose eliminated within 24 hr and the primary excretory metabolite is (b) (4) accounting for 44–68% of the dose (b) (4)). Sugammadex is indicated for single-dose administration, and continuous, or even substantial repeated administration will not occur. Therefore, duration of (b) (4) exposure is expected to be short after sugammadex administration.

Genotoxicity studies have been conducted using *in vitro* and *in vivo* models to evaluate the mutagenic and clastogenic property of (b) (4) in the past decades. (b) (4)
Overall, both positive and negative results have been obtained in *in vitro* genotoxicity tests. (b) (4) was found to be negative in mutagenesis in all Ames test conducted with and without activation by rat and hamster liver microsomal preparations using both plate and liquid suspension assays in different labs. Negative results were also reported evaluating unscheduled DNA synthesis (UDS) in rat hepatocytes and SHE cells. In addition, negative

results have been obtained in an *in vitro* micronucleus test and a transformation test in SHE cells as well as in the Drosophila sex-linked recessive lethal assay. Three mammalian gene mutation studies have been reported. No increase in mutation frequency was seen in a CHO/HPRT gene mutation assay with or without Aroclor 1254-induced rat liver S9 up to the concentration producing approximately 70% survival reduction. However, two mouse lymphoma L5178Y TK+/- studies conducted by different labs showed positive results with and without rat liver S9. As the WHO report indicated, the authors of these two studies did not state if there was any adjustment of pH. A single experiment conducted with CHO cells exposed to (b) (4) solutions adjusted to pH 7 at concentrations that reduced cloning efficiency by up to 58-65% found a concentration-related increase in the percentage of cells with chromosome aberrations, primarily chromatid breaks and exchanges in the presence and absence of rat liver S9. In *in vivo* models, (b) (4) was negative in chromosomal aberrations in the bone marrow of rats at single gavage dose of 1000 mg/kg or 5000 mg/L in the drinking water for 5 days and in dominant lethal assay in mice following acute (324 mg/kg) or repeated exposure (162 mg/kg for 5 days).

Generally, the Ames test is primarily used to detect the potential of point mutation while mouse lymphoma assay is theoretically capable of detecting both point mutation and chromosomal damage. The contradicting results that (b) (4) was negative in Ames test but positive in mouse lymphoma assay may suggest that (b) (4) is clastogenic. This was further confirmed by evaluation in *in vitro* chromosomal aberration assays. However, an *in vitro* micronucleus assay did not support this conclusion. In addition, an *in vivo* cytogenesis assay did not show chromosomal aberrations in rats. Furthermore, all other *in vitro* and *in vivo* genotoxicity assays including in vitro UDS, morphological transformation, and sex-linked recessive lethal assay and *in vivo* mouse dominant lethal assay showed negative results. Collectively, the weight-of-evidence evaluation of the genotoxicity potential of (b) (4) based on results of studies available in the public domain does not appear to indicate genotoxicity.

Studies in rats and mice for carcinogenicity evaluation indicated that (b) (4) is not likely to be carcinogenic. In a rat study, animals (50/sex/group) were given (b) (4) in the drinking-water at concentrations of 0, 120, 400 or 1200 mg/liter (0, 8, 27, or 78 mg/kg body weight per day, respectively) over 26 (males) or 28 (females) months (b) (4). The highest dose was chosen because of evidence of reduced water reduction at this dosing level. This study did not reveal any toxic changes or indications of a carcinogenic potential of (b) (4). It was reported that epidermal tumors were not observed in male mice (40 animals/group) after topical application of (b) (4) (25 µl 0.1% (b) (4), 3 times/week) for lifetime exposure (b) (4). In contrast, positive control (0.1% 3-methylcholanthrene) produced skin tumors in most treated animals. Another published carcinogenicity study of (b) (4) was conducted in female mice following SC injection of 20 µmol (approx 1.4 mg) in 50 µl trioctanoin, once a week for 52 weeks. Two of 30 mice receiving (b) (4) were found to have sarcomas developed at the site of application, while no tumors were observed in animals administered vehicle. The authors concluded that the results suggested that (b) (4) is a weak carcinogen in female Hsd:(ICR)Br mice. In addition, another study was also discussed by the WHO review report and other literature (b) (4) that topical application of 1 mg (14 µmol) for 1.5 years in female mice caused squamous cell carcinomas in 2 of the 30 animals. This study was never published due

to inconsistencies uncovered by an independent review of the study, as indicated in the WHO report. Both WHO reviewers and other authors indicated that the positive results of the latter two studies may be due to repeated direct insult of (b) (4) with highly irritating and corrosive properties ((b) (4)). This reviewer agrees with this explanation and furthermore believes (b) (4) does not appear to have significant carcinogenicity risk in human based on these data from animal studies.

Based on the evaluation above, additional studies are not needed to further investigate the general toxicology, genotoxicity and carcinogenicity potential of (b) (4) and the specifications proposed by the Sponsor are acceptable from the nonclinical standpoint.

**Recommendations**:
No action necessary. The specifications for (b) (4) are acceptable.

Reference List

(b) (4)

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/s/
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Adam Wasserman
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I concur.

**DEPARTMENT OF HEALTH AND HUMAN SERVICES**
**PUBLIC HEALTH SERVICE**
**FOOD AND DRUG ADMINISTRATION**
**CENTER FOR DRUG EVALUATION AND RESEARCH**

## Supervisory Pharmacologist Memorandum

# ADDENDUM

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|---|---|
| NDA NUMBER: | **22-225** |
| SERIAL NUMBER: | **000** |
| DATE RECEIVED BY CENTER: | **30-OCT-2007** |
| PRODUCT: | **(b) (4) ™; Sugammadex sodium** |
| INTENDED CLINICAL POPULATION: | **routine reversal of shallow or profound neuromuscular blockade induced by rocuronium or vecuronium, and immediate reversal of neuromuscular blockade at 3 minutes after administration of rocuronium.** |
| SPONSOR: | **Organon USA, a Division of Schering-Plough** |
| DOCUMENTS REVIEWED: | **N/A** |
| REVIEW DIVISION: | **Division of Anesthesia, Analgesia and Rheumatology Products (HFD-170)** |
| PHARM/TOX REVIEWER: | **Zengjun Xu, Ph.D., DABT** |
| PHARM/TOX SUPERVISOR: | **Adam Wasserman, Ph.D.** |
| DIVISION DIRECTOR: | **Bob Rappaport, M.D.** |
| PROJECT MANAGER: | **Allison Meyer** |

**Supervisory Pharmacologist Memorandum – ADDENDUM**

## Background

The initial primary nonclinical review and supervisory memo noted that no carcinogenicity evaluation was conducted by, nor required of, the Applicant due to the acute use nature of the drug product. Upon further consideration of the deposition of sugammadex into bone and the demonstration of an extended duration of retention with single administration (bone $t_{1/2(\beta)}$ mean of 172 days), an evaluation of the potential for bone carcinogenicity should be considered. This concern is especially relevant for the pediatric population in comparison to the adult population due to the higher concentration of drug which is believed will be retained in this tissue as well as the intrinsically high level of growth in this tissue prior to skeletal maturation and closure of the epiphyseal plates.

Primary bone tumors are not common in the adult or pediatric population; however, primary bone tumors represent the 6th most common malignant neoplasm in the children while in adolescents and young adults this represents the 3rd most common tumor. There is a greater propensity to develop tumors in the younger population compared with adults (Patel and Benjamin, 2008). Of primary malignant bone tumors, the most common version is osteosarcoma, though others such as Ewing's sarcoma, are observed in this population. Osteosarcoma is most often observed around the growth plate of long bones (Tang et al., 2008). The etiology is likely multi-factorial but several factors are known to play a significant role: radiation is considered the only proven exogenous risk factor (Longhi et al., 2006) but has a long delay of expression and results in osteosarcoma observed in adults. Genetic mutations and chromosomal rearrangements, which may be hereditary, have been described which underlie osteosarcoma. These include mutations in the retinoblastoma gene, the product of which is a growth suppressive protein; mutations in this gene increase the likelihood of development of osteosarcoma by 500X, as well as other genes (TP53, MDM2 and CDK4; C-myc and C-fos) which are also believed to be involved osteosarcoma development (Longhi et al., 2006; Kansara and Thomas, 2007; Tang et al., 2008). However, despite some genetic predisposition, the majority of osteosarcoma is not associated with a familial hereditary pattern which argues for other causes (Tang et al., 2008). Osteosarcoma, as a disease of impaired osteoblast differentiation has recently been implicated (Haydon et al., 2007). Although trauma had been considered as a potential etiology of primary bone cancer, this has largely been abandoned for lack of evidence (Longhi et al., 2006), though bone tumors are most commonly detected when evaluating patients with bone trauma which may or may not be caused by the bone tumor.

The active pharmaceutical ingredients in sugammadex (Org 25969, (b) (4)) as well as (b) (4) synthesis impurities, including (b) (4) impurities, were evaluated by the Applicant in standard genotoxicity assays which detect mutagenicity and clastogenicity. No evidence of genotoxicity was revealed, a finding which may be expected due to lack of cell entry suggested by the structure, size, and charge of the molecules under consideration.

The Applicant has provided data from a published study conducted in the rat which demonstrated that β-cyclodextrin administered at high dose through the oral route (dietary, up to 5%) over 104 weeks is not associated with the development of tumors (Toyoda et al., 1997). Data was also provided by the Applicant that hydroxypropyl-β-cyclodextrin, administered via the oral route in the mouse and rat was not carcinogenic in these species; however, some of the supplied data was not subject to peer review and was of uncertain quality: a manufacturer's "Data Sheet" for hydroxypropyl-β-cyclodextrin which cited two posters at a 1997 Society of Toxicology Meeting (not published), and a peer-reviewed study by Gould and Scott (2005) in the rat in which increased tumors were noted in pancreas and liver but were argued was species-specific and not representative of a risk in humans.

**Discussion & Recommendation**

The lack of apparent genotoxicity of sugammadex argues against a concern for potential tumor genesis through this mechanism; however, effects on hormones and growth factors in the local environment as a contributor to primary bone tumor development or promotion have not been ruled out. The submitted literature and reports does not provide significant support for or against the potential carcinogenicity of sugammadex. Although the chronic oral (gavage or dietary) route of administration was employed and systemic exposure to cyclodextrin assured, data was developed with different forms of cyclodextrins (β- versus γ-cyclodextrin of sugammadex), and neither form of this β-cyclodextrin (un-substituted or substituted) has been shown to have a similar deposition and retention in skeletal bone. In general, the Applicant's argument that the transient exposure to the substituted gamma-cyclodextrin sugammadex obviates a need to consider the carcinogenic potential of sugammadex is not persuasive. This should be the subject of greater investigation and the Applicant should provide data from in vitro and in vivo studies along with a well supported rationale which is persuasive that the risk of administration and long-term retention of sugammadex in the bones of pediatric patients will not confer a risk for bone tumor development in this sensitive population. Such data may come from in vitro evaluation of sugammadex on osteosarcoma cell lines, demonstration of lack of binding to, or effect on, osteocytes (or alternatively exclusive binding to hydroxyapatite), and perhaps most importantly histopathologic evaluation of skeletal bones in the requested long-term juvenile animal toxicity study (skeletal evaluation at maturity), or other studies which the Applicant may find appropriate. This evaluation is not considered necessary for approval of the adult indication due to the reduced sugammadex deposition percentage expected in the mature skeleton relative to the immature skeleton as well as the possible lower sensitivity of the adult relative to the pediatric population.

Therefore, the following additional comment should be provided to the Applicant as part of the response:

2. Although plasma levels of sugammadex rapidly decline with acute administration, the long retention of sugammadex in skeletal bone may be considered to be chronic exposure (i.e., greater than 6-months) and raises concern regarding the potential for development of tumors of this tissue, especially in the pediatric population which is known to develop primary bone tumors at rates which exceed

that in the adult population. You will need to provide data which may be derived from published literature, in vitro and/or in vivo studies, along with a well supported rationale which is persuasive that the risk of administration and long-term retention of sugammadex in the bones of pediatric patients will not confer a risk for bone tumor development in this population. Otherwise, an evaluation of carcinogenic potential of sugammadex may be required.

## References


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Tang N, Song WX, Luo J, Haydon RC, He TC. Osteosarcoma Development and Stem Cell Differentiation. *Clin Orthop Relat Res.* [Epub, 2008].

Tyoda K, Shoda T, Uneyama C, Takada K and Takahashi M. Carcinogenicity Study of β-Cyclodextrin in F344 Rats. *Food and Chemical Toxicol.* 35:331-336, 1997.

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/s/

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Adam Wasserman
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**PUBLIC HEALTH SERVICE**
**FOOD AND DRUG ADMINISTRATION**
**CENTER FOR DRUG EVALUATION AND RESEARCH**

# PHARMACOLOGY/TOXICOLOGY REVIEW AND EVALUATION

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|---|---|
| NDA NUMBER: | **22-225** |
| SERIAL NUMBER: | **000** |
| DATE RECEIVED BY CENTER: | **10/31/07** |
| PRODUCT: | (b) (4) **(Sugammadex sodium)/Org 25969** |
| INTENDED CLINICAL POPULATION: | **Adult patients with shallow or profound neuromuscular blockade induced by rocuronium or vecuronium** |
| SPONSOR: | **Organon USA Inc.** |
| DOCUMENTS REVIEWED: | **eCTD submission** |
| REVIEW DIVISION: | **Division of Anesthesia, Analgesia, and Rheumatology Products (HFD-170)** |
| PHARM/TOX REVIEWER: | **Z. Alex Xu, PhD, DABT** |
| PHARM/TOX SUPERVISOR: | **Adam Wasserman, PhD** |
| DIVISION DIRECTOR: | **Bob Rappaport, MD** |
| PROJECT MANAGER: | **Allison Meyer** |

Date of review submission to Division File System (DFS): 6-19-2008

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# EXECUTIVE SUMMARY

## I. Recommendations

A. Recommendation on approvability

Sugammadex may be approved from the pharmacology/toxicology perspective.

B. Recommendation for nonclinical studies

*For adult use:*

1) The effect of sugammadex on bone healing after fracture has not been evaluated in non-clinical models. A study in rat or other appropriate non-clinical models with bone fracture to evaluate the effect of sugammadex on the bone healing process is recommended.
2) A repeat-dose study in rats (≥ 14 days) with appropriate treatment-free recovery period is needed to examine the equivocal effects in reproductive organs of sugammadex in male rats and potential reversibility. Alterations in male reproductive organs were observed in the single-dose and 2-week repeat-dose study in rats treated with degraded drug substance batch which contained high levels of certain impurities/degradants.

*For pediatric use:*

Additional bone studies are needed before multiple-dose clinical trial in pediatric patients and/or for approval of the drug to be used in pediatrics:

a. A repeat-dose study (≥ 28-day) of sugammadex in the juvenile rat with multiple recovery periods of varying length ≥ 8 weeks is recommended to evaluate the mechanism and reversibility of the slight but lasting decreased bone length and body weight observed in the 28-day repeat-dose study in juvenile rats. Within this study, assays of μCT, bone turnover markers, and bone strength assessment are necessary with concurrent positive control in order to clarify the bone effects of sugammadex in juvenile rats.

b. The binding site of sugammadex will need to be conclusively determined and distribution of sugammadex within the bone will need to be further investigated in order to provide mechanistic information for apparent findings in the bone in animal studies. The sponsor hypothesized that sugammadex likely binds with hydroxyapatite, a compound of extracellular matrix in the bone, but there was no study conducted to directly investigate the binding site of sugammadex in the bone or tooth. The microautoradiography study conducted to examine the distribution of sugammadex in the bone does not appear to be adequate with deficiencies in method, small sample size, and inappropriate data presentation.

C. Recommendations on labeling

Labeling revision is recommended in the following sections. See section *Overall Conclusions and Recommendations* for detailed changes and rationale. It should be noted that all these recommended changes are preliminary. Subsequent revision is expected after discussion within the Division and negotiation with the sponsor.

**8.1 Pregnancy**

*Pregnancy Category B:*

Reproduction studies have been performed in rats and rabbits at doses up to 500 and 200 mg/kg/day, corresponding to 6× and 8× the human exposure based on AUC level at the 16 mg/kg clinical dose, respectively. These dose levels represent maximal feasible dosing levels based on the maximal practical injection volume, and no evidence of impaired fertility or embryofetal toxicity due to (b) (4) (sugammadex sodium) injection has been revealed. In a peri- and postnatal development study, neonatal mortality appeared to be slightly increased during the first 4 postnatal days after gestational exposure of (b) (4) The mechanism of this change is not known. Additional studies suggested that change in odor/taste due to in utero exposure may play a role in triggering cannibalization (see section 13.1.1). This effect is not likely to be clinically relevant. There are, however, no adequate and well-controlled trials in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

**8.3 Nursing Mothers**

(b) (4) is excreted into milk in rats. The maximum level reached at about 30 minutes after IV injection with a ratio of milk to plasma level approximately 1:1 at this time point. The elimination is slower in milk than that in blood. Excretion of (b) (4) in human milk has not been studied. Caution should be exercised when (b) (4) is administered to a nursing woman.

**13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility**

Carcinogenicity studies were not done in view of single dose use of (b) (4) (sugammadex sodium injection) in the clinical setting.

*In vitro* genotoxicity studies including Ames test and chromosomal aberration test in human peripheral blood lymphocytes with different manufactured batches of (b) (4) drug substance and special batches containing relatively high level of impurities have been conducted and the results were all negative. *In vitro* Ames and chromosomal aberration tests with a (b) (4) impurity (b) (4), (b) (4) also showed negative result. In addition, negative results were observed in *in vivo* micronucleus assay in mice and rats. These results indicate (b) (4) is not mutagenic and clastogenic

A fertility and early embryonic development study in Sprague-Dawley rats revealed that the No Observed Adverse Effect Level (NOAEL) of (b) (4) for effects on fertility and early embryonic development was 500 mg/kg/day. In addition, histopathological examination in the 4-week rat and dog toxicity studies of the male and female reproductive organs did not reveal any adverse effects on fertility. However, in a two-week repeat-dose study in rats with degraded drug substance batch containing high level of impurities/degradants, changes in reproductive organs were observed including decreased organ weight at doses ≥ 50 mg/kg and spermatogenic granuloma and ruptured epididymal duct at 200 mg/kg. The reversibility of these changes was not known. In an embryo-fetal development study in rats the NOAEL for embryofetal development was assessed to be >500 mg/kg/day. In rabbits (up to 200 mg/kg/day)

maternal toxicity, as evidenced by a decrease in maternal body weight, resulted in a slight decrease of fetal body weight. The effect on fetal body weight was also associated with slightly increased litter size, which could also explain the decreased fetal weight. The NOAEL for embryofetal toxicity in rabbits is considered to be at 20 mg/kg, but the NOAEL for teratogenicity is considered to be 200 mg/kg.

**13.2 Animal Toxicology and/or Pharmacology**

Bone and teeth retention of sugammadex occurred in rats after IV injection, with the mean $t_{1/2}$ of 172 and 8 days, respectively. The binding site is not known, and the distribution within these tissues has not been well-characterized. Studies in adult rats have shown that single-dose administration of 2000 mg/kg sugammadex slightly increased bone resorption, but had no effect on teeth color and appearance. In juvenile rats, 28-day administration of sugammadex at 120 and 500 mg/kg caused slightly decreased body weight and bone length which did not recover within an 8-week treatment-free period. Whether an association between these effects exists is not known. µCT assay showed alterations in bone parameters including such as increased trabecular bone mineral density and trabecular thickness at 500 mg/kg, along with decreased cortical thickness at both 120 and 500 mg/kg after Org 25969 administration, but the biological significance is not clear. Bone strength was not assessed in juvenile rat studies. Whitish discoloration was also observed in the incisors and disturbance of enamel formation was observed upon repeated dosing in the incisors and the molars at these dosing levels.

## II. Summary of nonclinical findings

### *A. Brief overview of nonclinical findings*

Org 25969 is a compound structurally belonging to the molecular family of γ-cyclodextrins (CDs). It is developed to bind to the hydrophobic region of the steroidal neuromuscular blocking (NMB) drugs rocuronium and vecuronium with high affinity resulting in reversal of neuromuscular blockade. The Org 25969 drug substance batches contains (b) (4) . Org 48302 was pharmacologically equivalent to Org 25969 and allowed to be considered part of the active pharmaceutical ingredient (API). (b) (4) were considered as impurities and studies were conducted to qualify the impurities and degradants.

Safety pharmacology

For evaluation of the effects of Org 25969 on the cardiovascular system, a hERG inhibition assay in HEK-293 cells demonstrated a 22% inhibition of hERG tail current at 1.5 mM of Org 25969. Higher concentrations of Org 25969 could not be evaluated due to an undesirable increase of the osmolarity of the (b) (4) system. An action potential duration (APD) assay in Purkinje fibers of dogs demonstrated that Org 25969 produced an increase in action potential duration ($APD_{50, 60,}$ and $_{90}$) for approximately 5% - 20% at 150 and 1500 µM under normal (1 Hz) and low stimulations (0.3 Hz); similar results were observed for Org 48302. In contrast, hydroypropyl-β-cyclodextrin and γ-cyclodextrin only slightly increased $APD_{90}$ of Purkinje fibers of dog at 1.5 mM concentration (< 5.0%) with no change in $APD_{50}$ and $APD_{70}$. An in vivo hemodynamic and electorcardiographic study with Org 25969 and rocuronium bromide in anesthetized

beagle dogs indicated that a dose of 1.8 mg/kg rocuronium followed after 2 min by administration of 250 mg/kg Org 25969 resulted in approximately 7% increase in corrected QT duration (both Bazett's and Fridericia's correction) at the end of a 5 min perfusion. These studies suggested a potential of Org 25969 for QT prolongation. The concentration/dose producing effects in these *in vivo and in vitro* studies roughly provided a 10-fold exposure margin for the proposed maximal human dosing level (16 mg/kg) based on plasma concentration. However, based on the small extent of the findings in these studies, the risk for QT prolongation may be negligible in human with the 16 mg/kg administration due to the small extent of the effects detected and large exposure margins as described above. The effect of Org 48302 on cardiovascular system is likely to be comparable to Org 25969. No CNS or respiratory effects were observed in rats following single-dose injection of Org 25969 at 500 mg/kg and 92 mg/kg, respectively.

ADME/PK

Org 25969 is indicated for IV injection therefore bioavailability is 100%; the systemic exposure levels appeared to be generally dose-proportional in rats, dogs, and rabbits. There was no apparent accumulation after multiple-dose administration and no remarkable difference found between male and female of any species. The elimination $t_{1/2}$ was short in these animal species (< 1 hour) and human (approximately 2 hours) by HPLC-MS assay. In addition, the distribution volume was relatively low in rats (~ 0.4 L/kg), dogs (~ 0.25 L/kg), rabbits (0.19 L/kg in F), and human (0.18 L/kg). The PK profile of Org 48302 appeared to be comparable to that of Org 25969. In contrast, a kinetics study using [$^{14}$C]-Org 25969 radioactivity assay demonstrated a very long $t_{1/2}$ in rats (~ 15 hours) and dogs (~ 8 hours). This is likely to be due to a difference in limit of quantitation (LOQ) between the liquid-chromatography-Mass Spectrometry (LC-MS) and radioactivity method, and the "tailing" of a long terminal elimination phase noted in the rat and dog is likely to represent slow elimination from a deeper compartment such as bone.

Distribution studies in the rat indicated that Org 25969 is rapidly distributed to the bone and is retained in these tissues for extended periods of time with mean $t_{1/2}$ of 172 days (range from 67 – 210 days). Retention of Org 25969 was also seen in teeth to a reduced degree as compared to bone with a $t_{1/2}$ of 8 days demonstrated in incisor. Injection of rocuronium immediately after sugammadex injection at a half molar dose diminished the extent of binding in bone and teeth by about 40%. The binding site and distribution of Org 25969 within these tissues have not been completely characterized. A micro-autoradiography study in young male adult rats showed labeled Org 25969 in periosteal and endosteal aspects of cortical bone and endosteal aspects of trabecular bone in femur and scapula, but not to growth plates. Bone formed subsequent to labeled dose administration did not contain radiolabeled Org 25969. This study did not provide sufficient detail to characterize the binding site of Org 25969 as cells or extracellular matrix. Binding to bone was not easily saturated and bone concentration (mg/g) increased in less than dose-proportional manner at doses up to 400 mg/kg, and the absorbed amount of Org 25969 in the bone and teeth after each individual administration (30 mg/kg) was generally decreased but absorption was still observed (approximately

50% of initial absorption ratio) on the last day of treatment in a 28-day multiple-dose study. At a dose of 30 mg/kg retention in pooled bones was 13% (juvenile), 3% (young adult), and 0.5% (aged rats), at 24h post dose. The increased bone retention in juvenile rats is probably due to rapid growth of bone containing multiple sites of bone resorption, formation and mineralization.

Distribution studies also demonstrated that accumulation was also observed in prostate and vagina, kidneys, and urinary bladder, but the amount was substantially reduced and appeared to be not different from other organs after 168 hours.

Org 25969 was found to cross the placenta and distributed into various fetal tissues with highest concentration in the femur (< 0.5% dose/gram) followed by urinary bladder and GI tract with no remarkable accumulation found in the vital organs such as the heart, brain, and lung in rats. The amount of Org 25969 in fetal blood was about 2-9% that of maternal blood at one hour after maternal administration, but the elimination was slower in the fetus than the dam. In addition, Org 25969 was found to be excreted into milk; maximal concentration was observed at 30 minutes after single-dose IV administration with the concentration similar to the plasma concentration at this time-point.

In vitro studies demonstrated that there was only a limited amount of Org 25969 uptake into hepatocytes, indicating hepatic metabolism may be minimal. Minimal metabolism likely occurs after Org 25969 administration in rats, dogs, and human. The primary metabolites were Org 198958-2 and Org 199425-1 in these species, which are also found in the Org 25969 drug substance. There were no remarkable unique metabolites in human as compared to rats and dogs. In both rats and dogs, Org 25969 excreted primarily though urine (78% in rats; 85% in dogs) with minor fecal excretion (< 7% in rats; <1% in dogs).

General toxicology:
Single-dose toxicology studies in mice and rats indicated that maximum non-lethal dose of Org 25969 in these species exceeds 2000 mg/kg with minor and transient clinical signs such as reduced activity, ptosis, and cramps observed after intravenous injection of this dose. A single-dose intravenous toxicity study in male and female rats was conducted to assess the acute toxicity profile of Org 25969 at 0, 200, 600, or 2000 mg/kg (containing 10.7, 32.1, and 107 mg/kg Org 48302). The target organs identified in this study were the kidney (tubular vacuolation at ≥ 600 mg/kg) and the lung (foamy alveolar macrophages at 2000 mg/kg). All changes were minimal and appeared to be reversible, indicating limited toxicologic significance. Therefore, the reversible NOAEL is considered to be at 2000 mg/kg, which provides an approximately 25-fold safety margin for the maximal proposed human dosing level of Org 25969 (16 mg/kg) and Org 48302 (1.12 mg/kg) based on AUC comparison.

Two 4-week toxicity studies were conducted in rats with the same dosing scheme (0, 30, 120, and 500 mg/kg Org 25969). The study design of these two studies were similar except that (1) the drug substance was batch U containing around 1.2% Org 48302 in the first study vs. batch AE containing around 5.4% Org 48302 in the second study; (2) the

recovery period was 3 weeks in the first study vs. 8 weeks for the second study. The second 4-week toxicity study was proposed to provide sufficient safety margin for the maximal dosing levels of Org 48302 as well as Org 25969 in human and examine the reversibility of the microscopic findings with a longer recovery period (8 weeks vs. 3 weeks). The findings in these two studies were comparable. Target organs identified after 4-week administration were lungs (minimal to marked foamy macrophages at 500 mg/kg), kidneys (minimal to moderate vacuolated cortical tubule cells at ≥ 120 mg/kg), and urinary bladder (minimal to moderate foamy cytoplasm in umbrella cells at ≥ 120 mg/kg). Changes in the lung and kidney showed complete or partial recovery, respectively, but the change in the urinary bladder was not reversible at 500 mg/kg after an 8-week recovery period. Based on these results, the NOEL of Org 25969 is considered to be at 30 mg/kg and reversible NOAEL is at 120 mg/kg in the 4-wk rat toxicity studies. The AUC levels of Org 25969 and Org 48302 in rats at 120 mg/kg Org 25969 (6.5 mg/kg Org 48302) were approximately equal to those in human with 16 mg/kg Org 25969 administration. It should be noted that although the change in urinary bladder at 500 mg/kg in the second 4-week toxicity study did not show evidence of recovery after an 8-week treatment-free period, the severity of this change did not increase with time and the finding was not associated with subsequent cellular damage or associated with changes in clinical pathology parameters, indicating limited toxicologic significance. This dosing level provides an approximately 5-fold exposure margin for the proposed maximal human dosing level of Org 25969 as well as Org 48302.

Single-dose and 2-week toxicity studies in rats administered a degraded Org 25959 drug substance batch were conducted to assess the acute toxicity profile of the degraded Org 25969 drug product in comparison with non-degraded drug product. The batch was especially prepared containing relatively high level of certain degradants. The results of these studies indicated that the toxic effects produced by degraded and non-degraded Org 25969 were generally comparable except for some changes associated with reproductive systems in male rats. In the single dose study which utilized degraded Org 25969 (0, 200, 600, and 2000 mg/kg) and non-degraded Org 25969 (2000 mg/kg), an increased number of spermatids (3/6 animals) and hypospermia (1/6 animals) in epididymides were observed at 2000 mg/kg with degraded but not non-degraded Org 25969. These changes were not seen in animals after a 2-wk recovery period. In the 2-wk repeat-dose study, decreased weight of seminal vesicle was observed at all doses in a dose-independent manner (-26%, -25%, and -20% for 50, 100, and 200 mg/kg vs. control), and ruptured epididymal duct (1/8 animals) and sperm granulomas (3/8 animals) in epididymides were found at 200 mg/kg. These changes were not seen in the 4-week repeat-dose study with non-degraded batches. Since there were no recovery groups in the repeat-dose study, it was not known whether these changes were reversible. Collectively, these changes suggested that degraded batch of Org 25969 drug substance which contains relatively high level of certain impurities or degradants including (b) (4) may cause effects on reproductive organs which were not seen treated with non-degraded batches. A NOAEL of 600 mg/kg based on the single-dose study provides sufficient safety margin for single-dose use of sugammadex, but further studies may be needed to further investigate these effects of degraded Org 25969 batch to support multiple dosing administration in human.

A 4-week IV toxicity study was also conducted in dogs to evaluate the toxicity of Org 25969. In this study, beagle dogs (4/sex/group) were treated with 0, 25, 80, and 250 mg/kg Org 25969 consecutively for 4-weeks. The results of this study indicated that intravenous administration of Org 25969 to beagle dogs for 4 weeks at the maximum feasible dose (MFD) did not result in remarkable systemic toxicity. Therefore, the NOAEL is identified as 250 mg/kg Org 25969 (3 mg/kg Org 48302), which provides around a 6-fold safety margin for the proposed maximal human Org 25969 dosing level of 16 mg/kg, and about 1-fold safety margin for proposed maximal human Org 48302 exposure based on AUC estimation with a 16 mg/kg clinical dose.

Overall, the general toxicity of Org 25969 and Org 48302 has been adequately characterized in rats and dogs, and the results of these studies appeared to provide sufficient evidence to support the safety of the proposed maximal dosing level of Org 25969 and Org 48302. In addition, levels of impurities/degradants in general toxicity studies adequately covered the maximal exposure levels in human with 16 mg/kg drug product for single-dose administration. Additional study may be needed to further investigate the potential effect of degraded Org 25969 drug substance on the reproductive system.

Genetic toxicology:
To investigate the genotoxicity potential of Org 25969, in vitro bacterial reverse mutation tests and chromosomal aberration tests in human peripheral blood lymphocytes have been conducted with batches L, T, V, and AE for the purpose of qualification of drug substance batches containing different levels of Org 48302 and impurities. One of the drug substance registration batches, AM, was also evaluated in the bacterial reverse mutation test because of a qualitatively different profile with respect to Org (b) (4) All these studies showed negative results indicating that Org 25969 is not mutagenic or clastogenic. In vivo clastogenic activity was assessed in mouse and rat micronucleus assays using a maximum IV single dose level of 1774 mg/kg and multiple dose level of 500 mg/kg for 4 weeks, respectively. Results from these studies did not show an increase in micronucleated polychromatic erythrocytes. Therefore, Org 25969 is not considered to be genotoxic.

As the second active ingredient which is allowed at (b) (4)% according to the drug substance specification, the genotoxic potential of Org 48302 was also investigated by the sponsor. In the in vitro bacterial reverse mutation test and the chromosomal aberration test, negative results were observed when tested up to 5 mg/plate and 5 mg/ml, respectively, indicating lack of mutagenicity and clastogenicity. The in vivo clastogenic potential has been assessed by examination of the bone marrow smears of rats dosed for 4 weeks with approximately 30 mg/kg Org 48302 and 500 mg/kg Org 25969. No *in vivo* clastogenicity was observed. Therefore, Org 48302 is not likely to be genotoxic.

Since the (b) (4) impurities were present in the drug substance with different levels in the batches tested, the genotoxic potential of these compounds were considered to be addressed to some extent. (b) (4) present in several drug

substance batches which contains [redacted] (b) (4) [redacted] (b) (4) for genotoxicity. An *in vitro* bacterial reverse mutation tests and a chromosomal aberration test were performed using maximum exposure levels (5 mg/plate or mg/ml, respectively) to investigate the potential of genotoxicity. Both genotoxicity tests indicated no mutagenic or clastogenic response. In addition, Org 25969 batch AJ, a batch in which the Org 25969 content was artificially decreased by chromatography resulting in an increase in the average content of [redacted] (b) (4) and impurities by [redacted] (b) (4) fold, was used to further investigate the genotoxic potential of [redacted] (b) (4) and impurities, and the results indicated no evidence of genotoxicity was present in this special batch AJ. To assess the genotoxic potential of degradation products, degraded drug product was prepared by accelerated conditions. Neither the bacterial reverse mutation test nor chromosomal aberration test revealed any genotoxic potential.

In summary, Org 25969, Org 48302, [redacted] (b) (4) and the [redacted] (b) (4) evaluated with in vitro and in vivo genotoxicity studies do not appear to indicate any potential for genotoxicity.

Carcinogenicity:
A carcinogenicity study is not needed since the drug is proposed for single-dose use and long-term exposure is not expected.

Reproductive toxicology:
In the segment I fertility study, both male and female rats (22/sex/group) were treated with Org 25969 at 0, (0.9% NaCl), 20, 100, and 500 mg/kg via intravenous bolus injection in the tail vein. The results showed slight decrease in body weight of female animals (~10%) indicating minimal maternal toxicity at 100 and 500 mg/kg. No Org 25969 treatment-related macroscopic or organ weight changes were observed in reproductive organs of male and female rats. There were no treatment-related changes in mating and fertility parameters including mating index, fertility index, pre-coital interval, live concepti number, and gestational index in Org 25969-treated groups as compared to control. The NOAEL for fertility and early embryonic development is considered to be at 500 mg/kg for rat based on the results of this study, corresponding to a 6-fold human AUC safety margin at the maximal human dose of 16 mg/kg.

Embryofetal development (segment II) studies were conducted in both rats and rabbits. In the rat study, pregnant animals were treated with Org 25969 at 0, 20, 100, and 500 mg/kg during fetal organogenesis. Minimal maternal toxicity was observed at 100 and 500 mg/kg represented by minimal decrease in body weight gain (~7%) likely due to slight decrease in food consumption (~7%) during the treatment period. Hysterectomy did not show any treatment-related change in pre- and post-implantation loss or live fetus numbers. There were no treatment-related changes in fetal soft tissue and skeleton except increased incidence of dilated renal pelvis in the offspring of dams treated with 500 mg/kg Org 25969 (6.5%) as compared to control (0.7%). However, this change is considered to be a variation but not malformation and the recorded value (6.5%) was still within the laboratory historical range (mean = 4.9%; range 0 – 8.5%) which suggests this is not related to drug. Therefore, the NOAEL for embryofetal developmental toxicity in

rats is considered to be at 500 mg/kg Org 25969 which corresponds to a 6-fold human AUC safety margin at the maximal human dose of 16 mg/kg.

In the rabbit embryofetal development study, pregnant New Zealand white rabbits were treated with Org 25969 at 0, 20, 65, 200 mg/kg during fetal organogenesis, and the animals were sacrificed at the end of gestation for embryofetal developmental toxicity evaluation. The results of the study showed a reversible dose-dependent decrease in body weight gain in dams during the treatment period at 20 (↓5.4%), 65 (↓16%) and 200 mg/kg (↓31%) as compared to the control, likely due to decreased food consumption. Macroscopic examination in dams indicated changes in gonads (conjunctive tissue, periovarian region: serous cysts) in 1/20 animals of all drug-treated groups, but not in control. This change was not dose-dependent, and has been reported to occur in animals of this strain at this age. Decrease in offspring body weight was also observed in all groups of animals with approximately 5%, 10%, and 14% at 20, 65, and 200 mg/kg, respectively, likely due to maternal body weight decrease or increased litter size in the treated groups as compared to the control. This reduced body weight is not considered to be adverse or directly related to drug on the fetus. Skeletal examination showed increased skeletal variation including increased percentage of incomplete ossification of $1^{st}$ to $4^{th}$ sternebra and $6^{th}$ sternebra, and unossified $1^{st}$ metacarpal at 200 mg/kg, likely to be due to the lower fetal weight in this group. These changes were within historical control value range except the incomplete ossification of $1^{st}$ and $4^{th}$ sternebra (12.6% vs. 1.4 – 11% in historical control). Since incomplete ossification of sternebra is considered variation but not malformation, this does not indicate teratogenicity. Malformations such as thoracic hemivertebra and misshapen thoracic vertebra, misaligned sternebra, and fused rib were found in one fetus in the high-dose group, which was not seen in the control. These were considered to be incidental because of the low incidence and similar malformations were also reported in animals of historical control. Overall, the primary change in maternal and offspring rabbits with Org 25969 administration during fetal organogenesis was a minor decrease in body weight, which is monitorable in the mother and of minimal toxicologic significance in the offspring; therefore, the NOAEL for embryofetal toxicity in rabbits is considered to be at 20 mg/kg, but the NOAEL for teratogenicity is considered to be 200 mg/kg, corresponding to approximately 8-fold safety margin for Org 25969 in human with the maximal 16 mg/kg administration based on AUC comparison.

In the segment III perinatal and postnatal development study, pregnant rats (22/group) were treated with vehicle or Org 25969 at 30, 120, 500 mg/kg from gestational day (GD) 6 to postnatal day (PND) 21 (corresponding to the beginning of organogenesis through parturition and subsequent pup weaning). No abnormalities were observed in dams ($F_0$ animals). There were no Org 25969-related effects on the mean number of implantation sites, the gestation or parturition duration, and the pregnancy rate was comparable between Org 25969 treated and control groups. Neonatal mortality appeared to be increased during PND0-4 in the high-dose (500 mg/kg) and mid-dose group (120 mg/kg). Initial examination indicated increased postnatal loss from the first litter check on day 0 to 4 post partum was found to be increased in the high dose group compared to control (19.2% vs. 10.4%) with observed total litter loss of 2, 0, 2, and 3 dams in groups of

control, 30, 120, and 500 mg/kg, respectively, during this period. However, in group 3, two dams had extremely small litter size (2 or 4 pups) as compared to others at the first litter check; while the implantation sites of these two animals were 13 and 18, respectively, indicating cannibalization occurred in these two dams immediately after birth and before the first litter check. In addition, there was one complete litter loss each in group 2 and group 3 before the first litter check after delivery, which is also most likely due to cannibalization since the body weight of these two animals were comparable with others. Collectively, there appeared to be increased postnatal loss in group 3 (120 mg/kg) and group 4 (500 mg/kg) from the birth on postnatal day 1 to postnatal day 4, most likely due to cannibalization. Histopathological examination of heart, lungs, liver, and kidneys of the dead or partially cannibalized $F_1$ pups did not identify any change that might cause pup death or trigger cannibalization. There were no remarkable abnormalities observed in the surviving $F_1$ pups during lactation period. In addition, there were no remarkable treatment-related changes in $F_1$ surviving animals in physical development, behavior evaluation, necropsy, and reproduction evaluation. Overall, the primary change was postnatal $F_1$ pup loss during postnatal day 0-4. The NOAEL for postnatal development was considered at 30 mg/kg.

To investigate the potential mechanism of increased postnatal loss observed in Org 25969 treated groups at 120 and 500 mg/kg, two follow-up studies were conducted. Results of the first study following similar dosing regimen in 22 pregnant rats/group showed no difference in neonatal death between control and 500 mg/kg groups, indicating that the increased postnatal loss of offspring in the regular prenatal and postnatal development study may be incidental. In addition, fetuses obtained by cesarian section from animals treated with 500 mg/kg Org 25969 showed good viability and a normal distribution of live and dead fetuses, which may indicate no intrinsic abnormalities within the fetuses to cause the dams to cannibalize their young. The purpose of the second follow-up study was to investigate whether ex-utero or in-utero exposure of Org 25969 was the possible cause of the increased incidence of cannibalism in animals with Org 25969 administration. To investigate effect of the ex-utero exposure of Org 25969, pups from two groups of pregnant females without any treatment during pregnancy were swabbed with saline or 0.5 mg/ml Org 25969 after birth and observation of maternal nesting and nursing behavior, neonatal mortality or maternal cannibalism were recorded. Results showed neonatal survival was comparable in the litters of Org 25969 swabbing as compared to the litters swabbed with saline (95.8% and 92.1%, respectively). To investigate in-utero exposure of Org 25969, pups from two groups of pregnant rats treated with 500 mg/kg Org 25969 and 0.9% NaCl during gestational day 6 to 21 were delivered by C-section on gestational day 21 and cross-fostered to untreated dams to observe for pup death and cannibalization from postnatal day 0-4. The results showed a slight increase in total pup death in the in-utero Org 25969 exposed litters as compared to the in-utero saline exposed litters (neonatal survival ratio: 79.4% vs. 85.1%). Collectively, results of these studies likely suggest that in-utero exposure of Org 25969 may play a role in triggering cannibalization due to change in odor or taste but not malformation or abnormality of pups. This effect in rats is not likely to be clinically relevant; therefore, 500 mg/kg is considered to be NOAEL for clinically relevant effects in peri- and postnatal development.

Juvenile Toxicity Evaluation

In support of pediatric trials, developmental toxicity studies in juvenile rats with single- and repeat-dose administration were conducted to further investigate the potential toxic effects of Org 25969 during the juvenile developmental period. In addition, bone and teeth effect of Org 25969 were specifically examined in juvenile rats because of the high amount of bone and teeth absorption in juvenile animals as observed in the distribution studies. In a single-dose treatment study, animals at ages of 7 days, 14 days, or 21 days were injected subcutaneously (for animals of 7 days old only) or intravenously with Org 25969 at 0 (0.9% NaCl), 30, 120, and 500 mg/kg. Reversible minimal to slight tubular vacuolation in kidney were observed upon14 days after dosing primarily at 120 and 500 mg/kg in animals administered drug at age 7 days. Minimal disruption of the enamel epithelium with presence of amorphous material was observed in the incisors of one female rat treated at age 21 days with 500 mg/kg on day 14 after dosing. The NOAEL is considered to be at 500 mg/kg Org 259659 for general toxicity and 120 mg/kg Org 25969 for the tooth effect. Another single-dose toxicity study showed slightly higher incidence of whitish discoloration at 500 mg/kg Org 259659 as compared to control.

The adverse effects of Org 25969 after multiple dose administration was investigated in juvenile rats following 4-week administration (n=10/sex/group) with an 8-week treatment-free period observation (n=12/sex/group). The dosing levels utilized in this study were 0, 30, 120, and 500 mg/kg Org 25969. Animals were administered drug or vehicle starting at day 7 after birth via SC injection for two weeks, and then switched to IV injection for 2 weeks. TK analysis showed that plasma levels of Org 25969 were comparable for males and females with a roughly proportional increase with dose escalation. Upon repeated dosing, plasma levels were lower at 34 days of age after the 28-day administration when compared with those following a single dose on Day 7 of age. The reason for this difference was not known. General toxicity effects in juvenile rats after 28-day administration were comparable to those observed in adult rats primarily including minimal to slight histopathological changes in the lungs, kidneys, and urinary bladder. Neurobehavioral examinations including motor activity, sensory function and reactivity, and learning and memory (Morris water maze) after treatment did not show any sign of abnormality. There was no body weight change during the treatment period, but minor decrease in body weight gain of both males (5-8%) and females (<5%) at 120 and 500 mg/kg Org 25969 were observed as compared to the control during the 8-week treatment free period. At the end of 8-week recovery period, the body weight of animals treated with Org 25969 at 120 and 500 mg/kg were still slightly lower than that of control in both males (4%) and females (3%). Additional observations in this study included an analysis of the effects of Org 25969 in teeth and bones. In bones, 4-week continuous administration of Org 25969 caused minor decreased ulna length (≤5%), femur length (≤3.5%) and weight (≤6.5%) with more severe effects at the end of 8-week recovery period at 120 and 500 mg/kg Org 25969 dose levels. It can not be determined whether the decrease of bone length was due to the slight body weight decrease. However, these results indicated that Org 25969 may have a potential to cause slightly smaller size and height lasting until the adulthood if administered during pediatric period. Minor but statistically significant changes in bone parameters of femur as assessed by

μCT including increased trabecular bone mineral density (9% in M, 18% in F versus control), bone volume/tissue volume of trabecular metaphysis (19% in M, 24% in F versus control) , and trabecular number (13-15%) and trabecular thickness (6-8%) at 500 mg/kg, along with dose-independently decreased cortical thickness at both 120 (9-10%) and 500 mg/kg (7-8%) after Org 25969 administration, which were reversible after 8-week treatment-free period. The biological significance of these changes is not clear. Bone turnover markers or bone strength were not evaluated. Histopathological examination found a very low incidence of chondromucinous degeneration in the Org 25969 120 and 500 mg/kg (one animal at each group) and fibrosis at 500 mg/kg (one animal) group in femur. Based on these results, the NOAEL for bone effect in juvenile is considered to be 30 mg/kg. In teeth, abnormal color (white marbled/mottled banding), malocclusion and/or overgrowth of the incisors occurred in the majority of animals receiving 500 mg/kg and some animals of 120 mg/kg, which generally became apparent following at least 2 weeks of treatment and persisted for up to five weeks into the recovery period. Histopathological examination showed a dose-dependently increased disruption of the enamel epithelial layer in the incisor of 1/20 (minimal severity), 0/20, 6/17 (5 minimal, 1 slight), and 15/20 rats (3 minimal, 5 slight, 6 moderate, 1 marked) at 0, 30, 120, and 500 mg/kg Org 25969, respectively. Deposition of amorphous material in the incisor enamel epithelial layer was observed at 120 (4/17, minimal) and 500 mg/kg (17/20, minimal to marked) Org 25969. Minimally to marked deposition of amorphous material in the molar enamel epithelial layer was also found in 5/10 males and 5/10 females of the 500 mg/kg group. Recovery was complete in 8 weeks for the changes in the molars but not fully complete for the incisors. The NOAEL was at 30 mg/kg Org 25969 for incisors but 120 mg/kg for molars.

Local tolerance: local tolerance and blood compatibility studies did not show a local toxic effect of Org 25969 and Org 48302.

Special toxicology:

*1. Special studies regarding potential effects on bone and teeth*

In a single-dose study, adult male and female rats (16/sex/group) were injected intravenously with 2000 mg/kg Org 25969 or 0.9% NaCl. Bone turnover markers (BTM) were measured on day 1, 7, 21, and 42 days after doing, and evaluation of μCT bone parameter and bone strength were conducted on day 21 and 42. Single-dose 2000 mg/kg administration caused a statistically significant change in bone parameters via μCT assay on day 21 after dosing as compared to the control, including decrease in trabecular bone volume/tissue volume (↓18% in female; ↓8% in male), trabecular number (↓10%), along with increased trabecular separation (↑31% in female; ↑11% in male). Trabecular bone mineral density also appeared to be decreased (female ↓18%, male ↓8%), although statistical significance was not reached. In addition, a bone indentation test showed decreased energy, maximal load, stiffness, and stress, indicating compromised bone integrity of the distal femur on day 21. BTM assay showed increased serum CTx and urine DPD, markers for bone resorption, primarily in females at this time-point. All these findings suggest a minor bone resorptive effect with greater severity in females than males. Decreased trabecular number and increased trabecular separation test were also

seen on day 42 after dosing, suggesting a prolonged bone effect. μCT assay and bone strength assessment were not conducted on day 0 and day 7 after dosing. Examinations of BTM did not suggest a remarkable change in bone during this period. Possible teeth discoloration due to Org 25969 was evaluated by determination of the color of the upper part of the lower incisors of the rats. There was no discoloration of incisor after a single-dose of Org 25969 at 2000 mg/kg. Overall, this study indicated that Org 25969 at 2000 mg/kg produced a resorptive effect in femur after 21 days of dosing but had no effect on teeth color after single-dose administration. The bone effect of Org 25969 was likely to be reversible since the changes in μCT parameters and BTM appeared to be less severe on day 42 as compared to those on day 21. In the second study of bone effect, rats were treated with Org 25969 at 0, 30, 120, and 500 mg/kg as a single-dose administration to explore lower doses than the initial study. μCT assay did not indicate any remarkable adverse effects in the bone, and therefore, NOAEL is considered to be at 500 mg/kg for bone effect in this study, corresponding to a 6-fold or 17-fold safety margin for human administration at 16 mg/kg based on AUC or bone concentration estimation. There was no effect on teeth color observed in either of the studies.

As a possible hormonal explanation for the reduced trabecular strength and volume, a PTH surge was observed in both rats and dogs upon Org 25969 administration. In the rat (30, 120, 500 mg/kg Org 25969) this increase was maximal at 3 min post dose (a statistically significant 4-fold increase at 500 mg/kg compared to controls), and returned to baseline by 30 min. Serum $Ca^{2+}$ was increased at 3 and 30 minutes after dosing and urine phosphate excretion was increased over 24 hours. In dogs, the PTH surge lasted about 15 min with Org 25969 doses ≥ 80 mg/kg. A transient effect is probably not relevant in the reduction of trabecular mass and strength since bone changes persisted through 3-6 weeks following single dose administration. However, it should be noted that the maximal dose examined for PTH release was 500 mg/kg, a dosing level without any observable changes in bone. The effect of Org 25969 on plasma PTH and $Ca^{2+}$/phosphate concentration after 2000 mg/kg, the dosing level that produced bone effect, was not examined.

*2. Immunogenicity:*

There were 3 studies conducted to examine the immunogenic property of Org 25969. First, a Plaque-Forming Cell (PFC) assay was conducted to evaluate the integrity of the T-cell dependent antibody response and any potential immunosuppressive effect. Sheep red blood cells (SRBCs) were injected into the rat after 2-week IV injection of Org 25969 at 30, 120, and 500 mg/kg and splenic cells were subsequently harvested and incubated with SRBCs. No decrease in plaque formation (which would indicate impaired immune response) was observed; however, Org 25969 treated groups showed slight increase in plaque formation in a dose-independent manner, suggesting immunostimulation. The second study was a popliteal lymph node assay (PLNA) assay conducted in mice to examine the potential for an immunostimulatory effect of Org 25969 after subcutaneous (SC) footpad injection at three different dose levels (0.3, 1.2 and 5 mg Org 25969/animal). The results showed a small but statistically increased [$^3$H]-thymidine incorporation in PLN at the high dose (278 mg/kg) (2-fold higher for Org 25969 compared with 26-fold higher for positive control) as compared to vehicle control,

indicating weak immuno-stimulation. The 3rd study was a local lymph node assay (LLNA) assay to examine skin contact hypersensitivity to Org 25969 after topical application on the ears of animals. The results showed no increase in [$^3$H]-thymidine incorporation in auricular lymph node suggestive of lymphocyte stimulation. However, skin absorption of Org 25969 has not been examined. Based on the results of the three immuno-toxicity studies submitted by the sponsor, it is concluded that Org 25969 is mildly immunostimulatory. However, these non-clinical studies provided little information to predict the immunotoxic effect of Org 25969 in human. It was not known whether anti-Org 25969 antibody was produced *in vivo* based on the results of these studies. Indeed, anti-Org 25969 production in human will be still difficult to predict even anti-Org 25969 antibodies had been observed in nonclinical studies because of species difference.

*B. Overview of the pharmacodynamic activities*

The pharmacodynamics of Org 25969 was examined using physicochemical techniques (isothermal titration microcalorimetry), in-vitro techniques (tissue bath studies), and *in-vivo* methods (muscle contraction in anaesthetized animals).

Isothermal microcalorimetry affinity binding assays indicated that Org 25969 bound to steroidal non-depolarizing neuromuscular blocking agents such as rocuronium and vecuronium with $K_A$ = 15.1 and 8.8 megamol$^{-1}$ respectively, but very limited-to-no (≤ 0.2 megamol$^{-1}$) affinity for non-steroidal non-depolarizing and depolarizing neuromuscular blocking agents such as succinylcholine and atracurium. These results indicated that Org 25969 may bind to rocuronium and vecuronium in vivo resulting in reversal of neuromuscular blockade.

Org 25969-induced reversal of neuromuscular block was demonstrated in the *in vitro* model of isolated mouse phrenic nerve-hemi-diaphragm preparation. The concentration of Org 25969 producing 50% reversal from a 90% neuromuscular blockade induced by rocuronium and vecuronium was 1.2 μM and 0.8 μM, respectively. In rats, a single-dose administration of Org 25969 remarkably enhanced the recovery time of blocking of M. gastrocnemius contraction induced by rocuronium and vecuronium as compared to saline. The examination on the M. gastrocnemius preparation in anesthetized guinea pigs indicated that cumulative administration of Org 25969 caused dose-dependent reversal of neuromuscular block. In contrast, administration of Org 25969 did not improve the recovery from neuromuscular blockade induced by the non-steroidal compounds succinylcholine, atracurium and d-tubocurarine, but produced limited recovery for mivacurium. In cats, Org 25969 improved the time of 90% recovery of M. tibialis contraction inhibition induced by rocuronium significantly. In monkeys, intravenous bolus injection of Org 25969 significantly enhanced the reversal time expressed as a Train-Of-Four (TOF) ratio of 0.9.

Using isothermal microcalorimetry, the affinity of Org 25969 (b) (4) for both rocuronium and vecuronium were examined. The results showed that (b) (4) contained in Org 25969 drug substance have different degrees of rocuronium and vecuronium binding affinity. Org 48302, the second active ingredient, showed about half

of Org 25969 binding affinity in isothermal microcalorimetry. Additional studies in mouse diaphragm preparation indicated that mixture of Org 25969 and Org 48302 containing up to 20% Org 48302 had similar potency to Org 25969 with 100% purity. Studies were conducted in anaesthetized guinea pigs that a mixture of 80% Org 25969 and 20% Org 48302 caused a similar speed of reversal as compared to 100% Org 25969. *In vivo* and *in vitro* studies also have shown that the affinities to rocuronium and vecuronium and potency for reversal were not significantly different when different manufacturing batches were examined.

*C. Overview of clinically relevant non-clinical findings*

Effect on bone:
Studies in adult rats indicated that Org 25969 caused slightly increased bone resorption at a high dose (2000 mg/kg). The effect appeared to be reversible and the NOAEL for bone effect was considered to be 500 mg/kg after single-dose administration, which provides a 6-fold or 17-fold safety margin for human administration at 16 mg/kg based on AUC or bone concentration estimation. However, the effect of Org 25969 on healing of bone fracture in non-clinical models and human has not been studied and therefore is not known.

In juvenile animals, the bone effect of Org 25969 has not been well-characterized. A 28-day repeat-dose toxicity study revealed a small but statistically significant decrease in bone length at 120 mg/kg and 500 mg/kg as compared to control, which may or may not be associated with observed body weight decrease in these groups as compared to control. This effect on bone length and body weight was not observed to recover after an 8-week treatment-free period, suggesting a lasting effect into adulthood following repeat-dose treatment during pediatric period. It is not known if this effect was primarily due to systemic or local bone exposure of Org 25969. μCT assay showed small alterations in bone parameters; however, the biological significance of these small changes is not clear. Bone strength assessment and bone turnover markers were not conducted in this study. Nevertheless, this study indicated that 28-day repeat-dose administration of 30 mg/kg produced no observed adverse effect which corresponds to approximately 22 μg·hr/ml in plasma AUC and 150 μg/g bone Org 25969. However, since the bone effect in juvenile rats has not been well characterized, it is uncertain whether AUC level or local bone concentration level shall be based for safety margin calculation. Therefore, additional non-clinical studies are needed to further characterize the bone effect of Org 25969 especially the mechanism of the lasting bone length decrease in the juvenile rat model to determine safety for pediatric use.

Effect on teeth
General toxicology studies in adult rat did not show discoloration effects on teeth after single-dose administration at a dose of 2000 mg/kg. Histopathologic examination of the teeth was not conducted, however. In juvenile rats, the 28-day repeat-dose study revealed incisor discoloration and disruption of the enamel-forming epithelium in incisors. In addition, deposition of amorphous material in the incisor and molar enamel epithelial

layer were observed. The NOAEL for incisor and molar changes is at 30 mg/kg and 120 mg/kg, respectively, with 28-day repeat-dose administration. With single-dose administration in the juvenile rat, the NOAEL for incisor change is at 120 mg/kg with minimal disruption of the enamel epithelium with amorphous material in the incisors of one animal at 500 mg while the NOAEL for molars is ≥ 500 mg/kg. Since adult human teeth have been fully mineralized and therefore do not have the ability to produce more enamel, these effects in juvenile may not reflect potential risk to the adult human. However, these effects in juvenile animals may have impact on the consideration for trial initiation in children. The incisors in rats are a good model for developing human teeth; the rat molars, which have only an extremely limited ability to continue growth, are a good model for permanent human teeth, as in individuals over the age of 7. Therefore, clinical study for pediatric patients is suggested to be initiated from patients greater than 7 years old based on safety concern on teeth effect of Org 25969 in pediatrics. Clinical trials with patients under 7 years old may be initiated if the safety of the maximal dosing level is supported by the NOAEL for incisor effect derived from these juvenile studies based on estimated tooth concentration of Org 25969.

Impurity qualification

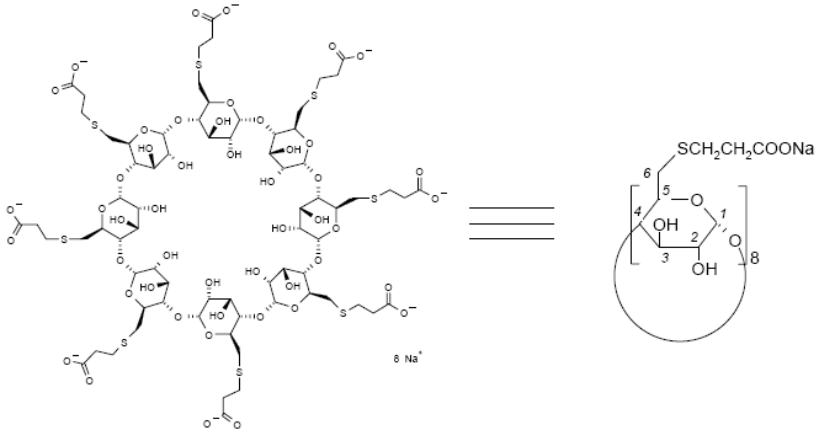
A degraded batch of Org 25969 drug substance which contains relatively high level of certain impurities or degradants including (b) (4) produced effects on reproductive organs which were not seen with non-degraded batches. Changes including increased number of spermatids and hypospermia in the single-dose treatment study were found at 2000 mg/kg and appeared to be reversible; the NOAEL for these effects is considered to be 600 mg/kg, which provides sufficient safety margins for these levels of impurities with single-use administration in human at 16 mg/kg sugammadex. However, a 2-week repeat-dose study demonstrated decreased weight of seminal vesicle at all doses in a dose-independent manner (-26%, -25%, and -20% for 50, 100, and 200 mg/kg vs. control), and ruptured epididymal duct (1/8 animals) and sperm granulomas (3/8 animals) were found at 200 mg/kg. Since there were no recovery groups in the repeat-dose study, it was not known whether these changes were reversible. These changes were not seen in the 4-week repeat-dose study with non-degraded batches, indicating the changes may be associated with impurities or degradants contained in the degraded batch. The findings in this 2-week study with degraded Org 25969 batch raised safety concern for multiple-dose administration in human because no NOAEL was identified in this study. This reviewer realized that a truly "multiple-dose" administration like the 2-week daily dosing may not occur in human, instead, the treatment of this drug may be more akin to intermittent treatment which might be considered single-dose with washout. However, warning should be included in the label to prevent any potential consecutive daily use before additional non-clinical studies are conducted to completely characterize the effect of the degraded batch or the individual impurities/degradants contained in this batch on reproductive system and provide sufficient safety margin for the maximal human dosing.

## *2.6 PHARMACOLOGY/TOXICOLOGY REVIEW*

### 2.6.1 INTRODUCTION AND DRUG HISTORY

**Drug**:

Trade name: (b) (4)
Generic name: Sugammadex
Code name: Org 25969
Chemical name: 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H (S)-(E)-(4-(5-Methoxy-pyrdin-3- yl)-1- methyl-but-3 enylJ-methyl-amine hemigalactarate salt
CAS registry number: N/A
Molecular formula/molecular weight:
Structure: Org 25969 is a modified γ-cyclodextrin (CD), (b) (4)




**Relevant INDs/NDAs/DMFs**: IND 68,029 (Org 25969)

**Drug class**: Not established, the drug substance is a modified cyclodextrin bound to neuromuscular blockade agents such as rocuronium or vecuronium for neuromuscular blockade reversal

**Intended indication**: Routine reversal of shallow or profound neuromuscular blockade (NMB) induced by rocuronium or vecuronium, and immediate reversal of at 3 minutes after administration of rocuronium

**Clinical formulation**: The drug product is a "clear colorless to slightly yellow aqueous solution for injection filled in (b) (4) (2 ml per vial) or (b) (4) (5 ml per vial) vials." It is filled in 2 mL and 5 mL vials at an active ingredient concentration of 100 mg/mL. The following table shows the composition of the drug product. There are no safety issues for the excipients in the drug product.

| Complete composition Org 25969 solution for injection 100 mg/mL | | |
|---|---|---|
| **Component** | **Function(s)** | **Quantity/mL** |
| Org 25969 + Org 48302 | Drug substance | 100 mg |
| Sodium hydroxide[3] | pH adjuster | q.s. to pH 7.5 |
| Hydrochloric acid | pH adjuster | q.s. to pH 7.5 |
| (b) (4) | | |

The drug substances in this drug product include Org 25969 and Org 48302. Org 25969, the active ingredient (b) (4), is a modified γ-CD manufactured in (b) (4) Org 48302 was found to be prominently presented in the drug substances (b) (4) s with a typical range (b) (4) %; the sponsor, therefore, included this (b) (4) compound as the secondary active ingredient in the Org 25969 drug substance after pharmacodynamics characterization. The acceptance criteria for all specified organic impurities in the Org 25969 drug substance was shown in the following table (left panel) as extracted from the sponsor's report. In addition, the specification of the drug product after release and during shelf life was also shown below (right panel) with acceptance criterion of the degradants. Non-clinical studies have been conducted to evaluate the toxicity of the active ingredients and specified organic impurities.

Drug substance

| Test | Method principle | Acceptance criterion |
|---|---|---|
| **Assay** | | |
| Total Org 25969 + Org 48302 content (b) (4) | HPLC | (b) (4) |
| Org 25969 content (b) (4) | HPLC | |
| Org 48302 content (b) (4) | HPLC | |
| Sodium content (b) (4) | ICP-AES | |
| **Impurities** | | |
| **Organic impurities:** | HPLC | |
| Specified identified: (b) (4) | | |
| Specified unidentified: (b) (4) | | |
| Unspecified (others) (b) (4) each individua each individual (other) | | |
| Total organic impurities (b) (4) | | |

Drug product

| Test | Method principle | Acceptance criterion | |
|---|---|---|---|
| | | Shelf life[1] | Release |
| **Description** | | | |
| Appearance | Visual | Aqueous liquid | |
| Color | Ph. Eur. (+VIS) [1] | Not more intense than BY3 | |
| **Identification** | | | |
| Active ingredient identical with standard | HPLC[2] | Org 25969 and Org 48302 present | |
| Active ingredient identical with reference spectrum (UV-DAD) | HPLC[2] | Org 25969 and Org 48302 present | |
| **Assay** | | | |
| Org 25969[2] + Org 48302[2] | HPLC[2] | (b) (4) mg/mL | mg/mL |
| Org 25969[2] | HPLC[2] | (b) (4) mg/mL | mg/mL |
| Org 48302[2] | HPLC[2] | (b) (4) mg/mL | |
| **Impurities** | | | |
| Degradation products[3] | HPLC[2] | | |
| Specified identified: (b) (4) | | (b) (4) | |
| Specified unidentified: (b) (4) | | | |
| Unspecified (others): Each individual | | | |
| Total degradation products | | | |

**Route of administration**: intravenous injection

**Proposed Dosage:** The sponsor is seeking approval of Sugammadex for routine and immediate NMB reversal. The proposed dosage is as following:

Routine Reversal:

- A dose of 4.0 mg/kg is recommended if recovery has reached 1-2 post tetanic counts (PTC) (profound blockade) following administration of rocuronium or vecuronium induced blockade.
- A dose of 2.0 mg/kg is recommended if spontaneous recovery has reached the reappearance of T2 (shallow blockade) following rocuronium or vecuronium induced blockade.

Immediate reversal:

- If there is a clinical need for immediate reversal at 3 minutes following administration of rocuronium, a dose of 16.0 mg/kg is recommended.

**Drug History**

The Sponsor has been developing drug substance Org 25969, a compound structurally belonging to the molecular family of cyclodextrins (CDs). Cyclodextrins contain a number of glucose monomers ranging from six to eight units in a ring, creating a cone shape, which makes these molecules be able to form host-guest complexes with hydrophobic molecules. Org 25969 has 8 side-chains each connected to a sugar unit of the CD, and the end of each side chain has a negatively charged group, resembling a donut with a lipophilic core and a hydrophilic outer surface. The lipophilic core is capable to attract and bind lipophilic molecules including the lipophilic steroidal part of the neuromuscular blocking agent (NMBA) rocuronium and vecuronium. Once rocuronium or vecuronium has entered the lipophilic core of Org 25969, these negatively charged groups are attracted to the positively charged ammonium group of rocuronium or vecuronium. As a result, tight binding is formed between the guest molecule (rocuronium or vecuronium) and the host cyclodextrin (Org 25969). Upon complexation with an aminosteroidal neuromuscular blocker such as rocuronium or vecuronium, Org 25969 potentially reduces the amount of neuromuscular blocking agent available to bind to nicotinic receptors in the neuromuscular junction, and thus results in the reversal of neuromuscular blockade (NMB). The drug is proposed for an indication in adults of routine reversal of shallow or profound neuromuscular blockade induced by rocuronium or vecuronium, and immediate reversal of neuromuscular blockade at 3 minutes after administration of rocuronium.

The original IND was submitted in August, 2003. During the development of the product, the sponsor found that there were (b) (4) (b) (4) of different batches of Org 25969 were varied, and (b) (4) Org 48302 with similar activity and pharmacological profile to that of Org 25969, is present at typical levels of (b) (4) (b) (4)% in all representative drug substance batches of Org 25969. The sponsor, therefore, add Org 48302 as an active entity in the drug substance along with Org 25969. Besides

Org 48302, the drug substance contains 10 specified identified and 4 specified unidentified impurities. The sponsor's strategy for assessment of the toxicological profiles of the active drug substances (Org 25969 and Org 48302) and impurities in *in vitro* and *in vivo* non-clinical models had been thoroughly discussed with the Agency during the drug development, and the Agency generally agreed to the sponsor's proposed plan. In addition, non-clinical drug distribution studies conducted by the sponsor during early drug development demonstrated long-term retention of Org 25969 in the bone and teeth of rats (average $T_{1/2}$ is approximately 172 days) during early drug development. The Agency consequently requested additional studies to further characterize bone retention and potential consequences. In response, the sponsor submitted protocols of adult and juvenile studies in a sensitive animal model, the rat, to address this issue. After review, the Agency generally concurred with the sponsor's proposal. The sponsor also sought to initiate clinical trials in pediatric patients under this IND, but this plan was not concurred by the Agency. Therefore, the sponsor asked for a deferral from pediatric studies at this time and only pursued the adult indication. The NDA of this drug product, Sugammadex, containing active ingredients Org 25969 and Org 48302 and a variety of (b) (4) (b) (4) impurities were submitted seeking for approval for marketing on October, 30 , 2007. In this review, Org 25969 generally refers to the Org 26969 drug substance, i.e. sugammadex.

**Disclaimer**: Tabular and graphical information are constructed by the reviewer unless cited otherwise.

**Studies reviewed within this submission:**

| Title | Report Designation | Location |
|---|---|---|
| ***Pharmacology Reports*** | | |
| Nonclinical pharmacology release report on ORG25969 | INT00008139 | eCTD |
| Non-clinical pharmacology release report on Org 25969 and (b) (4) | INT00008140 | eCTD |
| Nonclinical pharmacology release report on the pharmacology of different factory batches of ORG25969 | INT00012524 | eCTD |
| Nonclinical pharmacology release report on molecular interactions between steroidal neuromuscular blockers, Org 25969 and other drugs | INT00045465 | eCTD |
| ***Safety Pharmacology Reports*** | | |
| Org 25969: evaluation of effects on cardiac action potential in isolated canine Purkinje fibers | NL0028846 | eCTD |
| Org 25969: evaluation of effects on cardiac action potential in isolated canine Purkinje fibers | NL0045948 | eCTD |
| Org 25969: evaluation of effects on hERG current in stably transfected HEK-293 cells | NL0050096 | eCTD |
| An intravenous hemodynamic and electorcardiographic study with Org 25969 and Org 9426 in anesthetized beagle dog | NL0041993 | eCTD |
| Bronchopulmonary effects of Org 25969 in the conscious rat after single intravenous administration | NL0027441 | eCTD |
| An intravenous single dose neurobehavioral study in male Wistar rats | NL0048601 | eCTD |
| Hydroxypropyl-beta-cyclodextrin and gamma-cyclodextrin – | NL0062057 | eCTD |

| | | |
|---|---|---|
| Evaluation of effect on cardiac action potential in isolated canine Purkinje fibers | | |
| Org 48302 – Evaluation of effect on cardiac action potential in isolated canine Purkinje fibers | NL0059407 | eCTD |
| ***Pharmacokinetics Reports*** | | |
| A validation of the liquid chromatographic tandem mass spectrometry for the determination of Org 25969 in rat plasma | NL0027301, NL0056823, NL0063554, INT0009159, INT00037396 | eCTD |
| Validation of the LC-MS assay for the determination of Org 25969 in rat EDTA plasma and serum | INT00005927, INT00010075 | eCTD |
| A validation of the liquid chromatographic tandem mass spectrometry for the determination of Org 25969 in rabbit plasma | NL0043948, INT00016206 | eCTD |
| A validation of the liquid chromatographic tandem mass spectrometry for the determination of Org 25969 in dog plasma | NL0026185, NL0031075, NL0056828, INT00010457, INT00026946 | eCTD |
| Validation of the LC-MS assay for the determination of Org 25969 in dog EDTA plasma and serum | INT00011930, INT00022754 | eCTD |
| A validation of the liquid chromatographic tandem mass spectrometry for the determination of Org 25969 and Org 94226 in cat plasma | NL0035463, INT00007221 | eCTD |
| Validation of the LC-MS assay for the determination of Org 48302 in rat EDTA plasma | INT00006131, INT0009070 | eCTD |
| A validation of the liquid chromatographic tandem mass spectrometry for the determination of Org 9426 in dog plasma for the interference between Org 9426 and Org 25969 | NL0034322 | eCTD |
| Validation of the LC-MS assay for the determination of Org 9426 in dog EDTA plasma | INT00018080, INT00022770 | eCTD |
| An intravenous single dose TK study with Org 25969 batch AE containing Org 48302 in male and female Wistar rats | INT00007462 | eCTD |
| 3-week intravenous TK study with Org 25969 in male and female Beagle dogs | INT00025068, INT00047838 | eCTD |
| A study on the uptake of Org 25969 into rat-hepatocytes in vitro | NL0053972 | eCTD |
| An in vitro binding study of Org 25969 and/or Org 9426 to male rat, rabbit, cat, god and human plasma proteins | NL0046285 | eCTD |
| An in vitro binding study of Org 259696 to male rat, rabbit, cat, dog, and human erythrocytes and of Org 9426 with or without Org 259696 to male human erythrocytes | NL0047787 | eCTD |
| Bone retention kinetics in rat with Org 25969 (Sugammadex) after a single and multiple intravenous injections | INT00010275 | eCTD |
| A second bone retention study in male and female rats after single and multiple intravenous injection of [$^{14}$C]-Org 25969 (Org 259696-2) and Org 259696 (Sugammadex) | INT00033724 | eCTD |
| The distribution of radioactivity in bone after a single intravenous dose of [$^{14}$C]-Org 25969 to Wistar rats | INT00029186 | eCTD |
| A placental transfer study in pregnant Sprague-Dawley rats after intravenous administration of [$^{14}$C]-Org 259696, [$^{3}$H]-Org 9426, and [$^{3}$H]-Org 9426 followed by Org 25969 | NL0052819 | eCTD |

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| A placental transfer study including fetal tissue distribution in Sprague-Dawley rats after a single and multiple intravenous administration of Org 25969 | NL0025872 | eCTD |
| The distribution of radioactivity within fetuses and the proposition of Org 25969 and its metabolites in amnionic fluid after a single or multiple intravenous dose of [$^{14}$C]Org 259696 to pregnant Sprague-Dawley rats | INT00013814, INT0027787 | eCTD |
| A placental transfer study in pregnant New Zealand white rabbits after intravenous administration of [14C]-Org 25969, [3H]-Org 9426, and [3H]-Org 9426 followed by Org 25969 | NL0054636 | eCTD |
| A milk excretion study following a single intravenous dose of [14C]-Org 25969 to Sprague-Dawley rats | NL0051934 | eCTD |
| [14C]-Org 259696 and [3H]-Org 9426: an excretion balance and metabolite profiling study including tissue distribution after a single intravenous dose of [14C]-Org 25969, [3H]-Org 9426 or [3H]-Org 9426 followed by Org 25969 to male and female rats | NL0056358 | eCTD |
| [14C]-Org 25696 and [3H]-Org 9426: an excretion balance and metabolite profiling study of [14C]-Org 25969, [3H]-Org 9426 or [3H]-Org9426 followed by Org 25969 to male and female beagle dogs | NL0055283 | eCTD |
| ***Acute Toxicology Reports*** | | |
| An iv single dose acute toxicity study with Org 25969 in male CD-1 mice | NL0026309 | eCTD |
| A second iv single dose acute toxicity study with Org 25969 in male and female CD-1 mice | NL0041688 | eCTD |
| An iv single dose acute toxicity study with Org 25969 in male Wistar rats | NL0025853 | eCTD |
| A second iv single dose acute toxicity study with Org 25969 in male and female Wistar rats | NL0041666 | eCTD |
| Study 020397: a single dose iv toxicity study with Org 25969 (Batch T) in rats | NL0042813 | eCTD |
| Study 020396: Org 25969 (Batch V): single dose iv administration toxicity study in the rat with a 2-wk recovery period | INT00042732 | eCTD |
| An extended single dose iv toxicity study with Org 25969 (Batch AE) in male and female Wistar rats | NL0059572 | eCTD |
| An iv extended single dose acute toxicity study with degraded Org 25969 in Wistar rats | NL0059965 | eCTD |
| A single dose toxicity study with Org 25969 and Org 9426 using the intravenous route in beagle dogs | NL0034953, INT00011167 | eCTD |
| Org 25969 and Org 9426: A single-dose toxicity study in beagle dogs via iv injection | INT35242 | eCTD |
| A single dose toxicity study with Org 25969 and Org 9426 using the iv route in cats | NL0037029 | eCTD |
| An iv single dose study with Org 25969 and Org 9426 in cats | INT00014164, INT00033781 | eCTD |
| ***Repeat-dose Toxicology Reports*** | | |
| An iv two week toxicity and TK study with Org 25969 in Wistar rats | NL0028244, INT00020327 | eCTD |
| An iv two week toxicity study with degraded Org 25969 in Wistar rats | NL0058554 | eCTD |
| A 4-wk iv toxicity and TK study with Org 25969 in male and female Wistar rats followed by a 3-wk recovery period | NL0043521, INT00010805 | eCTD |

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| A 4-wk iv toxicity and TK study with Org 25969/Org 48302 in male and female Wistar rats followed by a 8-wk recovery period (second 4 wk study) | NL0059534, NL0067962, NL0068639 | eCTD |
| A 2-wk iv toxicity and TK study with Org 25969 in male and female beagle dogs followed by a 2-wk recovery period | NL0026533, INT00038026, INT00015286 | eCTD |
| A 4-wk iv toxicity and TK study with Org 25969 in male and female beagle dogs with 2 wk recovery | NL0043937, NL0050423, INT00020285 | eCTD |
| ***Genetic Toxicology Reports*** | | |
| A bacterial reverse mutation test (Ames Test) study with Org 25969 in Salmonella Typhimurium and Escherichia Coli | NL0027929 | eCTD |
| Ames test with Org 25969 in Salmonella Typhimurium and E. coli Ames test with Org 25969 (Batch T) in Salmonella Typhimurium and E. coli (including independent repeat) | NL0042807 | eCTD |
| Batch V: Reverse mutation in five histidine-requiring strains of Salmonella T. | NL0042175 | eCTD |
| Drug product Org 25969 batch AE: Reverse mutation in five histidine-requiring strains of Salmonella T | NL0059995 | eCTD |
| Ames test with Org 25969 (Batch AM) in Salmonella Typhimurium and E. coli (including independent repeat) | INT00005824 | eCTD |
| Org 25969 Batch AJ (impurity enriched): Reverse mutation in five histidine-requiring strains of Salmonella T. | NL0060035 | eCTD |
| Ames test with Org 25969 drug product containing degradation products in Salmonella Typhimurium and E. coli (including independent repeat) | NL0056306 | eCTD |
| Org 48302 batch B: reverse mutation in five his-requiring strains of Salmonella T. | NL0060048 | eCTD |
| Ames test with Org (b) (4) in salmonella T. and E. coli | NL0046279 | eCTD |
| A second Ames Test with Org (b) (4) in salmonella T. and E. coli | NL0050642 | eCTD |
| Evaluation of the mutagenic activity of broomcyclodextrine in salmonella T. reverse mutation assay and the E. coli mutation assay (with independent repeat) | INT00036687 | eCTD |
| A chromosome aberration test study with Org 25969 in human peripheral blood lymphocytes in vitro | NL0028999 | eCTD |
| Study 020398: Org 25969: induction of chromosome aberration in cultured human peripheral blood lymphocytes | NL0042249 | eCTD |
| Study 020399: Org 25969: induction of chromosome aberration in cultured human peripheral blood lymphocytes | NL0043003 | eCTD |
| Org 25969 batch AE drug product: induction of chromosome aberration in cultured human peripheral blood lymphocytes | NL0060033 | eCTD |
| Org 25969 Batch AJ (impurity enriched): induction of chromosome aberration in cultured human peripheral blood lymphocytes | NL0060039 | eCTD |
| A chromosome aberration test with Org 25969 drug product containing degradation products in human peripheral blood lymphocytes in vitro (with independent repeat) | NL0060000 | eCTD |
| Org 48302: Induction of chromosome aberrations in cultured human peripheral blood lymphocytes | NL0060042 | eCTD |
| Evaluation of the ability of Org (b) (4) to induce chromosome aberrations in cultured peripheral human lymphocytes | NL0047277 | eCTD |
| Evaluation of the ability of bromocyclodextrin aberrations in | INT00036688 | eCTD |

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| cultured peripheral human lymphocytes (with independent repeat) | | |
| Micronucleus test in bone marrow of the mouse with Org 25969 | NL0035188 | eCTD |
| Micronucleus test with Org 25969/Org 48302 in male and female Wistar rats | NL0060385 | eCTD |
| ***Reproductive and Developmental Toxicology Reports*** | | |
| An iv fertility and early embryo-fetal development study with Org 25969 in SD rats | NL0043955 | eCTD |
| An iv fertility and embryo-fetal development study with Org 25969 in SD rats | NL0043173 | eCTD |
| An iv embryo-fetal development study, including TK, with Org 25969 in New Zealand white rabbits | NL0043447, NL0049678, INT00026653, INT00038469 | eCTD |
| Study for effects on pre- and postnatal development including maternal function in the SD rat | NL0058898 | eCTD |
| An investigational peri- and postnatal development study with Org 25969 in SD rats | INT00030060 | eCTD |
| A second investigational peri- and postnatal development study with Org 25969 in SD rats | INT00030052 | eCTD |
| ***Juvenile Toxicology Reports (including bone and teeth study)*** | | |
| Single dose toxicity study in neonatal/juvenile rat by SC and iv administration | INT00038388 | eCTD |
| An extended single dose toxicity study in juvenile rats to assess the effects of Org 25969 on teeth | INT00032654 | eCTD |
| A two pilot juvenile toxicity and TK study with Org 25969 in male and female Wistar rats | INT00029851 | eCTD |
| Org 25969: toxicity study in the neonatal/juvenile rat by SC and iv administration | INT00038389 | eCTD |
| ***Local Toxicology Reports*** | | |
| A local tolerance study with Org 25969 in female New Zealand white rabbits using five routes of administration | NL0053500 | eCTD |
| An in vitro blood compatibility study if Org 25969 in the presence and absence of Org 9426 in male human blood | NL0050597 | eCTD |
| An in vitro blood compatibility study with Org 48302 in the presence and absence of Org 9426 in male human blood | NL0058927, NL0065038 | eCTD |
| ***Special Toxicology Reports*** | | |
| An investigational single dose toxicity study to assess the effects of Org 25969 on bone quality, structure and turnover in male and female Wistar rats | INT00015425 | eCTD |
| Second investigational single dose toxicity study to assess the effects of Org 25969 on bone quality, structure and turnover in male and female Wistar rats | INT00033963 | eCTD |
| The effect of a single iv dose org 25969 on PTH release and urinary $Ca^{2+}$ excretion in female Wistar rats | INT00029941 | eCTD |
| A popliteal lymph node assay (PLNA) with Org 25969 in female mice | NL0053614 | eCTD |
| determination of the potential immunomodulating effects of Org 25969 by mean of the plaque forming cell assay in a 2-wk iv dosing study in male and female Wistar rats | NL0047437 | eCTD |
| Assessment of contact hypersensitivity to Org 25969 in the | INT00036689 | eCTD |

| | | |
|---|---|---|
| mouse (local lymph node assay). | | |

**Studies not reviewed within this submission**: none

## 2.6.2 PHARMACOLOGY

### 2.6.2.1 Brief summary

Org 25969 is developed as a selective relaxant binding agent that binds to the hydrophobic molecular part of steroidal NMB drug rocuronium and vecuronium with high affinity resulting in reversal of neuromuscular blockade. This was a novel mechanism proposed for NMB reversal. The pharmacodynamics of Org 25969 was examined using physicochemical techniques (isothermal titration microcalorimetry), in-vitro techniques (tissue bath studies), in-vivo methods (muscle contraction in anaesthetized animals) and side effect profile studies. The pharmacodynamics of Org 25969 (b) (4) formed during Org 25969 synthesis were also examined and compared with that of Org 25969. In addition, the sponsor also compared the pharmacodynamic effect of various synthetic batches of the drug product which containing different composition of Org 25969, Org 48302, and (b) (4) impurities.

These studies have shown that Org 25969 was a potent and effective agent for the reversal of neuromuscular blockade induced by the steroidal neuromuscular blocking agents (NMBA) rocuronium, vecuronium and pipecuronium, but had very limited effect against non-steroidal neuromuscular blocking agents such as succinylcholine, atracurium, cis-atracurium and mivacurium. Org 25969 does not act as an acetylcholinesterase inhibitor. The Org 25969 (b) (4) also show a similar affinity for the steroidal neuromuscular blocking agents. Administration of Org 25969 or Org 259569 (b) (4) causes reversal of neuromuscular blockade in the *in-vitro* mouse diaphragm model and *in-vivo* models in rat, Guinea pig, cat, and rhesus monkeys. The studies of primary pharmacodynamics of manufactured batches of Org 25969 showed a similar NMB reversal effect in batch V, AE, AM, AN, AP, AQ, L00027721, L00027960, L00027961, and L00027962, indicating that small variation in composition of factory batches of Org 25969 are not likely to affect the ability of Org 25969 to encapsulate steroidal neuromuscular blocking agents like rocuronium and vecuronium.

Safety pharmacology studies were conducted to investigate the potential of adverse effects on vital organ systems such as the central nervous, cardiovascular, respiratory, and urinary systems. There were no CNS or respiratory effects observed in rats following single-dose Org 25969 administration at 500 mg/kg and 92 mg/kg, respectively. In vitro models (hERG inhibition assay, Purkinje fiber action potential assay) and in vivo cardiovascular assay in dogs suggested that Org 25969 may induce minor QT prolongation. Purkinje fiber action potential assay demonstrated that Org 25969 produced action potential inhibition represented by increase in $APD_{50}$, $APD_{70}$ and $APD_{90}$ for approximately 5% and 20% at 150 and 1500 μM; similar results were observed after Org 48302 treatment. The hERG assay in HEK-293 cells showed a 22% inhibition of hERG tail current at 1.5 mM of Org 25969. In vivo hemodynamic and electorcardiographic study with Org 25969 and rocuronium bromide in anesthetized beagle dogs indicated that a dose of 1.8 mg/kg rocuronium followed after 2 min by

administration of 250 mg/kg Org 25969 resulted in approximately 7% increase in corrected QT (both Bazett's and Fridericia's correction) at the end of a 5 min perfusion. These studies indicated that Org 25969 may have a potential for QT prolongation at the tested dosing levels. The dosing levels producing remarkable effects in these *in vivo and in vitro* studies roughly provided 10-fold exposure margin for the proposed maximal human dosing level (16 mg/kg) based on plasma level of Org 25969.

Due to its mechanism of action, Org 25969 has the potential to bind to other molecules (drugs or endogenous molecules). Isothermal microcalorimetry screening of a large number of molecules has been performed. This included drugs commonly used in anesthesia, drugs most commonly prescribed and molecules (both steroidal and non-steroidal) acting on steroidal receptors. Screening assays on binding of Org 25969 to about 300 compounds were conducted using isothermal microcalorimetry. In addition, functional effects of drug-drug PD interaction of selected drugs were examined in *in vitro* and *in vivo* models. Potential interaction was identified with drugs which have high affinity such as raloxifene and low affinity such as naloxone bound to Org 25969. Based on the results of non-clinical studies, a PK/PD model has been established to assess the PD interaction.

**2.6.2.2 Primary pharmacodynamics**

In vitro and in vivo non-clinical were conducted to examine the primary pharmacodynamics (PD) of Org 25969. All these studies were included in the submitted non-clinical pharmacology release reports (Document ID: INT00008139; INT00008140; 00012524).

Mechanism of action of Org 25969:

Org 25969 is compound belonging to γ-cyclodextrin family representing a novel mechanism to reverse neuromuscular block through the formation of complexes with a steroidal neuromuscular blocking agent such as rocuronium and vecuronium. The binding affinity was examined of Org 25969 bound to neuromuscular blocking agents were examined using microcalorimetry. Isothermal microcalorimetry is an analytical chemistry technique to determine the affinity of a test molecule for other molecules. When two different molecules form a complex, the amount of heat produced is proportional to the affinity of the molecules. Neuromuscular blocking agents can be subdivided into two classes: the depolarizing neuromuscular blocking agents (e.g. succinylcholine) and the non-depolarizing neuromuscular blocking agents (e.g. rocuronium, atracurium). Chemically, the non-depolarizing compounds can be subsequently subdivided into the steroidal neuromuscular blocking agents (e.g. rocuronium, vecuronium and pancuronium) and the nonsteroidal neuromuscular blocking agents (e.g. atracurium, cis-atracurium, mivacurium, tubocurarine and gallamine). The affinity expressed as association constant ($K_A$ in megamol$^{-1}$) of Org 25969 with these molecules was shown in the following table (extracted from the sponsor's report). The results indicated that relatively high affinity with steroidal non-depolarizing neuromuscular blocking agents, but very limited to none affinity with non-steroidal non-depolarizing and depolarizing neuromuscular blocking agents. Changing the pH within

the human physiological range of 7.2 – 7.6 did not remarkably alter the affinity of rocuronium and vecuronium for Org 25969.

**Table 2: Affinity of Org 25969 for neuromuscular blocking agents determined with isothermal microcalorimetry**

| compound | class | structure | $K_A$ value (megamol$^{-1}$) |
|---|---|---|---|
| | | | |
| Succinylcholine | depolarizing | non-steroidal | 0.000 |
| | | | |
| Pipecuronium | non-depolarizing | steroidal | 161.500 |
| Rocuronium | non-depolarizing | steroidal | 15.100 |
| Vecuronium | non-depolarizing | steroidal | 8.800 |
| Pancuronium | non-depolarizing | steroidal | 2.600 |
| | | | |
| Tubocurarine | non-depolarizing | non-steroidal | 0.193 |
| Alcuronium | non-depolarizing | non-steroidal | 0.009 |
| Mivacurium | non-depolarizing | non-steroidal | 0.009 |
| Cis-atracurium | non-depolarizing | non-steroidal | 0.005 |
| Atracurium | non-depolarizing | non-steroidal | 0.004 |
| Gallamine | non-depolarizing | non-steroidal | 0.000 |

Drug activity of Org 25969 related to proposed indication:

Org 25969-induced reversal of neuromuscular block was demonstrated in an *in vitro* model of isolated mouse phrenic nerve-hemi-diaphragm preparation. The diaphragm was split into two preparations after removal from a mouse. Each preparation was fixed to a tissue holder and placed in a buffer-filled tissue bath. The phrenic nerve is placed on an electrode allowing constant voltage electrical stimulation of the phrenic nerve. Each single pulse electrical stimulation of the nerve results in a twitch contraction of the preparation. Administration of a neuromuscular blocking agent (NMBA) causes decrease in twitch height; the effect of Org 25969 on NMBA-induced twitch height inhibition was then investigated after administration. The concentrations of Org 25969 causing 50% reversal are shown in the following (extracted from the sponsor's report). The results indicated that Org 25969 caused reversal of steroidal NMBA (pipecuronium, rocuronium, vecuronium, and pancuronium) induced twitch inhibition, but not non-steroidal NMBAs such as mivacurium, atracurium and succinylcholine.

**Table 3: Effect of Org 25969 on the neuromuscular blockade induced by 90% blocking doses of different neuromuscular blocking agents in the isolated mouse hemi-diaphragm preparation**

| | 90% blockade at (µM) | 50% reversal by Org 25969 at (µM) | maximum reversal (%) | at (µM) | 50% ratio RVA/NMB |
|---|---|---|---|---|---|
| pipecuronium | 0.30 | 0.1 ± 0.0 | 91.1 ± 1.8 | 0.2 | 0.3 ± 0.0 |
| rocuronium | 3.60 | 1.2 ± 0.8 | 95.1 ± 2.3 | 3.6 | 0.3 ± 0.2 |
| vecuronium | 0.85 | 0.8 ± 0.1 | 90.6 ± 5.1 | 1.4 | 0.9 ± 0.2 |
| pancuronium | 0.90 | 1.2 ± 0.3 | 60.4 ± 8.7 | 1.8 | 1.3 ± 0.3 |
| mivacurium | 8.00 | - | 0.0 ± 0.0 | 160.0 | - |
| atracurium | 45.00 | - | 4.7 ± 4.7 | 1400.0 | - |
| succinylcholine | 45.00 | - | 24.5 ± 8.8 | 900.0 | - |

Mean ± SEM; n = 4

Intravenous administration of magnesium can cause a decrease in twitch height. After spontaneous recovery from neuromuscular block, intravenous administration of magnesium can also result in re-occurrence of neuromuscular block. Antibiotics, especially aminoglycosides, are also known to potentiate and prolong the action of NMBAs. In this model, magnesium or aminoglycoside antibiotics such as neomycin or streptomycin administration potentiate the extent of NMBA-induced twitch height

inhibition, and thus, needed higher Org 25969 for complete reversal. Higher concentration Org 25969 was needed to prevent re-occurrence of neuromuscular block after reversal by Org 25969**.**

In rats, a single-dose administration of 1.0 µmol/kg Org 25969 remarkably enhanced the recovery time from blockade of M. gastrocnemius contraction induced by $3\times ED_{90}$ dose of rocuronium and vecuronium (50% recovery: ≤2.6 min) as compared to saline (50% recovery: approximately 5.6 min). In comparison to rats, Guinea pig is a more sensitive model for NMB and reversal test. The examination on the M. gastrocnemius preparation in anesthetized guinea pigs indicated that cumulative administration of Org 25969 caused dose-dependent reversal of neuromuscular block. A dose of 0.09 ± 0.02 µmol/kg (mean ± SEM; n = 3) Org 25969 resulted in 50% reversal. Maximum reversal was 97 ± 3 % (mean ± SEM; n = 3) of baseline twitch height and was obtained at a dose of 0.64 µmol/kg. In a second study in Guinea pigs paralyzed with rocuronium, Org 25969 caused a marked improvement in speed of recovery: 90% recovery was obtained after 2.86 ± 0.36 min; in contrast, 90% recovery was obtained at 5.22 ± 0.39 min after saline administration. Increasing the dose of Org 25969 to 70 nmol/kg reduced this time period to 1.03 ± 0.26 min (mean ± SEM; n = 4). At the higher dose of 460 nmol/kg Org 25969, this period shortened further to 0.63 ± 0.07 min (mean ± SEM; n = 4). Similar observations were observed for vecuronium and pancuronium as shown in the table below (extracted from the sponsor's table). No signs of residual blockade or re-occurrence of blockade were observed in the 30 minutes following the Org 25969 administration.

**Table 7: Effect of intravenous bolus injection of 460 nmol.kg$^{-1}$ Org 25969 on recovery from neuromuscular blockade induced by steroidal neuromuscular blocking agents**

| | spontaneous | 460 nmol.kg$^{-1}$ Org 25969 |
|---|---|---|
| Time to 90% recovery (min) | | |
| Rocuronium | 5.13 ± 0.54 | 0.63 ± 0.07 |
| Vecuronium | 21.15 ± 3.21 | 0.97 ± 0.08 |
| Pancuronium | 13.91 ± 3.39 | 0.78 ± 0.10 |
| Recovery Index (min) | | |
| Rocuronium | 2.54 ± 0.27 | 0.26 ± 0.04 |
| Vecuronium | 12.89 ± 3.06 | 0.36 ± 0.04 |
| Pancuronium | 7.36 ± 1.82 | 0.34 ± 0.05 |

Recovery Index: time between 25% and 75% recovery.
Mean ± SEM; n = 4.

In contrast, administration of Org 25969 did not improve the recovery from neuromuscular blockade induced by the non-steroidal compounds succinylcholine, atracurium and d-tubocurarine, but limited recovery was observed for mivacurium: time to 90% recovery improved from 66.4 ± 10.4 min to 29.6 ± 5.8 min. Studies in guinea pigs also demonstrated that Org 25969-induced reversal of rocuronium-induced NMB is likely to be independent of changes in renal perfusion and acid-base status. The potentiation effect of magnesium on Org 25969-induced NMB reversal was also demonstrated in Guinea pigs. The effect of magnesium was clearly potentiated and prolonged after complete reversal of rocuronium-induced neuromuscular blockade with 330 nmol/kg Org 25969. However, when a higher dose of Org 25969 was used (660 nmol/kg), the potentiation and prolongation of the effect of magnesium was remarkably reduced. Similar effects were also observed for meomycin.

In cats, Org 25969 (460 nmol/kg) improved the time of 90% recovery of M. tibialis contraction inhibition induced by rocuronium from 9.2 ± 2.9 min to 2.2 ± 0.5 min, and the effect was independent of renal clearance of Org 25969. The study in cats also showed that once reversal has occurred, not sufficient rocuronium can leave the complex with Org 25969 to cause re-occurrence of block.

Studies to investigate the NMB reversal effect of Org 25969 were also conducted in monkeys. An intravenous bolus injection of 230 or 460 nmol/kg Org 25969 significantly enhanced the reversal time expressed as a Train-Of-Four (TOF) ratio of 0.9, indicative of effective reversal of NMB, as shown in the following table (extracted from the sponsor's report).

**Table 8: Effect of administration of 230 or 460 nmol.kg$^{-1}$ Org 25969 on time to a TOF ratio of 0.9 (min)**

| | spontaneous | Org 25969 (nmol.kg$^{-1}$) | Org 25969-induced |
|---|---|---|---|
| rocuronium | 15.4 ± 2.2 | 230 | 3.7 ± 1.6# |
| | 14.5 ± 1.1 | 460 | 1.9 ± 0.5# |
| vecuronium | 25.0 ± 2.8 | 230 | 16.5 ± 4.1# |
| | 23.1 ± 1.8 | 460 | 4.4 ± 0.6# |
| 3-OH-vecuronium | 19.0 ± 3.6 | 230 | 2.5 ± 0.5# |
| | 18.6 ± 2.3 | 460 | 2.5 ± 0.9# |
| pancuronium | 37.6 ± 5.6 | 230 | 31.2 ± 6.3# |
| | 34.2 ± 4.9 | 460 | 19.7 ± 7.0# |

Mean ± SEM; n = 4 per group.
#: statistically significantly different from spontaneous recovery ($p<0.05$; paired t-test).

Primary Pharmacodynamics of Org 25969 (b) (4)

Using isothermal microcalorimetry, the affinity of Org 25969 (b) (4) for both rocuronium and vecuronium has been determined as shown in the table below (extracted from the sponsor's report). The results of these assays indicated Org 25969 (b) (4) contained in Org 25969 drug product have different degrees of rocuronium and vecuronium binding affinity. Org 48302, the second active ingredient, showed about half of Org 25969 binding affinity in isothermal microcalorimetry.

**Table 10: Affinity of Org 25969 and (b) (4) for rocuronium and vecuronium determined by isothermal microcalorimetry**

| Cyclodextrin | $K_A$ values (megamol$^{-1}$) rocuronium | vecuronium |
|---|---|---|
| Org 25969V [a] | 16.94 ± 0.63 | 10.29 ± 0.35 |
| (b) (4) | | |

Mean ± SEM; n = 3-6 determinations.
[a] The character behind the compound code indicates the batch code.
(b) (4)

*In vitro* isolated mouse phrenic nerve-hemi-diaphragm preparation was used to determine the potency and efficacy of these Org 25969 (b) (4). Cumulative administration of Org 25969 and Org (b) (4) caused a concentration-dependent reversal of neuromuscular blockade induced by rocuronium as shown in the table below (extracted from the sponsor's report). The results indicated that the most Org 25969 (b) (4) Org 48302 have showed effect on reversal of rocuronium-induced NMB. Similar results were observed for vecuronium.

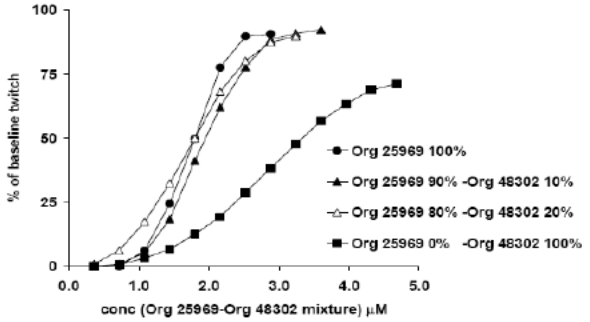
**Recovery from rocuronium-induced blockade of mouse hemi-diaphragm contractions after cumulative administration of Org 25969 or (b) (4)**

| Compound | n | 50% recovery at (μM) | maximum recovery (%) | maximum recovery at (μM) |
|---|---|---|---|---|
| Org 25969 | 6 | 1.78 ± 0.09 | 92.2 ± 3.6 | 2.64 ± 0.15 |
| (b) (4) | | | | |

Mean ± SEM
* The character behind the compound code indicates the batch code
#: statistically significantly different (p<0.05) from Org 25969 (one-way ANOVA, followed by Newman-Keuls procedure).

Additional studies in mouse diaphragm preparation also indicated that mixture of Org 25969 and Org 48302 containing up to 20% Org 48302 had similar potency to Org 25969 with 100% purity as shown below (extracted form the sponsor's report). In contrast, an Org 48302 batch with 100% purity had significantly lower potency.

**Figure 6: Effect of different mixtures of Org 25969 (Org 25969) and Org 48302 on rocuronium-induced (3.60 μM) blockade of mouse hemi-diaphragm contractions**




Comparison of the effects of NMB reversal was also conducted in vivo in guinea pigs. All Org 25969 (b) (4) caused rapid reversal of rocuronium-induced

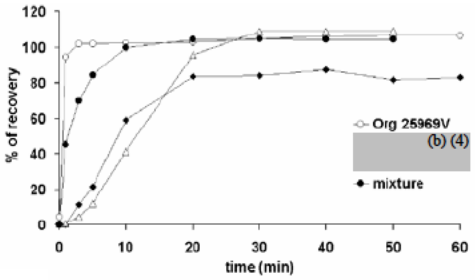
neuromuscular block, [redacted] (b) (4) which caused a delayed, but complete recovery, as shown below.

**Table 21 Reversal of rocuronium-induced neuromuscular block after intravenous bolus injection of 0.5 µmol/kg Org 25969 or** [redacted] (b) (4)

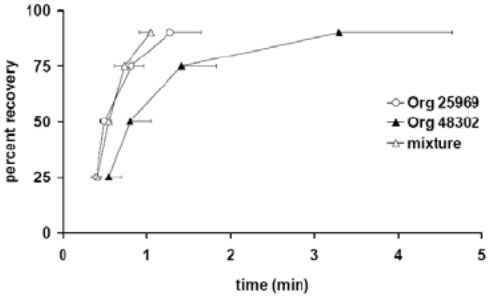
| compound | time to 25% recovery (min) | time to 50% recovery (min) | time to 75% recovery (min) | time to 90% recovery (min) |
|---|---|---|---|---|
| Org 25969 | 0.4 ± 0.1 | 0.5 ± 0.1 | 0.8 ± 0.1 | 1.3 ± 0.4 |
| [redacted] (b) (4) | | | | |

Mean ± SEM.#: significantly different ($p<0.05$) from Org 25969 (one-way analysis of variance, followed by Newman-Keuls procedure)

Follow-up studies were conducted to compare the reversal effect of rocuronium and vecuronium-induced NMB using combinations of 80% Org 25969 and 20% [redacted] (b) (4) in anaesthetized guinea pigs. The results of these studies indicated that a mixture of 80% Org 25969 and 20% [redacted] (b) (4) caused a slightly slower reversal speed than 100% Org 25969. In contrast, a mixture of 80% Org 25969 and 20% Org 48302 caused a similar speed of reversal as compared to 100% Org 25969.




Recovery of neuromuscular function after administration of Org 25969 (Org 25969V) [redacted] (b) (4) or an equi-molar dose of a mixture of 80% Org 25969 and 20% [redacted] (b) (4)




Recovery from rocuronium-induced neuromuscular block after administration of Org 25969 (Org 25969V), Org 48302 or an equi-molar dose of a mixture of 80% Org 25969 and 20% Org 48302 (0.5 µmol/kg)

Primary Pharmacodynamics of manufactured batches of Org 25969

There were no differences in affinity for vecuronium observed between the different batches of Org 25969. The affinity for rocuronium was similar with Ka value of approximately 25 megamol$^{-1}$ and slight difference in batch V (-20%) and batch L00027962 (+20%), as shown in the following table (extracted from the sponsor's report).

**Table 23: Affinity of different batches of Org 25969 for rocuronium determined by isothermal microcalorimetry**

| Org 25969 | rocuronium | vecuronium |
|---|---|---|
| | $K_A$ values ($megamol^{-1}$) | |
| Batch V | 19.46 ± 0.62 | 9.09 ± 0.54 |
| Batch AE | 25.82 ± 1.07# | 9.98 ± 0.46 |
| Batch AM | 24.13 ± 3.07 | 10.10 ± 0.16 |
| Batch AN | 24.47 ± 1.97 | 10.78 ± 0.80 |
| Batch AP | 26.47 ± 3.30# | 10.87 ± 0.54 |
| Batch AQ | 26.73 ± 1.49# | 11.29 ± 0.47 |
| Batch L00027721 | 28.03 ± 1.18# | 10.23 ± 0.88 |
| Batch L00027960 | 25.27 ± 1.47 | 11.54 ± 1.09 |
| Batch L00027961 | 24.70 ± 1.68 | 10.67 ± 0.27 |
| Batch L00027962 | 30.10 ± 3.16# | 12.07 ± 0.17 |

Mean ± SEM; n = 3 determinations
#: significantly different from Org 25969 batch V (p 0.05 level); one-way ANOVA followed by Newman-Keuls procedure

Results in mouse diaphragm assay indicated that the potency for rocuronium and vecuronium-induced NMB reversal in difference batches were similar with small variation within the variation range in repeated assays of batch V. The following table (extracted from the sponsor's table) shows the $ED_{50}$ of different batches for NMB reversal in comparison with the variation in the $ED_{50}$ of batch V in different assays. Similar results were observed in vecuronium.

**Table 24: Concentration of Org 25969 causing 50% recovery from rocuronium-induced blockade of mouse hemi-diaphragm contractions after cumulative administration of different batches of Org 25969**

| | batch of Org 25969 | | Org 25969 batch V | |
|---|---|---|---|---|
| | 50% recovery at (µM) | n | 50% recovery at (µM) | n |
| Batch AE | 1.01 ± 0.06 | 8 | 1.09 ± 0.05 | 8 |
| Batch AM | 1.16 ± 0.05 | 8 | 1.17 ± 0.05 | 8 |
| Batch AN | 1.39 ± 0.12 | 8 | 1.47 ± 0.18 | 8 |
| Batch AP | 1.11 ± 0.11 | 7 | 1.44 ± 0.17 | 7 |
| Batch AQ | 1.01 ± 0.10 | 8 | 1.02 ± 0.20 | 8 |
| Batch L00027721 | 1.19 ± 0.10 | 8 | 1.24 ± 0.08 | 8 |
| Batch L00027960 | 1.06 ± 0.12 | 8 | 1.12 ± 0.20 | 8 |
| Batch L00027961 | 1.08 ± 0.11 | 8 | 1.30 ± 0.12 | 8 |
| Batch L00027962 | 1.44 ± 0.19 | 8 | 1.47 ± 0.20 | 8 |

Mean ± SEM.
#: significantly different from batch V in matching preparation (p<0.05; unpaired t-test)

### 2.6.2.3 Secondary pharmacodynamics

Secondary pharmacodynamic effects of Org 25969 were investigated in various *in vitro* tissue culture models. In mouse hemi-diaphragm preparation possessing pre-junctional and post-junctional nicotinic receptors, muscarinic receptors, pre-junctional P/Q-type voltage-dependent calcium channels, sodium and potassium channels, as well as junctional acetylcholinesterases, administration of 36 µM Org 25969 had no effect on twitch height and resting tension. In mouse vas deferens preparation which contains pre-junctional α2-adrenoceptors, µ-, δ- and k-opioid, ORL1, cannabinoid CB2 and adenosine receptors, post-junctional α1-adrenoceptors, purinoceptors, pre-junctional N-type voltage-dependent calcium channels, post-junctional L-type voltage-dependent calcium channels, junctional ecto-ATPase, and pre-junctional noradrenalin uptake mechanisms, Addition of 36 µM Org 25969 to the tissue batch had no effect on resting tension and twitch height. The isolated spontaneously beating guinea pig right atrium contains

muscarinic M2 acetylcholine receptors, L-type calcium channels, Ih channels, sodium and potassium channels. Concentrations of Org 25969 in the range of 0.1 – 100 μM had no effect on beating rate, resting tension or contractions. In contrast, cumulative administration of either acetylcholine or methacholine caused a concentration-dependent decrease in beating rate. Isolated rat aortic ring preparations contain a1-adrenoceptors, 5-hydroxytryptamine 5-HT2 and histamine H1 receptors. Cumulative administration of (-)-adrenaline, (-)-noradrenalin, 5-hydroxytryptamine, dopamine or histamine results in contraction of the rings with the pEC50 values were 8.44, 8.25, 6.09, 6.05 and 3.34, respectively. Pretreatment with 36 μM Org 25969 did not modify the contractile response to (-)-adrenaline, (-)-noradrenalin, 5-hydroxytryptamine or dopamine. Rat iliac artery preparation contains pre-junctional a2-adrenoceptors, post-junctional a1-adrenoceptors, pre-junctional N-type voltage-dependent calcium channels, and post-junctional L-type voltage-dependent calcium channels. Constant current field stimulation of this preparation results in contractions. Addition of increasing concentrations of Org 25969 (1-100 μM) had no effect on resting tension and twitch height of the rat iliac artery preparation. Isolated guinea pig second order bronchus ring contains post-junctional muscarinic M3 acetylcholine and histamine H1 receptors. Cumulative administration of acetylcholine or histamine caused a concentration-dependent contraction. Pretreatment with 36 μM Org 25969 had no effect on this preparation and did not affect the contractile response to either acetylcholine or histamine. Overall, at the concentrations tested (in general between 0 to 100 μM), Org 25969 did not show interaction with the endogenous neurotransmitters or exogenous agonists added to the in vitro preparations, suggesting that Org 25969 do not likely interact with the receptors, enzymes, and re-uptake systems in these in vitro preparations. Standard binding screens with these molecules were not conducted.

In the *in vivo* animal models used for primary pharmacodynamics studies, treatment of Org 25969 had no effect on arterial blood pressure and heart rate in anesthetized rats, guinea pigs, cats, and monkeys. Similarly, during the testing of Org (b) (4) as reversal agent in anesthetized guinea pigs, no changes in blood pressure and heart rate was observed.

All these studies were included in submitted non-clinical pharmacology release reports (Document ID: INT00008139). In addition, an in vivo study in Guinea pigs was conducted to examine the effect of Org25969 and (b) (4) on hemodynamic parameters and the results showed that injection of Org 25969 or (b) (4) did not cause significant changes in blood pressure or heart rate ion deeply anaesthetized Guinea pigs (INT00008140).

### 2.6.2.4 Safety pharmacology

Neurological effects:

Neurological effects were examined in male Wistar rat following single-dose intravenous administration of 0 (saline), 20, 100, and 500 mg/kg Org 25969 (Batch U) (8 rats/group).

Clonidine (0.3 mg/kg) was used as positive control (NL0048601). Neurobehavioral, neurovegative or sensorimotor effects were assessed using a Functional Observational Battery (FOB), and effects on spontaneous locomotor activity were assessed using the Open-Field method within 2 hours after administration. The results of the study showed that there were no statistically significant and treatment-relevant effects observed in FOB test and Open-Field test as well as on body temperature in animals treated with Org 25969 at all doses as compared to control animals under the experimental conditions. In contrast, Clonidine, 0.3 mg/kg, induced its typical sedative, neurovegative and hypothermic effects, an effect on spontaneous locomotor activity characterized by statistically significant decreases in locomotor activity in the peripheral zone and an increase in the duration of immobility in the Open-Field arena. Overall, the results of the study in rat suggested no neurological effects at dose up to 500 mg/kg after single-dose administration.

Cardiovascular effects

In vitro and in vivo studies have been conducted to assess the effect of Org 25969 and/or Org 48302 in cardiovascular systems as shown in the following table.

| Type of study/ Test system | Species (sex, strain) | Dosing route | Dose levels and dose regimen | No. of prepa-rations or animals /group, gender | Parameters measured /assessments |
|---|---|---|---|---|---|
| In vitro studies | | | | | |
| *In vitro* cardiac effects: Purkinje fibers | Dog (male, beagle) | in vitro | Successively: Tyrode's (vehicle), $2.76\times10^{-7}$, $2.76\times10^{-6}$ and $2.76\times10^{-5}$ M Org 25969 [a] | 6 out of 3M [b] | RP, APA, $V_{max}$, $APD_{50}$, $APD_{70}$, $APD_{90}$ |
| *In vitro* cardiac effects: Purkinje fibers | Dog (male, beagle) | In vitro | Successively: Tyrode's (vehicle), $1.5\times10^{-5}$, $1.5\times10^{-4}$ and $1.5\times10^{-3}$ M Org 25969 [a] | 6 out of 6M [b] | RP, APA, $V_{max}$, $APD_{50}$, $APD_{70}$, $APD_{90}$ |
| *In vitro* effect on hERG $K^+$ channels in HEK-293 cells | Human embryonic kidney cell line stably transfected with human $I_{Kr}$ | in vitro | Phase 1: successively: Tyrode (vehicle), $1.5\times10^{-3}$ M Org 25969, Tyrode and E-4031 (pos. control) [c] | 6 | Amplitude of hERG tail current ($I_{Kr}$) |
| *In vitro* cardiac effects: Purkinje fibers | Dog (male, beagle) | In vitro | Successively: Tyrode's (vehicle), $1.5\times10^{-5}$, $1.5\times10^{-4}$ and $1.5\times10^{-3}$ M Org 48302 [a] | 6 out of 6M [b] | RP, APA, $V_{max}$, $APD_{50}$, $APD_{70}$, $APD_{90}$ |
| *In vitro* cardiac effects: Purkinje fibers | Dog (male, beagle) | In vitro | Successively: Tyrode's (vehicle), $1.5\times10^{-5}$, $1.5\times10^{-4}$ and $1.5\times10^{-3}$ M HP-β-CD or γ-CD [a] | 6+6 out of 7M [b] | RP, APA, $V_{max}$, $APD_{50}$, $APD_{70}$, $APD_{90}$ |
| In vivo studies | | | | | |
| Hemodynamic and cardiac effects: cumulative dose | Anesthetized dog (male, beagle) | IV | Phase 1, 2 and 3 as: saline (vehicle), 1.8 $mg.kg^{-1}$ rocuronium [d] and 1.8 $mg.kg^{-1}$ rocuronium followed 2 min later by 25 or 250 $mg.kg^{-1}$ Org 25969 given as infusion over 5 min, respectively [e] | 6M | Hemodynamic parameters and ECG parameters [f] |

In the first in vitro cardiac action potential assay in Purkinje fiber of dogs, Org 25969 at concentrations of 0.3, 3 and 30 µM (equal to 0.6. 6.0 and 60 µg/mL, respectively) had no statistically significant effect on action potential parameters under both normal (1 Hz) and low (0.33 Hz) stimulation rates. The results of the second Purkinje fiber assay showed that Org 25969 (batch V) induced dose-dependent increase in APD50, APD70, and APD90 at 150 and 1500 µM under normal (1 Hz) and low stimulations (0.33 Hz). There were no changes observed at 15 µM.

| | Normal Stimulation (1Hz) | | | Low stimulation (0.33 Hz) | | |
|---|---|---|---|---|---|---|
| | APD50 | APD70 | APD90 | APD50 | APD70 | APD90 |
| 15 μM | ↑0.8% | ↑1.0% | ↑0.9% | 0% | ↑1.2% | ↑1.3% |
| 150 μM | ↑7.5%* | ↑7.0%* | ↑6.2%* | ↑7.6%* | ↑7.1%* | ↑6.7%* |
| 1500 μM | ↑25.7%* | ↑22.7%* | ↑19.9%* | ↑21.7%* | ↑20.1%* | ↑17.6%* |

* $p<0.05$

The effect of Org 48302, the secondary active ingredient, on cardiac action potential was also assessed in isolated Purkinje fibers of dogs. The results indicated that Org 48302 induced increase in APD50, APD70, and APD90 similar to Org 25969 with a little less extent, as shown in the table below

| | Normal Stimulation (1Hz) | | | Low stimulation (0.33 Hz) | | |
|---|---|---|---|---|---|---|
| | APD50 | APD70 | APD90 | APD50 | APD70 | APD90 |
| 15 μM | ↑0.4% | ↑1.5% | ↑2.0% | ↑1.5% | ↑2.5% | ↑2.8% |
| 150 μM | ↑4.3% | ↑4.8%* | ↑4.9%* | ↑5.3% | ↑7.0% | ↑6.8% |
| 1500 μM | ↑16.5%* | ↑14.9%* | ↑14.1%* | ↑15.2%* | ↑16.2%* | ↑16.9%* |

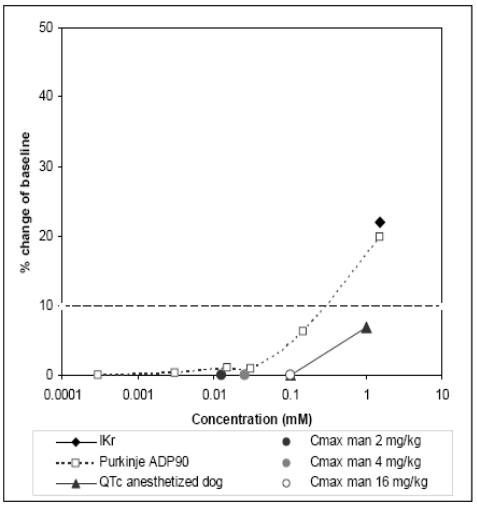
* $p<0.05$

In contrast, Hydroypropyl-β-cyclodextrin and γ-cyclodextrin only slightly increased APD90 of Purkinje fibers of dog at 1.5 mM concentration (<5.0%) with no change in APD50 and APD70. These in vitro cardiac action potential assays in dog Purkinje fibers suggested that Org 25969 and Org 48302 are more sensitive in Purkinje fiber APD prolongation than unmodified or different cyclodextrins.

To further evaluate the potential cardiovascular effects of Org 25969, a hERG channel inhibition assay was conducted in HEK-293 cells transfected with hERG-1 cDNA. The results showed that Org 25969 at 1.5 mM produced a noticeable inhibition of rapidly-activating delayed rectifier current IKr (22 ± 2%). IC50 (the concentration that induced 50% inhibition of hERG tail current) can not be determined since higher concentration could not be reached. hERG channel inhibition was not examined at lower concentration of Org 25969.

The cardiovascular effect of Org 25969 (batch U) was also examined in *in vivo* dog model. In a study to evaluate the reversal effects of Org 25969, two groups of male dogs (6/group) were 1.8 mg/kg Org 9426 followed by 25 mg/kg Org 25969 after 2 minutes (group1) or 1.8 mg/kg Org 9426 followed by 250 mg/kg Org 25969 after 5 minutes (group 2). Hemodynamic parameters including left intra-ventricular systolic pressure, left ventricular end diastolic pressure and aortic blood pressures; hear rate; aortic, coronary, renal, and iliac blood flows were measured. ECG parameters including PR, QRS, QT and RR intervals were also recorded. A slight and transient increase in corrected QT (QTcB of 0.318 to 0.340 msec and QTcF of 0.277 to 0.296 msec, which corresponds to an increase of 6.9%) was observed after Org 25969 treatment (phase 3) at 250 mg/kg. At 25 mg/kg, no change in QT or QTc-interval was observed.

: Overlay plots of concentration-effect data for $I_{Kr}$, $APD_{90}$ and QTc-interval in dog for Org 25969 related to the $C_{max}$ in man at 2, 4 and 16 mg·kg$^{-1}$




Note the Y-axis has been limited to 50% change to expand the figure. Mean Cmax levels at 2, 4 and 16 mg/kg are indicated by a filled, grey or open circle, respectively. The dashed line at 10% change indicates the degree of change typically regarded as physiologically relevant.

Overall, the results of these studies suggested that Org 25969 and/or Org 48302 may have the potential to induce QT prolongation in human. These included increase in $APD_{50}$, $APD_{70}$, and $APD_{90}$ at 150 (5%) and 1500 µM (50%) in *in vitro* Purkinje fiber action potential assays, inhibition of rapidly-activating delayed rectifier current IKr (22%) at 1500 µM in hREG channel assay, and transient increase in corrected QT interval (7%) at 250 mg/kg in the presence of Org 9626 (1.8 mg/kg) in an *in vivo* dog study. The sponsor has compared the Org 25969 concentrations in the *in vitro* assays and the $C_{max}$ in the *in vivo* study with the $C_{max}$ in human after Sugammadex administration with the proposed dosages, as illustrated in the figure below. Single-dose administration of 250 mg/kg Org 25969 in dogs produced an approximate $C_{max}$ of 2000 µg/ml while single-dose administration of 16 mg/kg in human produced an approximate $C_{max}$ of 200 µg/ml (100 µM), indicating a ≥ 10-fold exposure margin. These results suggested that Org 25969 may produce minimal QT prolongation at high concentration. The risk for QT prolongation may be negligible in human with the 16 mg/kg administration due to the small extent of the effects detected and large exposure margins as shown above

Pulmonary effects:

Rat whole body plethysmography study was conducted to evaluate the broncho-pulmonary effects of Org 25969 following single dose administration of 4.6, 23, or 92 mg/kg Org 25969 (batch L) (NL0027411). The results of this study indicated no broncho-pulmonary side effects detected with single dose Org 25969, up to 92 mg/kg in rats.

Abuse liability:
N/A

Other: N/A

### 2.6.2.5 Pharmacodynamic drug interactions

Due to the mechanism of action, Org 25969 could potentially bind to other molecules. The sponsor has proposed two potential mechanisms of drug-drug interaction. 1) a drug or endogenous molecule might interfere with the interaction between NMBA and Org 25969, thereby reducing the reversal action of Org 25969 (displacement); 2) Org 25969 might not only bind to NMBA, but also to another drug or endogenous molecules, thereby reducing the biological activity of the drug or endogenous molecules (capture). To investigate these interactions, the sponsor firstly measured the affinity of Org 25969 using isothermal microcalorimetry for ≥ 300 selected chemicals that may be co-administered with the drug product of Org 25969 including drugs commonly used during anesthesia, the most commonly prescribed drugs, and compounds (endogenous or exogenous) which act on steroidal receptors (either steroidal or non-steroidal). In comparison, raloxifene, and oral selective estrogen receptor modulator, showed an affinity similar to rocuronium while toremifene and norgestimate are similar to vecuronium. In addition, some other compound showed a lower affinity to Org 25969, as shown in the table below (part of the affinity table form the affinity screening study extracted from the sponsor's report). Isothermal microcalorimetry assay also showed that Org 25969 has very low affinity to divalent such as $Ca2^+$ and $Mg2^+$, and no affinity to monovalent cations such as $K^+$ and $Na^+$.

| compound | Ka value ($mol^{-1}$) |
|---|---|
| PIPECURONIUM | 190000000 |
| ROCURONIUM BROMIDE | 17890000 |
| RALOXIFENE HYDROCHLORIDE | 15700000 |
| RAPACURONIUM | 14700000 |
| VECURONIUM BROMIDE | 5720000 |
| NORGESTIMATE | 5470000 |
| TOREMIFENE | 5430000 |
| ETONOGESTREL | 2860000 |
| TIBOLONE | 2800000 |
| PANCURONIUM BROMIDE | 2620000 |
| DROSPERINONE | 2130000 |
| LEVONORGESTREL | 2000000 |
| TAMOXIFEN CITRATE SALT | 1830000 |
| PROGESTERONE | 1500000 |
| NORETHINDRONE ACETATE | 1460000 |
| ETHINYLESTRADIOL | 1080000 |
| MEDROXYPROGESTERONE ACETATE | 960000 |
| CLOMIPHENE CITRATE SALT | 852000 |
| BECLOMETHASONE DIPROPRIONATE | 770000 |
| ESTRADIOL | 750000 |
| GESTODENE | 672000 |
| DESOGESTREL | 574000 |
| FUSIDIC ACID | 314000 |
| CANRENOIC ACID POTASSIUM SALT | 302400 |
| FORMESTANE | 250000 |

Afterwards, the sponsor used mouse isolated phrenic nerve-hemi-diaphragm to examine functional effects of Org 25969 on reversal of neuromuscular block in the presence of selected chemicals. Three experimental protocols were applied and verified with 17-OH-rocuronium, a compound with high affinity bound for Org 25969 but without neuromuscular block effect, as illustrated blow.

*Experimental Protocol Type 1.*

- Administration of a single concentration of a neuromuscular blocking compound resulting in complete blockade in both hemi-diaphragm preparations.
- Cumulative administration of Org 25969, until almost complete recovery of twitch height was obtained.
- Administration of cumulative concentrations of either a non-blocking compound (e.g. 17-OH-rocuronium) or and equivalent amount of buffer solution.

*Experimental Protocol Type 2*

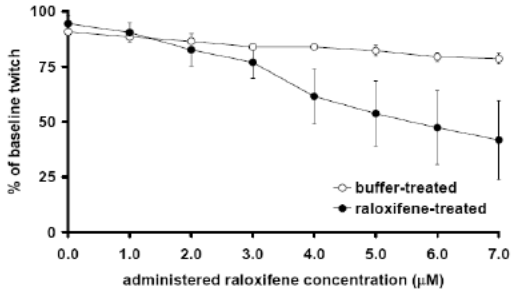
- Administration of a single concentration of either a non-blocking compound (e.g. 17-OH-rocuronium) or and equivalent amount of buffer solution.
- Administration of a single concentration of a neuromuscular blocking compound resulting in complete blockade in both preparations.
- Cumulative administration of Org 25969.

*Experimental Protocol Type 3*

- Administration of Org 25969 to both hemi-diaphragm preparations.
- Administration of 6.0 μM 17-OH-rocuronium to one preparation and an equivalent volume of distilled water to the other preparation.
- Cumulative administration of rocuronium.

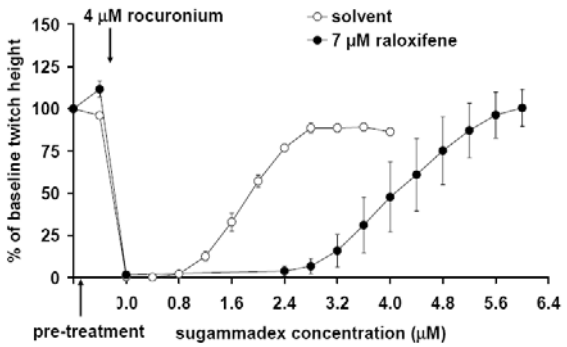
Raloxifene was a drug that has been selected for PD interaction analysis. Using the experimental protocol type 1, raloxifene up to 8 μM did not caused re-occurrence of neuromuscular block induced by rocuronium after the complete reversal by Org25969. In contrast, dose-dependent re-occurrence observed in vecuronium-induced neuromuscular block as shown below (extracted from the sponsor's report).

**Figure 17:** Effect of cumulative administration of raloxifene or equivalent amount of buffer after almost complete reversal of vecuronium-induced neuromuscular blockade with 2.0 μM Org 25969




Mean ± SEM; n = 4

Using experimental protocol type 2, pre-treatment of 7.0 μM raloxifene required higher concentration of Org 259696 for complete reversal of rocuronium-induced NMB as compared to the treatment group without raloxifene pre-treatment as shown below (extracted from the sponsor's report).

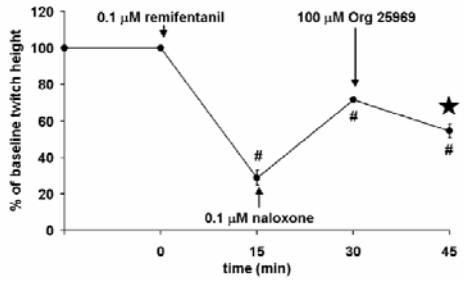



Using experimental protocol type 3, raloxifene and Org 25969 were added prior to addition of rocuronium, concentration of rocuronium required to induce NMB was decreased and the sensitivity of the tissue to rocuronium was increased since the binding site of Org 25969 had been occupied by raloxifene. Overall, the results of these assays indicated that presence of raloxifene decrease the potency of Org 25969 on reversal of NMB induced by rocuronium and vecuronium. Raloxifene is a selective estrogen receptor modulator (SERM) that belongs to the benzothiophene class of compounds used for the treatment of osteoporosis. Raloxifene is highly bound to plasma proteins (95%) and poorly soluble in water. When 7 μM raloxifene was added to the tissue bath containing plasma proteins in mouse diaphragm assay, the actual raloxifene concentration was only 1.7 μM. Administration of Org 25969 to a tissue bath containing raloxifene increased the amount of raloxifene molecules in solution, which complicated the interpretation of the results. As the Sponsor indicated, the peak plasma concentration of raloxifene in human after single oral administration is much lower (1 ng/ml or 2nM). Therefore, decrease of Org 25969 potency may not occur in human. Similar studies were not conducted in norgestimate and toremifene. Similar assays to acetaminophen, doxycyclin, remifentanil and spironolactone, which have a low affinity for Org 25969 ($\leq$ 0.2 megamol$^{-1}$), did no show any PD interactive effect with Org 25969. Other drugs with relatively high binding affinity ($\geq$1.5 megamol$^{-1}$) such as norgestimate, toremifene, etonogestrel, tibolone, drosperidine, levonorgestrel, tamoxifen citrate salt, and progesterone, as shown above, were not examined using these experimental protocols.

Administration of Org 25969 can potentially reduce the biological activity of other molecules besides NMBAs. However, it should be noted that, in clinical practice, administration of Org 25969 in the absence of a NMBA is extremely unlikely.

In a mouse isolated vas deferens preparation, opioid receptors are present and can be activated by the μ-opioid agonist such a remifentanil, resulting in a decrease in twitch height. Org 25969 did not cause a change in the effect of remifentanil, but decreased the antagonism of naloxone after administration, as shown below (extracted from the sponsor's report).

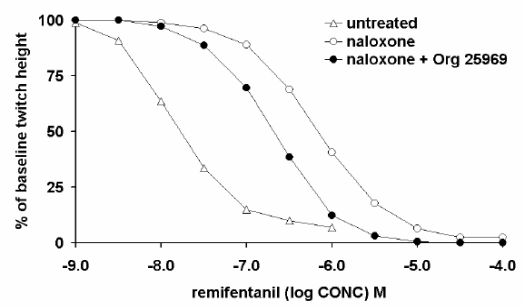
Figure 23: Effect of 0.1 μM remifentanil, 0.1 μM naloxone and 100 μM Org 25969 on mouse vas deferens contractions




Mean ± SEM; n = 8.
#: significantly (p<0.05) different from baseline (one-way analysis of variance, followed by Newman-Keuls procedure).
*: significantly (p<0.05) different from 0.1 μM naloxone

In addition, a cumulative concentration-response curve with remifentanil in the absence or presence of 0.1 μM naloxone or 0.1 μM naloxone + 100 μM Org 25969 showed a shift to the left of the cumulative concentration-response curve in the presence of naloxone + Org 25969, as shown below (extracted from the sponsor's report). All these indicated that administration of 100 μM Org 25969 seems to have more effect on the naloxone response than on the remifentanil response at identical concentration, despite the affinity of Org 25969 for remifentanil is much higher than for naloxone ($K_A$ values for remifentanil and naloxone are 0.0500 and 0.0184 megamol$^{-1}$, respectively). The reason for this effect is not known and the results of these assays indicated that Org 25969 may elicit PD interaction with drugs that have low binding affinity.

Figure 24: Cumulative concentration-response curves of remifentanil in untreated, 0.1 μM naloxone-pre-treated and 0.1 μM naloxone + 100 μM Org 25969-pre-treated preparations




n = 3 for untreated preparations, n=4 for 0.1 μM naloxone or 0.1 μM naloxone + 100 μM Org 25969-pre-treated preparations

Interaction of Org 25969 with atropine was also examined in rat tracheal rings after 30 μM carbachol administration and the results indicated that Org 25969 does not alter the effect of atropine on the relaxation of carbachol-induced contraction.

To investigate the possible interaction between a neuromuscular blocking agent, Org 25969 and another drug, the M. gastrocnemius muscle model in the anesthetized rat was used. Rats are relatively insensitive to neuromuscular blocking agents and are normally not used for in-vivo studies as the sponsor indicated. However, rats are often used for the testing of other drugs. In this study, vecuronium was selected as neuromuscular blocking agent, since vecuronium has a lower affinity for Org 25969 than rocuronium, and, therefore, is likely to be easier to displace from its complex with Org 25969 by other drugs. The results indicated that drugs listed below (extracted from the

Sponsor's report) with affinity ≤ 0.3 megamol$^{-1}$, unlike the positive control, 17-OH-rocuronium, did not displace a sufficient amount of vecuronium molecules from their complex with Org 25969 to cause a change in twitch height. Compounds with higher affinity to Org 25969 have not been examined in this animal model.

| | |
|---|---|
| 17-OH-rocuronium | 27.0000 |
| Vecuronium | 5.7200 |
| Canrenoic acid | 0.3024 |
| Remifentanil | 0.0500 |
| Dobutamine | 0.0381 |
| Methylprednisolone | 0.0348 |
| Naloxone | 0.0184 |

Gabexate, a serine protease inhibitor which is used for the treatment of disseminated intravascular coagulation and acute pancreatitis, has been shown to increase the spontaneous speed of recovery from vecuronium-induced NMB. However, administration of gabexate did not remarkably affect the Org 25969 induced recovery speed of NMB. The sponsor indicated this may be due to the low affinity of gabexate binding to Org 25969.

Overall, isothermal microcalorimetry demonstrated that certain drugs have similar binding affinity to Org 25969 to rocuronium and vecuronium, indicating potential for drug-drug PD interaction. *In vitro and in vivo* models indicated that not only drugs with high affinity to Org 25969 such as raloxifene but also drugs with low affinity such as naloxone may cause drug-drug interaction when co-administration with Org 25969 in different situation. However, the sponsor indicated that these interactions may not occur in human because the dosages used for human treatment produce much lower blood concentrations than those used in these assays. In addition, based on these results, the Sponsor developed PD/PK model to evaluate the drug-drug interaction. These studies have been submitted to the division before the NDA submission under IND 68,029. The division and the sponsor have discussed the appropriateness of the $K_A$ value cut-off of the interacting drugs and suggested the Sponsor to lower the cut-off value the PD/PK model to include drugs with low affinity with Org 25969 such as naloxone during the end-of-phase 2 meeting. With the NDA submission, the PD/PK model is considered to be acceptable according to the bio-pharmacology review team and the drug-drug PD interaction has been appropriately addressed.

## 2.6.3 PHARMACOLOGY TABULATED SUMMARY

None

## 2.6.4 PHARMACOKINETICS/TOXICOKINETICS

### 2.6.4.1 Summary

Most of the nonclinical kinetic data of Org 25969 have been collected in conjunction with toxicity or pharmacology studies in the preclinical species used. The main rodent and non rodent species used in the preclinical safety study package were rat and dog, respectively. As embryo-fetal development studies were conducted in rat and rabbit,

toxicokinetics, plasma protein and red blood cell binding, and placental transfer was determined in these species. The cat has been used in a single-dose Org 25969 toxicity study in the presence and absence of rocuronium (Org 9426), exposure data for both analytes were determined in conjunction with this preclinical safety study. Limited pharmacokinetics of Org 9426 in plasma and urine has been determined in guinea pig to support pharmacology experiments in this species. Studies comprising human samples for inter-species comparison were the protein and red blood cell (RBC) binding studies and plasma and urine metabolite profiling. In the *in vitro* distribution studies the binding of Org 25969 to plasma proteins and Red Blood Cells (RBCs) has been studied. In addition to the investigation of the tissue distribution and excretion balance of Org 25969 alone, the effect of Org 25969 on rocuronium tissue distribution and excretion balance was investigated in rats. In dogs, the excretion balance of Org 25969 and the effect of Org 25969 on the excretion balance of Org 9426 were studied. The effect of Org 25969 on rocuronium metabolism was not investigated as rocuronium is not metabolized to a significant extent. The effect of Org 25969 on rocuronium placental transfer in rats and rabbits was investigated to study the effect of Org 25969 on the extent of placental transfer and thus fetal exposure to rocuronium.

Org 25969 reached the peak level in the blood quickly after IV injection; the systemic exposure levels appeared to be generally dose-proportional in rats, dogs, and rabbits based on the results of TK analysis obtained using HPLC-MS assay. There was no apparent accumulation after multiple-dose administration and no remarkable difference between male and female. The elimination $T_{1/2}$ was short in these animal species (< 1 hour) and human (approximately 2 hours). In addition, the distribution volume was relatively low in rats (~ 0.4 L/kg), dogs (~ 0.25 L/kg), rabbits (0.19 L/kg in F), and human (0.18 L/kg). In contrast, Kinetics study using [$^{14}$C]-Org 25969 radioactivity assay demonstrated a very long T1/2 in the species examined (~ 15 hours in rats and ~ 8 hours in dogs). This may be due to a difference in LOQ between the LC-MS and radioactivity method, and the "tailing" of a long terminal elimination phase is likely to represent slow elimination from a deeper compartment such as the bone.

Distribution studies indicated that Org 25969 is rapidly distributed to the bone in rats and is retained in these tissues for extended periods of time with mean $T_{1/2}$ of 172 days (range from 67 – 210 days). Retention of Org 25969 was also seen in teeth with less amount as compared to bone as examined in incisor with $T_{1/2}$ of 8 days. Injection of rocuronium immediately after sugammadex injection at half molar dose diminished the extent of binding in bone and teeth by about 40%. Microautoradiography in young male adult rats showed labeled org 25969 in periosteal and endosteal aspects of cortex and endosteal aspects of trabecula in femur and scapula and on trabecular bone surfaces in femur. There was no binding to femoral growth plate or calcified cartilaginous zone. Bone formed subsequent to labeled dose administration did not contain compound org 25969. This study did not provide sufficient detail to characterize the binding site of Org 25969 as cells or extracellular matrix. It should be noted that the micro-autoradiography study did not appear to be with good quality due to large variation and small sample number. Binding to bone was not easily saturated and bone concentration (mg/g) increased in less than dose-proportional manner at doses up to 400 mg/kg, and the absorbed amount of

Org 25969/gram bone and teeth after each individual administration was generally decreased in a multiple-dose study. At a dose of 30 mg/kg retention in pooled bones was 13% (juvenile), 3% (young adult), and 0.5% (aged rats), at 24h post dose. The increased bone retention in juvenile rats is probably due to rapid growth of bone containing multiple sites of bone resorption, formation and mineralization.

Distribution studies also demonstrated that accumulation was also observed in male prostate and female vagina, kidneys, and urinary bladder, but the amount was substantially reduced and appeared to be indifferent from other organs after 168 hours.

Org 25969 was found to cross the placenta and distributed in various tissues with highest concentration in the femur (< 0.5% dose/gram) followed by urinary bladder and GI tract with no accumulation found in the vital organs such as the heart, brain, and lung. The amount of Org 25969 was about 2-9% that of maternal blood at one hour after maternal administration, but the elimination was slower in the fetus than the dam. In addition, Org 25969 was found to be excreted into milk; maximal concentration was observed at 30 minutes after single-dose administration with the concentration similar to the plasma concentration at this time-point.

In vitro studies demonstrated that there was only a limited amount of Org 25969 uptake into hepatocytes, indicating hepatic metabolism may be minimal if existing.
Minimal metabolism is likely to occur after Org 25969 administration in rats, dogs, and human. The primary metabolites were Org 198958-2 and Org 199425-1 in rats, dogs, and human, which are also found in Org 25969 drug substance. In both rats and dogs, Org 25969 excreted primarily though urine (78% in rats; 85% in dogs) with minor fecal excretion (< 7% in rats; <1% in dogs).

Since Org 48302 is considered the second active ingredient, plasma kinetics in comparison to Org 25969 have been studied in a single dose kinetic study in rat using Org 25969 batch AE in the dose range of 120 to 2000 mg/kg (7.2 to 120 mg/kg for Org 48302). The TK profile of Org 48302 was found to be comparable to that of the Org 25969 regarding systemic exposure/dose ratio, $T_{1/2}$, clearance, and volume distribution. The profiles of Org 48302 distribution and elimination were not conducted. Since the characteristics of most parenterally administered CDs are usually considered to be comparable, demonstration of comparable kinetic characteristics of Org 48302 and Org 25969 is considered a sufficient characterization of the distribution characteristics of Org 48302,

**2.6.4.2. Methods of analysis**

Levels of Org 25969 and Org 48302 were determined using validated assays.
Assays for determination of Org 25969 via liquid chromatographic assays with mass spectrometric detection (HPLC-MS) in rat, rabbit, dog, and cat plasma were validated. In the assay of cat plasma, the determination of Org 9426 was included. Besides, a validated assay to determine Org 48302 was used for rat. In dog studies, validated assays for determination of Org 9426 were used. To determine Org 9426 in plasma and urine in a guinea pig pharmacology experiment, an LC-MS/MS method (not GLP validated) was used. Additionally, [$^{14}$C]-Org 25969 and derived compounds were measured in various

matrices from preclinical species and human using an HPLC system with a flow-through radioactivity monitor or offline fraction collection, and the assay did not appear to be validated. An overview of validated bioanalytical assays is shown below as extracted from the sponsor's report. Assays underlined below were initially completed and used for the designated studies. Afterwards, the bioanalytical chromatography data of all series were re-evaluated based on findings during a French governmental inspection, and different values were obtained. Amendments were then submitted with corrected values attached. The TK parameter values shown in the following sections are based on the corrected values provided by the Sponsor.

| Species | Method | Analyte | LOQ (ng·mL$^{-1}$) | Used in study |
|---|---|---|---|---|
| rat | HPLC-MS [28] | Org 25969 | 183, adapted to 7130 | 2wk iv, NL0028244 [3]<br>4wk iv rat, NL0043521 [4] |
| | HPLC-MS [29] | Org 25969 | 1000 | SD iv rat, INT00007462 [1]<br>2 wk juvenile rat SC, INT00029851 [5]<br>4 wk juvenile rat SC/IV, INT00038389 [6] |
| | HPLC-MS [34] | Org 48302 | 200 | SD iv rat INT00007462 [1] |
| guinea pig | HPLC-MS [7] | Org 9426 (in urine and plasma) | 25 | IV infusion guinea pig, INT00008139 [7] |
| rabbit | HPLC-MS [30] | Org 25969 | 2000 | Embryo-fetal development study NL0043477 [8] |
| dog | HPLC-MS [31] | Org 25969 | 91.6, adapted to 183 | SD IV dog, NL0034953 [9]<br>2 wk iv dog, NL0026533 [12] |
| | HPLC-MS [32] | Org 25969 | 1000 | 3 wk iv Dog INT00025068 [13]<br>SD iv dog, INT00035242 [10] |
| | HPLC-MS [35] | Org 9426 | 9.56 | SD iv dog, NL0034953 [9] |
| | HPLC-MS [36] | Org 9426 | 100 | SD iv dog, INT00035242 [10] |
| cat | HPLC-MS [33] | Org 25969<br>Org 9426 | 410<br>19.1 | SD iv cat, NL0037029 [14] |

LOQ = lower limit of quantification

### 2.6.4.3. Absorption and PK/TK analysis

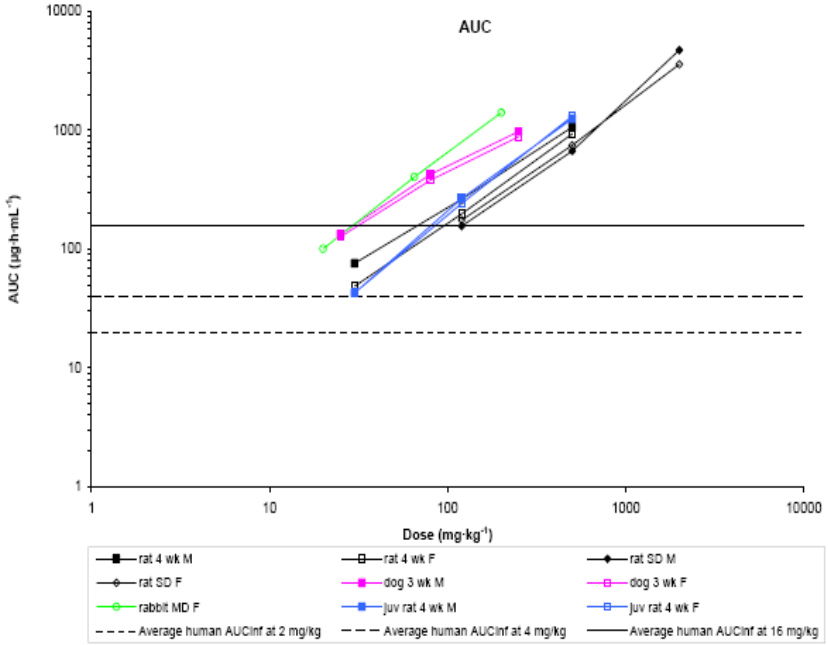
Intravenous (i.v.) kinetic data of Org 25969 were obtained in conjunction with toxicity studies in rats, pregnant rabbits, dogs and cats as shown below extracted from the sponsor's report.

| Species (strain) [a] | Dosage [b] / number of animals | Route/duration | Primary study parameter | Reference [d] |
|---|---|---|---|---|
| Toxicokinetic studies | | | | |
| Rat (Wistar) | 0, 120, 500 or 2000 mg·kg$^{-1}$, 6M+6F/group | IV, single dose | AUC, $C_0$, $t_{½}$, V, CL for both Org 25969 and Org 48302 | INT00007462 [1] |
| Rat (Wistar) | A: 8 mg·kg$^{-1}$ Org 25969<br>B+C: 2 mg·kg$^{-1}$ Org 9426 +/- 8 mg·kg$^{-1}$ Org 25969, 3F+3M/group | IV, single dose ([$^{14}$C]-Org 25969 alone, [$^{3}$H]-Org 9426 alone or [$^{3}$H]-Org 9426 followed by Org 25969) | AUC, $C_0$, $t_{½}$ (initial and terminal) ,$\lambda_z$ ,V, CL | NL0056358 [2] |
| Rat (Wistar) | 0, 1.8, 7.4 or 27.6 mg·kg$^{-1}$, 10M+10F/group | IV, 2 weeks | AUC, $C_0$, $t_{½}$, V, CL | NL0028244 [3]<br>amendment 1: INT00020327 [3] |
| Rat (Wistar) | 0, 30, 120 or 500 mg·kg$^{-1}$, 10M+10F/group | IV, 4 weeks | AUC, $C_0$, $t_{½}$, V, CL | NL00043521 [4]<br>amendment 1: INT00010805 [4] |
| Rat (Wistar, juveniles) | 0, 30, 120 or 500 mg·kg$^{-1}$, 40M+40F/group | SC, 2 weeks | AUC, $C_{max}$, $t_{½}$, V, CL | INT00029851 [5] |
| Rat (Wistar, juveniles) | 0, 30, 120 or 500 mg·kg$^{-1}$, 24M+24F/group | SC for 2 weeks, followed by IV for 2 weeks | AUC, $C_{max}$ or $C_0$, $t_{½}$, V, CL | INT00038389 [6] |
| Guinea pig (Dunkin-Hartley) | 12 nmol/kg/min rocuronium +/- 30 nmol/kg/min Org 25969, 6M/group | IV, infusion | $C_t$ at 0, 10, 20 and 30 min post Org 25969 infusion | INT00008139 [7] |
| Rabbit (NZW, pregnant) | 0, 20, 65 or 200 mg·kg$^{-1}$, 5F/group | IV, multiple dose after 9 days of dosing | AUC, $C_0$, $t_{½}$, V, CL | NL0043447 [8]<br>amendment 1: NL0049678 [8]<br>amendment 2/3: INT00026653 [8]<br>amendment 4: INT00038469 [8] |
| Dog (beagle, anesthetized) | 2.7 or 27.6 mg·kg$^{-1}$ divided in 3 subdoses alone or 2 min after | IV, single dose in 3 subdoses | Rough estimates of AUC, $C_0$, $t_{½}$, V, CL for Org 25969 and Org 9426 | NL0034953 [9]<br>amendment 1: INT00011167 [9] |

| | | | | |
|---|---|---|---|---|
| | 3x0.36 mg·kg$^{-1}$ rocuronium bromide, 4M+4F/group | | | |
| Dog (beagle, anesthetized) | 3 subdoses 30 min apart of 0.36, 3.6 or 18 mg·kg$^{-1}$ rocuronium bromide 2 min later followed by 0.92, 13 or 65 mg·kg$^{-1}$ Org 25969, 4M+4F/group | IV, single dose in 3 subdoses with rocuronium and Org 25969 approximately 2 min apart | Rough estimates of AUC, $C_{max}$, $t_{½}$ for Org 25969 and Org 9426 | INT00035242 [10] |
| Dog (beagle) | A: 8 mg·kg$^{-1}$ Org 25969 alone, B+C: 0.36 mg·kg$^{-1}$ Org 9426 +/- 8 mg·kg$^{-1}$ Org 25969, 3F+3M/group | IV, single dose ([$^{14}$C]-Org 25969 alone, [$^{3}$H]-Org 9426 alone or[$^{3}$H]-Org 9426 followed by Org 25969) | AUC, $C_0$, $t_{½}$ (initial and terminal) ,$\lambda_z$ ,V, CL | NL0055283 [11] |
| Dog (beagle) | 0, 1.8, 7.4 or 27.6 mg·kg$^{-1}$, 4M+4F/group | IV, 2 weeks | AUC, $C_0$, $t_{½}$, V, CL | NL0026533 [12] amendment 1: INT00015286 [12] amendment 2: INT00038026 [12] |
| Dog (beagle) | 0, 25, 80 or 250 mg·kg$^{-1}$, 3M+3F/group | IV, 3 weeks | AUC, $C_0$, $t_{½}$, V, CL | INT00025068 [13] amendment 1: INT00047838 [13] |
| Cat (anesthetized) | 2.7 or 27.6 mg·kg$^{-1}$ divided in 3 subdoses alone or 2 min after 3x0.5 mg·kg$^{-1}$ rocuronium bromide, 4M+4F/group | IV, single dose in 3 subdoses | Rough estimates of AUC, $C_0$, $t_{½}$, V, CL | NL0037029 [14] amendment 1: INT00014164 [14] amendment 2: INT00033781 [14] |

The pharmacokinetics of Org 25969 has been determined after single and multiple dosing administration. Generally, the dose range has been wide and exceeding the clinical dose range as shown below extracted from the sponsor's report.

**Figure 1 Overview of AUCs after single dose to preclinical species against dose and compared to human AUC at 2, 4 and 16 mg·kg$^{-1}$.**




Juv = juvenile; M = male; F = female; wk = number of weeks of the dosing period in the study; SD = single dose study.

Single dose $AUC_{0-inf}$ values were taken whenever available and reliably estimated. For pregnant rabbit only MD exposure data were available and for adult rat $AUC_{0-3h}$.

In the toxicokinetic studies in rat and cat, pooled sampling has been used to obtain an exposure estimate for the toxicity study, while individual curves and curves composed from 2 animals have been obtained in dog and rabbit studies. In juvenile rats, the single and multiple dose kinetics upon subcutaneous dosing has been assessed in pups at postnatal age of 1 to 3 weeks. In addition, IV multiple dose kinetics has been determined in 5-week old rats following 2 weeks SC plus 2 weeks IV dosing from postnatal day 7 onwards in juvenile animals. In general, the kinetic curves in the preclinical species were based on 7-10 time points of single, duplicate, triplicate (juveniles) or quadruplicate samples which are considered adequate for reliable exposure estimates. Detailed review of these studies will be presented in the individual toxicity study review. An overview of TK exposure parameters after single-dose administration in the pivotal toxicity and toxicokinetic studies is presented in following table as extracted form the Sponsor's report to compare with the parameters of human exposure. These results indicated that AUC and $C_{max}$ of Org 25969 increased proportionally with dose escalation at ≤ 500 mg/kg in adult and juvenile rats, ≤ 200 mg/kg in pregnant rabbits, and ≤ 250 mg/kg in dogs. In the rats, exposure after single-dose administration appeared to be slightly super-proportional between 500 to 2000 mg/kg dosing range. There was no substantial difference between males and females in these species tested. It should be noted that the bio-analysis of 4-wk dog study was not GLP compliant and the toxicokinetics used in the 4-week dog study was conducted in a separate 3-week toxicokinetic study in the dog (INT00025068) in order to generate GLP compliant exposure data in this species. There were two 4-week rat toxicity studies conducted using different batches (batch U for the first study (NL0043521) and batch AE for the second study (NL0059534)). The TK analysis of study NL0059534 was found to be not GLP compliant; therefore, results of TK data from study NL0043521 was used to generate GLP compliant exposure data for this study.

**Table 4: Overview of systemic exposure parameters Org 25969 in the main (toxico)kinetic studies**

| Study/species/dosing route | Dose ($mg \cdot kg^{-1}$) | AUC ($\mu g \cdot h \cdot mL^{-1}$) | | $C_0$ or $C_{max}$ ($\mu g \cdot mL^{-1}$) | |
|---|---|---|---|---|---|
| | | Male | Female | Male | Female |
| SD rat IV[1] | 120 | 156 | 175 | 411 | 500 |
| | 500 | 665 | 744 | 1899 | 2109 |
| | 2000 | 4699 | 3581 | 7516 | 12626 |
| 4-week rat IV [4] | 30 | 48.7 * | 48.1 | 240 * | 145 |
| | 120 | 262 | 197 | 961 | 880 * |
| | 500 | 1041 | 929 * | 6406 | 7669 * |
| 4-week juvenile rat SC/IV [6] | 30 | 42.7 | 42.8 | 69.3 | 100 |
| | 120 | 266 | 242 | 456 | 339 |
| | 500 | 1243 | 1306 | 1639 | 1992 |
| MD pregnant rabbit IV [8] | 20 | n.a. | 100.5 | n.a. | 137 |
| | 65 | | 403.7 | | 479 |
| | 200 | | 1400.8 | | 1519 |
| 3-week dog IV [13] | 25 | 134 | 127 | 252 | 232 |
| | 80 | 424 | 380 | 682 | 731 |
| | 250 | 971 | 879 | 1940 | 1923 |
| Human [40] | 2 | 20 | | 25 | |
| | 4 | 40 | | 50 | |
| | 16 | 160 | | 200 | |

Single dose values have been taken whenever available and reliably estimated.
* indicates that multiple dose data have been taken as the SD values were overestimates. For rabbit only MD exposure data were available and $C_0$ for juvenile rats was the estimate after multiple dosing as this was the only estimate available upon i.v. dosing. For AUC, the $AUC_{0-inf}$ has been taken except for adult rat for which $AUC_{0-3h}$ was used. $C_0$ was taken for all species except for the pregnant rabbit ($C_{0.08}$ is taken as the $C_{max}$ as these values were generally higher as compared to the extrapolated $C_0$ values).

Results obtained using HPLC-MS assay from these studies also indicated that the elimination rate ($t_{1/2}$) of Org 25969 was quick and the distribution volume (V) was relatively low in rat, rabbit, dog, and human as shown in the table below (extracted from the sponsor's report).

**Table 3: Comparative mean kinetic parameters of Org 25969 in rat, rabbit, dog and man**

| Species | Rat | | Rabbit | | Dog | | Man | |
|---|---|---|---|---|---|---|---|---|
| Dose | 30 mg·kg$^{-1}$ | | 20 mg·kg$^{-1}$ | | 25 mg·kg$^{-1}$ | | 4 mg·kg$^{-1}$ | |
| Gender | M | F | M | F | M | F | M | F |
| $AUC_{0\text{-}inf}$ (µg·h·mL$^{-1}$) | 48.7 | 48.1 | n.d. | 100.5 | 134 | 127 | 40 | 40 |
| $NAUC_{inf}$ (µg·h·mL$^{-1}$)/(mg·kg$^{-1}$) | 1.62 | 1.60 | n.d. | 5.02 | 5.17 | 4.91 | 10 | 10 |
| $C_0$ (µg·mL$^{-1}$) | 240 | 145 | n.d. | 137 | 252 | 232 | 50 | 50 |
| $NC_0$ (µg·mL$^{-1}$)/( mg·kg$^{-1}$) | 7.99 | 4.84 | n.d. | 6.85 | 9.73 | 9.00 | 12.5 | 12.5 |
| $t_{1/2}$ (h) | 0.4 | 0.6 | n.d. | 0.65 | 0.9 | 0.9 | 2 | 2 |
| CL (L·kg$^{-1}$·h$^{-1}$) | 0.40 | 0.62 | n.d. | 0.20 | 0.19 | 0.21 | 0.10 | 0.10 |
| V (L·kg$^{-1}$) | 0.3 | 0.5 | n.d. | 0.19 | 0.25 | 0.26 | 0.18 | 0.18 |

The dose levels taken for the preclinical species are those giving reliable estimates and closest to the Human Equivalent Dose (dose corrected by allometric scaling on body surface area) for the recommended dose level of 4 mg·kg$^{-1}$. The $AUC_{0\text{-}inf}$ is taken whenever available, but for man a rounded average values for exposure and kinetic parameters are taken. For rat, dog and rabbit the estimates for $C_0$, $NC_0$, $t_{1/2}$, CL, V should be taken as rough estimates as these were determined in kinetics associated with toxicity study studies.

Multiple-dose treatment in adult rats and dogs indicated no accumulation after 3 or 4-week administration. In juvenile rats, the systemic exposure after single dose administration on PND7 was higher than that after 4-wk administration from PND7 to PND35 represented by AUC and $C_{max}$ as shown below extracted from the sponsor's report. The reason for this difference is not known.

**Table 10: Mean kinetic parameters of 30, 120 and 500 mg·kg$^{-1}$ Org 25969 after single s.c. and multiple s.c./i.v. dosing for approximately 4 weeks in juvenile Wistar rats**

| Org 25969 kinetic parameters | Sex | Dose (mg·kg$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 30 | | 120 | | 500 | |
| | | SD | MD | SD | MD | SD | MD |
| $AUC_{(0\text{-}3)}$ (µg·h·mL$^{-1}$) | M | 39.8 | 21.0 | 237 | 94.7 | 1087 | 376 |
| | F | 41.5 | 21.7 | 217 | 85.0 | 1119 | 413 |
| $AUC_{inf}$ [b)] (µg·h·mL$^{-1}$) | M | 42.7 | 22.3 | 266 | 104 | 1243 | 416 |
| | F | 42.8 | - | 242 | 91.0 | 1306 | 448 |
| $C_{max}$ or $C_0$ [c)] (µg·mL$^{-1}$) | M | 33.3 | 69.3 | 145 | 456 | 570 | 1639 |
| | F | 33.5 | 100 | 134 | 339 | 592 | 1992 |
| $t_{max}$ (h) | M | 0.5 [1)] | n.a. | 1.0 | n.a. | 1.0 | n.a. |
| | F | 0.5 [1)] | | 0.5 [a)] | | 0.5 [a)] | |
| $t_{1/2}$ (h) | M | 0.7 | 0.3 | 0.9 | 0.3 | 0.8 | 0.4 |
| | F | 0.5 | - | 0.9 | 0.3 | 0.8 | 0.4 |
| CL (L·kg$^{-1}$·h$^{-1}$) | M | n.d. | 1.35 | n.d. | 1.15 | n.d. | 1.20 |
| | F | | - | | 1.32 | | 1.12 |
| V (L·kg$^{-1}$) | M | n.d. | 0.482 | n.d. | 0.548 | n.d. | 0.634 |
| | F | | - | | 0.560 | | 0.573 |

M = male; F = female; n.a. = not applicable; n.d. = not determined; - : could not be determined due to the irregular shape of the terminal portion of the curve (elimination phase)
[a)] : first sampling point after dosing, hence AUC and $C_{max}$ values might be underestimated
[b)] : AUC under the dosing interval ($AUC_{(0\text{-}24)}$) was estimated by $AUC_{inf}$
[c)] : $C_{max}$ was determined upon single s.c. dosing, $C_0$ was determined at the end of the study upon i.v. dosing. Therefore, these values can not be directly compared.

Since Org 48302 was added to be an active ingredient, the exposure of Org 48302 was examined and compared with Org 25969 using drug product batch AE containing approximately 90% Org 25969 and approximately 6% Org 48302 in a single-dose rat study with 120, 500, and 2000 mg/kg Org 25969 (INT00007462). The kinetic parameters

of Org 25969 and Org 48302 are shown in the following table as extracted from the sponsor's report. The results of this study indicated that the PK profiles of Org 25969 and Org 48302 are generally comparable.

**Table 5: Mean Org 25969 toxicokinetic parameters after single i.v. dosing of 120, 500 and 2000 mg·kg$^{-1}$ of Org 25969 and 7.2, 30 and 120 mg·kg$^{-1}$ of Org 48302 to Wistar rats.**

| Org 25969 toxico-kinetic parameters | Sex | Dose | | |
|---|---|---|---|---|
| | | 120 mg·kg$^{-1}$ Org 25969 7.2 mg·kg$^{-1}$ Org 48302 | 500 mg·kg$^{-1}$ Org 25969 30 mg·kg$^{-1}$ Org 48302 | 2000 mg·kg$^{-1}$ Org 25969 120 mg·kg$^{-1}$ Org 48302 |
| $AUC_{inf}$ [a)] (µg·h·mL$^{-1}$) | M | 156 | 665 | 4699 |
| | F | 175 | 744 | 3581 |
| $C_0$ (µg·mL$^{-1}$) | M | 411 | 1899 | 7516 |
| | F | 500 | 2109 | 12626 |
| $t_{1/2}$ (h) | M | 0.37 | 0.45 | 0.50 |
| | F | 0.44 | 0.72 | 0.46 |
| CL (L·kg$^{-1}$·h$^{-1}$) | M | 0.77 | 0.75 | 0.43 |
| | F | 0.69 | 0.67 | 0.56 |
| V (L·kg$^{-1}$) | M | 0.41 | 0.49 | 0.31 |
| | F | 0.43 | 0.70 | 0.37 |

Composite sampling design with n=6 rats/gender, generally 10 time points and n= 2 rats/time point; M = male; F = female

a) AUC under the dosing interval ($AUC_{(0-24)}$) was estimated by $AUC_{inf}$

**Table 6: Mean Org 48302 toxicokinetic parameters after single i.v. dosing of 120, 500 and 2000 mg·kg$^{-1}$ of Org 25969 and 7.2, 30 and 120 mg·kg$^{-1}$ of Org 48302 to Wistar rats.**

| Org 48302 kinetic parameters | Sex | Dose | | |
|---|---|---|---|---|
| | | 120 mg·kg$^{-1}$ Org 25969 7.2 mg·kg$^{-1}$ Org 48302 | 500 mg·kg$^{-1}$ Org 25969 30 mg·kg$^{-1}$ Org 48302 | 2000 mg·kg$^{-1}$ Org 25969 120 mg·kg$^{-1}$ Org 48302 |
| $AUC_{inf}$ [b)] (µg·h·mL$^{-1}$) | M | 12.7 | 54.4 | 438 |
| | F | 13.9 | 62.8 | 339 |
| $C_0$ (µg·mL$^{-1}$) | M | 36.1 | 174 | 759 |
| | F | 68.5 | 178 | 1081 |
| $t_{1/2}$ (h) | M | 0.39 | 0.36 | 0.69 |
| | F | 0.33 | 0.61 | 0.40 |
| CL (L·kg$^{-1}$·h$^{-1}$) | M | 0.57 | 0.55 | 0.27 |
| | F | 0.52 | 0.48 | 0.35 |
| V (L·kg$^{-1}$) | M | 0.32 | 0.29 | 0.27 |
| | F | 0.25 | 0.42 | 0.21 |

Composite sampling design with n=6 rats/gender, generally 10 time points and n= 2 rats/time point; M = male; F = female

b) AUC under the dosing interval ($AUC_{(0-24)}$) was estimated by $AUC_{inf}$

The sponsor conducted a 4-week dog toxicity study with TK analysis (NL0043937) as a pivotal study to support the safety of Org 25969 in human. However, the TK analysis was not GLP-compliant during the study. Therefore, the sponsor conducted a GLP-compliant 3-week TK study in dog to investigate the systemic exposure of Org 25969. Animals (3/sex/group) were treated with Org 25969 for 3 weeks at 25, 80, and 250 mg/kg and the TK parameters after single and 3-week treatment were shown in below as extracted from the sponsor's report.

**Table 17: Mean kinetic parameters of 20, 80 and 250 mg·kg$^{-1}$ of Org 25969 after single and multiple i.v. dosing for 3 weeks in beagle dogs**

| Org 25969 toxicokinetic Parameters [a)] | Sex | 25 mg·kg$^{-1}$ | | 80 mg·kg$^{-1}$ | | 250 mg·kg$^{-1}$ | |
|---|---|---|---|---|---|---|---|
| | | SD | MD | SD | MD | SD | MD |
| $AUC_{inf}$ | M | 134±2 | 108±9 | 424±40 | 425±16 | 971±9 | 932±12 |
| (µg·h·mL$^{-1}$) | F | 127±15 | 124±7 | 380±10 | 335±55 | 879±140 | 833±63 |
| $NAUC_{inf}$ | M | 5.17±0.10 | 4.31±0.41 | 5.19±0.48 | 5.11±0.16 | 3.72±0.05 | 3.59±0.14 |
| (µg·h·mL$^{-1}$)/(mg·kg$^{-1}$) | F | 4.91±0.62 | 4.84±0.29 | 4.56±0.22 | 4.11±0.75 | 3.35±0.55 | 3.22±0.20 |
| $C_0$ | M | 252±18 | 203±39 | 682±36 | 851±23 | 1940±290 | 1937±160 |
| (µg·mL$^{-1}$) | F | 232±23 | 224±11 | 731±46 | 739±40 | 1923±90 | 1993±74 |
| $NC_0$ | M | 9.73±0.97 | 8.07±1.60 | 8.35±0.62 | 10.3±0.2 | 7.43±1.12 | 7.46±0.75 |
| (µg·mL$^{-1}$)/(mg·kg$^{-1}$) | F | 9.00±1.07 | 8.74±0.40 | 8.78±0.75 | 9.04±0.67 | 7.31±0.25 | 7.71±0.50 |
| $t_{1/2}$ | M | 0.9±0.0 | 0.7±0.1 | 1.0±0.1 | 0.9±0.1 | 1.0±0.1 | 1.1±0.2 |
| (h) | F | 0.9±0.0 | 0.8±0.1 | 0.7±0.1 | 0.6±0.1 | 0.8±0.1 | 0.6±0.0 |
| CL | M | 0.19±0.00 | 0.23±0.02 | 0.19±0.02 | 0.20±0.01 | 0.27±0.00 | 0.28±0.01 |
| (L·kg$^{-1}$·h$^{-1}$) | F | 0.21±0.02 | 0.21±0.01 | 0.22±0.01 | 0.25±0.05 | 0.30±0.05 | 0.31±0.02 |
| V | M | 0.25±0.01 | 0.24±0.02 | 0.29±0.04 | 0.26±0.04 | 0.38±0.04 | 0.43±0.09 |
| (L·kg$^{-1}$) | F | 0.26±0.02 | 0.24±0.02 | 0.22±0.02 | 0.22±0.01 | 0.33±0.04 | 0.29±0.02 |

Values are means ± s.d. of n = 3 dogs/gender; SD = single dose; MD = multiple dose

a) AUC under the dosing interval ($AUC_{(0-24)}$) not given in the table, $AUC_{(0-24)}$ was estimated by $AUC_{inf}$

These results indicated no accumulation after 3-week administration in comparison to Org 25969 exposure after single-dose administration. Systemic exposure was dose-proportional from 25 mg/kg to 80 mg/kg, but slightly less than dose-proportional (~ 0.73) from 80 to 250 mg/kg. It should be noted that, in this study, batch AE containing around 90% Org 25969 and 6% Org 48302 was used, while batch U containing around 79% Org 25969 and 1.3% Org 48302 was used in the 4-week dog toxicity study. This difference should not affect the validity of the data from this study to evaluate the systemic exposure of 25969 in the 4-week dog toxicity study.

In addition to the kinetics studies in single and multiple toxicity and pharmacology studies, the sponsor has conducted specific studies to investigate the ADME of Org25969. In an excretion balance and metabolite profiling study in rats (see 2.6.4.6.1, Document ID: NL0056358), the plasma kinetics of 8 mg/kg [$^{14}$C]-Org 25969 was investigated after single IV administration. Mean plasma radioactivity pharmacokinetic data following a single IV dose of [$^{14}$C]-Org 25969 (8 mg/kg) to 3 male and 3 female rats are summarized in the following table extracted from the sponsor's report. Drug-related material was eliminated with estimated initial and terminal $t_{1/2}$ for approximately 15 min and 15 h, respectively. There were no major sex differences in the plasma kinetics of radioactivity. Plasma clearance of radioactivity was lower than the hepatic and renal plasma flow rates (3.3 and 2.2 L/h/kg, respectively) but was higher than the GFR (0.3 L/h/kg) in this species.

**Radioactivity kinetic parameters following a single i.v. dose of 8 mg·kg$^{-1}$ [$^{14}$C]–Org 25969 in rat**

| Kinetic parameter of [$^{14}$C] radioactivity | Male | Female |
|---|---|---|
| $C_0$ (µg eq/g) | 32.40 | 44.25 |
| $AUC_t$ (µg eq.h/g) | 12.17 | 17.44 |
| AUC (µg eq.h/g) [a)] | 12.61 | 17.98 |
| $\lambda_Z$ ($h^{-1}$) [a)] | 0.0477 | 0.0482 |
| Terminal $t_{½}$ (h) [a)] | 15 | 14 |
| Initial $t_{½}$ ($t_{½-\alpha}$; h) | 0.25 | 0.24 |
| CL (L/h/kg) [a)] | 0.649 | 0.460 |
| $V_{SS}$ (L/kg) [a)] | 1.680 | 1.149 |

a) Terminal rate constants could not be determined in accordance with acceptance criteria, so these and all derived data are approximations only

In addition, the plasma kinetics of rocuronium (Org 9426) was investigated after single IV doses of 2 mg/kg [$^3$H]–Org 9426 with and without the subsequent IV administration of 8 mg/kg non-radiolabeled Org 25969 5 min later to investigate the potential effects of the reversing agent (Org 25969) on the kinetics of the NMBA rocuronium (Org 9426) in this study. The mean plasma radioactivity pharmacokinetic data after a single IV dose of 2 mg/kg [$^3$H]–Org 9426 either alone or followed by an IV dose of 8 mg/kg. Org 25969 in 5 minutes after Org 9426 dosing are summarized by the sponsor as shown in the following table (extracted from the sponsor's report).

**Table 8: Radioactivity kinetic parameters in rat following a single i.v. dose 2 mg·kg$^{-1}$ [$^3$H]–Org 9426 alone or followed by 8 mg·kg$^{-1}$ Org 25969**

| Parameter of [$^3$H] radioactivity | [$^3$H]-Org 9426 alone | | [$^3$H]-Org 9426 and Org 25969 | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| $C_0$ (µg eq/g) | 3.594 | 3.461 | 3.566 | 3.245 |
| $AUC_t$ (µg eq.h/g) | 0.6252 | 0.6966 | 0.902 | 1.053 |
| AUC (µg eq.h/g) [b)] | 1.077 | 1.071 | 1.626 | 2.532 |
| $\lambda_Z$ ($h^{-1}$) [b)] | 0.0127 | 0.0158 | 0.0138 | 0.0081 |
| Terminal $t_{½}$ (h) [b)] | 55 | 44 | 50 | 85 |
| Initial $t_{½}$ ($t_{½-\alpha}$; h) | 0.11 [b)] | 0.12 [b)] | 0.16 | 0.17 |
| CL (L/h/kg) [b)] | 1.847 | 1.858 | 1.193 | 0.774 |
| $V_{SS}$ (L/kg) [b)] | 82.67 | 60.09 | 53.57 | 67.72 |

Eq = equivalents; $\lambda_Z$ = terminal elimination constant

b) The rate constants could not be determined in accordance with acceptance criteria, so these and all derived data are approximations only.

These results indicated that the extent of systemic exposure to [$^3$H]-Org 9426 associated radioactivity (AUC) was markedly increased (by about 50%) after Org 25969 was administered. Plasma clearance of [$^3$H]-Org 9426 was reduced when Org 25969 was administered after [$^3$H]-Org 9426.

Similar study was conducted in dogs. Mean plasma radioactivity pharmacokinetic data following a single IV dose of [$^{14}$C]–Org 25969 (8 mg/kg) to 3 male and 3 female dogs are summarized in Table below extracted from the sponsor's report.

**Radioactivity kinetic parameters following a single i.v. dose of 8 mg·kg$^{-1}$ [$^{14}$C]–Org 25969 in dog**

| Kinetic parameter of [$^{14}$C] radioactivity | Male | Female |
|---|---|---|
| $C_0$ (µg eq/mL) | 54.72 ± 1.31 | 55.06 ± 3.71 |
| $AUC_t$ (µg eq.h/mL) | 31.75 ± 3.55 | 33.81 ± 3.36 |
| AUC (µg eq.h/mL) [a)] | 32.37 ± 3.55 | 34.47 ± 3.28 |
| $\lambda_z$ (h$^{-1}$) [a)] | 0.0892 ± 0.0063 | 0.0847 ± 0.0138 |
| Terminal $t_{½}$ (h) [a)] | 7.8 | 8.2 |
| Initial $t_{½}$ ($t_{½-\alpha}$; h) | 0.8 | 0.7 |
| CL (L/h/kg) [a)] | 0.250 ± 0.027 | 0.237 ± 0.017 |
| $V_{SS}$ (L/kg) [a)] | 0.549 ± 0.071 | 0.513 ± 0.0073 |

Eq = equivalents; $\lambda_z$ = terminal elimination constant

[a)] AUC, terminal $t_{½}$, CL, $V_{SS}$ and terminal rate constants are approximations only

The results show that radioactive drug–related material was eliminated from dogs with a terminal half life of approximately 8 h and that there was no apparent gender difference in the plasma kinetics of radioactivity. The half-life for the initial phase of the decline in plasma radioactivity concentrations was estimated to be approximately 0.7 to 0.8 hours. Systemic clearance of radioactivity was lower than the hepatic and renal plasma flow rates in this species, while the volume of distribution at steady–state was similar to the total volume of body water. In the presence of Org 25969, AUC of 9426 was approximately 2-fold of that in the absence of Org 25969, and the initial t1/2 and CL were both approximately half of those observed when Org 9426 was presented alone, as shown in the table below (extracted from the sponsor's report).

**Table 16: Radioactivity kinetic parameters following a single i.v. dose of 0.36 mg·kg$^{-1}$ [$^{3}$H]–Org 9426 alone or followed by 8 mg·kg$^{-1}$ Org 25969**

| Kinetic parameter of [$^{3}$H] radioactivity | [$^{3}$H]–Org 9426 alone | | [$^{3}$H]–Org 9426 followed by Org 25969 | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| $C_0$ (ng eq/mL) | 886.7 ± 57.2 | 1033 ± 165 | 749.8 ± 109.4 | 846.0 ± 161.9 |
| $AUC_t$ (ng eq.h/mL) | 293.1 ± 12.4 | 320.5 ± 42.4 | 594.7 ± 68.5 | 595.0 ± 99.7 |
| AUC (ng eq.h/mL) [a)] | 361.0 ± 20.9 | 411.0 ± 26.1 | 639.1 ± 68.5 | 664.5 ± 59.5 |
| $\lambda_z$ (h$^{-1}$) [a)] | 0.0335 ± 0.0006 | 0.0351 ± 0.0153 | 0.0460 ± 0.010 | 0.0378 ± 0.0224 |
| Terminal $t_{½}$ (h) [a)] | 20.7 | 19.7 | 15.1 | 18.4 |
| Initial $t_{½}$ ($t_{½-\alpha}$; h) | 0.3 | 0.3 | 0.6 | 0.5 |
| CL (L/h/kg) [a)] | 0.992 ± 0.048 | 0.871 ± 0.067 | 0.562 ± 0.053 | 0.544 ± 0.035 |
| $V_{SS}$ (L/kg) [a)] | 12.3 ± 0.5 | 13.9 ± 10.8 | 2.84 ± 1.13 | 4.64 ± 2.93 |

Eq = equivalents; $\lambda_z$ = terminal elimination constant

[a)] AUC, terminal $t_{½}$, CL, $V_{SS}$ and terminal rate constants are approximations only

In comparison, there was a much longer terminal $t_{1/2}$ in rats (about 15 hr) and dogs (8 hours) determined by radioactivity assay as compared to those in rats and dogs (about 0.5 hours) determined by HPLC-MS. This may be due a difference in LOQ between the LC-MS method and radioactivity. This 'tailing' of a long terminal elimination phase may represent slow elimination from a deeper compartment such as the bone.

### 2.6.4.4. Distribution

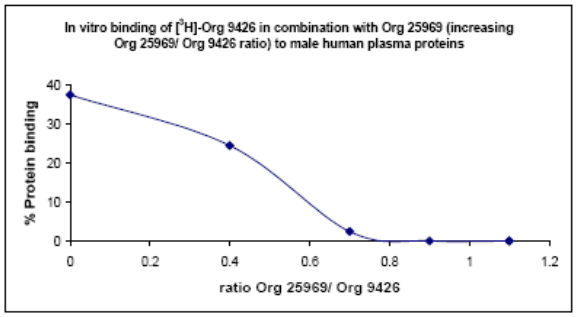
In vitro distribution studies

In vitro studies were conducted by the sponsor to examine the binding of Org 25969 to plasma and red blood cells (RBCs) and the effect of Org 25969 on the binding of Org 9426 to plasma proteins and RBCs. Org 25969 was radiolabeled with [$^{14}$C] while Org 9426 was radiolabeled with [$^{3}$H]; concentrations of Org 25969 used in these assays

covered the range of expected clinical use and the concentration of Org 9426 is comparable with the mean concentration used in a previous plasma protein binding study. In the plasma protein binding assay, the extent of binding of [$^{14}$C]-Org 25969 to plasma proteins was determined by equilibrium dialysis in plasma obtained from male rats, rabbits, cats, dogs and a human. plasma samples spiked with 0 -125 nmol/ml Org 25969/ 1.85 KBq/ml [$^{14}$C]-Org 25969 and or 4.3 nmol/ml Org 9426/1.85 KBq/ml [$^{3}$H] Org 9426 were dialyzed in duplicate against Na2HPO4/ KH2PO4 buffer (pH 7.4) spiked with equal concentrations Org 25969/[$^{14}$C]-Org 25969 at 37°C for 3 h, after which radioactivity in plasma and buffer was determined. The results showed negative binding values which are interpreted as zero, indicating that [$^{14}$C]-Org 25969 did not bind to plasma proteins. HPLC-analysis of human plasma spiked with [$^{14}$C]-Org 25969 showed that there was not co-elution of Org 25969 with protein. Org 9426 binds to plasma proteins. This study showed a concentration-independent 33.8 ± 4.4% binding for rabbit plasma proteins, which is comparable with protein binding results found in the past for other species according to the sponsor. Presence of Org 25969 decreased the binding of Org 9426 to plasma proteins in all the species examined. At approximately equimolar concentrations of Org 9426 and Org 25969 the extent of [$^{3}$H]-Org 9426 binding is zero as shown in the following table and example figure (extracted from the sponsor's report).

| Species | Ratio Org 25969/Org 9426 | Binding (%) (mean)* |
|---|---|---|
| Rat | 0 to1.1 | 38.2 to 0 |
| Rabbit | 0 to1.1 | 29.6 to 0 |
| Cat | 0 to1.1 | 43.1 to 0 |
| Dog | 0 to1.1 | 27.4 to 0 |
| Human | 0 to1.1 | 37.4 to 0 |

* Negative binding values are reported as zero (no binding)




In the RBC binding study, the extent of binding of [$^{14}$C]-Org 25969 was examined to RBCs from male rat, rabbit, cat, dog and human. Blood samples were (2.97 mL) was spiked with 0, 0.25, 1, 2.5, 25 and 250 nmol/mL Org 25969 with 3 KBq/ml [$^{14}$C]-Org 25969 (equivalent to a range of 0.43 to 0.49 nmol·mL-1). Incubations were performed at 37 °C for 60 minutes; radioactivity of the whole blood and plasma of the incubated samples was determined at 5 and 60 minutes after incubation. The results indicated that incubation did not affect the ratio of radioactivity (mean ± SD, n=6) in whole blood and plasma (R) of the species examined as shown in the example below (extracted from the sponsor's report), indicating that Org 25969 is not likely to bind with RBCs. Similarly, binding of Org 9426 was not observed, either.

**Table 8.1–5 In vitro binding of [$^{14}$C]-Org 25969 to male human erythrocytes**

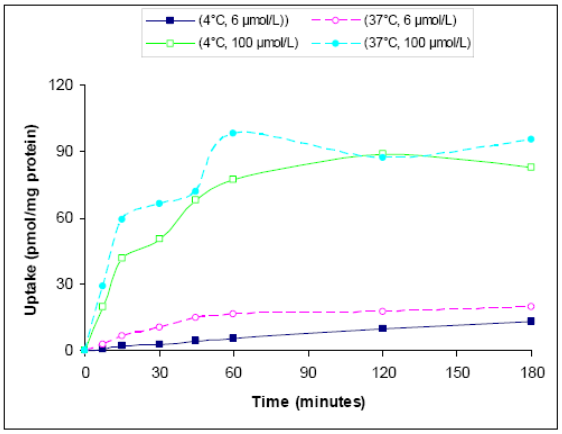
| [$^{14}$C]-Org 25969[1] (nmol·mL$^{-1}$) | Time (min) | H | Radioactivity Whole blood (Bq·mL$^{-1}$) | Radioactivity Plasma (Bq·mL$^{-1}$) | R | E[4] (%) |
|---|---|---|---|---|---|---|
| | 0[2] | 0.415 | | | | |
| 250 | 5 | | 2960 | 5216 | 0.56 | -4 |
| 25 | | | 2885 | 5140 | 0.55 | -7 |
| 2.5 | | | 2844 | 5085 | 0.55 | -6 |
| 1 | | | 2842 | 5096 | 0.56 | -5 |
| 0.25 | | | 2779 | 4939 | 0.57 | -3 |
| 0 | | | 2891 | 4981 | 0.58 | -1 |
| Mean ± SD | | | | | 0.56 ± 0.01 | -4 ± 2 |
| 250 | 60 | 0.405 | 2921 | 5357 | 0.55 | -7 |
| 25 | | 0.410 | 2757 | 5210 | 0.54 | -8 |
| 2.5 | | 0.420 | 2772 | 5172 | 0.54 | -8 |
| 1 | | 0.420 | 2846 | 5132 | 0.55 | -6 |
| 0.25 | | 0.405 | 2849 | 5094 | 0.55 | -6 |
| 0 | | 0.420 | 2891 | 4750 | 0.61 | 4 |
| Mean ± SD | | 0.413[3] | | | 0.56 ± 0.03 | -5 ± 5 |

An additional in vitro assay was to determine the uptake of [$^{14}$C]-Org 25969 into rat hepatocytes during 3 hour hepatocyte incubation with approximately 6 or 100 µM (12 or 200 µg/mL) [$^{14}$C]-Org 25969 / Org 25969 (39 MBq/L) for 3 h at 4 or 37°C. The rate of Org 25969 uptake into rat hepatocytes at 4°C and 37°C is shown in the following (extracted from the sponsor's report). These results indicated a very low uptake of Org 25969 (maximally 2.4%) into cultured hepatocytes in a less concentration proportional manner, likely due to the large size of Org 25969 molecule.

| Concentration Org 25969 | Incubation temperature | Incubation time (minutes) | Uptake Org 25969 (pmol/mg protein) | Rate of uptake Org 25969 (pmol/mg protein/h) | % uptake[1] |
|---|---|---|---|---|---|
| 6 µM | 4°C | 0 | 0.00 | 0.00 | 0.00 |
| | | 7.5 | 0.55 | 4.36 | 0.08 |
| | | 15 | 2.19 | 8.74 | 0.34 |
| | | 30 | 2.94 | 5.87 | 0.48 |
| | | 45 | 4.31 | 5.75 | 0.67 |
| | | 60 | 5.51 | 5.51 | 0.86 |
| | | 120 | 10.10 | 5.05 | 1.52 |
| | | 180 | 13.08 | 4.36 | 1.89 |
| 100 µM | 4°C | 0 | 0.00 | 0.00 | 0.00 |
| | | 7.5 | 19.67 | 157.34 | 0.18 |
| | | 15 | 41.54 | 166.14 | 0.33 |
| | | 30 | 50.44 | 100.88 | 0.47 |
| | | 45 | 67.95 | 90.60 | 0.58 |
| | | 60 | 77.05 | 77.05 | 0.66 |
| | | 120 | 88.78 | 44.39 | 0.68 |
| | | 180 | 82.89 | 27.63 | 0.82 |
| 6 µM | 37°C | 0 | 0.00 | 0.00 | 0.00 |
| | | 7.5 | 3.52 | 28.14 | 0.47 |
| | | 15 | 7.10 | 28.38 | 0.89 |
| | | 30 | 11.08 | 22.15 | 1.49 |
| | | 45 | 15.26 | 20.35 | 1.95 |
| | | 60 | 16.95 | 16.95 | 2.20 |
| | | 120 | 18.06 | 9.03 | 2.42 |
| | | 180 | 20.30 | 6.77 | 2.40 |
| 100 µM | 37°C | 0 | 0.00 | 0.00 | 0.00 |
| | | 7.5 | 28.99 | 231.94 | 0.29 |
| | | 15 | 58.91 | 235.62 | 0.49 |
| | | 30 | 66.56 | 133.12 | 0.55 |
| | | 45 | 71.74 | 95.66 | 0.54 |
| | | 60 | 97.83 | 97.83 | 0.76 |
| | | 120 | 87.09 | 43.54 | 0.61 |
| | | 180 | 95.16 | 31.72 | 0.67 |

[1] Expressed as % of total extracellular Org 25969 at t=0

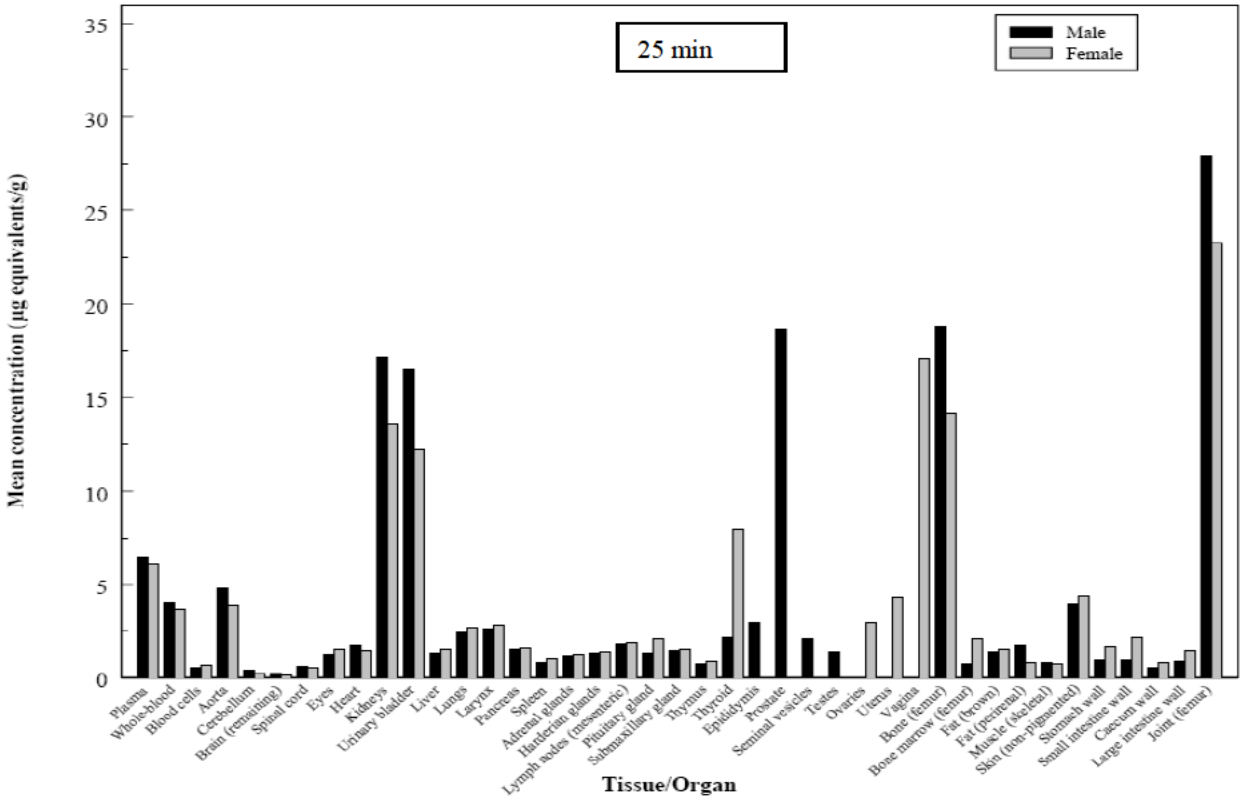
**The uptake of Org 25969 in rat hepatocytes at 4°C and 37°C at 6 and 100 µM**

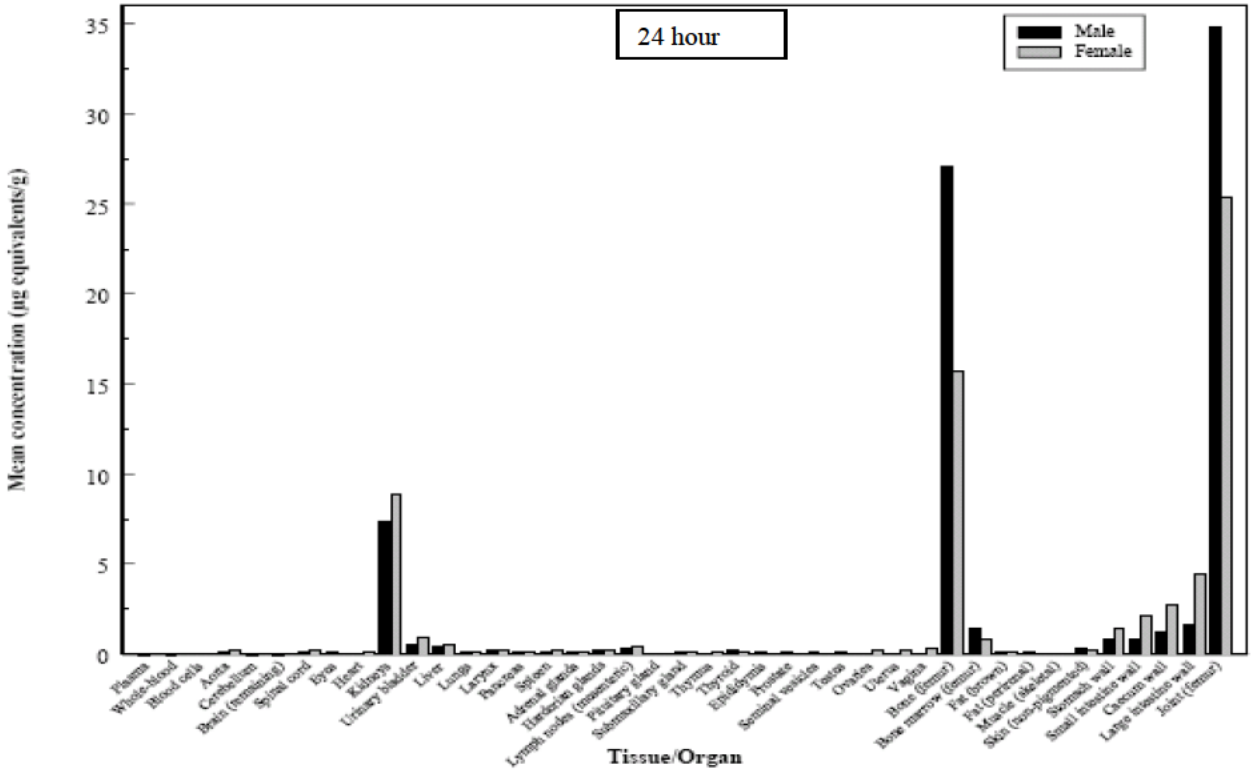


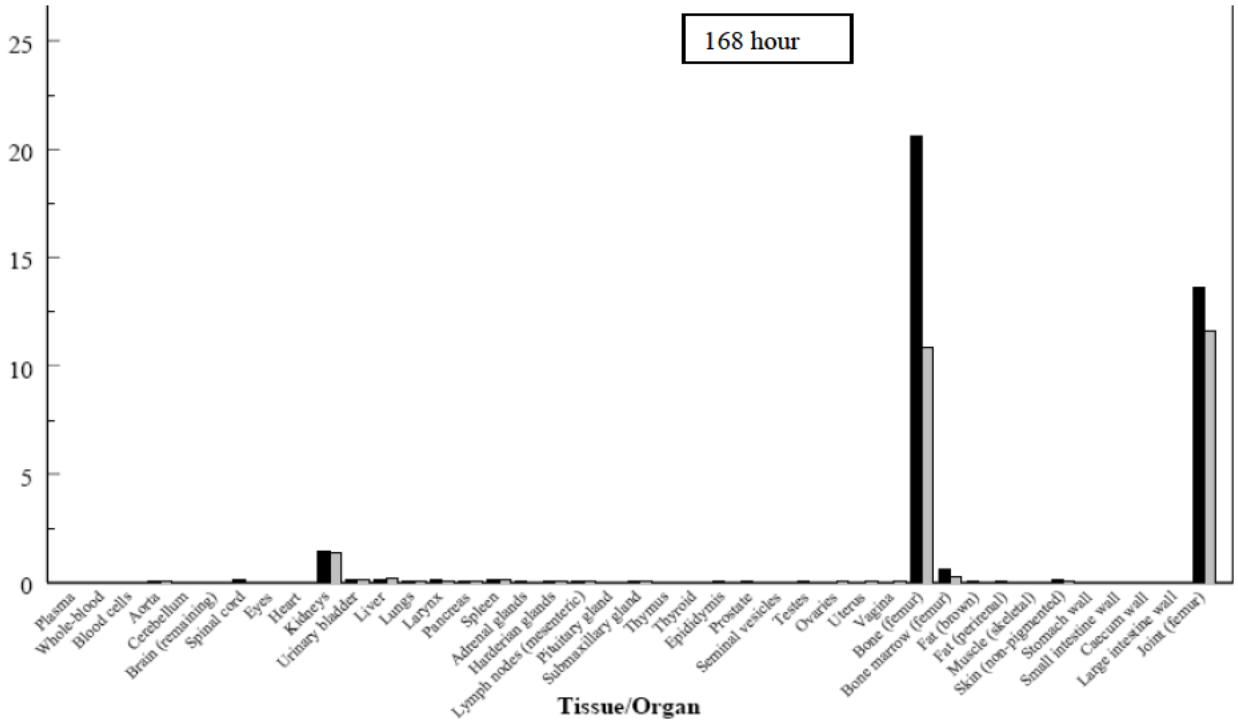

Overall, the in vitro distribution data indicated that Org 25969 does not bind to plasma proteins and erythrocytes in blood of the rat, rabbit, cat, dog, and human, but presence of Org 25969 in blood decreases Org 9426 binding to plasma proteins in a dose-dependent manner. In addition, there is limited amount of Org 25969 uptake by rat hepatocytes, indicating low rate of liver metabolism of this compound.

<u>In vivo distribution studies</u>

Tissue distribution was firstly examined in an excretion balance and metabolite profiling study including tissue distribution in rats (see section 2.6.4.6. Study1, Document ID: NL0056358). In this study, animals were treated with 8 mg/kg [$^{14}$C] Org 25969 and sacrificed at 25 minutes, 1, 5, 24, and 168 hours after dosing for radioactivity measurement in various tissue and organs. The results indicated that radioactivity was rapidly and widely distributed throughout the body after administration. Highest tissue concentrations were present in the femur joint and femur bone, followed by kidneys, urinary bladder in both males and females 25 minutes after administration. In addition, high concentration was also seen in the prostate in male rats and the vagina in females. [$^{14}$C] Org 25969 in all the organs and tissues examined were substantially reduced by 168 hr post-dose except in bone represented by femur bone and femur joint, indicating a slow elimination from the bone. Org 25969 did not readily penetrate the blood brain barrier. In addition, concentrations in the eyes of pigmented rats were similar to those in the albino strain, indicating that Org 25969 appeared to bind minimally to ocular melanin. The following figures extracted from the sponsor's report showed the tissue distribution of Org 25969 at 25 minutes and 168 hours after dosing.






These results indicated extended bone retention after Org 25969 in rats. Therefore, the sponsor has consequently conducted a series of distribution studies to investigate the bone and teeth retention and the placental transfer of Org 25969.

**1. Title: Bone retention kinetics in rat with Org 25969 (Sugammadex) after a single and multiple intravenous injection (INT00010275)**

The objectives of this study included:

- Assess tissue kinetics of [$^{14}$C]-Org 25969 in bones, teeth and cartilage, as well as in kidney, urinary bladder, liver and bone marrow after a single i.v. injection
- Assess or estimate half-life of Org 25969 in bone.
- Compare radioactivity levels in bone, teeth and cartilage after a single dose of [$^{14}$C]-labeled Org 25969 and after a dose of unlabelled Org 25969 followed by a dose of [$^{14}$C]-labeled Org 25969 in order to evaluate the saturation of bone and teeth accumulation.
- Assess the kinetics in kidney, urinary bladder, liver and bone marrow, after a single intravenous injection
- Assess urinary excretion of radioactivity after a single dose of [$^{14}$C]-labeled Org 25969 and after a dose of unlabelled Org 25969 followed by a dose of [$^{14}$C]-labeled Org 25969

Additionally, one of the femurs and scapula from one animal per group were collected for autoradiography to assess areas where retention occurs.

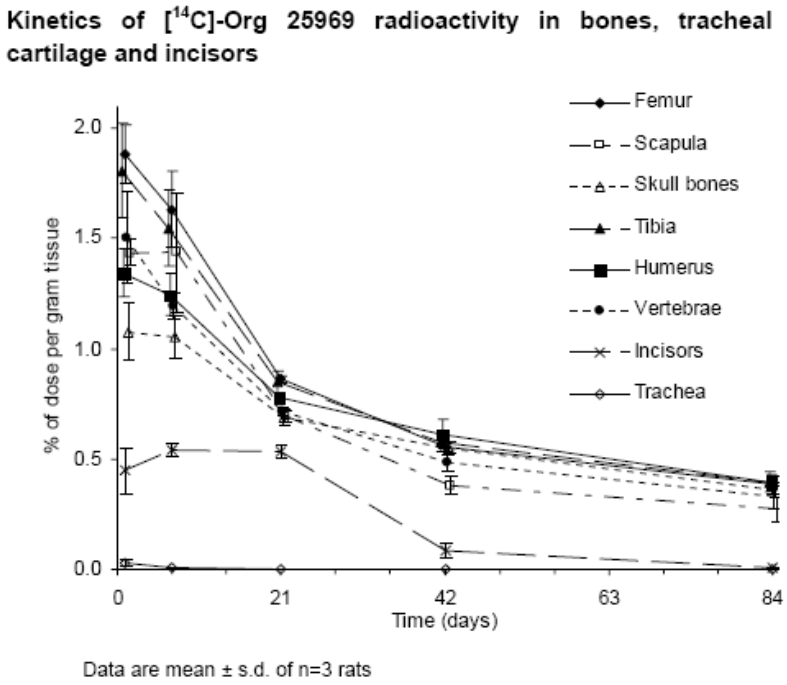
Male Wistar rats (body weight: 193-214 g) were intravenously injected with 8.6 mg/kg Org 25969 including 5.6 MBq/kg [$^{14}$C]-Org 25969 and sampled according to the following scheme (extracted from the Sponsor's report). The dosing level was chosen based on the distribution study conducted previously (Document ID: NL0056358).

| Group | Number of rats | Dose (mg·kg$^{-1}$); label (MBq·kg$^{-1}$) | Dosing Day | Autopsy[1] Time (h/d) | Balance |
|---|---|---|---|---|---|
| 1 | 3 | 8.6; 5.6 | 0 | 24 h | Urine (0-24 h)[3] |
| | | | | | Blood (30, 60 and 90 min after label, n=1) |
| 2 | 3 | 8.6; 5.6 | 0 | 7 d | Urine (0-7 d in 24 h periods) |
| 3 | 3 | 8.6; 5.6 | 0 | 21 d | |
| 4 | 3 | 8.6; 5.6 | 0 | 42 d | |
| 5 | 3 | 8.6; 5.6 | 0 | 84 d | |
| 6 | 3 | 8.6; 5.6 | 0[2],1 | 48 h | Urine (24-48 h)[3] |
| | | | | | Blood (30, 60 and 90 min after label, n=1) |

[1] From all animals in the study, the following tissues were collected: Bones (femur, scapula, skull bones, tibia, humerus, vertebrae (L4,5,6)), incisors, tracheal cartilage, kidney, urinary bladder (washed), liver and bone marrow.
[2] The first dose in group 6 contained only non-radioactive Org 25969. Autopsy time is expressed in hours after the first dose.
[3] Feces samples were collected but not assessed for radioactivity as the summed recoveries of urine and tissues collected accounted for more than 90% of the administered dose.

The samples were either dissolved in appropriate solvent such as potassium hydroxide, Solvable or Soluene 350®, and homogenized if needed and subsequently assessed for radioactivity by liquid scintillation counting (LSC), percentage of dosing per gram of tissue were calculated. Growth of tissues was observed during the course of the experiment, which contributed significantly to the dilution in radioactivity during the course of the study. Therefore, the radioactivity was fitted as percentage of the dose per whole tissue in time by first order kinetics using either a 1 or 2 compartment model

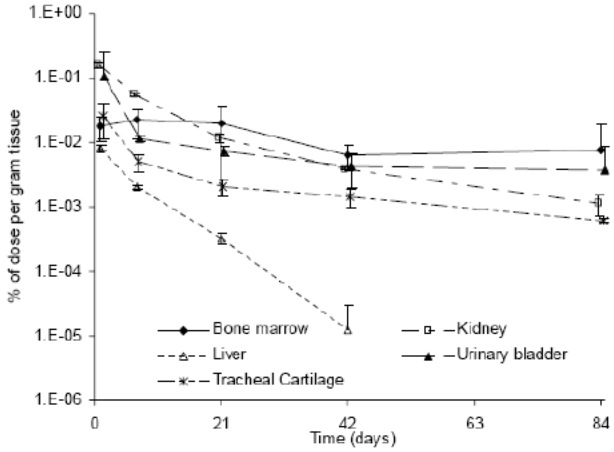
Concentrations of radioactivity in bones, incisors and tracheal cartilage between day 1 and 84 after dosing are given in the following figure (extracted from the sponsor's report).




Radioactivity per gram tissue at day 1 post dosing was the highest in femur (1.9%) followed by tibia (1.8%), vertebrae (1.5%), scapulae (1.4%), humerus (1.3%) and skull bones (1.1%). Ultimately, at day 84, the percentage of radioactivity in all these bones decreased to about 0.5% of dose radioactivity/gram. The accumulation of the radiolabeled Org 25969 lasted till to post-dosing day 21 in incisors, the highest level of radioactivity (0.5%) detected in the incisor was lower compared to bones. In contrast, levels of radioactivity were substantially lower in tracheal cartilage with a level of 0.03% on SD1 after dosing and $3.2 \times 10^{-5}$ % on SD84, which were in the range between urinary bladder, kidney and liver as shown below.

The percentage of the dose per gram tissue retrieved in bone marrow, kidney, liver, and urinary bladder was shown in the following figure. Tracheal cartilage was also included for comparison. The results indicated relatively low level of Org 25969 in these tissues (kidney - 0.16%, urinary bladder - 0.11%, bone marrow - 0.02%, and liver - 0.01% ) on SD1 as compared to those in bone and teeth. On SD84, the percentage of radioactivity in urinary bladder and bone marrow apparently achieved a steady state level at approximately 0.006% whereas levels in kidney and liver continuously decreased. Levels of radioactivity in liver were consistently lower compared to kidney, urinary bladder and bone marrow and at day 84 the radioactivity was no longer detectable.

Figure 1 e profile of the retention of radioactivity in bone marrow, kidney, liver, tracheal cartilage and urinary bladder (emptied) after a single intravenous injection of [$^{14}$C]-Org 25969




Notes: X-values were slightly scattered to enhance the resolution, error bars represent standard deviations, the Y-axis is expressed logarithmically

Radioactivity levels decreased from all tissues indicating elimination. The rate of elimination was quantified by the parameter estimates of the 1- or 2-compartment model. The results were shown below as extracted from the sponsor's report. Skull bones showed the longest estimated half-life ($t_{½}$: 210 days) while the shortest $t_{½}\beta$ was estimated for the liver (6 days). The other bones show elimination of radioactivity at an estimated $t_{½}\beta$ between 67 and 175 days while for incisors the $t_{½}\beta$ was estimated to be 8 days. The mean half life in bones was 172 days.

**Table 9.1-10 Estimated model parameters describing kinetics by a 1 or 2 compartment model with first order elimination from tissue, based on % dose per whole tissue.**

| Tissue | K[1] (% dose/gram/day) | $t½_\alpha$ | $t½_\beta$ | $k_{12}$ | $k_{21}$ | $K_A$ | Input |
|---|---|---|---|---|---|---|---|
| Femur | 0.019 | 0.69 | 175 | 0.78 | 0.21 | 0.54 | 1.7 |
| Scapulae | 0.081 | 0.69 | 67 | 0.81 | 0.13 | 0.24 | 1.1 |
| Skull[2] | 0.0033 | 210[2] | -[3] | -[3] | -[3] | 2.73 | 0.48 |
| Tibia | 0.012 | 0.8 | 252 | 0.63 | 0.19 | 0.55 | 1.2 |
| Humerus | 0.016 | 1 | 176 | 0.51 | 0.17 | 0.6 | 0.74 |
| Vertebrae[2] | 0.0045 | 154[2] | -[3] | -[3] | -[3] | 2.52 | 0.45 |
| Incisors | 0.13 | 0.92 | 8 | 0.21 | 0.5 | 0.1 | 0.5 |
| Trachea | 0.63 | 0.23 | 30 | 2.24 | 0.11 | 0.4 | 0.006 |
| Kidney | 0.19 | 1.84 | 17 | 0.15 | 0.083 | 1 | 0.55 |
| Liver | 0.41 | 0.33 | 6 | 1.21 | 0.58 | 1.98 | 0.19 |
| Urinary bladder | 0.72 | 0.32 | 104 | 1.43 | 0.02 | 0.54 | 0.1 |
| Bone Marrow[4] | 0.103 | 2.1 | 109 | 0.207 | 0.020 | 0.05 | 0.20 |

[1] K: elimination constant,
$t½_\alpha$: Ln (2) / k *(in 1-compartment model)*
$t½_\alpha$: Ln (2) / $((-a_1 + \sqrt{(a_1 \cdot a_1 - 4 \cdot a_0)}) / 2)$, *(in 2-compartment model)*
$t½_\beta$: Ln (2) / $((-a_1 - \sqrt{(a_1 \cdot a_1 - 4 \cdot a_0)}) / 2)$
$k_{12}$: distribution from compartment 1 to 2,
$k_{21}$: distribution from compartment 2 to 1,
$K_A$: absorption rate from a hypothetical depot,
Dose: initial % dose presented to the tissue
$a_1$: $-(K + k_{12} + k_{21})$
$a_0$: $K \cdot k_{21}$
[2] best described by a 1-compartment model
[3] not applicable in a 1-compartment model
[4] based on % radioactivity of the dose per gram tissue

This study further indicated no difference in radioactivity content of any tissue including the bones, incisors and trachea between animals exposed to a single dose of 8.6 mg/kg [$^{14}$C]-Org 25969 and the animals exposed to non-labeled 8.6 Mg/kg Org 25969 followed

by 8.6 mg/kg [$^{14}$C]-Org 25969 (group 6). This suggests that preloading with 8.6 mg/kg did not affect the systemic kinetics of Org 25969 or extent of tissue retention, and, thus, the binding of [$^{14}$C]-Org 25969 to bone and incisors does not appear to be saturated at 8.6 mg/kg. In addition, preloading with 8.6 mg/kg Org 25969 did not appear to affect the urinary excretion of [$^{14}$C]-Org 25969. Cumulative urinary excretion data revealed that 74% of the dose was excreted within 24 hours increasing to 75% at 2 days and to 78% at 7 days post-dosing, indicating retention of Org 25959 in body of rat.

After animal sacrifice, 1 femur and 1 scapula sample from groups at collection time of SD1, SD21, and SD84 were sectioned (5 µm thickness) longitudinally and mounted onto slides. Slides of bone samples (12 slides/sample) were examined qualitatively by (b) (4) to determine the distribution of radioactivity in the femur and scapula by microautoradiography (INT00029186). One femur and one scapular sample from untreated rats were used as control. The slides containing sections of femur and scapula were coated with photographic emulsion, placed into a light-free box to expose and then developed and counterstained for qualitative evaluation of the distribution of silver granules. The results of the study demonstrated that in all three animals that received a single intravenous dose of [$^{14}$C]-Org 25969 (8.6 mg/kg; 5.6 MBq/kg), there was a band of deposition of silver granules at or close to the periosteal and endosteal aspect of the cortex and endosteal aspect of trabeculae in scapula and femur. No silver deposition was seen in growth plates or in the most recently formed trabeculae immediately adjacent to them. However, among the 3 animals examined, growth plate was not identifiable in either femur or scapula of the animal sacrificed on day 1 after dosing, was only identifiable in scapula but not in femur of the animal sacrificed on day 21 after dosing, was identifiable in both scapular and femur of the animal sacrificed on day 84 after dosing. Therefore, additional evidence is needed in support of the conclusion of Org 25969 absence in growth plate. The deposition of silver granules was at or adjacent to sites of active bone formation when the [$^{14}$C]-Org 25969 was administered. Bone formed subsequent to this area showed no silver deposition. The sections did not provide sufficient detail to characterize the binding site of Org 25969 as cells or extracellular matrix. There were no discernible differences in extent and location of binding between cortical and trabecular bone. It should be noted that there were no differences in intensity of silver granule deposition between animals sacrificed on day 1 and day 84 after dosing detectable by this method, although significant difference existed as shown above. The sponsor indicated that it may be "due to the limitations of the methodology (there was sometimes variation in intensity between sections of the same tissue from the same animal)." Based on the identified deficiencies and small sample size in this study, additional study with other method or improved microautoradiography may be needed to further examine the distribution of org 25969 within the bone and teeth.

2. **Study title:** A second bone retention study in male and female rats after single and multiple intravenous injections of [$^{14}$C]-Org 25969 (Org 5969-2) and Org 25969 (Sugammadex)

This is a study to further explore the bone retention of Org 259696 in rats. The objectives of this study including:

- Assess the extent of Org 25969 binding in bone and teeth following a range of single doses of [$^{14}$C]-Org 25969 and unradiolabeled Org 25969 in rats in order to investigate whether the extent of binding is linear to the dose;
- Assess the accumulation of Org 25969 binding in bone and teeth following repeated daily dosing of [$^{14}$C]-Org 25969 and Org 25969 for up to 4-weeks in young adult rats to assess the existence of saturation and estimate the maximum extent of binding;
- Assess whether age, and thus bone growth rate, modulates the extent of Org 25969 binding in bone and teeth of rats after single dose of [$^{14}$C]-Org 25969 (Org 25969-2) and Org 25969;
- Assess whether the presence of rocuronium (Org 9426) modulates the extent of Org 25969 binding in bone and teeth in young adult rats.

Male and female juvenile, young adult and aged Wistar rats were intravenously injected with Org 25969 containing [$^{14}$C]-Org 25969 and sampled according to the following scheme:

| Group | Rats[1] | Body weight range (gram) | | Dose ($mg \cdot kg^{-1}$; $MBq \cdot kg^{-1}$) | Regimen[2] | Sacrifice time[3] |
|---|---|---|---|---|---|---|
| | | M | F | | | |
| 1 | 3M + 3F | 266-273 | 197-229 | 30; 3 | SD | 24 hours |
| 2 | 3M + 3F | 276-285 | 187-195 | 120;12 | SD | 24 hours |
| 3 | 3M + 3F | 271-290 | 206-220 | 400; 40 | SD | 24 hours |
| 4 | 10M + 10F | 281-428 | 201-266 | 30; 9[4] | MD | 3, 7, 14, 21, 28 days[5] |
| 5 ("aged" rats) | 3M + 3F | 453-546 | 268-282 | 30; 3 | SD | 24 hours |
| 6 | 3M + 3F | 317-370 | 207-239 | 30; 3 + 4[6] | SD | 24 hours |
| 7 (juvenile rats) | 3M + 3F | 13 | 16 | 30; 15 | SD | 24 hours |

[1] M =male and F = female
[2] SD = single dose and MD = multiple dose (daily dosing).
[3] From all animals in the study, the following tissues were collected: Bones (femur, scapula, skull bones, tibia, humerus, vertebrae L4, 5, 6 and ossicles), incisors and molars.
[4] Initial doses were with unlabelled Org 25969, only the last dose of the multiple doses contained [$^{14}$C]-Org 25969.
[5] Animals were sacrificed 24 hours after the last dosing, 2M and 2F animals per time point.
[6] 30 $mg \cdot kg^{-1}$ Org 25969 and 3 $MBq \cdot kg^{-1}$ [$^{14}$C]-Org 25969 immediately followed by 4 $mg \cdot kg^{-1}$ Org 9426.

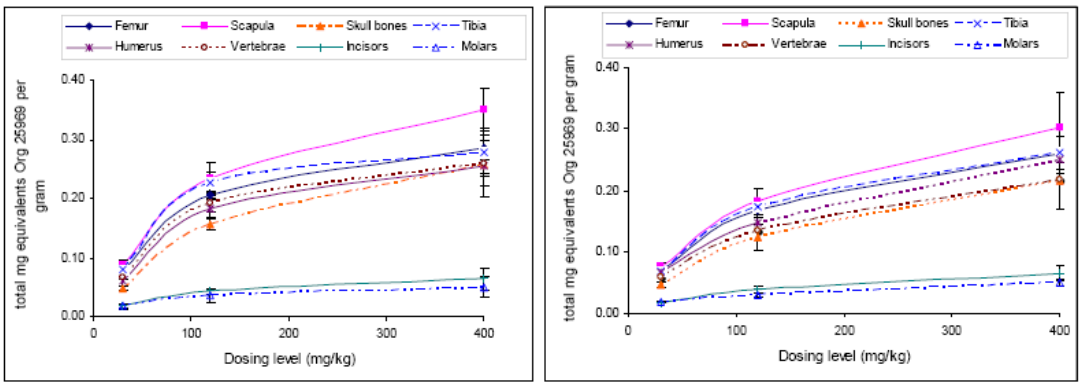
Aged rats used in group 5 were approximately 11 month and juvenile animals used in group were 7 days old. The young adult animals used in other groups were within the weight range of 160-180 g for males and 140-160 g for females.

Animals were sacrificed at the time-points indicated in the table above, and bone (femur, scapular, skull bones, tibia, humerus, ossicles, and vertebrae L4, 5, 6) and teeth (incisors, molars) samples were collected. The samples were dissolved in potassium hydroxide and subsequently assessed for radioactivity by liquid scintillation counting (LSC). All data were expressed as percentage of the dose and as percentage of the dose per gram tissue.

The results of this study showed that after a single dose of 30, 120, and 400 mg/kg Org 25969 to male and female young adult Wistar rats, the percentage of the radioactivity dose per gram tissue in bones, incisors and molars decreased with increasing dose levels, however the absolute amount absorbed (mg equivalents Org 25969) increases with increasing dosing levels as is shown in the figures extracted from the sponsor's report

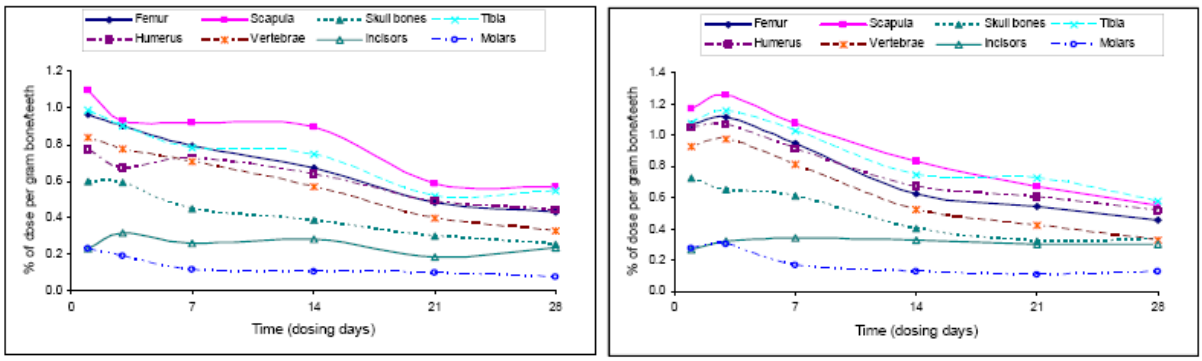
below. This indicates a less than dose-proportional increase after single dose administration.

**Figure 2: The binding of a single dose of [$^{14}$C]-Org 25969 to bone, incisors and molars as a function of dose level in young adult male (left) and female (right) rats**




The multiple dose groups were daily dosed with unlabeled 30 mg/kg Org 25969 and only the last day (day 3, 7, 14, 21 and 28, respectively) with 30 mg/kg Org 25969 including 9 MBq/kg [$^{14}$C]-Org 25969. This allowed measurement of the degree of Org 25969 binding over the last day of dosing. The results were illustrated in the figure below as extracted from the sponsor's report.

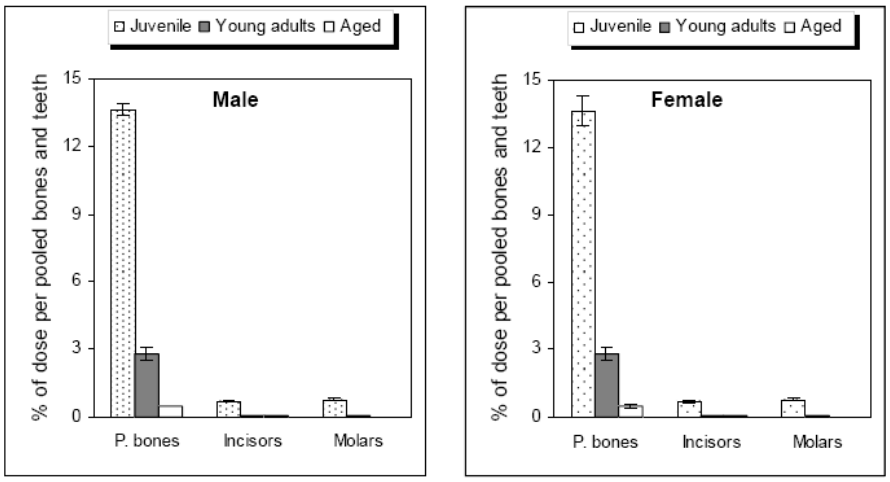
**Figure 3: The extent of [$^{14}$C]-Org 25969 binding to bone, incisors and molars as a function of dose regimen in young adult male (left) and female (right) rats**




The absorbed amount of radioactivity per gram bones (except ossicles) and teeth was generally decreased with multiple dose administration. The amount of radioactivity per gram bone ranged from approximately 0.6- 1.2% in rats after 1$^{st}$ day dosing. After 3-day dosing, the absorbed amount of radioactivity to bone gradually decreased per dosing day to radioactivity values per gram bone of 0.3-0.6% on day 28 of dosing for both male and female young adult Wistar rats. Nevertheless, at the end of the 28-day dosing period the bones were still able to absorb a part of the Org 25969 administered. This gradual decrease in the amount of Org 25969 absorbed may represent saturation of bone, decreasing growth rate with increasing age (see below) or a combination of both. The

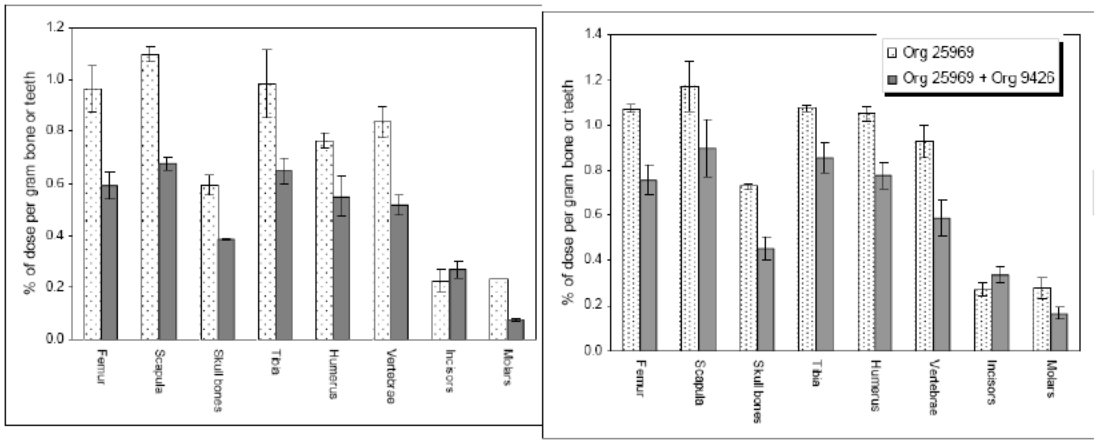
amount of Org 25969 absorbed by rat incisors appeared to consistent from day 3 to day 28, which may be due to the continuous growth of the incisors.

The percentage of the dose retrieved per pooled bones, incisors and molars were used for comparison between the extent of binding of Org 25969 in bone, incisors and molars in juvenile, young adult and aged Wistar rats. The percentage of the dose found in pooled bones, incisors and molars of juvenile, young adult and aged Wistar rats, respectively, after a single intravenous injection of 30 mg/kg (including 3 MBq/kg [$^{14}$C]-Org 25969 (young adult and aged) or 15 MBq/kg [$^{14}$C]-Org 25969 (juvenile)) decreased with age is shown in following figures as extracted from the sponsor's report.




The percentages of the dose in pooled bones of male and female juvenile Wistar rats were 13.6% and 13.0%, respectively, and were significantly lower in pooled bones of young adult and aged Wistar rats to 2.8% (for male and female young adult rats) and 0.5% (for "aged" male rats) and 0.4% (for "aged" female rats), respectively. The percentages of the dose found in incisors and molars were much lower than in pooled bones and ranged in juvenile Wistar rats from 0.6% to 0.8%. The percentage of the dose found in incisors and molars also decreased with age. The levels found in the incisors of the aged Wistar rats were relatively higher than found in their molars, but this was not observed for the juvenile and young adult Wistar rats.

A single intravenous injection of 4 mg/kg Org 9426 (half molar dose of 30 mg/kg Org 25969) after a single intravenous injection of 30 mg/kg Org 25969 containing 3 MBq/kg [$^{14}$C]-Org 25969 to young adult Wistar rats resulted in a decrease in the percentages of the dose bound to bones and molars (about 36% and 67% for males and 31% and 41% for females, respectively), indicating the presence of the neuromuscular blocker may prevent binding of [$^{14}$C]-Org 25969 to bone and molars. Interestingly, this effect was not noted in the incisors. The results were illustrated in the following figures (left panel: male; right panel: female) as extracted from the sponsor's report.




**3. Study title:** A placental transfer study in pregnant Sprague-Dawley rats after intravenous administration of [$^{14}$C]-Org 259696, [$^{3}$H]-Org 9426, and [$^{3}$H]-Org 9426 followed by Org 25969 (NL0052819)

The objective of this study was to assess the concentration of radioactivity in plasma, uterus, and placenta of pregnant Sprague-Dawley rats, and embryonic or fetal tissues/fluids after single dose administration of [$^{14}$C]-Org 25969, [$^{3}$H]-Org 9426, or [$^{3}$H]-Org 9426 followed by Org 25969 after 3 minutes.

Animals were treated as indicated in the table below (extracted from the sponsor's report).

| Group No. | Group size | Dosing [$^{14}$C]-Org 25969 (mg·kg$^{-1}$) | | Dosing [$^{3}$H]-Org 9426 (mg·kg$^{-1}$) | | Dosing [$^{3}$H]-Org 9426 and Org 25969 (mg·kg$^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|
| | | GD | Dose | GD | Dose | GD | Dose Org 9426 | Dose Org 25969 |
| 1 | 3 | 11 | 20 [a)] | | | | | |
| 2 | 3 | 16 | 20 [a)] | | | | | |
| 3 | 4 | | | 10 | 0.3 [b)] | | | |
| 4 | 4 | | | 16 | 0.3 [b)] | | | |
| 5 | 4 | | | | | 10 | 0.3 [b)] | 2 [c)] |
| 6 | 4 | | | | | 16 | 0.3 [b)] | 2 [c)] |

GD = gestation day
[a)] equals the low dose level of the embryo-fetal development study
[b)] sub-efficacious dose for practical reasons
[c)] represents a molar ratio of Org 25969 : Org 9426 of 1.4:1

Animals of group were sacrificed at different time-points after dosing (groups 1 and 2 at 10, 30 and 60 min with one rat/time-point; groups 3 to 6 at 10 and 30 min with 2 rats/time-point). The following tissues/fluids as extracted from the sponsor's report were collected and examined for radioactivity.

| Group No. | Tissues/fluids determined for radioactive content |
|---|---|
| 1,3,5 | Maternal plasma, uterus, embryo [a,b] |
| 2,4,6 | Maternal plasma, uterus, amnionic fluid[a], placentas[a], fetal carcass[c] (including head)[a] |

[a] 3 per dam (if feasible)
[b] the complete contents of the amnion
[c] fetal blood and fetal liver were could not be collected due to technical difficulty

After administration of Org 25969 (20 mg/kg/4.7 MBq/kg) on gestational day 11 and 16, a gradual decrease was observed in radioactivity levels in maternal plasma from 10 to 60 minutes with overall level <1% dose/g. In contrast, radioactivity in amnionic fluid at GD 16 (<0.003%) was much lower than that in the maternal plasma but appeared to be increased from 10 to 60 minutes after dosing, as shown below (extracted from the sponsor's report). However, it should be noted, these data were derived from one animal/time-point, the reliability of these data are questionable.

**Table 8-4 Total radioactivity in maternal plasma and amnionic fluid after a single intravenous injection of 20 mg·kg$^{-1}$ [$^{14}$C]-Org 25969 on GD11 and GD16**

| Animal no. | Time (min) | Plasma (Bq·g$^{-1}$) | % of the dose·g$^{-1}$ | Amnionic fluid (Bq·g$^{-1}$) | % of the dose·g$^{-1}$ |
|---|---|---|---|---|---|
| GD11 | | | | | |
| 1001 | 10 | 10047.36 | 0.87 | - | - |
| 1002 | 30 | 3841.03 | 0.35 | - | - |
| 1003 | 60 | 939.48 | 0.09 | - | - |
| GD16 | | | | | |
| 2001 | 10 | 9804.03 | 0.69 | 5.07 | 0.0004 |
| 2002 | 30 | 3711.26 | 0.25 | 20.69 | 0.0014 |
| 2003 | 60 | 980.18 | 0.08 | 27.91 | 0.0021 |

-not collected

The radiolabeled Org 25969 in maternal embryo, uterus, placenta, and fetuses were shown below as extracted from the sponsor's report. After 20 mg/kg [$^{14}$C]-Org 259696 administration, limited radioactivity levels were detected as expressed as % dose/g or organ in embryo (≤0.024%), fetal carcass (0.012%), uterus (≤0.36%/g, ≤1.11%/organ), and placenta (≤0.054%).

**Total radioactivity in maternal tissues and embryos and fetuses expressed as percentage of the dose after a single intravenous injection with 20 mg·kg$^{-1}$ [$^{14}$C]-Org 25969 on GD 11 and GD16**

| Animal No. | Time (min) | Embryo % dose·embryo$^{-1}$ | Uterus % of the dose·g$^{-1}$ | Uterus % dose·organ$^{-1}$ | Placenta % dose·organ$^{-1}$ |
|---|---|---|---|---|---|
| GD11 | | | | | |
| 1001 | 10 | 0.0244±0.0013 | 0.0596* | 0.0663* | - |
| 1002 | 30 | 0.0098±0.0010 | 0.2325* | 0.1655* | - |
| 1003 | 60 | 0.0037±0.0006 | 0.0108* | 0.0127* | - |
| | | Fetal carcass % dose·carcass$^{-1}$ | | | |
| GD16 | | | | | |
| 2001 | 10 | 0.0012±0.0003 | 0.3628±0.0027 | 1.1119±0.0083 | 0.0540±0.0076 |
| 2002 | 30 | 0.0012±0.0000 | 0.1891±0.0046 | 0.5039±0.0122 | 0.0221±0.0047 |
| 2003 | 60 | 0.0010±0.0001 | 0.0912±0.0012 | 0.3057±0.0039 | 0.0084±0.0021 |

* no homogenates prepared single measurement
- not collected

The [$^{3}$H]-Org 9426 radioactivity level in embryo or fetus in animals of groups 5 and 6 with treatment of Org 9426 and Org 25969 were comparable to those of groups 3 and 4 with treatment of Org 9426 only, indicating complexation with Org 25969 does not increase transfer of Org 9426 over the placenta. Whether binding of Org 9426 and Org 25969 affects the transfer of Org 25969 was not examined.

**4. Study Title:** A placental transfer study including fetal tissue distribution in Sprague-Dawley rats after a single and multiple intravenous administration of Org 25969 (INT00025872)

The objectives of the study were to assess the concentration of radioactivity in plasma, blood, and placenta of pregnant rats and in the tissues/fluids of fetus at various time-points after a single or multiple IV administration.

Pregnant Sprague Dawley rats were intravenously injected with Org 25969 (batch U) including [$^{14}$C]-Org 25969 according to the following scheme:

| Group | No. rats | Dose (mg·kg$^{-1}$); label (MBq·kg$^{-1}$) | Dosing[1] (number of dosings) | Experimental activity | Sacrified at time (h) |
|---|---|---|---|---|---|
| 1 | 3 | 500; 2.3 | GD 6-20 (15) | maternal TK, fetal tissue | 1 |
| 2 | 3 | 500;2.3 | GD 16-20 (5) | maternal TK, fetal tissue | 1 |
| 3 | 7[2] | 500; 2.3 | GD 6-20 (15) | maternal TK, fetal tissue | 0, 2, 4, 6, 7, 24 |
| 4 | 2[3] | 30; 2.5 | GD 20 (1) | maternal TK, fetal tissue | 1 |
| 5 | 3 | 500; 2.3 | GD 20 (1) | maternal TK, fetal tissue | 1 |

[1] sacrifice on GD 20
[2] extra rats were pregnant. These rats were added to group: 7h (n=1) and 24 h (n=2).
[3] one rat was excluded due to misdosing, n=2 evaluated

The animals were sacrificed at the time-points after administration indicated in the table above. Maternal blood was collected, and the uterus and fetuses were excised. From three fetuses, the corresponding placentas and amniotic fluid, liver, blood, bone, kidney, urinary bladder, lungs, brains and carcass were collected, and radioactivity were measured. It should be noted that in group 3, there was only one animal/time-point.

Comparison of the radioactivity concentration in fetal and maternal blood suggesting a slower elimination in fetus in contrast to maternal elimination within 24 hours after Org 25969 administration, as shown in the following table extracted from the sponsor's report. The amount of Org 25969 in fetal blood was about 2-9% that of maternal blood at one hour after administration. The radioactivity level was < 0.01% of the dose/gram blood in fetus.

| Animal No | # dosings | Time | % radioactivity of the dose per gram blood | | Fetal to maternal blood ratio (x100) | | |
|---|---|---|---|---|---|---|---|
| | | | Fetal blood | Maternal blood | % | mean | sd |
| 1006 | 15 | 1 | 0.0049 | 0.08 | 6.03 | 3.84 | 1.90 |
| 1010 | 15 | 1 | 0.0032 | 0.11 | 2.91 | | |
| 1012 | 15 | 1 | 0.0051 | 0.20 | 2.57 | | |
| 2002 | 5 | 1 | 0.0050 | 0.16 | 3.11 | 2.87 | 0.83 |
| 2004 | 5 | 1 | 0.0053 | 0.15 | 3.56 | | |
| 2006 | 5 | 1 | 0.0021 | 0.11 | 1.94 | | |
| 3004 | 15 | 0 | 0.0027 | 1.1727 | 0.23 | | |
| 3008 | 15 | 2 | 0.0024 | 0.0155 | 15.58 | | |
| 3010 | 15 | 4 | 0.0008 | 0.0034 | 23.62 | | |
| 3012 | 15 | 6 | 0.0005 | 0.0012 | 37.87 | | |
| 3014 | 15 | 7 | 0.0005 | 0.0008 | 56.33 | | |
| 3002 | 15 | 24 | 0.0003 | 0.0004 | 81.13 | | |
| 3016 | 15 | 24 | 0.0003 | 0.0005 | 69.44 | | |
| 4002 | 1 | 1 | 0.0084 | 0.09 | 9.20 | 5.59 | |
| 4006 | 1 | 1 | 0.0034 | 0.18 | 1.97 | | |
| 5002 | 1 | 1 | 0.0034 | 0.18 | 1.92 | 2.37 | 0.60 |
| 5004 | 1 | 1 | 0.0023 | 0.11 | 2.14 | | |
| 5006 | 1 | 1 | 0.0041 | 0.14 | 3.05 | | |

The data of this study also indicated that fetal femur (the only bone assessed) had the highest radioactivity concentration (≤ 0.5% dose/gram) comparison to the other tissues examined after multiple dose administration as shown below (extracted from the sponsor's report). As compared to multiple dosing, a single dose treatment generally

resulted in consistently lower mean fetal radioactivity levels per gram tissue, which may suggest accumulation of radioactivity with multiple dose administration. However, there was no apparent difference in fetal tissue levels between 5 and 15 dosing suggesting that steady state may be achieved after approximately 5 daily dosages.

**Table 9-4 % Radioactivity of the intravenous dose of Org 25969 per gram fetal or maternal tissue after intravenous injection during different intervals of pregnancy**

| Animal no. | No. dosings | Dose $mg \cdot kg^{-1}$ | Amniotic fluid | Fetal Brains | Fetal blood | Fetal femur | Fetal kidney | Fetal urinary | Fetal liver | Fetal lungs | Fetal carcass | Placenta | Uterus |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1006 | 15 | 500 | 0.0019 | 0.0009 | 0.0049 | 0.2395 | 0.0057 | 0.0195 | **0.0442** | 0.0015 | 0.0644 | 0.0943 | 0.2114 |
| 1010 | 15 | 500 | 0.0016 | 0.0010 | 0.0032 | 0.2816 | 0.0046 | 0.0109 | 0.0012 | 0.0015 | 0.0145 | 0.0455 | 0.2767 |
| 1012 | 15 | 500 | 0.0031 | 0.0011 | 0.0051 | 0.2428 | 0.0063 | 0.0124 | 0.0021 | 0.0025 | 0.0218 | 0.1462 | 0.3415 |
| mean | | | 0.0022 | 0.0010 | 0.0044 | 0.2546 | 0.0055 | 0.0143 | 0.0158 | 0.0019 | 0.0336 | 0.0953 | 0.2765 |
| sd | | | 0.0008 | 0.0001 | 0.0011 | 0.0234 | 0.0008 | 0.0046 | 0.0246 | 0.0006 | 0.0270 | 0.0504 | 0.0651 |
| 2002 | 5 | 500 | 0.0026 | 0.0010 | 0.0050 | 0.3610 | 0.0067 | 0.0122 | 0.0021 | 0.0022 | 0.0205 | 0.1529 | 0.3918 |
| 2004 | 5 | 500 | 0.0020 | 0.0010 | 0.0053 | 0.3276 | 0.0072 | 0.0146 | 0.0020 | 0.0021 | 0.0235 | 0.1553 | 0.5684 |
| 2006 | 5 | 500 | 0.0027 | 0.0006 | 0.0021 | X | 0.0036 | 0.0078 | 0.0007 | 0.0014 | 0.0126 | 0.0951 | 0.2700 |
| mean | | | 0.0024 | 0.0009 | 0.0042 | 0.3443 | 0.0058 | 0.0115 | 0.0016 | 0.0019 | 0.0189 | 0.1344 | 0.4101 |
| sd | | | 0.0004 | 0.0002 | 0.0018 | 0.0236 | 0.0020 | 0.0035 | 0.0007 | 0.0004 | 0.0056 | 0.0341 | 0.1500 |
| 3002 | 15(24h) | 500 | 0.0027 | 0.0006 | 0.0003 | 0.2446 | 0.0031 | 0.0012 | 0.0005 | 0.0003 | 0.0192 | 0.0269 | 0.0644 |
| 3004 | 15(0h) | 500 | 0.0014 | 0.0008 | 0.0027 | 0.2556 | 0.0035 | 0.0050 | 0.0012 | 0.0013 | 0.0113 | 0.4329 | 0.7921 |
| 3008 | 15(2h) | 500 | 0.0021 | 0.0011 | 0.0024 | X | 0.0055 | 0.0103 | 0.0014 | 0.0013 | 0.0313 | 0.0557 | 0.1811 |
| 3010 | 15(4h) | 500 | 0.0029 | 0.0008 | 0.0008 | 0.3634 | 0.0037 | 0.0053 | 0.0012 | 0.0008 | 0.0196 | 0.0309 | 0.1982 |
| 3012 | 15(6h) | 500 | 0.0042 | 0.0007 | 0.0005 | 0.5277 | 0.0031 | 0.0024 | 0.0007 | 0.0009 | 0.0165 | 0.0453 | 0.1474 |
| 3014 | 15(7h) | 500 | 0.0021 | 0.0009 | 0.0005 | 0.2242 | 0.0027 | 0.0035 | 0.0009 | 0.0004 | 0.0166 | 0.0379 | 0.1854 |
| 3016 | 15(24h) | 500 | 0.0014 | 0.0006 | 0.0003 | 0.1450 | 0.0019 | 0.0021 | 0.0006 | 0.0002 | 0.0135 | 0.0239 | 0.3518 |
| Mean 24h | | | 0.0020 | 0.0006 | 0.0003 | 0.1948 | 0.0025 | 0.0016 | 0.0005 | 0.0003 | 0.0164 | 0.0254 | 0.2081 |
| 4002 | 1 | 30 | 0.0022 | 0.0011 | 0.0084 | 0.3863 | 0.0090 | 0.0185 | 0.0021 | 0.0031 | 0.0170 | 0.1413 | 0.3797 |
| 4006 | 1 | 30 | 0.0042 | 0.0004 | 0.0034 | 0.2294 | 0.0041 | 0.0123 | 0.0009 | 0.0009 | 0.0062 | 0.1037 | 0.2170 |
| mean | 1 | | 0.0032 | 0.0008 | 0.0059 | 0.3078 | 0.0065 | 0.0154 | 0.0015 | 0.0020 | 0.0116 | 0.1225 | 0.2983 |
| 5002 | 1 | 500 | 0.0009 | 0.0003 | 0.0034 | 0.0933 | 0.0036 | 0.0047 | 0.0007 | 0.0012 | 0.0066 | 0.1267 | 0.2079 |
| 5004 | 1 | 500 | 0.0011 | 0.0003 | 0.0023 | 0.1116 | 0.0024 | 0.0061 | 0.0003 | 0.0005 | 0.0053 | 0.0861 | 0.1445 |
| 5006 | 1 | 500 | 0.0016 | 0.0005 | 0.0041 | 0.1415 | 0.0045 | 0.0089 | 0.0009 | 0.0015 | 0.0088 | 0.1012 | 0.1871 |
| mean | | | 0.0012 | 0.0004 | 0.0033 | 0.1155 | 0.0035 | 0.0066 | 0.0007 | 0.0011 | 0.0069 | 0.1047 | 0.1798 |
| sd | | | 0.0003 | 0.0001 | 0.0009 | 0.0244 | 0.0010 | 0.0021 | 0.0003 | 0.0005 | 0.0018 | 0.0205 | 0.0323 |

A follow-up whole body autoradiography study (INT00013814) was conducted to investigate the tissue distribution of [$^{14}$C]-Org 25969 in rat fetuses sacrificed at 0, 1, 2, 4, 6, 24 hours after 15 daily i.v. doses from GD 6-20 of pregnant dams (one dam/time-point). One sagittal section (30 µm) was obtained at four different levels throughout the carcass including liver, lungs, kidney, heart, thymus, bone, brain, spinal cord, eye, adrenal gland, spleen, skeletal muscle, brown fat, urinary bladder and gastrointestinal tract. The results of this study showed that radioactivity were present in the rat fetal tissues and organs. The radioactivity was largely confined to bone (e.g. rib, jawbone, skull, vertebrae, tibia and fibula) and excretory organs (urinary bladder contents and gastrointestinal tract). The radioactivity in the urinary system and gastrointestinal tract was observed from approximately 4 h after dosing. No accumulation of radioactivity is observed in vital organ systems such as heart, brain or lung. In addition, radioactive components present in the amniotic fluid of pregnant rats killed at selected times after single and repeated daily intravenous doses of [$^{14}$C]-Org 25969 were separated by HPLC

and quantified by off-line fraction collection/liquid scintillation counting. Specific metabolites were not detected.

**5. Study title:** A placental transfer study in pregnant New Zealand white rabbits after intravenous administration of [$^{14}$C]-Org 25969, [$^{3}$H]-Org 9426, and [$^{3}$H]-Org 9426 followed by Org 25969 (NL0054636) (batch V)

Animals were treated as shown below (extracted from the sponsor's report).

Animals were treated as indicated in the table below.

| Group number | Compound | Dose level | Group size (females) | Dosing on day[a] |
|---|---|---|---|---|
| 1 | [$^{14}$C]-Org 25969 | 20 mg·kg$^{-1}$ (1 MBq·kg$^{-1}$) | 3 | 10 |
| 2 | [$^{14}$C]-Org 25969 | 20 mg·kg$^{-1}$ (1 MBq·kg$^{-1}$) | 5[b] | 17 |
| 3 | [$^{3}$H]-Org 9426 | 0.02 mg·kg$^{-1}$ (3.3 MBq·kg$^{-1}$) | 4 | 10 |
| 4 | [$^{3}$H]-Org 9426 | 0.02 mg·kg$^{-1}$ (3.4 MBq·kg$^{-1}$) | 4[c] | 17 |
| 5 | [$^{3}$H]-Org 9426 within 3 minutes followed by Org 25969 | 0.02 mg·kg$^{-1}$ (3.3 MBq·kg$^{-1}$)<br>0.2 mg·kg$^{-1}$ | 4 | 10 |
| 6 | [$^{3}$H]-Org 9426 within 3 minutes followed by Org 25969 | 0.02 mg·kg$^{-1}$ (3.3 MBq·kg$^{-1}$)<br>0.2 mg·kg$^{-1}$ | 4 | 17 |

[a] day of gestation [b] animal 2005 was dosed 8 mL instead of 10.8 mL due to shortage of dose formulation. As the animal was not pregnant this does not affect the integrity of the study. [c] the dosing volumes were either too low (animal 4001; 8.33 mL instead of 9.8 mL) or too high (animal 4002; 10.84 mL instead of 8.2 mL). As the individual doses were calculated this does not affect the integrity of the study.

The drugs were injected intravenously via the marginal ear vein. The injection volume per injection was 2 mL/kg. The rabbits were sacrificed by at approximately 10, 30 or 60 min after dosing (group 1 and 2; one rabbit per time point) or at 10 or 30 min (group 3 to 6; two rabbits per time point), and the following tissues/fluids were measure for radioactivity.

| Group No. | Tissues/fluids determined for weight and radioactive content |
|---|---|
| 1, 3, 5 | Maternal plasma, uterus, embryo[a,b] |
| 2, 4, 6 | Maternal plasma, uterus, amnionic fluid[a], placentas[a], fetal blood[a], fetal liver[a], fetal carcass (including head)[a] |

[a] 3 per dam

[b] = the complete contents of the amnion

After administration of Org 25969 (20 mg/kg/1 MBq/kg) on gestational day 10 and 17, a gradual decrease was observed in radioactivity levels in maternal plasma from 10 to 60 minutes with ≤ 0.13% dose/g at 10 minutes. In contrast, radioactivity in amnionic fluid gestational at GD 17 were much lower than plasma radioactivity level of Org 25969 (0.0002%). However, it should be noted, these data were derived from one animal/time-point, the reliability of these data are questionable. The radiolabeled Org 25969 in maternal embryo, uterus, placenta, and fetuses were shown below as extracted from the sponsor's report. After 20 mg/kg [$^{14}$C]-Org 259696 administration, limited radioactivity levels were detected in these tissues.

Table 8.1-4 Total radioactivity in maternal tissues and embryos and fetuses expressed as percentage of the dose after a single intravenous injection with 20 mg·kg$^{-1}$ [$^{14}$C]-Org 25969 on GD 10 and GD 17

| Animal No. | Time (min) | Embryo (%dose·embryo$^{-1}$) | | | Uterus (%dose·g$^{-1}$) | Uterus (% dose·organ$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| GD 10 | | | | | | | |
| 1001 | 10 | 0.0196 | | | 0.0379 | 0.5802 | |
| 1002 | 30 | 0.0126 | | | 0.0338 | 0.4534 | |
| 1003 | 60 | 0.0085 | | | 0.0221 | 0.3915 | |
| | | Fetal carcass (%dose·fetal carcass$^{-1}$) | Fetal liver (% dose·organ$^{-1}$) | Fetal blood (% dose·g$^{-1}$) | Uterus (%dose·g$^{-1}$) | Uterus (% dose·organ$^{-1}$) | Placenta (%dose·organ$^{-1}$) |
| GD 17 | | | | | | | |
| 2001 | 10 | not pregnant | | | 0.0604 | 0.7192 | |
| 2002 | 10 | 0.0001 | 0.0000[a] | 0.0001[a] | 0.0264 | 1.0048 | 0.0186 |
| 2003 | 30 | not pregnant | | | 0.0358 | 0.3340 | |
| 2004 | 30 | 0.0003 | 0.0000[a] | 0.0005 | 0.0194 | 0.9716 | 0.0161 |
| 2005 | 60 | not pregnant | | | 0.0144 | 0.1796 | |

In addition, Org 25969 was found to be excreted into milk in rats in a milk extraction study conducted by the sponsor. In this study, 3 dams were treated with 20 mg/kg [$^{14}$C]-Org 25969 (4.8 MBq/kg) on postnatal day 9, as shown below (extracted from the sponsor's report). Oxytocin was given to facilitate milk collection. Milk and plasma samples were collected at 15, 30 and 60 minutes after dosing, and radioactivity was measured.

| | | *Dosing* | *Sampling* | | | *Sampling* |
|---|---|---|---|---|---|---|
| *Time (min)*[a] | *T=-15* | *t=0* | *t=15* | *t=30* | *T=45* | *T=60* |
| Org 25969 (iv) | | 20 mg·kg$^{-1}$ (~4.8 MBq·kg$^{-1}$) | Plasma, milk | Plasma, milk | | Plasma,milk |
| Oxytocin (sc) | 3 IU | | | | 3IU | |

The results of the study showed small amount excretion of radiolabeled Org 25969 into milk with maximum concentration observed at 30 min after dosing as shown below (extracted from the sponsor's report).

| Time (min) | **Plasma** Mean ± sd % dose radioactivity·g$^{-1}$ | **Milk** Mean ± sd % dose radioactivity·g$^{-1}$ |
|---|---|---|
| 15 | 0.44 ± 0.03 | 0.06 ± 0.02 |
| 30 | 0.24 ± 0.02 | 0.22 ± 0.05 |
| 60 | 0.10 ± 0.01 | 0.16 ± 0.00 |

n=3

### 2.6.4.5. Metabolism

An in vitro study in rat hepatocytes, as described in Section 2.6.4.4, demonstrated there was only a limited amount of Org 25969 uptake into hepatocytes (maximally 2.4% at 6 µM and ≤ 1% at 100 µM). This suggested that significant liver metabolism *in vivo* may not occur, thus, the sponsor did not conduct *in vitro* metabolism studies to compare metabolites profiles in human and species used in non-clinical studies.

In vivo studies have been conducted to determine the metabolite profiling in selected plasma, urine, and feces samples in rats and dogs as part of the mass balance and tissue distribution studies with 8 mg/kg [$^{14}$C]-Org25969 single-dose administration (See section 2.6.4.6).

In rats, 8 peaks were identified possibly representing metabolites (M1-M8) based on retention time and the proportion of these components in plasma samples at 25 minutes and 1 hour and urine samples during 0-24 hours are shown in the following tables as extracted from the sponsor's report. Plasma samples collected after 1 hour were not applicable for metabolite profiling detection due to the low radioactivity.

**Plasma**

| Sex | Time | % of extract radioactivity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | M1 | M2 | M3 | M4 | M5 | M6 [a] | M7 [b] | M8 [c] | Other [d] |
| M | 25 min | ND | 1.2 | 2.4 | 3.5 | 3.1 | 15.0 | 13.1 | 59.4 | 2.3 |
| | 1 h | ND | ND | ND | ND | ND | 8.8 | 9.9 | 83.1 | 0.0 |
| F | 25 min | ND | 2.5 | 4.9 | 8.3 | 7.6 | 12.1 | 24.6 | 37.7 | 2.3 |
| | 1 h | ND | ND | ND | ND | ND | 8.8 | 9.8 | 80.3 | 1.1 |
| *Mean retention time (min)* | | - | *11.5* | *12.5* | *13.5* | *14* | *17* | *18* | *21* | - |

**Urine**

| Sex | Time | % of extract radioactivity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | M1 | M2 | M3 | M4 | M5 | M6 [a] | M7 [b] | M8 [c] | Other [d] |
| M | 0-3 h | ND | ND | ND | ND | 3.1 | 8.2 | 9.2 | 75.0 | 4.5 |
| | 3-6 h | ND | 1.4 | 1.9 | 3.1 | 4.4 | 9.6 | 10.9 | 62.9 | 5.8 |
| | 6-24 h | ND | ND | ND | ND | ND | 5.4 | 8.7 | 53.2 | 32.7 |
| F | 0-3 h | ND | ND | ND | ND | 2.9 | 7.0 | 7.9 | 79.5 | 2.7 |
| | 3-6 h | ND | ND | ND | 4.9 | 4.6 | 10.2 | 11.9 | 48.6 | 19.8 |
| | 6-24 h | 11.5 | ND | ND | ND | ND | 6.2 | 4.7 | 61.9 | 15.7 |
| *Mean retention time (min)* | | *4.5* | *11* | *12.5* | *13.5* | *14* | *16* | *17.5* | *21* | - |

Samples of 3 rats/gender were pooled.
ND Not detected, below lower LOQ
[a] Chromatographically consistent with Org 198958-2
[b] Chromatographically consistent with Org 199425-1
[c] Chromatographically consistent with [$^{14}$C]-Org 25969
[d] Between-peak radioactivity, not associated with any clearly defined radioactive component(s)

The data showed that the major component in plasma and urine at each time was M8, which co-eluted with unchanged Org 25969, representing ≥ 50% proportion. In addition, M6 and M7 were presented at all sample collection periods in plasma and urine with a proportion of 9-15% and 10-25% in plasma and 5-10% and 5-12% in urine, respectively. The proportion of M8 in urine corresponded to a total of 55% to 59% of the radioactive dose while M6 and M7 corresponding to 5-7%, respectively, during 0-24 hours. In feces, the proportion of the fecal radioactivity in the form of M8 was in a range of 46-57%, corresponding to totals of 2%-3% of the radioactive dose respectively during 0-48 h post-dose. The proportions of the minor components M6 and M7 in feces (co-eluting with Org 198958-2 and Org 199425-1, respectively) were similar at each time and ranged from 9-11% and 5-8%, corresponding to totals of <0.7% and <0.5% of the dose, respectively, during 0-48 h post dose. In addition, similar patterns of component proportions were found in rat amniotic fluid. Component M1 to M5 with low proportions were poorly resolved and not consistently present at all time-points and in all samples examined, which may not represent potential metabolites, therefore, the sponsor did not identify the chemical property of these components.

It was not clear whether components of M6 and M7 were metabolites of Org 25969 since Org 198958-2 and Org 199425-1 are presented in the dose formulation used in this study.

The [$^{14}$C]-Org 25969 dose formulation analysis before animal dosing showed that the proportions of M6, M7 and M8 were 4.6%, 5.8%, and 73.0%, respectively. In addition, the [$^{14}$C]-Org 25969 stock used to prepare the dose formulation (stored in water at about 4°C) was also analyzed after storage for a similar length of time to the test samples and the proportions of M6 and M7 were 10.2% and 13.4% respectively. These relative amounts in dose formulation and stock solution are lower as compared to the proportions of M6 and M7 observed in plasma, urine and feces samples; therefore, minor in vivo metabolism of Org 25969 to Org 198958-2 and Org 199425-1 (structures shown below: these two compounds are different enantiomers with same structure ) cannot be excluded.

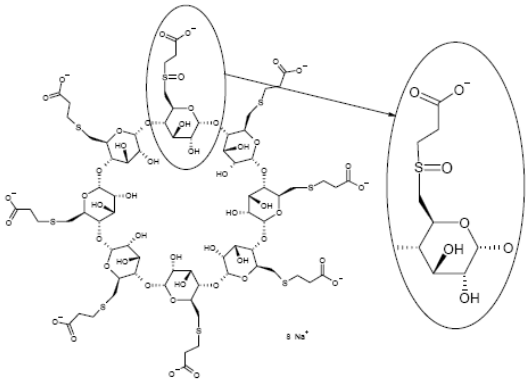


**Figure 9: Structural formula of Org 198958-2 and Org 199425-1**

In dogs, the separated potential radio-metabolites were assigned identities (M1–M4) on the basis of their retention times, although not all were present in every sample and some were present in trace quantities only. The proportions of the components in plasma and urine were shown below as extracted from the sponsor's report.

**Plasma**

| Sex | Time | % of extract radioactivity | | | | |
|---|---|---|---|---|---|---|
| | | M1 | M2 [a] | M3 [b] | M4 [c] | Other [d] |
| M | 5 min | ND | 4.7 | 15.4 | 72.6 | 7.3 |
| | 20 min | 8.1 | 6.3 | 16.1 | 59.7 | 9.8 |
| | 40 min | 10.6 | 10.6 | 16.5 | 60.6 | 1.7 |
| | 1 h | 13.9 | 10.5 | 13.2 | 56.6 | 5.8 |
| | 2 h | 5.7 | 20.1 | 17.1 [f] | 55.7 [e] | 1.4 |
| F | 5 min | 4.7 | 8.4 | 19.3 | 63.4 | 4.2 |
| | 20 min | ND | 3.5 | 12.8 | 76.6 | 7.1 |
| | 40 min | 11.6 | 11.1 | 18.5 | 56.7 | 2.1 |
| | 1 h | 18.1 | 10.7 | 21.1 | 45.7 | 4.4 |
| | 2 h | 14.6 | 12.8 | 20.0 | 47.6 | 5.0 |
| *Mean retention time (min)* | | *9.5 – 14.5* | *14.5 – 16.0* | *16.0 – 18.0* | *19.0 – 23.0* | – |

Samples of 3 dogs/gender were pooled.
ND Not detected, below lower LOQ
[a] Chromatographically consistent with Org 198958-2
[b] Chromatographically consistent with Org 199425-1
[c] Chromatographically consistent with [$^{14}$C]-Org 25969
[d] Between-peak radioactivity, not associated with any clearly defined radioactive component(s)
[e] Poor chromatography, M4 was split in two accounting for 41% and 59% of the peak
[f] Poor chromatography, M3 was split into two accounting for 47% and 53% of the peak

**Urine during 6 – 24 hours**

| Animal number and sex | % of extract radioactivity | | | | |
|---|---|---|---|---|---|
| | M1 | M2 [a] | M3 [b] | M4 [c] | Other [d] |
| 1M | ND | 5.1 | 7.9 | 83.9 | 3.1 |
| 2M | 3.6 | 4.1 | 5.8 | 81.2 | 5.3 |
| 3M | 4.0 | 9.5 | 12.5 | 75.6 | 0.0 |
| 4F | 2.4 | 6.0 | 8.1 | 85.6 | 0.0 |
| 5F | ND | 7.7 | 8.5 | 88.4 | 0.0 |
| 6F | ND | 6.1 | 6.3 | 87.8 | 0.0 |
| *Mean retention time (min)* | *10.6–13.9* | *15.0* | *16.5* | *19.5* | – |

No urine was produced over the first 6 h following dosing.

ND Not detected, below lower LOQ

[a] Chromatographically consistent with Org 198958-2

[b] Chromatographically consistent with Org 199425-1

[c] Chromatographically consistent with [$^{14}$C]-Org 25969

[d] Between-peak radioactivity, not associated with any clearly defined radioactive component(s)

The data show that the major component at each time was M4 in plasma, which co–eluted with unchanged Org 25969. The proportion of this component was in the range 56 – 73% for male dogs and 46 – 77% for females. The proportions of the minor components M2 and M3, which co-eluted with the monosulphoxides Org 198958–2 and Org 199425–1 respectively, were ranged from about 5–20% and 13–21% respectively. The changes in the proportions of M2 (increase) and M4 (decrease) from 5 minutes to 2 hours after administration were likely to be time-dependent; this was not seen in that of M3. In urine samples collected from 6-24 hours, M4 accounted for ≥ 75% of the total activity while M2 and M3 corresponding to 5-12%. M1 component identified in dogs was not a discrete peak and is likely to comprise a number of minor components; therefore, the sponsor did not characterize the chemical nature of this component.

The [$^{14}$C]–Org 25969 dose formulation analysis demonstrated the proportions of M2, M3 and M4 in dose formulation were 3.1%, 4.6% and 80.6%, respectively. The [$^{14}$C]–Org 25969 stock solution used to prepare the dose formulation analyzed after storage for a similar length of time to the test samples, the proportions of M2, M3 and M4 were 9.5%, 12.6% and 64.5% respectively. These relative amounts in stock solution are (slightly) lower as compared to the proportions of M6 and M7 observed in plasma and urine samples; therefore, like in rats, minor metabolism of Org 25969 to Org 198958–2 and Org 199425–1 can not be excluded in dogs.

In human, metabolic profiling assay following single-dose administration of [$^{14}$C]-Org 25969 (4 mg/kg, containing about 1.85 MBq of radioactivity/subject) to 6 healthy male subjects also demonstrated that Org 25969 was the major component and Org 198958–2 and Org 199425–1 were also presented at levels ≤ 18% in plasma and urine samples. In addition, this study also showed a high urinary recovery of unchanged Org 25969 (96.1%), indicating the minimal role of metabolism if existed. It should be noted that the current data submitted by the sponsor could not determine whether Org 25959 in vivo metabolism are present in either rats, dogs, or human. However, this may not affect the validity of rats and dogs as appropriate models for safety assessment of Org 25969 in human since there were no remarkable difference in the major components in plasma and urine between human and the non-clinical models. Complete characterization of the Org 25969 in these models may not be critical since the metabolism may be very minor or not present which may not contribute to potential adverse effects of Org 25969 after administration based on the data submitted.

### 2.6.4.6. Excretion

1. **Study Title:** [$^{14}$C]-Org 259696 and [$^{3}$H]-Org 9426: an excretion balance and metabolite profiling study including tissue distribution after a single intravenous dose of [$^{14}$C]-Org 25969, [$^{3}$H]-Org 9426 or [$^{3}$H]-Org 9426 followed by Org 25969 to male and female rats (NL0056358)

The primary objectives of this study were to determine the plasma kinetics, quantitative tissue distribution and routes and rates of excretion of [$^{14}$C]-Org 25969 after single intravenous administration to male and female rats. In addition, the plasma kinetics, quantitative tissue distribution and excretion of radioactivity of Org 9426, was investigated after single intravenous doses of [$^{3}$H]-Org 9426 (2 mg/kg) to male and female rats, with and without the subsequent intravenous administration of non-radiolabeled Org 25969 (8 mg/kg) in 5 minutes in order to investigate the potential effects of the Org 25969 on the disposition of Org 9426. Tissue distribution studies were carried out on albino and pigmented rats. Finally the tissue distribution of [$^{3}$H]-Org 9426 was investigated following intravenous administration of non-radiolabeled Org 25969 (8 mg/kg) 15 minutes after [$^{3}$H]-Org 9426 administration, at which time neuromuscular block had spontaneously reversed.

After dose administration, blood, plasma, urine, feces, cage wash and tissues/organs, dose sites and residual carcasses were collected and retained as appropriate. Radioactivity was measured by liquid scintillation analysis. Metabolite profiling was carried out on plasma, urine and feces samples obtained after administration of [$^{14}$C]-Org 25969 by high performance liquid chromatography using a radioactivity detector and radioactive components in representative samples were identified by liquid chromatography-mass spectrometry.

Plasma radioactivity pharmacokinetic data following a single intravenous dose of [$^{14}$C]-Org 25969 (8 mg/kg) to male and female rats is summarized in the following table as extracted from the sponsor's report.

| Parameter | Male | Female |
|---|---|---|
| $C_0$ (µg eq/g) | 32.40 | 44.25 |
| $AUC_t$ (µg eq.h/g) | 12.17 | 17.44 |
| AUC (µg eq.h/g)* | 12.61 | 17.98 |
| $\lambda_Z$ ($h^{-1}$)* | 0.0477 | 0.0482 |
| Terminal $t_{½}$ (h)* | 15 | 14 |
| Initial $t_{½}$ ($t_{½-\alpha}$; h) | 0.25 | 0.24 |
| CL (L/h/kg)* | 0.649 | 0.460 |
| $V_{SS}$ (L/kg)* | 1.680 | 1.149 |

* Terminal rate constants could not be determined in accordance with acceptance criteria, so these and derived data are approximations only

[$^{14}$C]-Org 25969 was eliminated with an estimated terminal half-life of approximately 15 hours and an estimated initial half-life of about 15 minutes after intravenous

administration of in rats. There were no major sex differences in the plasma kinetics of radioactivity. Plasma clearance of radioactivity was lower than the hepatic and renal plasma flow rates but was higher than the glomerular filtration rate in this species while the volume of distribution at steady-state was greater than the total volume of body water. Radioactivity was excreted primarily in the urine after intravenous administration of [$^{14}$C]-Org 25969 to rats as shown in the table below as extracted from the sponsor's report.

| Excretion | % administered dose | |
|---|---|---|
| | [$^{14}$C]-Org 25969 (8 mg/kg) | |
| | Male | Female |
| Total urinary excretion (0 – 168 hours) | 78.48 ± 3.36 | 78.86 ± 3.74 |
| Total faecal excretion (0 – 168 hours) | 4.22 ± 1.06 | 6.82 ± 2.72 |
| Total in cage wash (0 – 168 hours) | 2.12 ± 0.72 | 2.21 ± 1.50 |
| Residual bone (168 hours) | 2.71 ± 0.20 | 2.63 ± 0.08 |
| Residual carcass (168 hours) | 7.34 ± 0.62 | 4.68 ± 0.61 |
| Total recovery | 94.86 ± 2.30 | 95.19 ± 1.00 |

There were no apparent sex-related differences in the routes or rates of excretion of radioactivity. Although urinary excretion was very rapid initially, radioactivity continued to be excreted by this route at 168 hours post-administration, by which time about 78% of the dose had been recovered in the urine. Fecal excretion of radioactivity amounted to only about 5% of the dose. About 8-10% of the dose remained in the carcass and residual skeletal bone at 168 hours.

Metabolite profiles of plasma and urine determined by radio-HPLC were very similar to that of the dose formulation itself, confirming that little if any biotransformation of the reversing agent occurred in the rat. Two of the minor radioactive components, (b) (4) co-chromatographed with the (b) (4).

Plasma radioactivity pharmacokinetic data in rats after a single intravenous administration of [$^{3}$H]-Org 9426 (2 mg/kg) either alone or followed 5 minutes later by an intravenous dose of non-radiolabeled Org 25969 (8 mg/kg) are summarized in the following table as extracted from the sponsor's report:

| Parameter | [$^{3}$H]-Org 9426 alone | | [$^{3}$H]-Org 9426 and Org 25969 | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| $C_0$ (µg eq/g) | 3.594 | 3.461 | 3.566 | 3.245 |
| $AUC_t$ (µg eq.h/g) | 0.6252 | 0.6966 | 0.902 | 1.053 |
| AUC (µg eq.h/g)* | 1.077 | 1.071 | 1.626 | 2.532 |
| $\lambda_Z$ ($h^{-1}$)* | 0.0127 | 0.0158 | 0.0138 | 0.0081 |
| Terminal $t_{½}$ (h)* | 55 | 44 | 50 | 85 |
| Initial $t_{½}$ ($t_{½-\alpha}$; h) | 0.11* | 0.12* | 0.16 | 0.17 |
| CL (L/h/kg)* | 1.847 | 1.858 | 1.193 | 0.774 |
| $V_{SS}$ (L/kg)* | 82.67 | 60.09 | 53.57 | 67.72 |

* The rate constants could not be determined in accordance with acceptance criteria, so these and derived data are approximations only

There were no substantial difference (≥ 2 fold) between males and females. The calculated concentrations of radioactivity at zero time ($C_0$) were approximately the same for male and female rats after administration of [$^3$H]-Org 9426 alone or with Org 25969. The estimated terminal half-life of $^3$H-radioactivity increased in females when Org 25969 was administered 5 minutes after [$^3$H]-Org 9426 administration. The extent of systemic exposure to radioactivity ($AUC_t$) was markedly increased (by about 50%) after the reversing agent was administered. After administration of [$^3$H]-Org 9426 alone, the estimated plasma clearance of radioactivity was similar to the hepatic and renal plasma flow rates, while its estimated volume of distribution ($V_{ss}$) was much greater than the volume of total body water. Plasma clearance was reduced when Org 25969 was administered after [$^3$H]-Org 9426, whereas the volume of distribution was apparently unchanged in females but lower in males.

Similar patterns of tissue distribution of radioactivity were obtained after intravenous administration of [$^3$H]-Org 9426 alone (2 mg/kg) or followed, 5 or 15 minutes later, by an intravenous dose of the reversing agent, Org 25969. This indicates that Org 25969 had no notable effect on the quantitative tissue distribution of Org 9426 at the dose levels used, irrespective of whether it was administered before or after spontaneous reversal of neuromuscular block. In all cases, the tissue concentrations were broadly similar in male and female rats and the greatest tissue concentrations were present in the gastrointestinal tract, liver, urinary bladder and kidneys (*ie* the main organs of excretion), and accordingly the contents of the small intestine contained means of about 20-50% of the dose at 1 hour after dosing. High concentrations of radioactivity were also associated with the pituitary gland, vagina, aorta, thyroid, pancreas, larynx and prostate. Lowest concentrations, in the presence and absence of reversing agent, were generally found in the tissues of the central nervous system, *ie* cerebellum, remaining brain and spinal cord, indicating that Org 9426 does not readily penetrate the blood-brain barrier, and in bone, whole-blood and blood cells. At 168 hours post-dose, concentrations were still measurable in all tissues, and those in the pituitary gland, urinary bladder, eyes and kidneys were among the highest. Radioactivity levels in the eyes of albino rats were not particularly high, but they declined relatively slowly and were still quite high at 168 hours post-dose. The limited tissue data obtained for pigmented rats were in broad agreement with those of the albino strain, although the results for the eyes indicated that there was some binding of Org 9426-related material to ocular melanin. The mean concentrations in the eyes of pigmented rats were 2 to 3-fold higher than those in albino rats at 168 hours post-dose. The concentrations in pigmented and non-pigmented skin were, however, similar in pigmented rats (and were also similar to the mean concentrations in albino animals) which indicates that binding to melanin was not extensive.

| Excretion | % of administered dose | | | |
|---|---|---|---|---|
| | [$^3$H]-Org 9426 alone (2 mg/kg) | | [$^3$H]-Org 9426 (2 mg/kg) and Org 25969 (8 mg/kg) | |
| | Male | Female | Male | Female |
| Total urinary excretion (0 – 168 hours) | 10.27 ± 0.37 | 9.75 ± 1.03 | 14.43 ± 2.58 | 14.09 ± 5.69 |
| Total faecal excretion (0 – 168 hours) | 81.11 ± 1.57 | 78.92 ± 2.85 | 82.33 ± 2.38 | 82.12 ± 4.27 |
| Total in cage wash (0 – 168 hours) | 0.27 ± 0.08 | 0.44 ± 0.18 | 0.52 ± 0.11 | 0.39 ± 0.16 |
| Residual carcass (168 hours) | 0.55 ± 0.09 | 0.53 ± 0.06 | 0.48 ± 0.03 | 0.50 ± 0.04 |
| Total recovery | 92.19 ± 1.41 | 89.65 ± 2.33 | 97.77 ± 1.67 | 97.10 ± 6.33 |

During 0 - 168 hours after intravenous administration of [$^3$H]-Org 9426 to albino rats, both in the presence and absence of Org 25969 (administered 5 minutes after the blocking agent), a mean of about 80% of the dose was excreted in the feces and fecal excretion continued for at least 7 days after dosing, suggesting that biliary excretion and/or intestinal secretion played an important role in the disposition of Org 9426 in this species. In the absence of the reversing agent, a mean of 10% of the dose was excreted in the urine, whereas in the presence of Org 25969, this increased to 14%. Org 25969 had no marked effect on the biliary or intestinal secretion of [$^3$H]-Org 9426 and/or its metabolites. There were no apparent sex-related differences in the routes and rates of excretion of radioactivity.

2. **Study title:** [$^{14}$C]-Org 25969 and [$^3$H]-Org 9426: an excretion balance and metabolite profiling study of [$^{14}$C]-Org 25969, [$^3$H]-Org 9426 or [$^3$H]-Org 9426 followed by Org 25969 to male and female Beagle dogs (NL0055283)

In this study, the plasma kinetics, excretion and metabolism of [$^{14}$C]–Org 25969 have been investigated after single intravenous administration to dogs at 8 mg/kg (0.10 MBq/kg). In addition the plasma kinetics and excretion of the neuromuscular blocking agent, Org 9426, have been investigated after single intravenous doses of [$^3$H]–Org 9426 (0.36 mg/kg; 0.34 MBq/kg) to the same dogs, with and without the subsequent intravenous administration of the reversing agent, Org 25969 (8 mg/kg), during the period of neuromuscular block in order to investigate the potential effects of the reversing agent on the disposition of the blocking agent. After dose administration, blood, plasma, urine, feces and cage wash were collected. Metabolite profiling of Org 25969 was carried out on plasma, urine and feces samples obtained after administration of [$^{14}$C]–Org 25969 by high performance liquid chromatography using a radioactivity detector.

Mean plasma radioactivity pharmacokinetic data following a single intravenous dose of [$^{14}$C]–Org 25969 (8 mg/kg) to three male and three female dogs are summarized in the following table as extracted from the sponsor's report. The results showed that radioactive drug–related material was eliminated with a terminal half– life of approximately 8 hours after intravenous administration of [$^{14}$C]–Org 25969 to dogs, and there were no apparent sex differences in the plasma kinetics of radioactivity. Systemic clearance of radioactivity was lower than the hepatic and renal plasma flow rates in this species, while the volume of distribution at steady–state was similar to the total volume of body water.

| Parameter | Male | Female |
|---|---|---|
| $C_0$ (µg eq/mL) | 54.72 ± 1.31 | 55.06 ± 3.71 |
| $AUC_t$ (µg eq.h/mL) | 31.75 ± 3.55 | 33.81 ± 3.36 |
| AUC (µg eq.h/mL)* | 32.37 ± 3.55 | 34.47 ± 3.28 |
| $\lambda_Z$ ($h^{-1}$)* | 0.0892 ± 0.0063 | 0.0847 ± 0.0138 |
| Terminal $t_{½}$ (h)* | 7.8 | 8.2 |
| Initial $t_{½}$ ($t_{½-\alpha}$; h) | 0.8 | 0.7 |
| CL (L/h/kg)* | 0.250 ± 0.027 | 0.237 ± 0.017 |
| $V_{SS}$ (L/kg)* | 0.549 ± 0.071 | 0.513 ± 0.0073 |

* Terminal rate constants could not be determined in accordance with acceptance criteria, so they and derived data are approximations only

The excretion was rapid and primarily in urine (approximately 87%) after i.v. administration as shown in the following table (extracted from the sponsor's report).

**Table 23: Excretion of radioactivity in dog following a single i.v. dose of 8 mg·kg$^{-1}$ [$^{14}$C]– Org 25969**

| Excretion | % of the administered dose [$^{14}$C]-Org 25969 (8 mg·kg$^{-1}$) | |
|---|---|---|
| | Male | Female |
| Total urinary excretion (0 – 168 h) | 87.91 ± 3.11 | 87.20 ± 2.05 |
| Total fecal excretion (0 – 168 h) | 0.33 ± 0.26 | 0.80 ± 0.06 |
| Total in cage wash (0 – 168 h) | 2.14 ± 2.15 | 1.98 ± 1.32 |
| Total recovery | 90.38 ± 1.07 | 89.99 ± 1.03 |

Metabolite profiles of plasma and urine determined by radio-HPLC were very similar to that of the dose formulation itself, confirming that little if any biotransformation of Org 25969 occurred in the dog. Two of the minor radioactive components, (b) (4) co-chromatographed with the (b) (4)

The excretion of radioactivity after i.v. administration of [$^3$H]–Org 9426 to dogs either alone or followed, 10 minutes later, by non-radiolabel led Org 25969 is summarized in the following table (extracted from the sponsor's report)

**Table 24: Excretion of radioactivity in dog following a single i.v. dose of 0.36 mg·kg$^{-1}$ [$^3$H]–Org 9426 alone or followed by 8 mg·kg$^{-1}$ Org 25969**

| Excretion | % administered dose | | | |
|---|---|---|---|---|
| | [$^3$H]–Org 9426 alone (0.36 mg·kg$^{-1}$) | | [$^3$H]–Org 9426 (0.36 mg·kg$^{-1}$) and Org 25969 (8 mg·kg$^{-1}$) [a] | |
| | Male | Female | Male | Female |
| Total urinary excretion (0–168 h) | 14.04 ± 0.81 | 12.89 ± 0.62 | 32.52 ± 0.65 | 32.59 ± 4.02 |
| Total fecal excretion (0–168 h) | 71.04 ± 2.31 | 72.20 ± 2.92 | 54.86 ± 1.22 | 55.47 ± 4.69 |
| Total in cage wash (0–168 h) | 2.53 ± 0.87 | 4.04 ± 2.32 | 2.63 ± 1.25 | 2.60 ± 0.60 |
| Total recovery | 87.61 ± 1.54 | 89.13 ± 2.02 | 90.01 ± 1.40 | 90.66 ± 1.57 |

[a] Org 25969 was dosed 10 min after Org 9426

The results revealed that the main route of excretion of radioactivity after i.v. administration of [$^3$H]–Org 9426 to dogs was via the feces, indicating that biliary excretion and/or intestinal secretion played a major role in the elimination of rocuronium in this species. The mean proportion of the dose excreted in urine increased from 13% (males and females combined) after administration of [$^3$H]–Org 9426 alone to 33% when Org 25969 was administered 10 min after the NMBA and the mean proportion of the

dose excreted in feces consequently decreased from 72% to 55%. There was no apparent gender related difference in the routes or rate of excretion.

### 2.6.4.7. Pharmacokinetic drug interaction

None

## 2.6.5 PHARMACOKINETICS TABULATED SUMMARY

None

## 2.6.6 TOXICOLOGY

### 2.6.6.1 Overall toxicology summary

General toxicology:
Among the general toxicity studies the sponsor submitted in this application, 4-wk studies with Org 25969 in rats and dogs are considered to be the pivotal studies for the NDA. A single-dose Org 25969 toxicity study with appropriate recovery evaluation and appropriate histopathological examination submitted are considered to be critical to support the safety of the drug products in the human for single-dose administration and clinical trials. These studies were reviewed by Dr. Adam Wasserman. In addition, a single-dose and 2-wk repeat-dose studies with degraded Org 25969 in rats were submitted to investigate the toxicity of degraded drug product and compare with the non-degraded drug products in order to qualify drug substance impurities and degradants. These studies were also reviewed by Dr. Adam Wasserman. The full review by Dr. Adam Wasserman is inserted in 2.6.6.2 Single-dose toxicity and 2.6.6.3 Repeat-dose toxicity sections. Other studies including 2-wk repeat-dose toxicity studies with Org 25969 in rats and dogs and single-dose toxicity study with Org 25969 in mice, rat, dog and cat were conducted with lower doses that may not cover the systemic exposure in human or lack histopathological examination and/or recovery groups. These studies were not considered to be pivotal and thus are only summarized in the single- and repeat-dose toxicity section. It should be noted that the findings in these non-pivotal toxicity studies were generally consistent with what were found in the pivotal studies.

An extended ingle-dose intravenous toxicity study with Org 25969 (Batch AE) in male and female Wistar rats was conducted to assess the acute toxicity profile of Org 25969 at 0, 200, 600, or 2000 mg/kg (containing 10.7, 32.1, and 107 mg/kg Org 48302) and to evaluate recovery of any findings in a 2-wk treatment-free period. The parameters measured in this study were in accordance with the OECD protocol except that toxicokinetics analysis and ophthalmology were not conducted. The target organs identified in this study were the kidney and the lung. In the kidney, minimal tubular vacuolation were observed at ≥ 600 mg/kg with trend toward recovery being still evident at 2000 mg/kg after 14-day treatment-free. The lungs were noted to have increased incidence of foamy alveolar macrophages at 2000 mg/kg. This change was mild in

nature but did not reverse over 14-day treatment-free period. The NOEL of Org 25969 in rats after single-dose administration is considered to be at 200 mg/kg.

Another single-dose toxicity study was conducted to assess the acute toxicity profile of the degraded drug product Org 25969 and compared with non-degraded Org 25969. Test articles used for the study were specially prepared from a mix of drug substance batches U, W and Z (43.77, 9.03, and 45.49 mg/mL, respectively). Five groups of animals (6/sex/group) were administered with 0, degraded Org 25969 at 200, 600, or 2000 mg/kg, or non-degraded Org 25969 at 2000 mg/kg in this study. This study indicated that the toxic effects produced by degraded and non-degraded Org 25969 were comparable and the incidence and severity of findings were similar. Target organs identified with single-dose administration were the lung and kidney with likely mild hemolysis reflected in the presence of blood in urine. Similar incidence and severity of findings was noted with both the degraded and non-degraded form of Org 25969, NOEL is 200 mg/kg and reversible NOAEL is at 600 mg/kg for degraded Org 25969 based on the results of this study.

In a two-week rat study to assess the toxicity of degraded Org 25969, animals were treated with 0, 50, 100, and 200 mg/kg degraded Org 25969 for 14 consecutive days. Target organs after 2-week degraded Org 25969 administration were identified to be the lung (foamy alveolar macrophage at 200 mg/kg), kidney (cortical tubular vacuolation and tubular basophilia at all doses), and urinary bladder (umbrella cells with foamy cytoplasmic vacuolation at all doses). NOAEL was not identified due to the renal and urinary bladder finding at all dose levels. In the toxicity studies with degraded Org 25969, examinations showed changes in male reproductive organs. In a single dose study which utilized higher dose levels of degraded Org 25969 (0, 200, 600, and 2000 mg/kg) vs. non-degraded Org 25969 (2000 mg/kg), increased number of spermatids (3/6 animals) and hypospermia (1/6 animals) were observed at 2000 mg/kg with degraded but not non-degraded Org 25969. These changes were not seen in animals after 2-wk recovery period. In the 2-wk repeat-dose study, decreased weight of seminal vesicle were observed at all doses in a dose-independent manner (-26%, -25%, and -20% for 50, 100, and 200 mg/kg vs. control), and ruptured epididymal duct (1/8 animals) and sperm granulomas (3/8 animals) were found at 200 mg/kg. Since there were no recovery groups in the repeat-dose study, it was not known whether these changes were reversible.

Two 4-week toxicity studies were conducted in rats with administration of 0, 30, 120, and 500 mg/kg Org 25969 by the same lab. The dosing regimen and the study design of these two studies were similar except that (1) the drug substance was batch U containing in the first study vs. batch AE in the second study; (2) the recovery period was 3 weeks in the first study vs. 8 weeks for the second study. The secondary 4-week toxicity study was proposed to provide sufficient safety margin for the dosage of Org 48302 in human and examine the reversibility of the adverse findings with a longer recovery period (8 weeks vs. 3 weeks). The adverse findings in these two studies were comparable. Both studies were reviewed, but a full review report was only completed on the second study while the results of the first study were summarized. Target organs identified after 4-week administration were lungs (minimal to marked foamy macrophages at 500 mg/kg),

kidneys (minimal to moderate vacuolated cortical tubule cells at ≥ 120 mg/kg), and urinary bladder (minimal to moderate foamy cytoplasm in umbrella cells at ≥ 120 mg/kg). The change in the lung was completely reversible because similar change was not observed after an 8-week recovery period. The change in the kidney was partially reversible while change in the urinary bladder was not reversible at 500 mg/kg after 8-week recovery period. In addition, clinical pathology examination found mild changes including decreased RBC and HCT, PT, platelets, and creatinine levels, as well as increased neutrophils at 500 mg/kg. All these changes were recovered after 8-week treatment-free period. Based on these results, the NOEL of Org 25969 is considered to be at 30 mg/kg and reversible NOAEL is at 120 mg/kg in the 4-wk rat toxicity studies. It should be noted that the results of the TK analysis in the second 4-wk study was discarded because the bioanalysis part was outside of GLP scope. Instead, the sponsor decided to use the results of the first 4-wk TK analysis to estimate the systemic exposure of Org25969 in the second 4-wk toxicity study. It is accepted because the TK analysis indicated that the $C_{max}$ and AUC levels of Org 25969 were increased in proportion to the escalation of the dosing levels and there was no apparent accumulation after 4-wk administration. The sponsor also conducted single-dose PK study of Org 25959 batch AE (see Section 2.6.4.3 Absorption); the results indicated that Org 48302 had a similar PK profile as compared with Org 25969. Therefore, the systemic exposure level of Org 48302 in the second 4-wk study can also be estimated based on the first 4-wk tox study. In the second 4-week toxicity study in rats, special histopathological stains on un-decalcified bone (von Kossa) were included to assess potential histopathological consequences in a semi-quantitative manner. Von Kossa staining and regular histopathological investigations on bone did not indicate a qualitative effect on bone morphology and mineralization.

A 4-week iv toxicity study was also conducted in dogs to evaluate the toxicity of Org 25969 in a non-rodent species. In this study, beagle dogs (4/sex/group) were treated with 0, 25, 80, and 250 mg/kg Org 25969 (batch U) consecutively for 4-weeks to investigate the toxicity of Org 25969. In addition, recovery groups of control and 250 mg/kg (1/sex/group) concurrently treated to investigate the reversibility of potential toxicity findings; there were no recovery groups for 25 and 80 mg/kg groups. Parameters examined in this study were in accordance with OECD protocol. The results of this study indicated that intravenous administration of Org 25969 25, 80, or 250 mg/kg/day to beagle dogs for 4 weeks resulted in minimal systemic toxicity when compared with control animals receiving vehicle. The only notable finding which appeared to be treatment related was a reduction in red blood cell parameters (RBC, HCT, HGB ↓17% vs. controls) at the 250 mg/kg/day dose level in male dogs at the end of 4-week treatment which was not associated with a compensatory increase in reticulocytes and which appeared to return to control or pre-dosing levels with a 2-week treatment-free recovery period. Female dogs did not demonstrate a meaningful change in red cell parameters. Although a 17% decrease in red cell parameters is considered to represent a potential adverse effect level in males, this reduction was not sufficient to elicit a compensatory reticulocyte elevation and was reversible. Therefore, in agreement with the Sponsor, the NOAEL is identified as 250 mg/kg/day. It should be noted that the maximal practical injection volume (2.5 ml for dogs) was reached in this study. Since the maximal

concentration is 100 mg/ml for Org 25969, 250 mg/kg is considered to be the maximum feasible dose (MFD). Toxicokinetics were conducted for the study but were found to be conducted outside of GLP and the resulting data in error; therefore, no direct TK can be ascribed from this study but the Sponsor considers the data from a 3-week PK study in the dog with identical dose levels to be informative. This is accepted since PK data from the 3-week dog study (see Section 2.6.4.3 Absorption) indicated that there were no apparent accumulation after repeat-dose administration and plasma concentration of Org 25969 was general increased in a dose-proportional manner.

Genetic toxicology:

In vitro and in vivo genotoxicity studies were conducted to characterize the genotoxic potential of the active ingredients Org 25969 and Org 48302, and [redacted] (b) (4) impurities of the drug product.

To investigate the genotoxicity potential of Org 25969, in vitro bacterial reverse mutation tests and chromosomal aberration tests in human peripheral blood lymphocytes have been conducted with batches L, T, V, and AE for the purpose of qualification of drug substance batches. One of the drug substance registration batches, AM, was also evaluated in the bacterial reverse mutation test because of a qualitatively slightly different profile with respect to Org 25969-[redacted] (b) (4) All these studies showed negative results indicating that Org 25969 is not mutagenic or clastogenic. In vivo clastogenic activity was assessed in mouse and rat micronucleus assays using a maximum i.v. single dose level of 1774 mg/kg and multiple dose level of 500 mg/kg for 4 weeks, respectively. In mice, the high dose level was intended to be 2000 mg/kg active entity, however due to improper corrections for [redacted] (b) (4) and [redacted] (b) (4) impurity content the actual Org 25969 dose was slightly lower as advised for a single dose limit test from OECD protocol. Since there were no toxic effects observed at this dose level (1774 mg/kg) and genotoxicity of γ-cyclodextrin is not expected due to the relatively large size of molecule, full study using 3 dosing levels were not conducted. This is consistent with the OECD protocol. No clastogenicity was observed in this study. In rats, bone marrow smears taken from rats dosed with 500 mg/kg Org 25969 and 30 mg/kg Org 48302 for 4 weeks (Document ID: NL0059543) did not show an increase in micronucleated polychromatic erythroblasts. Therefore, Org 25969 is not considered to be genotoxic.

As the second active ingredient which is present at [redacted] (b) (4) % according to the drug substance specification, the genotoxic potential of Org 48302 was also investigated by the sponsor. In the in vitro bacterial reverse mutation test and the chromosomal aberration test, no positive results observed when tested up to 5 mg/plate and 5 mg/ml, respectively, indicating lack of mutagenicity and clastogenicity. The in vivo clastogenic potential has been assessed by examination of the bone marrow smears of rats dosed for 4 weeks with 30 mg/kg Org 48302 and 500 mg/kg Org 25969. No in vivo clastogenicity was observed. Therefore, Org 48302 is not likely to be genotoxic.

As the [redacted] (b) (4) present in several drug substance batches, [redacted] (b) (4) contains a structural alert [redacted] (b) (4)) for genotoxicity, bacterial reverse mutation tests and a

chromosomal aberration test were performed using maximum exposure levels (5 mg/plate or mg/ml, respectively) to investigate the potential of genotoxicity. Both genotoxicity tests indicated no mutagenic or clastogenic response was observed. In addition, the (b) (4) synthesis intermediate was evaluated for in vitro mutagenic and clastogenic potential as part of a study package to evaluate the toxicity of this intermediate in the framework of a chemical notification. It was not genotoxic in vitro.

In addition, Org 25969 batch AJ, a batch in which the Org 25969 content was artificially decreased by chromatography resulting in an increase in the content of (b) (4) and impurities by on average approximately a factor (b) (4), was used to further investigate the genotoxic potential of (b) (4) and impurities, and the results indicated no evidence of genotoxicity was present in this special batch AJ. To assess the genotoxic potential of degradation products, degraded drug product was prepared by accelerated degradation. Neither the bacterial reverse mutation test chromosomal aberration test revealed any genotoxic potential.

Carcinogenicity:
Carcinogenicity evaluation was not conducted and is not needed since the drug is proposed for single-dose use and long-term exposure is not expected.

Reproductive toxicology: Reproductive and developmental studies have been conducted to investigate the fertility and early embryonic developmental toxicity in rats (segment I), the embryo-fetal developmental toxicity in rats and rabbits (segment II), and perinatal and postnatal developmental toxicity in rats (segment III).

In the segment I fertility study, both male and female rats (22/sex/group) were treated with Org 25969 (batch U) at 0, (0.9% NaCl), 20, 100, and 500 mg/kg via intravenous bolus injection in the tail vein. For males, animals were treated on day 29 prior to mating and continued after mating until sacrifice. For females, Org 25969 was given from day 14 prior to mating through the mating period until gestational day 5 (GD5). The males were sacrificed after mating, and the pregnant females were sacrificed on GD15. Macroscopic examinations were conducted in both male and females with special attention on the reproductive organs. The weight of these organs was also recorded. Hysterectomy was performed in females and fertility/mating parameters were calculated. The results of this study showed light decrease in body weight of female animals (about 10%) indicating maternal toxicity at 100 and 500 mg/kg. Macroscopic post-mortem examination found no Org 25969 treatment-related changes in male or female reproductive organs. Organ weight of these tissues in Org 25969 treated groups were not significantly different from that of the control, histopathological examination was not conducted. There were no change in mating index and fertility parameters including mating index, fertility index, pre-coital interval, live concepti, and gestational index in Org 25969 treated groups as compared to control. The NOAEL for fertility and early embryonic development is considered to be at 500 mg/kg for rat based on the results of this study.

The segment II embryofetal development studies were conducted in both rats and rabbits. In the rat study, pregnant animals (22/sex/group) were treated with Org 25969 (batch U) at 0, 20, 100, and 500 mg/kg during organogenesis from GD6 to GD17. Animals were sacrificed on GD20 and subjected to hysterectomy. The weight of ovaries of all females was also recorded. The ovaries and uterus were examined to determine the number of corpora lutea, number and distribution of dead and live fetuses, number and distribution of early and late resorptions, and the number and distribution of implantation sites uterine scars). After hysterectomy, macroscopic post-mortem examination of the principle thoracic and abdominal organs, and a gross evaluation of placentas was also performed. Fetuses were examined for body weight, sex ratio, and external alterations including all visible structures, surfaces and orifices of all groups. Skeletal and soft tissue malformation and variation were examined at control and high dose group. Minimal maternal toxicity was observed at 100 and 500 mg/kg represented by a minimal decrease in body weight gain (approximately 7%) during the first-week treatment (GD 6-9) and decrease in food consumption (approximately 7%) during the treatment period (GD6-18). There were no treatment-related changes in pre- and post-implantation loss, and live fetus numbers in hysterectomy assay. There were no treatment-related changes in fetal examination except increased dilated renal pelvis in animals of 500 mg/kg (6.5%) as compared to control (0.7%) - examination was not conducted at 30 and 150 mg/kg. However, this change was not considered to be adverse since this change is considered a variation but not abnormality and the recorded value (6.5%) was still within the historical range of the laboratory (mean = 4.9%; range 0 – 8.5%). TK analysis was not conducted in this study, but data from the PK/TK analysis in the general toxicity and PK studies in this species is considered to be appropriate to estimate the systemic exposure in pregnant rats. Overall, the primary change in the pregnant rats with Org 25969 administration during organogenesis was minor body weight decrease in which is monitorable and of little toxicologic significance without evidence of effect on embryofetal development. Therefore, the NOAEL for embryofetal developmental toxicity in rats is considered to be at 500 mg/kg

In the rabbit study, pregnant New Zealand white rabbits (20/group) were treated with Org 25969 (batch U) at 0, 20, 65, 200 mg/kg during organogenesis from GD6 to 18, and the animals were sacrificed on GD29 for embryofetal developmental toxicity evaluation. The parameters examined were similar to those measured in the rat segment II study. The results of the study showed a dose-dependent decrease in body weight gain in dams during the treatment period at 20 (↓5.4%), 65 (↓16%) and 200 mg/kg (↓31%) as compared to the control, likely due to decreased food consumption. This change was reversible as represented by compensatory increase in body weight gain during the treatment free period from GD 19 to 29. Macroscopic examination in dams indicated changes in gonads (conjunctive tissue, periovarian region: serous cysts) in 1/20 animals of all drug-treated groups, but not in control. This change was not dose-dependent, and reportedly occurs in animals of this strain at this age, suggesting incidental change. Decrease in offspring body weight was also observed in all groups of animals with approximately 5%, 10%, and 14% at 20, 65, and 200 mg/kg, likely due to maternal toxicity. Skeletal examination showed increased skeletal variation including increased percentage of incomplete ossification of 1$^{st}$ to 4$^{th}$ sternebra and 6$^{th}$ sternebra, and

unossified 1st metacarpal at 200 mg/kg (examination was not conducted at 20 and 65 mg/kg), likely to be at least partly due to the lower fetal weight in this group. These changes are within historical control value range except the incomplete ossification of 1st and 4th sternebra (12.6% vs. 1.4 – 11% in historical control). Overall, these skeletal variations may be due to the lower body weight and are considered to be of no major toxicological significance. Skeletal examination also showed malformation such as thoracic hemivertebra and misshapen thoracic vertebra, misaligned sternebra, and fused rib in one fetus at high-dose group, which was not seen in the control. This change is considered to be incidental because of the low incidence and similar changes are reported in normal animals of historical control. Overall, there were no remarkable adverse effects identified in both dam animals and pups. The NOAEL for embryofetal toxicity in rabbits is considered to be at 200 mg/kg based on the results of this study. The maximum feasible dose (MFD) in rabbit was considered to be reached in this study since the maximal injection volume is 2 ml and the maximal concentration of Org 25969 drug substance is 100 mg/ml. TK assays were conducted in the same animals in this study to evaluate the systemic exposure of Org 25969 in rabbit. Ten of 20 animals in each Org 25969 treated group were selected to have blood samples collected on GD14 and plasma concentration was determined. Both Cmax and AUC level increased in a dose-proportional manner in pregnant rabbits, and the NOAEL is corresponding to AUC0-∞ level 1400 μg · hr/ml

The segment III studies were designed to detect adverse effects of Org 25969 on pregnant/lactating female ($F_0$) and on the development of the conceptus and the offspring ($F_1$) following exposure of Org 25969 to the $F_0$ animals. Observations were continued through sexual maturity of the F1 generation and assessment of the reproductive performance of F1 adults. In this study, pregnant rats (22/group) were treated with saline vehicle or Org 25969 (batch W) at 30, 120, 500 mg/kg from GD6-PND21 (post-natal day 21). In addition, one satellite group of 5 vehicle-control animals was added to serve as dams to deliver age- and gender-matched controls for pups of the drug treated groups which died or were euthanized during the first 5 days post partum. These controls were necessary for histopathological evaluation of deceased pups. All females survived until the scheduled sacrifice and no clinical signs attributable to Org 25969 treatment were noted. Slightly reduced food consumption was noted between days 6 and 21 of pregnancy (-4.9% compared to saline control). Mean absolute maternal weights and body weight gain were similar in all groups during pregnancy and lactation and no abnormalities were noted in the dams at necropsy. There were no Org 25969-related effects on the mean number of implantation sites, gestation and parturition noted and pregnancy rate was comparable between Org 25969 treated and control groups. In addition, there were no treatment-related macroscopic changes observed in any Org 25969 treated groups as compared to control at PND21. Neonatal mortality appeared to be increased during PND0-4 in the high-dose (500 mg/kg) and mid-dose group (120 mg/kg). Initial examination indicated increased postnatal loss from the first litter check on day 0 to 4 post partum was found to be increased in the high dose group compared to control (19.2% vs. 10.4%) with observed total litter loss of 2, 0, 2, and 3 dams in groups of control, 30, 120, and 500 mg/kg, respectively, during this period. However, in group 3, two dams had extremely small litter size (2 or 4 pups) as compared to others at the first

litter check; while the implantation sites of these two animals were 13 and 18, respectively, indicating cannibalization occurred in these two dams immediately after birth and before the first litter check. In addition, there was one complete litter loss each in group 2 and group 3 before the first litter check after delivery, which is also most likely due to cannibalization since the body weight of these two animals were comparable with others. Collectively, there appeared to be increased postnatal loss in group 3 (120 mg/kg) and group 4 (500 mg/kg) from the birth on postnatal day 1 to postnatal day 4, most likely due to cannibalization. Histopathological examination of heart, lungs, liver, and kidneys of the F1 pups of dead or partially cannibalized did not identify the potential mechanism of postnatal loss during PND0-4. There were no remarkable abnormalities observed in the surviving $F_1$ pups during the lactation period. In addition, there were no remarkable treatment-related changes in F1 surviving animals in physical development, behavior evaluation, necropsy, and reproduction evaluation. Overall, the primary change was postnatal $F_1$ pup loss during PND0-4. The mechanism was unknown. The sponsor indicated that the postnatal loss during PND0-4 may be due to treatment-related increase in cannibalization. This reviewer agreed this possibility should not be excluded. The NOAEL for postnatal development was considered to be 30 mg/kg.

To investigate the potential mechanism of increased postnatal loss in Org 25969 treated groups at 120 and 500 mg/kg, two follow-up studies were conducted. In the first study, 22 pregnant rats were treated from GD6 to PND4 with saline or 500 mg/kg Org 25969 to compare the postnatal loss of offspring during PND0-4. The results showed no difference in neonatal death between control and treated groups, indicating that increasing of postnatal loss in the regular prenatal and postnatal development study may be of incidental change. In addition, fetuses obtained by cesarian section from animals treated with 500 mg/kg Org 25969 showed good viability and a normal distribution of live and dead fetuses, which may indicate no intrinsic abnormalities within the fetuses that caused the dams to cannibalize their young. However, this study also attempted to determine the most sensitive period during GD6 to PND4 causing increased neonatal mortality by treating pregnant rats with 500 mg/kg from GD6-10, GD11-15, GD16-21, and PND0-4 and comparing postnatal loss during PND0-4 with the control group that treated with saline from GD6-PND4. The results showed that the group treated from GD16-21 had a higher neonatal mortality rate than the control as 5/21 females in this Org 25969 treated group were observed to have cannibalized the majority of their litters vs. 1/19 placebo females in the control group. The reason for this finding was not known. The sponsor indicated that increased cannibalism in this group was likely to be due to stress since these animals had not been accustomed completely to the dosing procedure (6-day treatment; GD16-21); however, drug treatment may play also play a role on this change. However, this can not explain the findings in the regular peri- and postnatal study since all treatment to animals were started from GD 6. The purpose of the second follow-up study was to investigate whether ex-utero or in-utero exposure of Org25969 is the possible cause of the increased incidence of cannibalism in animals with Org 25969 administration. To investigate effect of the ex-utero exposure of Org 25969, pups from two groups of pregnant females (22-24/group) without any treatment during pregnancy were swabbed with saline or 0.5 mg/ml Org 25969 after birth and observation of maternal nesting and nursing behavior, neonatal mortality or maternal cannibalism were recorded.

Results showed neonatal survival was comparable in the litters in which the pups were swabbed with 0.5 mg/kg Org 25969 as compared to the litters in which the pups were swabbed with saline (95.8% and 92.1%, respectively). To investigate in-utero exposure of Org 25969, pups in from two groups of pregnant rats treated with 500 mg/kg Org 25969 (23/group) and 0.9% saline (22/group) from GD6-21 were delivered by C-section on GD21 and cross fostered to untreated dams to observe for pup death and cannibalization from PND0-4. The results showed a slightly increase in total fetal death in the in-utero Org 25969 exposed litters as compared to the in-utero placebo exposed litters (neonatal survival ratio 79.4% vs. 85.1%). These results likely suggest slightly increased cannibalization due to in-utero Org 25969 exposure. However, the mechanism for this effect was not known. The sponsor hypothesized that cannibalism may be triggered by the presence (e.g. smell or taste) of Org 25969 in the fetal urine. Although this possibility can not be excluded, this study did not provide direct evidence to support this hypothesis.

Juvenile studies

In support of pediatric trials, developmental toxicity studies in juvenile rats with single- and repeat-dose administration were conducted to further investigate the potential toxic effects of Org 25969 during the juvenile developmental period. In a single-dose treatment study, animals at age of 7 days, 14 days, and 21 days (16/sex/group) were injected subcutaneously (for animals of 7 days old) or intravenously with Org 25969 at 0 (0.9% NaCl), 120, and 500 mg/kg; eight rats were sacrificed 24 hours after dosing to examine the acute effects of Org 25969 in juvenile animals and the other were sacrificed 14 days after dosing to investigate the reversibility of the potential changes. There were no remarkable clinical signs, mortalities, changes in body weight and parameters of clinical pathology were considered to be treatment-related. Reversible minimal to slight tubular vacuolation in kidney were observed in animals of age 7 days and 14 days at 500 mg/kg; this change were also seen in single- and repeat-dose toxicity studies in rats. Similar changes were occasionally seen at 120 mg/kg. All these changes appeared to be reversible. In addition, minimal disruption of the enamel epithelium with presence of amorphous material was observed in the incisors of one female rat treated of age 21 days at 500 mg/kg on day 14 after dosing. The NOAEL is considered to be 120 mg/kg in this study due to teeth effect. The second single-dose toxicity study in juvenile rats were designed to investigate the adverse effects of Org 25969 in teeth and bone. Juvenile Wistar rats were dosed once on day 7 of age with either 0 (0.9% NaCl), 30, 120 or 500 mg/kg of Org 25969 via subcutaneous (SC) injection. Three animals/sex/group/time-point were sacrificed on SD 1, 3, 7, 14, 21, 28, 35, or 42, and incisor and bone samples were collected. Mild whitish discoloration of incisors (mottled appearance) was observed at all groups including control with 2 (mild), 4 (minimal: 1; mild: 2; more severe: 1), 2 (mild), and 7 (minimal: 1; mild: 5; more severe: 1) at 0, 30, 120, and 500 mg/kg. Since white discoloration of incisors was also observed in control animals, the relevance of this effect to Org 25969 is difficult to determine. However, the incidence and severity of whitish discoloration was slightly higher at 30 and 500 mg/kg but not at 120 mg/kg, which suggested this change is likely to be treatment-related at 500 mg/kg.

In a preliminary two-week toxicity study in juvenile, rats at age of 7 days were SC injected with vehicle control (0.9% NaCl), or Org 25969 at doses of 30, 120, and 500 mg/kg/day for 2-weeks, and the toxicity and toxicokinetic parameters were determined. There were no treatment-related clinical signs and body weight change observed in groups treated with Org 25969 as compared to control animals. In addition, there were no treatment related gross necropsy findings and organ weight changes in contrast to control. Histopathological examination found minimal to moderate changes in lung, kidney, and urinary bladder which were comparable to what was found in adult rats. Reversibility of these changes was not examined in this study. Blood samples were collected for TK analysis at 0 (pre-dose sample), 2.5, 5, 10, 20, 40 min, 1, 3, 7, and 24 hours after 2-week dosing at day 1 and day 14. TK assay indicated there were no substantial difference between males and females. Systemic exposure was generally dose-proportional. The AUC levels in animals at age of 21 days after multiple dosing for 2-weeks were slightly lower than those in animals on day 7 after single dose. The AUC levels appeared to be comparable to those in adult rats from the 4-week repeat-dose treatment; the $C_{max}$ levels appeared to be lower in juvenile animals both at age of 7 days and 21 days likely due to the difference in route of administration: SC for juvenile animals and IV for adults.

The adverse effects of Org 25969 after multiple dose administration were further investigated in a 4-week juvenile rat study. The dosing levels utilized in this study were 0, 30, 120, and 500 mg/kg Org 25969. Animals (22/sex/group) were administered drug or vehicle at day 7 after birth via SC injection for two weeks, and then switched to IV injection for 2 weeks. Ten males and ten females per group were sacrificed after the termination of dosing and 12/sex/group were sacrificed after an eight-week recovery period. TK analysis was conducted in this study. For the single-dose TK phase, additional animals groups (24/sex/group) treated with Org 25969 were allocated to the study for blood samples for assessment of toxicokinetic parameters after a single dose on day 7 of age. For the multiple-dose TK phase, blood samples were collected from the recovery phase animals after the termination of treatment. Plasma levels of Org 25969 were comparable for males and females with a roughly proportional increase with increasing dose. Upon repeated dosing, plasma levels were lower at 34 days of age after 28-day administration when compared with those following a single dose on Day 7 of age. The reason was not known. The sponsor suggested this was probably due to the increased glomerular filtration rate (GFR) at 34 days of age. This explanation does not appear to be reasonable. This reviewer agreed that the difference in GFR may play important role in the systemic exposure of Org 25969. However, a comparison with the adult TK data indicated that systemic exposure levels of Org 25969 appeared to be closer to those in animals at age of 7 days. It is believed that the glomerular filtration function in rats is more mature and similar to that in adult at age of 34 days than age of 7 days. Therefore, GFR difference is not likely to be the reason for the difference in the systemic exposure in animals of 7 days old and 34 days old. General toxicity effects in juvenile rats after 28-day administration were comparable to those observed in adult rats primarily including minimal to slight histopathological changes in the lungs, kidneys, and urinary bladder. Neurobehavioral examinations including motor activity, sensory function and reactivity, and learning and memory (Morris water maze) after treatment did not show

any sign of abnormality. There was no body weight change during the treatment period, but minor decrease in body weight gain of both males (5-8%) and females (<5%) at 120 and 500 mg/kg were observed as compared to the control during the 8-week treatment free period. At the end of 8-week recovery period, the body weight of animals treated with Org 25969 at 120 and 500 mg/kg were still slightly lower than that of control in both males (4%) and females (3%). Significantly increased in liver weight was observed in males at 120 mg/kg (10%) and 500 mg/kg (14%) while increase in females was minimal (≤5%). Significant increase in lung and bronchi weight was observed at 500 mg/kg in both males (32%) and females (18%); increase was also observed at lower doses without statistical significance. In addition, weight of seminal vesicle was decreased at all doses (18% to 20%) with statistical significance at 500 mg/kg. All the changes in organ weight were reversible after 8 week recovery and were not seen with single dose administration in juvenile rats at 500 mg/kg. Particularly, effects of Org 25969 in teeth and bones were evaluated in this study. In teeth, abnormal color (white marbled/mottled banding), malocclusion and/or overgrowth of the incisors in the majority of animals receiving 500 mg/kg (21 of 22 animals per sex), which were generally became apparent following at least 2 weeks of treatment and persisted for up to five weeks into the recovery period. These changes were also seen in some animals of 120 mg/kg (2/22 per sex). Histopathological examination showed dose-dependently increased disruption of enamel epithelial layer in the incisor with 1/20 (minimal), 0/20, 6/17 (5 minimal, 1 slight), and 15/20 (3 minimal, 5 slight, 6 moderate, 1 marked) at 0, 30, 120, and 500 mg/kg, respectively. Deposition of amorphous material in the incisor enamel epithelial layer were observed at 120 (4/17, minimal) and 500 mg/kg (17/20, minimal to marked). Minimally to markedly deposition of amorphous material in the molar enamel epithelial layer were found in 5/10 males and 5/10 females of the 500 mg/kg/day group. Recovery was complete in 8 weeks for the changes in the molars but not fully complete for the incisors (1/24 at 120 mg/kg and 2/24 at 500 mg/kg). The NOAEL for tooth effect was at 30 mg/kg. In bones, 4-week continuous administration of Org 25969 in juvenile rats from Day 7 of age elicited minor but statistically significant changes in bone parameters of femur as assessed by μCT including transiently increased trabecular BMD (9% in M, 18% in F), bone volume/tissue volume trabecular metaphysis (19% in M, 24% in F) , and trabecular number (13-15%) and trabecular thickness (6-8%) at 500 mg/kg, along with dose-independently decreased cortical thickness at both 120 (9-10%) and 500 mg/kg (7-8%). The biological significance of these is not clear. Histopathological examination found a very low incidence of chondromucinous degeneration in the 120 and 500 mg/kg (one animal at each group) and fibrosis at 500 mg/kg (one animal) in femur. Moreover, 4-week continuous administration of Org 25969 caused minor decreased ulna length (< 5%), femur length (< 5%) and weight with more severe effect on Day 91 (end of recovery period) at 120 and 500 mg/kg. The association of this change to body weight decrease was unknown. In addition, decrease in polar moment of inertia, a parameter indicative of the ability of bone to resist torsion, was observed on Day 91 in all groups of males and 500 mg/kg group of female. All these may suggest a minimal prolonged effect of bone growth. Overall, all these change may be indicative of minor but lasting effect on bone growth at 500 mg/kg as well as at 120 mg/kg; this effect was not likely to be secondary to the minimal body weight decrease. In this study, 30 mg/kg is considered to be NOAEL.

Special toxicology:

*1. Special studies regarding potential effects on bone and teeth*

Tissue distribution studies using [$^{14}$C]-Org 25969 indicated that the test compound appeared to be retained bones in the skeletal system and in teeth with average $T_{1/2}$ of 176 days. In the second 4-week toxicity study in rats, Von Kossa staining and regular histopathological investigations on bone did not indicate a qualitative effect on bone morphology and mineralization. The sponsor thus conducted two dedicated studies to investigate the potential effects of Org 25969 on bone quality, structure, and turnover following single dose IV administration in Wistar rats. The effects on teeth were also evaluated in these studies.

In the first study, male and female rats (16/sex/group) were injected intravenously with Org 25969 (batch AE) at 0 (0.9% NaCl) and 2000 mg/kg for a single-dose administration. Bone turnover markers (BTM) were measured on day 1, 7, 21, and 42 days after administration. In addition, eight/sex/group animals were sacrificed on day 21 or 42 for evaluation of μCT and bone strength. Single-dose 2000 mg/kg administration caused statistically significant change in bone parameters via μCT assay on day 21 after dosing as compared to the control, including decrease in trabecular bone volume/tissue volume (18% in female; 8% in male), trabecular number (↓10%), along with increased trabecular separation (31% in female; 11% in male). Trabecular bone mineral density also appeared to be decreased (female ↓18%, male ↓8%), although statistical significance was not reached. In addition, bone strength assessment indicated decreased femur stiffness (femur indentation test: ↓19% in female, ↓47% in male; 3-point bending test: ↓10%) or stress (femur indentation test: ↓27% in female, ↓50% in male) on day 21 after dosing. In addition, indentation test showed decreased energy, maximal load, indicating compromised bone strength of the distal femur on day 21. BTM assay showed increased serum CTx and urine DPD, markers for bone resorption, primarily in females at this time-point. All these suggest a minor bone resorptive effect with greater severity in females than males. Decreased trabecular number and increased trabecular separation test lasted up to day 42 after dosing, suggesting a prolonged bone effect. Decreased stiffness in the femoral mid shaft by femur 3-point bending test and increased stiffness of the trabecular bone in the marrow cavity of the distal femur by indentation test on day 43 after dosing, further indicative of bone effect by Org 25969. Stiffness and stress in the marrow cavity of distal femur was significantly increased as compared to control on day 42 after dosing, which may suggest a compensatory response. μCT assay and bone strength assessment were not conducted on day 0 and day 7 after dosing. Examinations of BTM did not suggest a remarkable change in bone during this period. Possible teeth discoloration due to Org 25969 were evaluated by determination of the color of the (upper part) of the lower incisors of the rats. There was no effect of discoloration on incisor after single-dose of 25969 at 2000 mg/kg. Overall, this study indicated that Org 25969 at 2000 mg/kg produced resorptive effect in femur after 21 days of dosing but no effect on teeth discoloration after single-dose administration. The bone effect of Org 25969 was likely to be reversible since the changes in μCT parameters and BTM appeared to be less severe as compared to those on day 21.

In the second study of bone effect, rats were treated with Org 25969 at 0, 30, 120, and 500 mg/kg for single-dose administration. Animals (3/sex/group/time-point) were sacrificed on day 1, 3, 7, 14, 21, 28, 35, and 42 after dosing for bone samples collection. Femurs were subjected to μCT assay. The results of the study did not indicate any remarkable adverse effects in the bone, and therefore, NOAEL is considered to be at 500 mg/kg in this study. A PTH surge was observed in both rats and dogs upon sugammadex administration at >80 and >120 mg/kg. In the rat (30, 120, 500 mg/kg) this increase was maximal at 3 min post dose (4x at 500 mg/kg with statistical significance). PTH returned to baseline by 30 min. Serum Ca was increased for 30 min and urine P excretion was increased over 24h. In dogs, the PTH surge lasted about 15 min based on analysis on samples obtained from the 3-week TK dog study. Sponsor concluded this transient effect is probably not relevant in the reduction of trabecular mass and strength since bone changes persisted through 3-6 weeks following single dose administration.

*2. Immunogenicity*

There were 3 studies conducted to examine the immunogenic property of Org 25969. PFC study was firstly conducted to evaluate the potential immunosuppressive effect after 2-week IV injection of org 25969 at 30, 120, and 500 mg/kg. However, Org 25969 treated groups showed slight increase in PFC formation in a dose-independent manner, suggesting immuno-stimulation. The second study was a popliteal lymph node assay (PLNA) assay conducted in mice to examine the stimulatory effect of Org 25969 after subcutaneous (SC) footpad injection at three different dose levels (0.3, 1.2 and 5 mg/animal). The results showed a small but statistically increased [$^3$H]-thymidine incorporation in PLN at 500 mg/kg (2× for Org 25969 vs. 26× for positive control) as compared to vehicle control, indicating weak stimulation. The 3$^{rd}$ study was a local lymph node assay (LLNA) assay to examine skin contact hypersensitivity to Org 25969 after topical application on the ears of animals. The results showed negative in [$^3$H]-thymidine incorporation in auricular lymph node. However, skin absorption of Org 25969 has not been characterized; therefore the validity of the study is questionable.

**2.6.6.2 Single-dose toxicity**

The sponsor has submitted 2 IV single-dose toxicity studies in CD-1 mice with administration of Org 25969 up to 2000 mg/kg. In the first study (NL0026309), animals were treated with Org 25969 at 100 mg/kg and no abnormalities in clinical signs, body weight, and gross necropsy examination were found. In the second mouse study (NL0041688), animals (6/sex/group) were treated with Org 25969 at 0 (water control) and 2000 mg/kg, and there were no mortalities, change in body weight, and abnormalities in gross necropsy examination and injection site histopathological examination found after administration. Mild clinical signs including reduced activity, ptosis, less extensive grooming in one male mouse were observed and were not considered to be Org 25969 treatment related. Therefore, it can be concluded that maximum non-lethal dose in CD-1 mice after a single intravenous dose of Org 25969 exceeds 2000 mg/kg. The sponsor also conducted a toxicity study in anesthetized cats (NL0037029). The study was designed to asses the local and systemic toxicity of Org 25969 in the absence or presence of Org 9426 (Rocuronium). Org 25969 and Org 9426 were both administered via a bolus, IV injection in 3 sub-doses with ½ hour apart, followed by a 14-day observation period. Cats

(4/sex/group) were treated with 3 ×1 mg/kg or 3 × 10 mg/kg Org 25969, alone (group 1 and 2) or following 3 × 0.5 mg/kg Org 9426 (group 3 and 4). Two cats (1/sex) was received control vehicle (0.9% NaCl) concurrently. In-life observations including mortality, physical examination, and body weight and food consumption measurement were conducted. Neuromuscular function was examined for the efficacy of Org 25969. Cardiovascular assays including ECG and blood pressure, clinical pathology parameter assays, and blood gas analysis were performed. In addition, macroscopic and microscopic pathology examination was also conducted. The study showed that Org 25969 induced a dose-related NMB reversal. Macroscopic examination found lesions in the skeletal muscle and skin including discoloration and edema in the Org 25969 treated animals in a dose-independent manner. Histological examination indicated myonecrosis in the skeletal muscle. Ovary cyst was observed in Org 25969 treated groups histologically correlated with unremarkable mature follicles. The Sponsor has indicated that these changes were likely to be incidental or due to catheterization or other anesthetic drugs co-administered. These changes were not seen human clinical trials. A single-dose study in dogs was conducted with similar dose regimen to that in the cat study described above except that the dosage of rocuronium was 1.08 mg/kg (3 × 0.36 mg/kg) (NL0034953). There were no remarkable adverse effects observed in this study. In the second single-dose dog toxicity study (INT00035242), animals were treated with Org 9426 followed by Org 25969 after 2 minutes in a single day with the following scheme as extracted from the sponsor's report:

| | Exposure Level[a] 3 times a day in a single day | | Number of Animals | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total[b] | | Toxicokinetics | | Clinical Pathology Pretest and Termination | | Necropsy Termination Day 4 | | Microscopic Pathology | |
| Group | Dose Org 9426 (mg/kg) | Dose Org 25969 (mg/kg) | M | F | M | F | M | F | M | F | M | F |
| 1 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 2 | 3 x 0.36 | 3 x 0.92 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 3[c] | 3 x 3.6 | 3 x 13 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 4[c] | 3 x 18 | 3 x 65 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |

Administration of doses 3 × 3.6 mg/kg and 3 × 18 mg/kg Org 9426 resulted in transient dose-related decreases in blood pressure and heart rate values which were reversible following administration of Org 25969. At the highest dose of 3x18 mg/kg Rocuronium, transient QT/QTc prolongation occurred secondary to decreases in heart rate that was reversible following administration of Org 25969. Transient decreases in PR intervals at 3x65 mg/kg Org 25969 were also observed and considered secondary to increases in heart rate observed at this dose. One of 8 animals dosed with 3x18 mg/kg rocuronium plus 3x65 mg/kg Org 25969 had an increase in blood urea nitrogen (BUN), creatinine and kidney weight with no corresponding microscopic changes observed in the kidney. The NOAEL in this dog study was considered to be at 3x3.6 mg/kg rocuronium plus 3x13 mg/kg Org 25969.

In rats, there were 6 single-dose toxicity studies conducted. The first two studies (NL0025853; NL0041666) were exploratory studies without histopathology examination conducted and the results indicated that Org 25969 did not produce any adverse effect at 100 mg/kg. and mild clinical signs including spasm in the legs, staggered gait, reduced activity, ventral and/or lateral recumbency and labored breathing, and concluded that maximum non-lethal in male and female Wistar rats after a single intravenously dose of

Org 25969 exceeds 2000 mg/kg. In the third and forth study, animals were treated with Org 25969 at 0, 100, and 250 mg/kg using batch T and batch V (NL0042813; NL0042813), respectively; animals were examined for clinical observations, clinical pathology, and histopathology on day 1 and day 14 after dosing. The results indicated that both Org 25969 drug product batch T and V did not cause effects with toxicology significance at dose level up to 250 mg/kg following single-dose administration. The fifth and sixth rat studies were designed to assess the toxicity of non-degraded and degraded Org 25969 at a dose level up to 2000 mg/kg. The review of these two studies was conducted by Dr. Adam Wasserman and inserted in the following.

### STUDY TITLE: AN EXTENDED SINGLE-DOSE INTRAVENOUS TOXICITY STUDY WITH ORG 25969 (BATCH AE) IN MALE AND FEMALE WISTAR RATS

**Study purpose:** Objectives were to assess the acute toxicity profile of Org 25969 in Wistar rats with Single intravenous administration of batch AE at 0, 200, 600, or 2000 mg/kg and to evaluate recovery of any findings in a 2-week treatment-free period. The effects of Org 25969 on bone were also evaluated.

**Key study findings**:
Intravenous single-dose administration of Org 25969 (Batch AE) at 0, 200, 600, or 2000 mg/kg/day to rats with acute and 14-day recovery group sacrifice revealed a NOAEL of 200 mg/kg/day.

- Clinical signs of cramps noted in males and females at 2000 mg/kg/day acutely, which recovered shortly after administration
- Target organs identified were the **kidney** ≥ 600 mg/kg/day with evidence of **renal tubular vacuolation**, minimal in severity, and which trended towards recovery being still evident at 2000 mg/kg/day after 14-days treatment-free. The **lung** was also noted to have **increased incidence of foamy alveolar macrophages** at 2000 mg/kg/day in both males and females. This was mild in nature but did not reverse over the 14-day treatment-free period.

** TK was not conducted but RD toxicokinetic studies (4-week rat tox w/3 wk recovery) had data which indicated no accumulation and an AUC which dose-normalized was 2 (µg*h/mL)/mg. A NOAEL of 200 mg/kg would be expected to be associated with an $AUC_{0-24}$ of ~400 µg*h/mL (or ~24,000 µg*min/mL); this would provide coverage for human use (AUC = 10,143 µg*min/mL) at 16 mg/kg dose level.

**Study no.**: NL0059572
**Volume #, and page #**: Original submission, section 4.2.3.1 (electronic)
**Conducting laboratory and location**: N.V. Organon, Department of TDD, Nistelrooise baan 3, NL-5374 RE Schaijk, The Netherlands
**Date of study initiation**: Final report issued February 2005
**GLP compliance**: Yes
**QA report**: Yes

**Drug, lot #, and % purity**: Org 25969 Batch AE (#TY-006-1); 89.3% Org 25969, 5.35% Org 48302

| TEST | SPECIFICATION | RESULT |
|---|---|---|
| Related substances | For information only (nr, d=3, u/d=1) | (b) (4) Unspecified degradation products (b) (4) Total in DS = (b) (4) |
| Identification | Org 25969 present (r, d=1, u/d=1) | Org 25969 present |
| Sterility | Sterile | Sterile |
| LAL-test | To be determined | < 12.5 EU/mL |

## METHODS

### *Test System*

**Species/strain**: Wistar rats, Hsd/Cpb:WU (spf) supplied by (b) (4)
**Age**: Not specified, weight suggests ~ 8 weeks of age
**Weight**: 236 - 291 g ♂; 152 – 195 g ♀

### *Study Design*

| Group No. | Group size and sex | | Dose (mg·kg$^{-1}$) | Treatment |
|---|---|---|---|---|
| | Male | Female | | |
| 1 (placebo-PL1) | 12* | 12* | 0 | Vehicle |
| 2 (low-dose, DS2) | 12* | 12* | 200 | Org 25969 |
| 3 (mid-dose, DS3) | 12* | 12* | 600 | Org 25969 |
| 4 (high-dose, DS4) | 12* | 12* | 2000 | Org 25969 |

* 6 animals per group/sex were sacrificed approximately 24h after dosing. The remaining 12 animals in each group (6 males and 6 females), were sacrificed approximately 14 days after dosing.

**Doses**: 0 (vehicle), 200, 600 or 2000 mg/kg/day Org 25969 (*containing 0, 10.7, 32.1, or 107 mg/kg/day Org 48302*)
**Route, formulation, volume, and infusion rate**: Intravenous infusion, vehicle is physiological saline (batch 1452D17/01l06b)
**Number/sex/group or time point (main study)**: 6 rats/sex/group (main)
**Satellite groups used for toxicokinetics or recovery**: 6 rats/sex/group (recovery at 2 weeks post-dosing)
**Unique study design, husbandry or methodology issues**: A total dosing volume of 20 ml/kg was administered as 2 separate IV injections of 10 ml/kg 1 – 1.5 hr apart on SD0.

#### METHODS: OBSERVATIONS AND TIMES

| Observational Parameter | Methods |
|---|---|
| Toxicokinetics | Not conducted |
| Clinical Observations | Morbidity and mortality during in-life period. Evaluated regularly for behavioral and physical abnormalities during dosing and post-dosing periods. *Reviewer's comment: Sponsor does not define "regularly" though data suggests this was done daily at least.* |
| Body Weights | 1x/week pre-dosing, SD0 (prior to dosing), SD1, and at least once a week during the observation period. During post-dosing period body weight was determined once a week. |

<table>
<tr><td>Food Consumption</td><td>Recorded weekly per cage (2 animals of same sex/cage) through in-life period by weighing amounts given and remaining and dividing by number of days between weightings of food.<br>Reviewers comment: Group housing is not the ideal estimate of body weight response to test article.</td></tr>
<tr><td>Ophthalmology</td><td>Not conducted</td></tr>
<tr><td>Clinical Pathology</td><td>Hematology, clotting evaluation, biochemistry and urinalysis performed on all animals sacrificed approximately 24 hr after dosing at the end of the post-dosing period and of animals sacrificed at unscheduled times where possible. Animals were fasted overnight though allowed access to water.<br>- hematology, biochemistry and clotting samples taken from abdominal aorta<br>- animals placed individually in metabolism cages and urine collected overnight (approx 18 hr prior to sacrifice)<br>Hematology parameters:
<table>
<tr><td>Red blood cells (RBC)</td><td>☒</td><td>Lymphocytes (LYM*)</td><td>☒</td></tr>
<tr><td>Hematocrit (HCT)</td><td>☒</td><td>Monocytes (MONO*)</td><td>☒</td></tr>
<tr><td>Hemoglobin (HGB)</td><td>☒</td><td>Neutrophilic granulocytes (NEU*)</td><td>☒</td></tr>
<tr><td>Mean corpuscular volume (MCV)</td><td>☒</td><td>Eosinophilic granulocytes (EOS*)</td><td>☒</td></tr>
<tr><td>Mean corpuscular hemoglobin (MCH)</td><td>☒</td><td>Basophilic granulocytes (BASO*)</td><td>☒</td></tr>
<tr><td>Mean corpuscular hemoglobin conc.(MCHC)</td><td>☒</td><td>Platelets (PLT)</td><td>☒</td></tr>
<tr><td>Red cell distribution width (RDW)</td><td>☒</td><td>Mean Platelet Volume (MPV)</td><td>☒</td></tr>
<tr><td>Reticulocytes (RTC*)</td><td>☒</td><td>Platelet Distribution Width (PDW)</td><td>☒</td></tr>
<tr><td>White blood cells (WBC)</td><td>☒</td><td>Platelet packed cell volume (PCT)</td><td>☒</td></tr>
</table>
Clotting parameters:
<table>
<tr><td>Activated partial thromboplastin time (APTT)</td><td>☒</td><td>Prothrombin time (PTT)</td><td>☒</td></tr>
</table>
Biochemistry parameters:</td></tr>
</table>

| | | |
|---|---|---|
| Albumin | (Bromocresol green method) | ☒ |
| Albumin/Globulin ratio | (Calculated: albumin/(total protein - albumin) | ☐ |
| Alkaline phosphatase | (IFCC method) | ☒ |
| Calcium | (O-cresolphthalein method) | ☒ |
| Chloride | (ISE method) | ☒ |
| Cholesterol | (CHOD-PAP method) | ☒ |
| Creatine kinase (CK) | (NVKC method) | ☒ |
| Creatinine | (Jaffe method without deproteinization) | ☒ |
| Globulin | (Difference between total proteins and Albumin) | ☒ |
| Glucose | (GOD-PAP method) | ☒ |
| Glutamic oxalacetic transaminase | (IFCC method) | ☒ |
| Glutamic pyruvic transaminase | (IFCC method) | ☒ |
| Gamma-glutamyl transaminase | (Szasz method) | ☐ |
| HDL-cholesterol | (PEG-modified enzyme method) | ☐ |
| Hemoglobin in plasma | (TMB method) | ☐ |
| Phosphate | (Molybdate UV method) | ☒ |
| Potassium | (ISE method) | ☒ |
| Sodium | (ISE method) | ☒ |
| Total bilirubin | (DPD method) | ☒ |
| Total protein | (Biuret method) | ☒ |
| Triglycerides | (GPO-PAP method) | ☒ |
| Urea-N | (Urease UV method) | ☒ |

Urinalysis parameters:

| | | |
|---|---|---|
| Volume | (Measured in graduated flask) | ☒ |
| pH | (Hanna pH-meter model 302, -log[H+]) | ☒ |
| Protein | (Roche Diagnostics Hitachi 904 analyzer, pyrogallol red method at 37 °C) | ☒ |
| Glucose | (Roche Diagnostics Hitachi 904 analyzer, GOD-PAP method at 37 °C) | ☒ |
| Osmolality | (Advanced osmometer model 3D3, freezing point depression method) | ☒ |
| Blood | (Roche Combur 3 test E) | ☒ |

| | | |
|---|---|---|
| Sodium | (Roche Diagnostics Hitachi 904 analyzer, ISE method) | ☒ |
| Potassium | (Roche Diagnostics Hitachi 904 analyzer, ISE method) | ☒ |
| Chloride | (Roche Diagnostics Hitachi 904 analyzer, ISE method) | ☒ |
| Creatinine | (Roche Diagnostics Hitachi 904 analyzer, Jaffe method without deproteinization at 37°C) | ☒ |
| Urea-N | (Roche Diagnostics Hitachi 904 analyzer, Urease UV method at 37°C) | ☒ |
| Phosphate | (Roche Diagnostics Hitachi 904 analyzer, Molybdate UV method at 37°C) | ☒ |
| Calcium | (Roche Diagnostics Hitachi 904 analyzer, O-cresolphthalein method at 37°C) | ☒ |

*Reviewers comment: Notable that they are not evaluating inorganic phosphorus levels which is a parameter which appeared to alter dose-dependently in the prior single-dose study with Batch AE. The Sponsor is not conducting hormone evaluation (including bone turnover marker) as previously conducted in the single-dose rat study (negative).*

| | |
|---|---|
| Terminal Procedures | Rats were sacrificed by exsanguination from abdominal aorta under isoflurane in |

| | |
|---|---|
| | anesthesia. |
| Gross Pathology | All animals examined for gross anatomical abnormalities based on a macroscopic examination. |
| Organ Weights | Organs listed (in histopathology table below) were weighed and an absolute weight and relative to body weight for each organ was calculated from pre-sacrifice weight. |
| Histopathology | Tissues and organs preserved in 4% buffered formaldehyde solution except eyes (including optic nerves) which were preserved in modified Davidson's fluid and the left femur which was fixed in Burkhardt's fixative. Except left femur, organs and tissues were embedded in paraffin wax, cut to 5 µm sections and stained with H&E. A myelogram was not performed.<br>All animals sacrificed had bone marrow samples obtained from femur. 2 slides/animal were prepared from each animal, fixed, dried and archived for use as needed. All stained sections were examined from animals of placebo (PL1 and high dose (HD1 and HD2) groups sacrificed and any animals that died unscheduled. Lungs (with bronchi), femur (right) and joint, kidneys and urinary bladder of all the animals of the low (LD) and mid-dose (MD) groups. These sections were examined from animals sacrificed at end of dosing and post-dosing periods. Results were peer-reviewed. |

| Organ/Tissue/Cavity | Weight | Macroscopy | Microscopy |
|---|---|---|---|
| Autopsy Code | ST/SU | All | All |
| Abdominal cavity | | ☒ | |
| Adrenal glands | ☒* | ☒ | ☒ |
| Aorta | | ☒ | ☒ |
| Bone marrow | | ☒ | ☒ |
| Brain | ☒ | ☒ | ☒ |
| Cecum | | ☒ | ☒ |
| Colon | | ☒ | ☒ |
| Cranial cavity | | ☒ | |
| Diaphragm | | ☒ | ☒ |
| Duodenum | | ☒ | ☒ |
| Epididymides | ☒* | ☒ | ☒ |
| Esophagus | | ☒ | ☒ |
| External features | | ☒ | |
| Eyes | | ☒ | ☒ |
| Fallopian tubes | | ☐ | ☐ |
| Femur & Joint | | ☒ | ☒ |
| Gall bladder | | ☐ | ☐ |
| Harderian glands | | ☒ | ☒ |
| Heart | ☒ | ☒ | ☒ |
| Identification | | ☒ | |
| Ileum | | ☒ | ☒ |
| Intestines, MALT | | ☒ | ☒ |
| Jejunum | | ☒ | ☒ |
| Kidneys | ☒* | ☒ | ☒ |
| Lacrimal glands, extraorbital | | ☒ | ☒ |
| Liver | ☒ | ☒ | ☒ |
| Lungs and bronchi | | ☒ | ☒ |
| Lymph node, mesenteric | | ☒ | ☒ |
| Lymph node, popliteal | ☒ | ☒ | ☒ |
| Mammary glands | | ☒ | ☒ |
| Nerve, optic | | ☒ | ☒ |
| Nerve, sciatic | | ☒ | ☒ |
| Oral cavity | | ☒ | |
| Ovaries | ☒* | ☒ | ☒ |
| Pancreas | | ☒ | ☒ |
| Pituitary gland | ☒ | ☒ | ☒ |
| Prostate gland | ☒ | ☒ | ☒ |

| | | | |
|---|---|---|---|
| Rectum | | ☒ | ☒ |
| Salivary glands, Parotid | | ☒ | ☒ |
| Salivary glands, Sublingual | | ☒ | ☒ |
| Salivary glands, Submandibular | | ☒ | ☒ |
| Seminal vesicles & Coagulating glands | ☒ | ☒ | ☒ |
| Skeletal muscles | | ☒ | ☒ |
| Skin and subcutaneous tissues | | ☒ | ☒ |
| Spinal cord | | ☒ | ☒ |
| Spleen | ☒ | ☒ | ☒ |
| Sternum | | ☒ | ☒ |
| Stomach | | ☒ | ☒ |
| Testis | ☒ * | ☒ | ☒ |
| Thoracic cavity | | ☒ | ☐ |
| Thymus | ☒ | ☒ | ☒ |
| Thyroid glands & Parathyroid glands | ☒ * | ☒ | ☒ |
| Tongue | | ☒ | ☒ |
| Trachea | | ☒ | ☒ |
| Treatment site(s) | | ☒ | ☒ |
| Urinary bladder | | ☒ | ☒ |
| Uterus | ☒ | ☒ | ☒ |
| Vagina | | ☒ | ☒ |
| Vertebral column | | ☒ | ☐ |

* Paired organs will be weighed together.

ST = Sacrificed at termination of dosing period animals

SD = Spontaneously dead animals

SU = Unscheduled sacrificed animals

SP = Postdosing animals

| | |
|---|---|
| Statistics | Wilcoxin signed rank test, Covariance analysis, Permutation test, Student t-test utilized as appropriate for determining statistical significance between drug-treated and vehicle-treated animals. |

***Study Deviations/Impact***:

- Due to an error in the protocol, the following text was omitted: Section 1.2 Adresses - Lead Quality Assurance and section 1.9 Lead Quality Assurance;
- The cages were not allocated to the groups in an ascending mean body weight order. Randomization of the animals took place according to the instructions of the Study Director;
- Erroneously the body weight of male Wistar rats was not determined following the first day of dosing but on the second day following dosing. In addition, no animals were weighed in the second week of the observation period prior to sacrifice;
- Due to absence of the organ during autopsy, the pituitary gland of animal M1001 and F1008 was not weighed;

*Reviewers comment: Sponsor believes these deviations did not influence the outcome of the study and/or hamper interpretation. Clearly the body weight of male rats is not considered to be properly evaluated though no change in bw was noted on SD2.*

**RESULTS**

**Toxicokinetics**

Not conducted.

**Mortality**

No animals died during the study.

**Clinical signs**
Clinical signs not observed ≤ 600 mg/kg. At 2000 mg/kg, cramps noted in 1/6 males and 1/6 females for 1 minute. Abduction of hindlimbs was noted in 2/6 females. All signs noted after the initial (first) dose with no other abnormalities noted prior to, during, or after second dose on SD0 or during remainder of the study. *Reviewers comment: The Sponsor states this is due to rate and viscosity of the formulation as injected. It is not clear which vein the drug/vehicle is administered, likely this is the tail vein. The Sponsor does not believe this response is due to test article but no evidence presented to support this contention.*

**Body weights**

Body weight gain was observed to decrease transiently in females of the 200 and 600 mg/kg/day group compared to control group. There was no dose-response as no observation was noted in the 2000 mg/kg/day group and by SD7 absolute and relative body weights were similar across groups.

| | **Males** | | | **Females** | | |
|---|---|---|---|---|---|---|
| | ***Absolute Body Weight*** | | | | | |
| | SD-1-SD0 | SD1-SD2 | SD7 | SD-1-SD0 | SD1-SD2 | SD7 |
| Placebo | 270 | 274 | 297 | 177 | 183 | 193 |
| 200 | 265 | 269 | 290 | 174 | 173 | 181 |
| 600 | 269 | 273 | 294 | 172 | 173 | 184 |
| 2000 | 274 | 278 | 301 | 174 | 177 | 186 |
| | ***Relative Body Weight (% baseline)*** | | | | | |
| Placebo | 100 | 102 | 111 | 100 | 104 | 109 |
| 200 | 100 | 101 | 109 | 100 | 101* | 105 |
| 600 | 100 | 102 | 109 | 100 | 101* | 107 |
| 2000 | 100 | 101 | 109 | 100 | 102 | 107 |
| * p<0.05 compared with relative body weight in controls | | | | | | |

**Food consumption**
Food consumption was decreased during the post-dosing period during the second week of observation in both genders at 200 mg/kg/day (95% of controls males, 94% of control females). Although these reductions in food consumption parallel to a certain degree the reduction in body weight gain noted in 200 mg/kg/day females (statistically significant) and males (not statistically significant) described above, there was no dose-response observed and therefore the relationship to treatment is not apparent.

**Hematology**
No treatment-related hematologic changes were observed. An elevated MCHC level noted at the end of the 2-week recovery period in all treated male groups was the result of a low mean value for the males of the placebo group at recovery sampling. Decreased red cell distribution width for 200 mg/kg/day females at the end of the recovery period was without dose-response or observations in other red cell parameters. Decreased mean platelet distribution width was noted in 2000 mg/kg/day males at the end of the recovery period but was mild (-1.7%), not observed at the acute sampling period, and was without similar observation in females or changes in platelet numbers, mean platelet volume or other packed platelet volume .

No treatment-related changes in clotting parameters were observed at the acute or recovery sampling time-point.

**Clinical chemistry**

No clear treatment-related effects on clinical chemistry were observed at any dose level. Hepatic parameters such as ALP, Glutamate dehydrogenase, and Cholesterol were observed to be significantly changed in Org 25969-treated groups though this was sporadic, not dose-responsive, not consistent between males and females, and was not maintained during the recovery period. Alkaline phosphatase increased in males at 200 mg/kg/day but decreased in females at this dose and was not significantly different from controls at higher dose levels. Glutamate dehydrogenase was decreased compared with controls in males at ≥ 600 mg/kg/day; however, as a mitochondrial leak enzyme a decrease in this parameter is not considered toxicologically significant. Though female rats appeared to have higher levels compared with controls at levels ≥ 600 mg/kg/day (12.1 and 13.0 mmol/L) these values were not generally different as that found in control females at the end of the recovery period (12.5 mmol/L). Cholesterol was significantly increased in females at 2000 mg/kg/day (+10%*) and though this value was still higher in this group at the end of the recovery (+9.3%) it was not statistically significant and the increases are marginal in both cases. Increased inorganic phosphorus value for females at 600 mg/kg/day and males and females at 2000 mg/kg/day on SD1 demonstrated full recovery by SD14 and its toxicologic significance is likely minimal. The mild increase in albumin in the 2000 mg/kg/day males at the end of the recovery period is considered without toxicologic significance by this reviewer.

| **Clinical Chemistry Findings** | | **0** | | **200** | | **600** | | **2000** | |
|---|---|---|---|---|---|---|---|---|---|
| | | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** |
| ALP (U/L) | SD1 | 204 | 117 | 254* (+24%) | 92* (-21%) | 223 | 109 | 211 | 105 |
| | SD14 | 159 | 166 | 172 | 163 | 80 | 86 | 84 | 75 |
| Glutamate dehydrogenase (U/L) | SD1 | 7.2 | 8.2 | 7.2 | 5.7 | 5.9* | 12.1 | 6.1* | 13.0 |
| | SD14 | 6.6 | 12.5 | 6.0 | 11.1 | 7.2 | 13.9 | 6.4 | 14.9 |
| Cholesterol (mmol/L) | SD1 | 2.07 | 1.48 | 1.77 | 1.45 | 2.13 | 1.37 | 1.79 | 1.63* (+10%) |
| | SD14 | 1.72 | 1.49 | 1.45 | 1.51 | 1.63 | 1.75 | 1.72 | 1.53 (+9.3%) |
| Inorganic phosphorus (mmol/L) | SD1 | 2.78 | 2.36 | 28.2 | 2.44 | 2.97 | 2.60* (+10%) | 3.11* (+12%) | 2.69 (+14%) |
| | SD14 | 2.53 | 2.24 | 2.43 | 2.01 | 2.42 | 2.07 | 2.51 | 2.17 |
| Albumin (g/L) | SD1 | 36.7 | 41.0 | 36.6 | 40.3 | 36.1 | 41.1 | 36.8 | 42.0 |
| | SD14 | 37.6 | 41.3 | 38.8 | 41.6 | 38.8 | 42.1 | 39.7* (+6%) | 40.8 |

No alterations in CK indicative of muscle damage was noted at the end of treatment or recovery. No changes in testosterone or estradiol were observed though mildly increased deoxypyrdinoline values for males at 600 mg/kg/day only were described at the end of the recovery period. As there was no dose-response or observation at 2000 mg/kg/day and was not seen acutely, this change is considered incidental.

**Urinalysis**

Sporadic statistically significant findings were noted though without evidence of dose-response or observation in both male and female rats. No treatment-related effects were noted ≤ 600 mg/kg/day in either gender. At 2000 mg/kg/day, mildly decreased potassium/creatinine ratio as well as inorganic phosphorus/creatinine ratio was noted in males with full recovery at the end of 14-day treatment-free period.

**Organ weights**

No treatment-related effects on organ weight were noted. Mild reduction in testes weight (maximum -6%) in males ≥ 600 mg/kg/day was not observed in recovery animals and is of uncertain toxicologic significance.

**Gross pathology**

No treatment-related observations noted.

**Histopathology**
Treatment-related findings were not observed at 200 mg/kg/day in either male or female rats.

***Lung***
Evidence of increased alveolar foam cells (macrophages) was noted at 2000 mg/kg/day in both males and females, was minimal in severity but remained evident in the SD14 recovery group. No evidence was noted at 600 mg/kg/day in either gender.

***Kidney***
At 600 mg/kg/day, males and females had evidence of minimal tubular cell vacuolation which increased in incidence, though not severity at 2000 mg/kg/day. Some evidence of recovery was noted with no evidence after 14-days in the 600 mg/kg/day animals though a lower incidence remained in the 2000 mg/kg/day recovery group.

| Histopathologic Findings | | 0 | | 200 | | 600 | | 2000 | |
|---|---|---|---|---|---|---|---|---|---|
| | | M | F | M | F | M | F | M | F |
| **Lung** Increase in foamy alveolar macrophages | SD1 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 4/6 (+) | 2/6 (+) |
| | SD14 | 1/6 (+) | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 4/6 (+) | 2/6 (+) |
| **Kidney** Tubular cell vacuolation | SD1 | 0/6 | 0/6 | 0/6 | 0/6 | 2/6 (+) | 3/6 (+) | 5/6 (+) | 3/6 (+) |
| | SD14 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 3/6 (+) | 2/6 (+) |

## STUDY TITLE: AN INTRAVENOUS EXTENDED SINGLE-DOSE ACUTE TOXICITY STUDY WITH DEGRADED ORG 25969 IN WISTAR RATS

### Study purpose:
The objectives of this study were:

- to assess the acute toxicity of degraded drug product Org 25969 upon single intravenous administration in male and female Wistar rats[5],
- to compare the acute toxicity of degraded and non-degraded drug product Org 25969 upon single intravenous administration in male and female Wistar rats at the high dose level of 2000 $mg.kg^{-1}$.

### Key study findings:
- Rats were intravenously administered degraded Org 25969 at dose levels of 0, 200, 600, 2000 mg/kg/day or non-degraded Org 25969 at 2000 mg/kg/day. The toxicity of degraded Org 25969 was comparable at the equivalent 2000 mg/kg/day dose level to non-degraded Org 25969. Liver enzyme elevations (ALT) and blood in urine were noted in both degraded and non-degraded drug groups. Target organs identified with single-dose administration were the lung and kidney with likely mild hemolysis reflected in the presence of blood in urine. Similar incidence and severity of findings was noted with both the degraded and non-degraded form of Org 25969.

**Study no.**: Report # NL0059965
**Volume #, and page #**: Original submission, section 4.2.3.1 (electronic)
**Conducting laboratory and location**: N.V. Organon, Department of TDD, Nistelrooise baan 3, NL-5374 RE Schaijk, The Netherlands
**Date of study initiation**: Final report March, 2005
**GLP compliance**: Yes
**QA report**: Yes
**Drug, lot #, and % purity**:

**METHODS**

***Test System***

**Species/strain**: Wistar Han rat (Han Crl: WI (Glx/BRL/Han) IGS) from (b) (4) (b) (4).
**Age**: 7-8 weeks at initiation
**Weight**: ♂: 246-291g, ♀164-193 g

***Study Design***

| Group No. | Group size males | Group size females | Dose ($mg \cdot kg^{-1}$) | Treatment |
|---|---|---|---|---|
| 1 (placebo) | 12* | 12* | 0 | Vehicle |
| 2 (LD, Low Dose) | 12* | 12* | 200 | Degraded Org 25969 |
| 3 (MD, Mid Dose) | 12* | 12* | 600 | Degraded Org 25969 |
| 4 ($HD_1$, High Dose) | 12* | 12* | 2000 | Degraded Org 25969 |
| 5 ($HD_2$, High Dose) | 12* | 12* | 2000 | Non-degraded Org 25969 |

* 6 animals per group/sex were sacrificed approximately 24h after dosing. The remaining 12 animals in each group (6 males and 6 females), were sacrificed approximately 14 days after dosing.

**Doses**: 0, or degraded Org 25969 at 200, 600, or 2000 mg/kg/day, or Non-degraded Org 25969 at 2000 mg/kg/day
**Route, formulation, volume, and infusion rate**: IV, 100 mg/mL stock formulation for the 2000 mg/kg/day group, or diluted in vehicle (isotonic saline solution) for 200 and 600 mg/kg/day groups. A total of 20 mL/kg of the formulation/vehicle was administered over the day comprised of 4X 5 ml/kg infusions separated by 1-1.5 hr intervals.
**Number/sex/group or time point (main study)**: 6 animals/sex/group
**Satellite groups used for toxicokinetics or recovery**:6 animals/sex/group used as recovery sacrifice at SD14
**Sampling times**:
**Unique study design, husbandry or methodology issues**:

**Test article:** Test articles used for the study were designated as TY004 (undegraded) and TY003 (degraded). The drug substance was a mix of drug substance batches U, W and Z (43.77, 9.03, and 45.49 mg/mL, respectively). The mixture of these solutions resulted in a solution designated as TY003-1 which was at a concentration of 100 mg/mL Org 25969. With the exception of a sample of the solution which was kept as a reference, TY003-1 was autoclaved at 121°C for 30 minutes and was then split to TY003-1-1 and TY003-1-2. TY003-1-1 was stored for 7 months at 2-8°C and was the reference (labeled TY-003-1) to compare to TY003-1-2 which was stored at 60°C for 2 months. The latter degraded solution was relabeled TY004-1 and was then stored at 2-8°C for a further 4+ months prior to study initiation.

**Administration**: Test article/vehicle was given as 4X 5 ml/kg injection with 1-1.5 hr intervals between infusions. *Reviewers comment: This is a high injection volume for this species. 1-5 ml/kg is considered an ideal volume (Toxicologist's Pocket Handbook; Diehl et al., A Good Practice Guide to the Administration of Substances and Removal of Blood, including routes and volumes Journal of Applied Toxicology, p15-23 2001), with max*

*injection volume considered to be in the 10-20 ml/kg range as a slow infusion. Technically this is a daily injection volume total and therefore is likely without serious consequences.*

**Observations and times**

| Observational Parameter | Methods |
|---|---|
| Toxicokinetics | Not conducted |
| Clinical Observations | Morbidity and mortality during in-life period. Evaluated regularly for behavioral and physical abnormalities during dosing and post-dosing periods.<br>*Reviewer's comment: Sponsor does not define "regularly" though data suggests this was done daily at least.* |
| Body Weights | 2x/week pre-dosing, SD0 (prior to dosing), SD1, and 2X/week during the observation period. Only individual and group mean values for animals sacrificed after the 2-week recovery period are reported in tables. |
| Food Consumption | Recorded weekly per cage (2 animals of same sex/cage) through in-life period by weighing amounts given and remaining and dividing by number of days between weightings of food.<br>*Reviewers comment: Group housing is not the ideal estimate of body weight response to test article.* |
| Ophthalmology | Not conducted |
| Clinical Pathology | Hematology, clotting evaluation, biochemistry and urinalysis performed on all animals sacrificed approximately 24 hr after dosing at the end of the post-dosing period and of animals sacrificed at unscheduled times where possible. Animals were fasted overnight though allowed access to water.<br>- hematology, biochemistry and clotting samples taken from retro-orbital sinus<br>- animals placed individually in metabolism cages and urine collected overnight (approx 18 hr prior to sacrifice)<br>Hematology parameters:<br>Red blood cells (RBC) ☒ Lymphocytes (LYM*) ☒<br>Hematocrit (HCT) ☒ Monocytes (MONO*) ☒<br>Hemoglobin (HGB) ☒ Neutrophilic granulocytes (NEU*) ☒<br>Mean corpuscular volume (MCV) ☒ Eosinophilic granulocytes (EOS*) ☒<br>Mean corpuscular hemoglobin (MCH) ☒ Basophilic granulocytes (BASO*) ☒<br>Mean corpuscular hemoglobin conc.(MCHC) ☒ Platelets (PLT) ☒<br>Red cell distribution width (RDW) ☒ Mean Platelet Volume (MPV) ☒<br>Reticulocytes (RTC*) ☒ Platelet Distribution Width (PDW) ☒<br>White blood cells (WBC) ☒ Platelet packed cell volume (PCT) ☒<br>Clotting parameters:<br>Activated partial thromboplastin time (APTT) ☒ Prothrombin time (PTT) ☒<br>Biochemistry parameters: |

| | | |
|---|---|---|
| Aspartate aminotransferase (AST) | (IFCC method) | ☒ |
| Alanine aminotransferase (ALT) | (IFCC method) | ☒ |
| Glutamate dehydrogenase (GLDH) | (DGKC method) | ☒ |
| Creatine kinase (CK) | (IFCC method) | ☒ |
| Alkaline phosphatase (ALP) | (IFCC method) | ☒ |
| Gamma-glutamyl transferase(GGT) | (Szasz method) | ☒ |
| Total bilirubin(BIL-T) | (DPD method) | ☒ |
| Icterus index (I) | (Measurement after dilution with saline) | ☒ |
| Triglycerides (TG) | (GPO-PAP method) | ☒ |
| Cholesterol (CHOL) | (CHOD-PAP method) | ☒ |
| HDL-cholesterol | (PEG-modified enzyme method) | ☐ |
| Phospholipids | (Enzymatic method) | ☐ |
| Lipemia index (L) | (Measurement after dilution with saline) | ☒ |
| Creatinine (CREA) | (PAP method) | ☒ |
| Urea (UREA) | (Urease UV method) | ☒ |
| Glucose (GLUC) | (Hexokinase method) | ☒ |
| Total protein (TP) | (Biuret method) | ☒ |
| Albumin (ALB) | (Bromocresolgreen method) | ☒ |
| Globulin (GLOB) | (Calculated: total protein-Albumin) | ☒ |
| Albumin/Globulin ratio (AGRA) | (Calculated: albumin/(total protein - albumin) | ☒ |
| Calcium (Ca) | (O-cresolphtalein method) | ☒ |
| Inorganic phosphorus (PHOS) | (Molybdate UV method) | ☒ |
| Sodium (Na) | (ISE method) | ☒ |
| Potassium (K) | (ISE method) | ☒ |
| Chloride (CL) | (ISE method) | ☒ |
| Hemolysis index (H) | (measurement after dilution with saline) | ☒ |

Hormone parameters:

| | | |
|---|---|---|
| Testosterone (TES) | Chemiluminicent enzyme immunoassay | ☒ |
| Estradiol (E2) | Chemiluminicent enzyme immunoassay | ☒ |
| Deoxypyridinoline (DPD) | Chemiluminicent enzyme immunoassay | ☒ |

| | | |
|---|---|---|
| Volume (Quantity) | (Graduated flask) | ☒ |
| Osmolality (OSM) | (freezing-point depression method) | ☒ |
| PH (pH) | (pH-meter) | ☒ |
| Glucose (GLUC) | (Quantitative, Hexokinase method at the Modular) | ☒ |
| Protein (UCTP) | (Quantitative, Brenzethonium chloride method at the Modular) | ☒ |
| N-acetyl-Beta-D-Glucosaminidase (NAG) | (Quantitative, 3-cresolsulfonphtalinein method at the Modular) | ☒ |
| Creatinine (CREA) | (Quantitative, PAP method at the Modular) | ☒ |
| Hemoglobin (HGB) | (Semi-quantitative, Roche Combur$^5$ test® D, score 0, +, ++, +++, ++++) | ☒ |
| Ketones (KET) | (Semi quantitative, Roche Combur$^5$ test® D, score 0, +, ++, +++) | ☒ |
| Sodium (Na) | (Quantitative, ISE method at the Modular) | ☒ |
| Potassium (K) | (Quantitative, ISE method at the Modular) | ☒ |
| Chloride (CL) | (Quantitative, ISE method at the Modular) | ☒ |
| Calcium (Ca) | (Quantitative, ISE method at the Modular) | ☒ |
| Inorganic phosphorus (PHOS) | (Molybdate UV method at the Modular) | ☒ |

Urinalysis parameters:

| | | |
|---|---|---|
| Volume (Quantity) | (Graduated flask) | ☒ |
| Osmolality (OSM) | (freezing-point depression method) | ☒ |
| PH (pH) | (pH-meter) | ☒ |
| Glucose (GLUC) | (Quantitative, Hexokinase method at the Modular) | ☒ |
| Protein (UCTP) | (Quantitative, Brenzethonium chloride method at the Modular) | ☒ |
| N-acetyl-Beta-D-Glucosaminidase (NAG) | (Quantitative, 3-cresolsulfonphtalinein method at the Modular) | ☒ |
| Creatinine (CREA) | (Quantitative, PAP method at the Modular) | ☒ |
| Hemoglobin (HGB) | (Semi-quantitative, Roche Combur$^5$ test® D, score 0, +, ++, +++, ++++) | ☒ |
| Ketones (KET) | (Semi quantitative, Roche Combur$^5$ test® D, score 0, +, ++, +++) | ☒ |
| Sodium (Na) | (Quantitative, ISE method at the Modular) | ☒ |
| Potassium (K) | (Quantitative, ISE method at the Modular) | ☒ |
| Chloride (CL) | (Quantitative, ISE method at the Modular) | ☒ |
| Calcium (Ca) | (Quantitative, ISE method at the Modular) | ☒ |
| Inorganic phosphorus (PHOS) | (Molybdate UV method at the Modular) | ☒ |

| | |
|---|---|
| Terminal Procedures | Rats were sacrificed by exsanguination from abdominal aorta under isoflurane in $N_20/0_2$ anesthesia. |
| Gross Pathology | All animals examined for gross anatomical abnormalities based on a macroscopic examination. |
| Organ Weights | Organs listed (in histopathology table below) were weighed and an absolute weight and relative to body weight for each organ was calculated from pre-sacrifice weight. |
| Histopathology | Tissues and organs preserved in 4% buffered formaldehyde solution except eyes (including optic nerves) which were preserved in modified Davidson's fluid and the left femur which was fixed in Burkhardt's fixative. Except left femur, organs and tissues were embedded in paraffin wax, cut to 5 µm sections and stained with H&E.<br>Left femur was discarded without evaluation after data from the 2nd 4-week toxicity study in the rat which evaluated bone effects was completed.<br>All stained sections were examined from animals of placebo (PL1 and high dose (HD; DS4) groups sacrificed at end of dosing and at end of recovery period.<br>Lungs (with bronchi), femur (right) and joint, kidneys and urinary bladder of the animals of the low (LD; DS2) and mid-dose (MD; DS3) were examined at end of dosing and post-dosing period. Results were peer-reviewed. |

| Organ/Tissue/Cavity | Weight | Macroscopy | Microscopy | Microscopy |
|---|---|---|---|---|
| Autopsy Code | ST/SU/SP | All | ST/SD/SU | SP |
| Abdominal cavity | | ☒ | | |
| Adrenal glands * | ☒ | ☒ | ☒ | ☐ |
| Aorta | | ☒ | ☒ | ☐ |
| Bone marrow | | ☒ | ☒ | ☐ |
| Brain | ☒ | ☒ | ☒ | ☐ |
| Cecum | | ☒ | ☒ | ☐ |
| Colon | | ☒ | ☒ | ☐ |
| Cranial cavity | | ☒ | | |
| Diaphragm | | ☒ | ☒ | ☐ |
| Duodenum | | ☒ | ☒ | ☐ |
| Epididymides * | ☒ | ☒ | ☒ | ☐ |
| Esophagus | | ☒ | ☒ | ☐ |
| External features | | ☒ | | |
| Eyes | | ☒ | ☒ | ☐ |
| Femur & Joint (left and right) | | ☒ [1] | ☒ [2] | ☒ [3] |
| Gall bladder | | ☐ | ☐ | ☐ |
| Harderian glands | | ☒ | ☒ | ☐ |
| Heart | ☒ | ☒ | ☒ | ☐ |
| Identification | | ☒ | | |
| Ileum | | ☒ | ☒ | ☐ |
| Intestines, MALT | | | ☒ | ☐ |
| Jejunum | | ☒ | ☒ | ☐ |
| Kidneys * | ☒ | ☒ | ☒ | ☒ |
| Lacrimal glands, extraorbital | | ☒ | ☒ | ☐ |
| Liver | ☒ | ☒ | ☒ | ☐ |
| Lungs and bronchi | | ☒ | ☒ | ☒ |
| Lymph node, mesenteric | | ☒ | ☒ | ☐ |
| Lymph node, popliteal | ☒ | ☒ | ☒ | ☐ |
| Mammary glands | | ☒ | ☒ | ☐ |
| Nerve, optic | | ☒ | ☒ | ☐ |
| Nerve, sciatic | | ☒ | ☒ | ☐ |
| Oral cavity | | ☒ | | |
| Ovaries * | ☒ | ☒ | ☒ | ☐ |
| Pancreas | | ☒ | ☒ | ☐ |
| Pituitary gland | ☒ | ☒ | ☒ | ☐ |

| Organ/Tissue/Cavity | Weight | Macroscopy | Microscopy | Microscopy |
|---|---|---|---|---|
| Autopsy Code | ST/SU/SP | All | ST/SD/SU | SP |
| Prostate gland | ☒ | ☒ | ☒ | ☐ |
| Preputial & clitoral glands | | ☒ | ☐ | ☐ |
| Rectum | | ☒ | ☒ | ☐ |
| Salivary glands, Parotid | | ☒ | ☒ | ☐ |
| Salivary glands, Sublingual | | ☒ | ☒ | ☐ |
| Salivary glands, Submandibular | | ☒ | ☒ | ☐ |
| Seminal vesicles/Coagulating glands | ☒ | ☒ | ☒ | ☐ |
| Skeletal muscles | | ☒ | ☒ | ☐ |
| Skin and subcutaneous tissues | | ☒ | ☒ | ☐ |
| Spinal cord | | ☒ | ☒ | ☐ |
| Spleen | ☒ | ☒ | ☒ | ☐ |
| Sternum | | ☒ | ☒ | ☐ |
| Stomach | | ☒ | ☒ | ☐ |
| Testes * | ☒ | ☒ | ☒ | ☐ |
| Thoracic cavity | | ☒ | | |
| Thymus | ☒ | ☒ | ☒ | ☐ |
| Thyroid glands & Parathyroid glands * | ☒ | ☒ | ☒ | ☐ |
| Tongue | | ☒ | ☒ | ☐ |
| Trachea | | ☒ | ☒ | ☐ |
| Treatment site(s) | | ☒ | ☒ | ☐ |
| Urinary bladder | | ☒ | ☒ | ☒ |
| Uterus | ☒ | ☒ | ☒ | ☐ |
| Vagina | | ☒ | ☒ | ☐ |
| Vertebral column | | ☐ | ☐ | ☐ |

* Paired organs will be weighed together.
ST = Sacrificed at termination of dosing period animals
SD = Spontaneously dead animals
SU = Unscheduled sacrificed animals
SP = Postdosing animals

| | |
|---|---|
| Statistics | Wilcoxin signed rank test, Covariance analysis, Permutation test, Student t-test utilized as appropriate for determining statistical significance between drug-treated and vehicle-treated animals. |

***Study Deviations/Impact***:

- individual values and means for body weight and food consumption are reported in tables per group per sex only for the animals sacrificed approximately 2 weeks after dosing; consequently statistical analysis could not be performed on SD1 (24 hr post-dosing. *Reviewers comment: The Sponsor considers this to have had no impact on the conclusions of the study. Although some mild and transient reductions in decreased weight gain have been reported in prior single-dose study, I do not believe this seriously impacts the study results.*
- Animal M5517 (HD♂) received only 3 administration (instead of 4) over a 1-1.5 h interval. *Reviewers comment: 5 animals still remained at this dose level (2000 mg/kg/day)*
- Covariance analysis could not be performed for most of the parameters in groups of animals sacrificed at SD1 (24 hr after dosing) due to deviations. *Reviewers comment: Although Sponsor considers this has no impact on the conclusion of the study (comparison of degraded vs. non-degraded Org 25969 toxicity) this is a significant concern overall. However, this reviewer agrees it is still possible to obtain a general comparison of degraded vs. non-degraded toxicity.*

**RESULTS**

**Toxicokinetics**
Not conducted

**Mortality**
3 rats died during the study, all in the high dose groups (2 in HD1 [degraded] and 1 in HD2 [non-degraded]). M4103 and M4415 died on SD2 and SD8, respectively. F5312 died on SD8.

All 3 deaths were considered non-treatment related and were related to blood sampling errors (M4415 and F4312) or restraint device (M4103).

Animals M4415 and F5312 had clinical values utilized during for study analysis while M4103 did not (due to earlier death on study). Necropsy of decedents revealed reddish areas on surface of one lung lobe in M4103 but no histopathology evaluation was done. M4415 (HD1) had reddish foci on surface of thymus

and congested liver. Histopathologic evaluation indicated slight perivascular eosinophilic cell infiltration and an increased number of foamy alveolar macrophages in lungs (minimal increase) and a slight cortical tubular cell vacuolation in kidneys. Similar liver and kidney findings were noted in F5312, though severity of cortical tubular cell vacuolation was rated as minimal in severity.

**Clinical signs**
No treatment-related clinical signs were observed.

**Body weights**
Absolute body weight (compared to control) was increased 3% ($p<0.05$) vs. controls in the HD2 group (non-degraded 2000 mg/kg/day) though no difference was seen when comparing weight gain relative to starting body weight for this group (+ 21%) versus HD1 (+18%) and control animals (+21%). *Reviewers comment: The difference is small and is in a positive direction and so is not considered toxicologically significant*

**Food consumption**
No effects on food consumption were noted in degraded Org 25969 treatment groups vs. controls; however, a small increase in food consumption (+8%; $p\leq0.01$) was noted during the second week of the study in HD2 males. This compares to a +5% increase in the HD1 males. An increase was not observed in females. *Reviewers comment: This seems to explain the increased weight in the HD2 group noted previously. Still consider this a non-toxicologically significant finding.*

**Hematology**
Parameters which demonstrate a possible treatment-related change with *degraded* Org 25969 include:

- ↑Reticulocytes in HD1F; there was no dose-dependency in males
- ↑Neutrophils (absolute); a dose-dependent increase was observed with degraded Org 25969 ≥ 600 mg/kg/day. An increase was observed in the non-degraded HD2M but was not observed in HD2F.
- ↑Neutrophils (relative); An increase was observed in the HD1M and a non-dose-dependent increase in all treated females. An increase was not observed in HD2M but was observed in HD2F.

*Note: SD1 statistics could not be conducted (see Deviations)*

| | | Males (% change) | | | | | Females (% change) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SD** | **0** | **200** | **600** | **2000** | **2000** | **0** | **200** | **600** | **2000** | **2000** |
| Retic (g/L) | SD1 | 392 | +5 | **+14** | -4 | -2 | 252 | 0 | +1 | **+20** | +7 |
| | SD14 | 346 | +1 | -2 | **+11** | -6 | 254 | **+13** | +9 | +6 | **+24** |
| WBC (g/L) | SD1 | 6.2 | +6 | **+19** | -2 | **+22** | 4.2 | -10 | -5 | +9 | +7 |
| | SD14 | 6.6 | -3 | -3 | -3 | **-17** | 3.6 | +3 | -3 | -3 | **+22** |
| Lymphos (g/L) | SD1 | 4.49 | +10 | **+25** | -9 | **+32** | 3.13 | -12 | -9 | +6 | -9 |
| | SD14 | 4.87 | +1 | 0 | -3 | **-17** | 2.67 | +10 | -4 | -1 | **+21** |
| Lymphos (rel) | SD1 | 73.8 | +2 | +3 | **-11** | +5 | 74.6 | -3 | -5 | -2 | -3 |
| | SD14 | 74.5 | +2 | +2 | 0 | -1 | 74.1 | +7 | -2 | +1 | -2 |
| Neuts (g/L) | SD1 | 1.1 | -9 | +9 | **+36** | **+18** | 0.7 | 0 | **+14** | **+14** | 0 |
| | SD14 | 1.1 | **-27** | **-27** | -9 | **-27** | 0.6 | **-33** | **+16** | **-17** | **+33** |
| Neuts (rel) | SD1 | 17.9 | -14 | -7 | **+46** | -2 | 15.9 | **+15** | **+30** | **+16** | **+19** |
| | SD14 | 16.3 | **-20** | **-21** | -7 | -9 | 15.3 | **-17** | **+25** | +4 | **+20** |

No changes in blood clotting were noted that were considered by Sponsor or reviewer to be treatment-related. Sporadic values were statistically significant but demonstrated neither dose-dependency or consistency between gender.

**Clinical chemistry**

Notable treatment-related alterations on SD1 were observed in hepatic enzymes AST and ALT as shown in the table below. AST was increased primarily in the non-degraded HD2M and HD2F. ALT was noted to be increased in all males treated with non-degraded or degraded drug while this elevation was observed in females at ≥ 600 mg/kg. These elevations were slightly greater in the HD2 vs. HD1 groups. All values returned to control levels (or below) by SD14.

*Note: SD1 statistics could not be conducted (see Deviations)*

| | | Males (% change) | | | | | Females (% change) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SD** | **0** | **200** | **600** | **2000** | **2000** | **0** | **200** | **600** | **2000** | **2000** |
| GOT/AST (U/L) | SD1 | *126* | +25 | +14 | +9 | **+31** | *125* | -9 | +19 | +12 | **+57** |
| | SD14 | *98* | -18 | -11 | -8 | +3 | *78* | 0 | +6 | +3 | +10 |
| GPT/ALT (U/L) | SD1 | *37* | **+43** | **+40** | **+32** | **+59** | *24* | 0 | **+29** | **+25** | **+88** |
| | SD14 | *35* | -31* | -22 | -6 | +14 | *20* | -5 | -20* | -15 | -5 |

Sporadic values of other parameters were altered and statistically significant but demonstrated neither dose-dependency nor consistency between genders. No clear changes in renal parameters were observed.

**Urinalysis**

| | | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SD** | **0** | **200** | **600** | **2000** | **2000** | **0** | **200** | **600** | **2000** | **2000** |
| Blood (incidence) | SD1 | 2 | 2 | 3 | 6 | 4 | 1 | 1 | 2 | 4 | 6 |
| | SD14 | 1 | 0 | 2 | 1 | 1 | 2 | 0 | 0 | 1 | 0 |
| Urea-N | SD1 | | - | - | ↑ | | | - | - | ↑ | ↑ |
| | SD14 | | - | - | - | | | - | - | - | - |
| Chloride | SD1 | | | | ↓ | ↓ | | | | ↓ | ↓ |
| | SD14 | | ↓ | ↓ | | | | ↓ | ↓ | ↓ | ↓ |
| Sodium | SD1 | | | | ↑ | ↑ | | | ↑ | ↑ | ↑ |
| | SD14 | | | | ↑ | | | | ↑ | ↑ | |
| Potassium | SD1 | | | | ↓ | ↓ | | | ↓ | ↓ | ↓ |
| | SD14 | | | | ↓ | | | ↓ | ↓ | | ↓ |

Increased blood in the urine of both males and females administered degraded Org 25969 is considered treatment-related. This increase is also observed in the non-degraded HD2, is comparable to the degraded HD1 incidence and is not observed with a 2-week recovery period.

Other mild alterations in urinalysis characterized by ↑urea, ↓chloride, ↑sodium and ↓potassium are considered by this reviewer and sponsor to be treatment-related. Several parameters (chloride, sodium, and potassium) still appeared to be altered at the conclusion of the 2-week treatment-free period.

**Organ weights**

No changes in organ weight were noted at the end of SD1. Increased liver weight (absolute) was noted in HD2M (+6%) at the recovery sacrifice and is not considered to be evidence of a treatment-related effect by this reviewer.

**Gross pathology**

No gross macroscopic changes were noted that appeared to be treatment-related.

**Histopathology**
Clear treatment-related histopathologic alterations are shown in the following table.

| Target Organ/ Finding | SD | Severity | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 200 | 600 | 2000 | 2000 | 0 | 200 | 600 | 2000 | 2000 |
| **Lung** | | | | | | | | | | | | |
| ↑ number of foamy alveolar macrophages | SD1 | Total | 0/5 | 0/6 | 2/6 | 5/6 | 4/5 | 1/6 | 0/6 | 0/6 | 3/6 | 4/6 |
| | | Minimal | | | 2 | 4 | 4 | 1 | | | 2 | 4 |
| | | Slight | | | | 1 | | | | | 1 | |
| | SD14 | Total | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 1/6 | 0/6 | 0/6 | 1/6 | 1/6 |
| | | Minimal | | | | | | 1 | | | 1 | 1 |
| **Kidney** | | | | | | | | | | | | |
| Vacuolation of cortical tubular epithelium | SD1 | Total | 0/5 | 0/6 | 2/6 | 5/6 | 5/6 | 0/6 | 0/6 | 2/6 | 6/6 | 4/6 |
| | | Minimal | | | 2 | 5 | 3 | | | 2 | 6 | 4 |
| | | Slight | | | | | 2 | | | | | |
| | SD14 | Total | 0/6 | 0/6 | 0/6 | 1/6 | 2/6 | 0/6 | 0/6 | 0/6 | 0/6 | 1/6 |
| | | Minimal | | | | 1 | 2 | | | | | 1 |

Other changes which were not clearly treatment related (though LD and MD animals were not evaluated) when comparing control vs. HD1 and HD2 groups included:
**Lungs**: granulomatous lesion (slight) in 1 HD1M on SD1 and in 1 HD2F (marked) on SD14.
**Liver**: bile duct epithelial hypertrophy in 1 HD1M on SD1; microglanulomata in 1 HD1M and 1 HD1F on SD1 (none in HD2M or HD2F on SD1 but in 1 HD2M on SD14)
**Testes**: testicular changes (increased number of residual bodies in 1 HD1M (M#4311), depletion of elongated spermatids and vacuolation in germinal epithelium in 1 HD1M (M#4625), and degeneration of germinal epithelium(M#4623) in 1 HD1M on SD1
**Epididymides**: increased number of spermatids (3/6 HD1M (M#4207, 4623, 4625); 0/5 HD2M), hypospermia (1/6 HD1M; 0/5 HD2M) on SD1. No findings in any male group on SD14.

Additionally, one HD1F had slight urothelial hyperplasia. This was attributed to pyelonephritis and bilateral urothelial hyperplasia in the kidneys of this animal.

### Histopathology inventory (optional)

### 2.6.6.3 Repeat-dose toxicity

### Rat studies

The sponsor had conducted repeat-dose toxicity studies in rats and dogs. In an initial two-week study in rats, animals were treated for Org 25969 at 0 (0.9% NaCl), 2 mg/kg, 8 mg/kg, or 30 mg/kg. There were no remarkable treatment-related toxic effects observed in clinical signs, body weight and food consumption, clinical pathology parameters, and gross necropsy examinations after consecutive 14-day treatment; histopathology examination was not conducted in this study. The second 2-week study in rats was to assess the toxic effects of degraded Org 25969 drug product; the study was reviewed by Dr. Adam Wasserman and the review is inserted in the following.

## STUDY TITLE: AN INTRAVENOUS TWO WEEK TOXICITY STUDY WITH DEGRADED ORG 25969 IN WISTAR RATS

**Study purpose:** To toxicologically qualify degradation products and/or impurities of Org 25969 drug product upon repeated intravenous administration for approximately 2 consecutive weeks in male and female Wistar rats.

**Key study findings**:

- Target organs of degraded Org 25969 were **lung** (foamy alveolar macrophages), **kidney** (cortical tubular vacuolation, tubular basophilia), and **urinary bladder** (umbrella cells with foamy cytoplasmic vacuolation). Renal and urinary findings were observed at all dose levels in both males and females, lung findings were at the HD level only.
- Other potential target organs identified included the seminal vesicle (↓weight in all treated male groups), epididymides (ruptured epididymal duct and sperm granulomas), liver (hepatic necrosis in 1 HD♂), and synvoial epithelium (↑foam cells), lymph node (↑foam cells)
- **No NOAEL identified due to renal and urinary bladder findings at all dose levels in both males and females**.

**Study no.**: Report #58554
**Volume #, and page #**: 4.2.3.2.1; eCTD submission
**Conducting laboratory and location**: N.V. Organon, Department of TDD, Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands
**Date of study initiation**: July 2004; Final report March, 2005
**GLP compliance**: Yes
**QA report**: Yes
**Drug, lot #, and % purity**: (Degraded) Org 25969; Batch codes U, W, and Z formulated as TY003-1 which was subdivided into TY003-1-2 and stored at 60°C for 2 months to degrade and relabeled TY004-1. TY004-1 was used in the study as the test article.

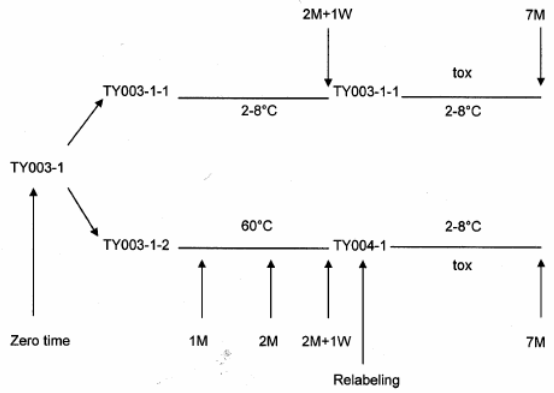


Fig 1.: Overview of the analytical time points for each batch

Total degradation products: (b) (4) %. Specific chemical analysis of degradation products were identified as RRTs at end of this report.

**METHODS**

***Test System***

**Species/strain**:Wistar rat (Han Crl: WI (Glx/BRL/Han) IGS) were supplied by (b) (4)

**Age**: 7-8 weeks at initiation
**Weight**: Males 256-301 g; Females 166-209 g

***Study Design***

| Group No. | Group size males | Group size females | Dose ($mg \cdot kg^{-1}$ per day) | Treatment |
|---|---|---|---|---|
| 1 (placebo) | 8 | 8 | 0 | Vehicle |
| 2 (LD, Low Dose) | 8 | 8 | 50 | Degraded Org 25969 |
| 3 (MD, Mid Dose) | 8 | 8 | 100 | Degraded Org 25969 |
| 4 (HD, High Dose) | 8 | 8 | 200 | Degraded Org 25969 |

**Doses**: 0 (saline), 50, 100 or 200 mg/kg Degraded Org 25969 x 2 weeks
**Route, formulation, volume, and infusion rate**: Intravenous at ~ 2 ml/kg depending on body weight; formulated as 100 mg/mL for HD and diluted with saline (vehicle) to 50 or 25 mg/mL concentrations for MD and LD.
**Number/sex/group or time point (main study)**: 8 rats/sex/group
**Satellite groups used for toxicokinetics or recovery**: None
**Unique study design, husbandry or methodology issues**: Housed individually. Blood for clinical pathology obtained by puncture of retro-orbital sinus under anesthesia. No acute sacrifice conducted, animals were terminated on SD14.

**Observations and times**

| Observational Parameter | Methods |
|---|---|
| Toxicokinetics | Not conducted |
| Clinical Observations | Observed for morbidity and mortality daily during the in-life period. Regularly observed (not further defined) during the 24 hr period after dosing. Samples of skin lesions in spare animal M039 were taken for further investigations. |
| Body Weights | 2X/week |
| Food Consumption | 1X/week with daily FC determined by dividing total consumed per number of days between evaluation |
| Ophthalmology | Not conducted |
| Clinical Pathology | Obtained from blood/urine samples from all animals at the end of the 2-week dosing period. Blood obtained from puncture of the retro-orbital sinus.<br><br>Hematologic parameters<br>Basophilic granulocytes ☒ Mean corpuscular volume ☒<br>Eosinophilic granulocytes ☒ Monocytes ☒<br>Hematocrit ☒ Neutrophilic granulocytes ☒<br>Hemoglobin ☒ Red blood cells ☒<br>Lymphocytes ☒ Red cell distribution width ☒<br>Mean corpuscular hemoglobin ☒ Reticulocytes ☒<br>Mean corpuscular hemoglobin conc. ☒ White blood cells ☒<br><br>Coagulation parameters<br>Mean thrombocytes volume ☒ Thrombocytes ☒<br>Thrombocytes distribution width ☒ Thrombocyte packed cell volume (thrombocrit) ☒<br>Activated partial thromboplastin time ☒ Prothrombin time ☒ |

Biochemistry parameters

| | | |
|---|---|---|
| Albumin | (Bromocresol green method) | ☒ |
| Albumin/Globulin ratio | (Calculated: albumin/(total protein - albumin)) | ☐ |
| Alkaline phosphatase | (IFCC method) | ☒ |
| Calcium | (O-cresolphthalein method) | ☒ |
| Chloride | (ISE method) | ☒ |
| Cholesterol | (CHOD-PAP method) | ☒ |
| Creatine kinase | (NVKC method) | ☒ |
| Creatinine | (Jaffe method without deproteinization) | ☒ |
| Globulin | (Difference between total proteins and Albumin) | ☒ |
| Glucose | (GOD-PAP method) | ☒ |
| Glutamic oxalacetic transaminase | (IFCC method) | ☒ |
| Glutamic pyruvic transaminase | (IFCC method) | ☒ |
| Gamma-glutamyl transaminase | (Szasz method) | ☐ |
| HDL-cholesterol | (PEG-modified enzyme method) | ☐ |
| Hemoglobin in plasma | (TMB method) | ☐ |
| Phosphate | (Molybdate UV method) | ☒ |
| Potassium | (ISE method) | ☒ |
| Sodium | (ISE method) | ☒ |
| Total bilirubin | (DPD method) | ☒ |
| Total protein | (Biuret method) | ☒ |
| Triglycerides | (GPO-PAP method) | ☒ |
| Urea-N | (Urease UV method) | ☒ |

Urinalysis parameters

| | | |
|---|---|---|
| Volume | (Measured in graduated flask) | ☒ |
| pH | (Hanna pH-meter model 302, -log[H+]) | ☒ |
| Protein | (Roche Diagnostics Hitachi 904 analyzer, pyrogallol red method at 37 °C) | ☒ |
| Glucose | (Roche Diagnostics Hitachi 904 analyzer, GOD-PAP method at 37 °C) | ☒ |
| Osmolality | (Advanced osmometer model 3D3, freezing point depression method) | ☒ |
| Blood | (Roche Combur 3 test E) | ☒ |
| Sodium | (Roche Diagnostics Hitachi 904 analyzer, ISE method) | ☒ |
| Potassium | (Roche Diagnostics Hitachi 904 analyzer, ISE method) | ☒ |
| Chloride | (Roche Diagnostics Hitachi 904 analyzer, ISE method) | ☒ |
| Creatinine | (Roche Diagnostics Hitachi 904 analyzer, Jaffe method without deproteinization at 37°C) | ☒ |
| Urea-N | (Roche Diagnostics Hitachi 904 analyzer, Urease UV method at 37°C) | ☒ |
| Phosphate | (Roche Diagnostics Hitachi 904 analyzer, Molybdate UV method at 37°C) | ☒ |
| Calcium | (Roche Diagnostics Hitachi 904 analyzer, O-cresolphthalein method at 37°C) | ☒ |
| N-acetyl beta-D-glucosaminidase (NAG) | (Roche Diagnostics Hitachi 904 analyzer, cresolsulfonphtalein method at 37°C) | ☒ |

The ratio of chloride/creatinine was also calculated for all animals of the study.

| | |
|---|---|
| Terminal Procedures:<br>Gross Pathology<br>Organ Weights<br>Histopathology | Animals sacrificed by exsanguination under isoflurane anesthesia. Necropsy performed on all animals. Organ weights were obtained in the following table with absolute and relative (to body weight) values calculated. Tissue sections listed in following table were obtained and those from the control and HD groups evaluated histopathologically after fixation in 4% buffered formaldehyde (except testes and eyes which were fixed in Davidson's solution) and were stained with H&E. Kidney, urinary bladder, and lung which are considered to be possible target organs were examined in LD and MD group animals. Bone marrow samples from femur was taken and slide preparations made and fixed.<br>*Reviewers comment: Only the kidney, bladder and lung was evaluated in low- and mid-dose groups. Presence of a higher value in the HD vs. controls did not by protocol require evaluation of lower dose groups. Also, this was not peer-reviewed according to Sponsor.* |

| Organ/Tissue/Cavity<br>Autopsy Code | Weight<br>ST/SU | Macroscopy<br>All | Microscopy<br>ST/SD/SU | Microscopy<br>SP |
|---|---|---|---|---|
| Abdominal cavity | | ☒ | | |
| Adrenal glands | ☒* | ☒ | ☒ | ☐ |
| Aorta | | ☒ | ☒ | ☐ |
| Bone marrow | | ☒ | ☒ | ☐ |
| Brain | ☒ | ☒ | ☒ | ☐ |
| Cecum | | ☒ | ☒ | ☐ |
| Colon | | ☒ | ☒ | ☐ |
| Cranial cavity | | ☒ | | |
| Diaphragm | | ☒ | ☒ | ☐ |
| Duodenum | | ☒ | ☒ | ☐ |
| Epididymides | ☒* | ☒ | ☒ | ☐ |
| Esophagus | | ☒ | ☒ | ☐ |
| External features | | ☒ | | |
| Eyes | | ☒ | ☒ | ☐ |
| Fallopian tubes | | ☐ | ☐ | ☐ |
| Femur & Joint | | ☒ | ☒ | ☐ |
| Gall bladder | | ☐ | ☐ | ☐ |
| Harderian glands | | ☒ | ☒ | ☐ |
| Heart | ☒ | ☒ | ☒ | ☐ |
| Identification | | ☒ | | |
| Ileum | | ☒ | ☒ | ☐ |
| Intestines, MALT | | ☒ | ☒ | ☐ |
| Jejunum | | ☒ | ☒ | ☐ |
| Kidneys | ☒* | ☒ | ☒ | ☐ |
| Lacrimal glands, extraorbital | | ☒ | ☒ | ☐ |
| Liver | ☒ | ☒ | ☒ | ☐ |
| Lungs and bronchi | | ☒ | ☒ | ☐ |
| Lymph node, mesenteric | ☐ | ☒ | ☒ | ☐ |
| Lymph node, popliteal | ☒ | ☒ | ☒ | ☐ |
| Mammary glands | | ☒ | ☒ | ☐ |
| Nerve, optic | | ☒ | ☒ | ☐ |
| Nerve, sciatic | | ☒ | ☒ | ☐ |
| Oral cavity | | ☒ | | |
| Ovaries | ☒* | ☒ | ☒ | ☐ |
| Pancreas | ☐ | ☒ | ☒ | ☐ |
| Pituitary gland | ☒ | ☒ | ☒ | ☐ |
| Prostate gland | ☒ | ☒ | ☒ | ☐ |
| Rectum | | ☒ | ☒ | ☐ |
| Salivary glands, Parotid | | ☒ | ☐ | ☐ |
| Salivary glands, Sublingual | | ☒ | ☒ | ☐ |
| Salivary glands, Submandibular | | ☒ | ☒ | ☐ |
| Seminal vesicles & Coagulating glands | ☒ | ☒ | ☒ | ☐ |
| Skeletal muscles | | ☒ | ☒ | ☐ |
| Skin and subcutaneous tissues | | ☒ | ☒ | ☐ |
| Spinal cord | | ☒ | ☒ | ☐ |
| Spleen | ☒ | ☒ | ☒ | ☐ |
| Sternum | | ☒ | ☒ | ☐ |
| Stomach | | ☒ | ☒ | ☐ |
| Testis | ☒* | ☒ | ☒ | ☐ |
| Thoracic cavity | | ☒ | ☐ | ☐ |
| Thymus | ☒ | ☒ | ☒ | ☐ |
| Thyroid glands & Parathyroid glands | ☒* | ☒ | ☒ | ☐ |
| Tongue | | ☒ | ☒ | ☐ |
| Trachea | | ☒ | ☒ | ☐ |
| Treatment site(s) | | ☒ | ☒ | ☐ |
| Urinary bladder | | ☒ | ☒ | ☐ |
| Uterus | ☒ | ☒ | ☒ | ☐ |
| Vagina | | ☒ | ☒ | ☐ |
| Vertebral column | | ☒ | ☐ | ☐ |

*Paired organs will be weighed together.
ST = Sacrificed at termination of dosing period animals
SD = Spontaneous dead animals
SU = Unscheduled sacrificed animals
SP = Post-dosing animals

| | |
|---|---|
| Statistical Evaluation | Depending on the statistical characteristics of the data for the numerical parameters, if feasible, one of the following tests was performed to assess significant differences between the drug-treated and the vehicle-treated animals (covariance = start body weight):<br>Wilcoxon signed rank test,<br>Covariance analysis,<br>Permutation test,<br>Student *t* test. |

***Study Deviations/Impact***: Test article parent (TY003-1) was put on accelerated stability for 2 months instead of 2.5 months as specified in the protocol to generate TY004-1. Data from 2-month accelerated stability is available in the table at the end of the report. *Reviewers comment: This does not appear to impact the conclusions of the study.*

**RESULTS**

**Toxicokinetics**

Not conducted.

**Mortality**
No deaths were observed.

**Clinical signs**
No treatment-related clinical observations were observed.

**Body weights**
A mild decrease (-18% vs. control; p≤0.05) in body weight gain was noted after SD9 in HD♂. No findings in females.

**Food consumption**
A mild decrease in mean food consumption (-5.7% vs. control; p≤0.05) was noted in the second week in HD♂. This correlates with the reduction in body weight gain observed in this group. No observations in females.

**Hematology**
An increased reticulocyte level (absolute) was increased in the treated females which was statistically significant in the HD♀ (+30% vs. controls, p≤0.05) at the end of the treatment period. No difference was noted in males. No other hematologic parameters were notably altered in a treatment- or dose-related fashion.

**Blood clotting**
Mildly increased thrombocrit values were noted in the MD♀ (vs. controls; p ≤ 0.05) and HD♀ (n.s.s.) vs. controls.

**Clinical chemistry**
***ALT***: ↑increased in a single male animal M#4407 (141 U/L vs. control mean of 24 ± 5 U/L). No significant differences between group means of males or females. No other notable individual ALT outliers.
***ALP***: Non-statistically significant increase in ALP in HD♀ (+23%) vs. controls. No changes in males.
***Potassium***: Decreased mean levels in HD♀ (-8% vs. controls; p≤0.05)
***CK***: Increased mean CK activity in HD males (+17% vs. controls; n.s.s.) whereas there was decreased mean activity in HD♀ (-34% vs. controls; p≤0.05)

Sporadic values of other parameters were altered (↓Urea-nitrogen, ↓Creatinine, ↑Phosphate, ↑Globulin) and statistically significant but demonstrated neither dose-dependency nor consistency between genders.

**Urinalysis**
A treatment-related decrease in mean Chloride level was noted in the HD♂ (vs. controls; p≤0.05) which when corrected by creatinine values was not statistically significant.

**Organ weights**
***Thymus:*** Increased absolute and relative weight of thymus was noted in HD♀ (+16% vs. controls; p≤0.05). No treatment-related effect on the thymus in males.

***Seminal vesicles***: Decreased absolute (-26%, -25%, -20% for LD, MD, and HD vs. controls) and relative (-23%, -30%, -17% vs. controls) weight of seminal vesicles compared with controls in all male Org 25969-

treated groups. *Reviewers comment: Dose dependency is not observed; however, testicular histopathologic effects were noted in previous studies though no microscopic changes were noted in the present study. Therefore the relationship to treatment is not clear.*

**Gross pathology**

No treatment-related macroscopic observations were found.

**Histopathology**

| Target Organ/ Finding | Study Day | | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 50 | 100 | 200 | 0 | 50 | 100 | 200 |
| **Lung** | | | | | | | | | | |
| ↑alveolar Macrophages | SD14 | Total | 1/8 | 0/8 | 1/8 | **6/8** | 0/8 | 0/8 | 1/8 | **3/8** |
| | | *Min* | | | | *3* | | | *1* | |
| | | *Slight* | | | | | | | | *2* |
| | | *Mod* | | | | *3* | | | | *1* |
| **Kidneys** | | | | | | | | | | |
| ↑tubular basophilia | SD14 | Total | 0/8 | 0/8 | 0/8 | **1/8** | 0/8 | **2/8** | 0/8 | **2/8** |
| | | *Slight* | | | | *1* | | *2* | | *1* |
| | | *Mod* | | | | | | | | *1* |
| Dilation of renal pelvis | SD14 | Slight | 0/8 | 0/8 | 0/8 | **1/8** | 0/8 | 0/8 | 0/8 | 0/8 |
| Tubular cells with basophilic, foamy cytoplasm | SD14 | Total | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | **1/8** |
| Vacuolated cortical tubular cells | SD14 | Total | 0/8 | **5/8** | **7/8** | **8/8** | 0/8 | **7/8** | **8/8** | **8/8** |
| | | *Min* | | *4* | *3* | | | *5* | *3* | *1* |
| | | *Slight* | | *1* | *3* | *7* | | *2* | *5* | *5* |
| | | *Mod* | | | *1* | *1* | | | | *2* |
| **Urinary bladder** | | | | | | | | | | |
| Foamy cytoplasm, umbrella cells | SD14 | Total | 0/8 | **2/8** | **4/8** | **8/8** | 0/8 | 0/8 | **5/8** | **8/8** |
| | | *Min* | | *2* | *1* | *2* | | | *5* | *5* |
| | | *Slight* | | | *3* | *6* | | | | *3* |
| Karyomegalia | SD14 | Total | 0/8 | 0/8 | 0/8 | **1/8** | 0/8 | 0/8 | 0/8 | 0/8 |
| Urothelial hyperplasia | SD14 | Total *Minimal* | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 1/8 | 0/8 |
| **Liver** | | | | | | | | | | |
| Hepatocellular necrosis | SD14 | Total | 0/8 | - | - | 1/8 | 0/8 | - | - | 0/8 |
| **Jejunum** | | | | | | | | | | |
| granuloma | SD14 | Total | 0/8 | - | - | 1/8 | 0/8 | - | - | 0/8 |
| **Epididymides** | | | | | | | | | | |
| Ruptured epididymal duct | SD14 | Total | 0/8 | - | - | 1/8 | | | | |
| Sperm granulomas | SD14 | Total | 0/8 | - | - | 3/8 | | | | |
| **Popliteal lymph node** | | | | | | | | | | |
| Foam cell aggregation | SD14 | Total | 0/8 | - | - | 3/8 | 0/8 | - | - | 0/8 |

Bold values are those which are thought to represent evidence of a test-article-related effect;
-, no evaluation conducted

***Lung:*** The Sponsor notes the increased incidence of foamy macrophages in the lung of HD♂ and HD♀ group animals.

***Kidney:*** Clear treatment- and dose-related increases in "fine cortical tubular cell vacuolation" (p. 33) described by Sponsor which is diffuse in females but of greater area in males was noted. The Sponsor notes that observed clinical chemistry parameters and urinary parameters do not suggest this is a degenerative change (no changes in electrolytes, Urea-N, protein, NAG).
*Reviewers comment: A minority of rats in the HD groups also demonstrated evidence of tubular damage including ↑tubular basophilia, and tubular cells with basophilic foamy cytoplasm.*

***Urinary Bladder:*** Umbrella cells were noted in all ***treated*** male groups and females ≥ MD with "fine, foamy cytoplasmic vacuolation" (p. 33) of minimal to slight severity. Karyomegaly was noted in 1/8 HD♂ (not found in other treatment or control groups).

***Reproductive tract***: Not a single organ but several changes noted in these tissues including the sperm granulomas in 3 HD♂, a ruptured epididymal duct in 1 HD♂, a ↓seminal vesicle weight (absolute and relative) in all treatment groups compared with control. Microscopic evaluation of seminal vesicle was performed and no histopathologic correlate was found.

**Other changes that may be treatment-related**: ***Synovial epithelial cells*** with foam cytoplasm in 2 HD♂, ***Liver:*** hepatocellular necrosis in 1 HD♂ (M#4101; not the animal with elevated ALT); ***Seminal vesicles***: ↓weight in all treated male groups compared with controls. ***Lymph node***: Popliteal lymph node with increased foam cells in 3/8 HD♂.

**Other Evaluations**
Degradation/Impurity batch analysis for:
TY003-1 (parent batch) and TY004-1 (degraded batch)

Table 1: Impurity content results of batch TY003-1-1

| | Zero time | | 2 months + 1 week 2-8°C | 7 months 2-8°C |
|---|---|---|---|---|
| RRT | Drug substance (%) | TY003-1-1 (%) | TY003-1-1 (%) | TY003-1-1 (%) |
| | | | | (b) (4) |

Nd= not detected.
* : degradation products to be specified.

Table 2: Impurity content results of batch TY003-1-2 / TY004-1

| | Zero time | | 1 month 60°C | 2 months 60°C | 2 months + 1 week 60°C | 4 months + 3 weeks 2-8°C |
|---|---|---|---|---|---|---|
| RRT | Drug substance (%) | TY003-1-2 (%) | TY003-1-2 (%) | TY003-1-2 (%) | TY003-1-2 (%) | TY004-1 (%) |
| | | | | | | (b) (4) |

TY003-1-2 stored for 2 months+1week at 60°C = TY004-1 at zero time.
Nd= not detected.
TY004-1 stored for 4 months + 3 weeks at 2-8°C was first stored at 60°C for 2 months + 1 week (total storage time is 7 months).
* : degradation products to be specified.

Two 4-week toxicity studies were conducted in rats. In these 4-wk studies, Org 25969 was administered IV to male and female Wistar rats via a tail vein for 4 consecutive weeks at dosages of 0, 30, 120 or 500 mg/kg; toxic effects were examined after immediate termination of treatment and the reversibility of the toxic effects was also investigated in animals of recovery groups. The second study, however, had a recovery period of 8 weeks as compared to 3 weeks in the first study purportedly to investigate the reversibility of the adverse findings with 4-week administration of Org 25969 after a longer treatment-free period. In addition, the drug substance used in the second 4-wk study was batch AE which contains relatively high level of the second active ingredient Org 48302 as compared to batch U (containing 1.3% Org 48302) used in the first study purportedly to assess the potential toxicity of Org 48302 in rats at a level higher than those that will be used in human. These two 4-wk studies were conducted in the same lab facility and the finding following administration in these two studies were comparable; therefore, only a full review report was completed for the second study. This review report was conducted by Dr. Adam Wasserman and inserted in the following.

### STUDY TITLE: A 4-WEEK INTRAVENOUS TOXICITY AND TOXICOKINETIC STUDY WITH ORG 25969/ORG 48302 IN MALE AND FEMALE WISTAR RATS FOLLOWED BY AN 8-WEEK RECOVERY PERIOD (SECOND 4-WEEK STUDY)

**Study purpose: (per Sponsor, p. 2)**

- To study the toxicity of Org 25969 with it's related cyclodextrin Org 48302 in Wistar rats with daily intravenous administration for four consecutive weeks.
- To determine the Org 25969 and 48302 plasma concentrations after a single intravenous dose and after multiple daily intravenous dosing for approximately three weeks;
- The TK parameters of Org 25969 and 48302 associated with the plasma concentrations and compare the TK characteristics and relate them to toxicologic findings
- To evaluate the effects of Org 25969/48302 on bone, considering the retention of Org 25969 in this tissue;
- To determine the reversibility of effects at the high dose during a recovery period of 8 weeks.
- To obtain bone marrow samples for a micronucleus test that will be performed and reported separately

**Key study findings**:

Rats were administered Org 25969, containing 5.99% Org 48302, at doses of 0, 30, 120 or 500 mg/kg/day for 4-weeks with 8-week treatment-free period to assess reversibility. Major findings were:

Clinical Pathology (all recovered)

- Mild inhibitory effect on red cell parameters (↓RBC, ↓HCT, with compensatory ↑Retics) at 500 mg/kg/day in both males and females
- Mild ↑neutrophils, primarily in the 500 mg/kg/day males
- Mild ↓PT in 500 mg/kg/day males
- Mild ↓platelets in 500 mg/kg/day males

- Mild ↓creatinine levels in 500 mg/kg/day females

Histopathology (partial or no recovery)
- Lungs: ↑foamy macrophages (minimal to marked) in 500 mg/kg/day males and females (near total recovery)
- Kidney: vacuolated cortical tubule cells (minimal to moderate) at ≥ 120 mg/kg/day in both males and females (partial recovery)
- Urinary bladder: foamy cytoplasm (minimal to moderate) in umbrella cells at ≥ 120 mg/kg/day in both males and females (no recovery)

Based on histopathologic findings the NOAEL in the study is 30 mg/kg/day. Toxicokinetics data for the particular study could not be determined as they fell outside GLP standards and were rejected. Data from the prior 4-week toxicology and toxicokinetic study in the rat (NL#0043521) which used identical dosing may be informative. In that study, the NOAEL of 30 mg/kg/day was associated with an end-of-study $C_0$ of approximately 195 µg/mL (rough estimation) and an $AUC_{0-3}$ of 48 µg*h/mL (predicted from $AUC_{0-\infty}$ data).

**Study no.**: Final Report NL0059534
**Volume #, and page #**: 4.2.3.2.1, eCTD
**Conducting laboratory and location**: Test facility: N.V. Organon, Department of TDD, Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands; Bioanalytical test site: Organon S.A. Dept. of Toxicology and Drug Disposition, 22, rue Henri Goudier, 63203 Riom, Cedex, France; Pathology Group (special staining and slide preparation of bone): Pathology Group, General Pharmacology, N.V. Organon, Molenstraat 110, NL-5342 CC Oss, The Netherlands.
**Date of study initiation**: July 19, 2004, final report date March, 2005.
**GLP compliance**: Yes
**QA report**: Yes
**Drug, lot #, and % purity**: Org 25969 Batch AE, 81.7% purity (5.99% Org 48302), preparation coded TY006-1.

**METHODS**

***Test System***

**Species/strain**: Wistar rat (Hsd/Cpb:WU (spf-bred) supplied by (b) (4)

**Age**: Not stated; likely 7-8 weeks as these weights are in line with previous studies reported at this age at initiation

**Weight**: Males: 228-269 g (301-393g positive controls for MN test); Females: 141-189 g (209-244 g positive controls for MN test).

***Study Design***

| Group No. | Group size and sex | | Dose Org 25969 | Treatment |
|---|---|---|---|---|
| | Female | Male | (mg/kg/day) | |
| 1 (placebo-PL1) | 20* | 20* | 0 | Vehicle |
| 2 (low-dose, DS2) | 10 | 10 | 30 | Org 25969 |
| 3 (mid-dose, DS3) | 10 | 10 | 120 | Org 25969 |
| 4 (high-dose, DS4) | 20* | 20* | 500 | Org 25969 |
| 5 (positive control micronucleus test) | 6 | 6 | 50 | MMS** |

* 10 animals per sex (Groups 1 and 4) were allocated to the post-dosing period.

** Methylmethanesulphonate, single intraperitoneal dose

**Doses**: 0 (physiologic saline), 30, 120 or 500 mg/kg/day Org 25969 or 50 mg/kg MMS
**Route, formulation, volume, and infusion rate**: i.v. administration through tail vein, 5 ml/kg
**Number/sex/group or time point (main study)**: 10 animals/sex/group
**Satellite groups used for toxicokinetics or recovery**: 10 animals/sex/placebo and HD group
**Unique study design, husbandry or methodology issues**: A subset of positive control animals for the micronucleus test were administered a single i.p. dose of 50 mg/kg methylmethanesulfonate (MMS) in a volume of 2 ml/kg approximately 24 hr prior to sacrifice.

**Observations and times**

| Observational Parameter | Methods |
|---|---|
| Toxicokinetics | Plasma obtained and $C_0$ and AUC values for both Org 25969 and Org 48302 were determined. Dose-normalized values ($NC_0$ and NAUC were calculated). As Org 48302 represented 5.99% of the Org 25969 dose given, the doses of Org 48302 given were effectively 1.8, 7.2, and 30 mg/kg for the Org 25969 doses 30, 120 and 500 mg/kg. Blood collection for bioanalysis was collected at start of dosing and during 4th week of dosing period for 2 animals/sex/time-point and regimen from the 30 and 120 mg/kg groups and 4 animals per sex/time-point and regimen for the 500 mg/kg group at 0h, 2.5 min, 5 min, 10 min, 20 min, 45 min, 1.5 h, 3 h, 7 h, and 24 h. Org 25969 and Org 48302 levels were measured using LC-MS/MS. RESULTS OF TOXICOKINETICS IN THE STUDY ARE NOT REPORTED DUE TO TECHNICAL ERRORS. |
| Clinical Observations | Animals observed for morbidity and mortality during in-life period, observed regularly on day of treatment and at least once a day during remaining observation period during dosing and post-dosing periods. |
| Body Weights | Recorded at least 1X/week during pre-dosing period, once at the start of dosing and twice a week thereafter during the dosing period. During the post-dosing period body weight was determined 1X/week. |
| Food Consumption | Weekly per cage evaluation with daily determination through dividing number of days in between weighing of food. |
| Ophthalmology | All animals examined prior to initiation of dosing. Controls and HD animals examined during SW4. Exam comprised "general impression, observation a vue of eye lids, conjunctiva, epiphora and eye ball, and observation with an indirect binocular ophthalmoscope of cornea, anterior chamber, iris, lens, vitreous body and fundus. |
| Clinical Pathology | Hematology, clotting, biochemistry and UA were obtained from all animals sacrificed at the end of the dosing period or at the end of the post-dosing period. Animals were fasted overnight prior to sampling though water was available.<br><br>Toxicokinetic blood sampling was obtained from all treated animals after a single dose as well as during the last week of the dosing period (*Reviewer's comment: Not apparently the final dosing day*). 2 animals per time-point per dosing group, per sex for the LD and MD groups and 4 animals per time point, |

per sex for the HD group. Vehicle-treated animals also had the same volume of blood removed to be consistent with treatment of Org 25969-treated animals.

Final blood samples for hematology and blood biochemistry evaluation collected from abdominal aorta under isoflurane/NO2/O2 anesthesia. Urine collected overnight in metabolism cages (18 hr duration).

**Hematology parameters:**

| | | | |
|---|---|---|---|
| Red blood cells (RBC) | ☒ | Lymphocytes (LYM*) | ☒ |
| Hematocrit (HCT) | ☒ | Monocytes (MONO*) | ☒ |
| Hemoglobin (HGB) | ☒ | Neutrophilic granulocytes (NEU*) | ☒ |
| Mean corpuscular volume (MCV) | ☒ | Eosinophilic granulocytes (EOS*) | ☒ |
| Mean corpuscular hemoglobin (MCH) | ☒ | Basophilic granulocytes (BASO*) | ☒ |
| Mean corpuscular hemoglobin conc.(MCHC) | ☒ | Platelets (PLT) | ☒ |
| Red cell distribution width (RDW) | ☒ | Mean Platelet Volume (MPV) | ☒ |
| Reticulocytes (RTC*) | ☒ | Platelet Distribution Width (PDW) | ☒ |
| White blood cells (WBC) | ☒ | Platelet packed cell volume (PCT) | ☒ |

*Parameters were reported with addition 1 (=absolute value) or 2 (=relative value).

**Clotting parameters:**

| | | | |
|---|---|---|---|
| Activated partial thromboplastin time (APTT) | ☒ | Prothrombin time (PTT) | ☒ |

Data are reported as individual values and means per sex per group in a table.

**Biochemistry parameters**:

| | | |
|---|---|---|
| Aspartate aminotransferase (AST) | (IFCC method) | ☒ |
| Alanine aminotransferase (ALT) | (IFCC method) | ☒ |
| Glutamate dehydrogenase (GLDH) | (DGKC method) | ☒ |
| Creatine kinase (CK) | (IFCC method) | ☒ |
| Alkaline phosphatase (ALP) | (IFCC method) | ☒ |
| Gamma-glutamyl transferase(GGT) | (Szasz method) | ☒ |
| Total bilirubin(BIL-T) | (DPD method) | ☒ |
| Icterus index (I) | (Measurement after dilution with saline) | ☒ |
| Triglycerides (TG) | (GPO-PAP method) | ☒ |
| Cholesterol (CHOL) | (CHOD-PAP method) | ☒ |
| HDL-cholesterol | (PEG-modified enzyme method) | ☐ |
| Phospholipids | (Enzymatic method) | ☐ |
| Lipemia index (L) | (Measurement after dilution with saline) | ☒ |
| Creatinine (CREA) | (PAP method) | ☒ |
| Urea (UREA) | (Urease UV method) | ☒ |
| Glucose (GLUC) | (Hexokinase method) | ☒ |
| Total protein (TP) | (Biuret method) | ☒ |
| Albumin (ALB) | (Bromocresolgreen method) | ☒ |
| Globulin (GLOB) | (Calculated: total protein-Albumin) | ☒ |
| Albumin/Globulin ratio (AGRA) | (Calculated: albumin/(total protein - albumin) | ☒ |
| Calcium (CA) | (O-cresolphtalein method) | ☒ |
| Inorganic phosphorus (PHOS) | (Molybdate UV method) | ☒ |
| Sodium (Na) | (ISE method) | ☒ |
| Potassium (K) | (ISE method) | ☒ |
| Chloride (CL) | (ISE method) | ☒ |
| Hemolysis index (H) | (measurement after dilution with saline) | ☒ |

**Hormone parameters:**

| | | |
|---|---|---|
| Testosterone (TES) | Chemiluminicent enzyme immunoassay | ☒ |
| Estradiol (E2) | Chemiluminicent enzyme immunoassay | ☒ |

**Urinalysis parameters**:

<table>
<tr><td></td><td>
<table>
<tr><td>Volume (Quantity)</td><td>(Graduated flask)</td><td>☒</td></tr>
<tr><td>Osmolality (OSM)</td><td>(freezing-point depression method)</td><td>☒</td></tr>
<tr><td>PH (pH)</td><td>(pH-meter)</td><td>☒</td></tr>
<tr><td>Glucose (GLUC)</td><td>(Quantitative, Hexokinase method at the Modular)</td><td>☒</td></tr>
<tr><td>Protein (UCTP)</td><td>(Quantitative, Brenzethonium chloride method at the Modular)</td><td>☒</td></tr>
<tr><td>N-acetyl-Beta-D-Glucosaminidase (NAG)</td><td>(Quantitative, 3-cresolsulfonphtalinein method at the Modular)</td><td>☒</td></tr>
<tr><td>Creatinine (CREA)</td><td>(Quantitative, PAP method at the Modular)</td><td>☒</td></tr>
<tr><td>Hemoglobin (HGB)</td><td>(Semi-quantitative, Roche Combur$^5$ test® D, score 0, +, ++, +++, ++++)</td><td>☒</td></tr>
<tr><td>Ketones (KET)</td><td>(Semi Quantitative, Roche Combur$^5$ test® D, score 0, +, ++, +++)</td><td>☒</td></tr>
<tr><td>Sodium (Na)</td><td>(Quantitative, ISE method at the Modular)</td><td>☒</td></tr>
<tr><td>Potassium (K)</td><td>(Quantitative, ISE method at the Modular)</td><td>☒</td></tr>
<tr><td>Chloride (CL)</td><td>(Quantitative, ISE method at the Modular)</td><td>☒</td></tr>
<tr><td>Calcium (CA)</td><td>(Quantitative, ISE method at the Modular)</td><td>☒</td></tr>
<tr><td>Inorganic phosphorus (PHOS)</td><td>(Molybdate UV method at the Modular)</td><td>☒</td></tr>
</table>
Additional UA parameters: Creatinine excretion and glucose/creatinine, protein/creatinine, NAG/creatinine, sodium/creatinine, potassium/creatinine and chloride/creatinine were calculated.
</td></tr>
<tr><td>Terminal Procedures<br>Gross Pathology<br>Organ Weights<br>Histopathology</td><td>Rats were euthanized by abdominal aorta exsanguination under urethane anesthesia. Animals underwent a necropsy and gross abnormalities noted. Organ weights were obtained as indicated in the tissue list following and calculated as absolute and relative to body weight.<br>Organs fixed in 4% buffered formaldehyde except eyes which was fixed in Davidson's fluid and right femur which was fixed in Burkhardt's fixative.<br>Tissues were processed to 5 µM sections and stained with H&E. Femurs from 5 animals from both gender of the placebo and HD group were sent to Pathology Group of the General Pharmacology Group, N.V. Organon, Oss, The Netherlands for subsequent von Kossa staining.</td></tr>
</table>

| Organ/Tissue/Cavity<br>Autopsy Code | Weight<br>ST/SU | Macroscopy<br>All | Microscopy<br>ST/SP |
|---|---|---|---|
| Abdominal cavity | | ☒ | |
| Adrenal glands* | ☒ | ☒ | ☒ |
| Aorta | | ☒ | ☒ |
| Bone marrow | | ☒ | ☒ |
| Brain | ☒ | ☒ | ☒ |
| Cecum | | ☒ | ☒ |
| Coagulating glands | | ☒ | ☒ |
| Colon | | ☒ | ☒ |
| Cranial cavity | | ☒ | |
| Diaphragm | | ☒ | ☒ |
| Duodenum | | ☒ | ☒ |
| Epididymides* | ☒ | ☒ | ☒ |
| Esophagus | | ☒ | ☒ |
| External features | | ☒ | |
| Eyes | | ☒ | ☒ |
| Femur & Joint | | ☒ | ☒ |
| Gall bladder | | ☐ | ☐ |
| Harderian gland | | ☒ | ☒ |
| Heart | ☒ | ☒ | ☒ |
| Identification | | ☒ | |
| Ileum | | ☒ | ☒ |
| Intestines, MALT | | ☒ | ☒ |
| Jejunum | | ☒ | ☒ |
| Kidneys* | ☒ | ☒ | ☒ |
| Liver | ☒ | ☒ | ☒ |
| Lungs and bronchi | | ☒ | ☒ |
| Lymph node, mesenteric | | ☒ | ☒ |
| Lymph node, right popliteal | | ☒ | ☐ |
| Mammary glands | | ☒ | ☒ |
| Nerve, optic | | ☒ | ☒ |
| Nerve, sciatic | | ☒ | ☒ |
| Oral cavity | | ☒ | |
| Ovaries* | ☒ | ☒ | ☒ |
| Pancreas | | ☒ | ☒ |
| Pituitary gland | ☒ | ☒ | ☒ |
| Preputial/clitoal glands | | ☒ | ☐ |
| Prostate gland | ☒ | ☒ | ☒ |
| Rectum | | ☒ | ☒ |
| Salivary glands, Parotid | | ☒ | ☒ |
| Salivary glands, Sublingual | | ☒ | ☒ |

| Organ/Tissue/Cavity<br>Autopsy Code | Weight<br>ST/SP/SU | Macroscopy<br>All | Microscopy<br>SP/ST |
|---|---|---|---|
| Salivary glands, Submandibular | | ☒ | ☒ |
| Seminal vesicles | ☒ | ☒ | ☒ |
| Skeletal muscle | | ☒ | ☒ |
| Skin and subcutaneous tissues | | ☒ | ☒ |
| Spinal cord | | ☒ | ☒ |
| Spleen | ☒ | ☒ | ☒ |
| Sternum | | ☒ | ☒ |
| Stomach | | ☒ | ☒ |
| Testes* | ☒ | ☒ | ☒ |
| Thoracic cavity | | ☒ | |
| Thymus | ☒ | ☒ | ☒ |
| Thyroid glands & Parathyroid glands* | ☒ | ☒ | ☒ |
| Tongue | | ☒ | ☒ |
| Trachea | | ☒ | ☒ |
| Treatment site(s) | | ☒ | ☒ |
| Urinary bladder | | ☒ | ☒ |
| Uterus | ☒ | ☒ | ☒ |
| Vagina | | ☒ | ☒ |
| Vertebral column | | ☐ | ☐ |

* Paired organs will be weighed together
ST = Sacrificed at the end of the dosing period
SP = Sacrificed at the end of the post-dosing period

| | |
|---|---|
| Other Evaluations | ***Bone marrow micronucleus***: The left femur was obtained from all animals sacrificed at the end of the dosing period and of all animals of group 5 (positive control group) and marrow collected for a bone marrow micronucleus test reported separately. |
| Statistical analysis | One of the following tests was performed to assess significant differences |

| | |
|---|---|
| | between the drug-treated and the vehicle-treated animals (covariable = start body weight):<br>Wilcoxon signed rank test, Covariance analysis, Permutation test, Student t-test.<br>-- note the covariable does not appear to have been used in any test |

***Study Deviations/Impact***: (from Sponsor, verbatim, p.11-12):
This study was performed as outlined in the protocol and its amendments, with the following
exceptions:
- The right femur was fixed in Bürkhardt's fixative instead of the left femur (from the left femur bone marrow was taken for the micronucleus test at the end of the dosing period, as a consequence the right femur was also kept in Bürkhardt's fixative at the sacrifice at the end of the post-dosing period).
- With respect to allocation the following was noted after start of dosing:
The cages were not allocated to the blocks in an ascending body weight order. For the cages of either sex used for allocation the mean body weight was determined and the cages were allocated to the blocks in an ascending order for the deviation of the mean body weight of the cage from the mean body weight per sex.
As a consequence, the difference in mean body weight between the cages were the highest for the last block, and the body weights per sex differed slightly between the groups.
*Reviewer's comment: Agree with Sponsor that these deviations are not likely to have affected the integrity or conclusions of the study.*

From study report 0067962 (Amendment #1): Date January 2006.
The Bioanalysis part of the study is excluded from the GLP scope and references to TK should be omitted. The QC samples were not integrated in a justified way. Reevaluation of the calculated concentrations resulted in QC concentrations that were out of the acceptance limits. This will cause the rejection of the bioanalytical results.
*Reviewer's comment: The inability to use toxicokinetic data collected during the study does not fatally compromise the results of the study; however, interpretation of exposure to Org 25969 and Org 48302 is complicated. The Sponsor uses data from a previous 4-week toxicology and toxicokinetic study in the rat (study report #0043521) with identical Org 25969 dosing as the current study; no information on 48302 levels are present in this study, however..*

**RESULTS**

**Toxicokinetics**

***Note***: Due to deviations from GLP in the current study, the following TK table of Org 25969 kinetics is not derived from samples obtained during this study but from study #43521, a previous 4-week tox and TK study in rat which used identical dosing to this study.

| kinetic parameters * | Sex | 30 mg·kg$^{-1}$ | | 120 mg·kg$^{-1}$ | | 500 mg·kg$^{-1}$ | |
|---|---|---|---|---|---|---|---|
| | | SD | MD | SD | MD | SD | MD |
| $AUC_{(0-3)}$ (µg·h·mL$^{-1}$) | M | 75.6# | 48.7[1)] | 262 | 230 | 1041 | 1174 |
| | F | 48.1 | 38.9[1)] | 197 | 183 | 1160# | 929 |
| $NAUC_{(0-3)}$ (µg h·mL$^{-1}$)/(mg·kg$^{-1}$) | M | 2.52# | 1.62[1)] | 2.18 | 1.92 | 2.08 | 2.35 |
| | F | 1.60 | 1.30[1)] | 1.64 | 1.53 | 2.32# | 1.86 |
| $AUC_{inf}$ (µg·h·mL$^{-1}$) | M | 75.9# | 50.9 | 263 | 232 | 1059 | 1212 |
| | F | 48.7 | - | 198 | - | - | - |
| $NAUC_{inf}$ (µg·h·mL$^{-1}$)/(mg·kg$^{-1}$) | M | 2.53# | 1.70 | 2.19 | 1.93 | 2.12 | 2.42 |
| | F | 1.62 | - | 1.65 | - | - | - |
| $C_0$ (µg·mL$^{-1}$) | M | 1609# | 240 | 961 | 510 | 6406 | 7669 |
| | F | 145 | 149 | 1973 | 880 | 13724# | 4073 |
| $NC_0$ (µg·mL$^{-1}$)/(mg·kg$^{-1}$) | M | 53.6# | 7.99 | 8.01 | 4.25 | 12.8 | 15.3 |
| | F | 4.84 | 4.96 | 16.4 | 7.33 | 27.5# | 8.15 |
| $t_{1/2}$ (h) | M | 0.43 | 0.35 | 0.40 | 0.43 | 0.65 | 1.10 |
| | F | 0.60 | - | 0.43 | - | - | - |
| CL (L·kg$^{-1}$·h$^{-1}$) | M | 0.40 | 0.59 | 0.46 | 0.52 | 0.47 | 0.41 |
| | F | 0.62 | - | 0.61 | - | - | - |
| V (L·kg$^{-1}$) | M | 0.24 | 0.29 | 0.26 | 0.32 | 0.44 | 0.65 |
| | F | 0.53 | - | 0.37 | - | - | - |

SD: single dose ; MD: multiple dose; M: male ; F: female; - : not reportable value; * : After multiple dosing, AUC under the dosing interval ($AUC_{(0-24)}$) was estimated by $AUC_{inf}$
# : slightly overestimated
1) : $AUC_{(0-1.5)}$ and $NAUC_{(0-1.5)}$ instead of $AUC_{(0-3)}$ and $NAUC_{(0-3)}$

Org 25969: TK data for Org 25969 demonstrates a dose-dependent increase in exposure with increasing dose, no accumulation is apparent, and when AUC is dose-normalized levels are linear from an AUC standpoint. $C_0$, however, appears to be quite variable though this increases with dose as would be expected. Normalized $C_0$ varies between genders and between SD and MD evaluation. *Reviewer's comment: This likely reflects the uncertainty surrounding the first few time-points for evaluation which are magnified as data is tracked back to t=0.* $T_{1/2}$ is short, clearance rapid, and the volume of distribution relatively restricted.

***Note***: The following table of Org 48302 study is reproduced from this study which, though omitted from revised final report due to significant technical errors in bioanalytical procedures (QC issues, see Study Deviations, above), may be useful for drawing general impressions of the similarity between 25969 and 48302 kinetics.

| | | Dose | | | | | |
|---|---|---|---|---|---|---|---|
| kinetic parameters Org 48302* | Sex | 30 mg·kg$^{-1}$ Org 25969 1.8 mg·kg$^{-1}$ Org 48302 | | 120 mg·kg$^{-1}$ Org 25969 7.2 mg·kg$^{-1}$ Org 48302 | | 500 mg·kg$^{-1}$ Org 25969 30 mg·kg$^{-1}$ Org 48302 | |
| | | SD | MD | SD | MD | SD | MD |
| $AUC_{inf}$** (µg·h·mL$^{-1}$) | M | - | - | 12.4 | 9.65 | 41.2 | - |
| | F | 1.71 | - | 7.70 | 8.39 | 32.1 | 55.3 |
| $NAUC_{inf}$** (µg·h·mL$^{-1}$)/(mg·kg$^{-1}$) | M | - | - | 1.72 | 1.34 | 1.37 | - |
| | F | 0.947 | - | 1.07 | 1.17 | 1.07 | 1.84 |
| $C_0$ (µg·mL$^{-1}$) | M | 12.7 | 9.40 | 34.6 | 39.6 | 92.5 | 173 |
| | F | 5.73 | 6.95 | 26.4 | 42.9 | 99.3 | 155 |
| $NC_0$ (µg·mL$^{-1}$)/(mg·kg$^{-1}$) | M | 7.08 | 5.22 | 4.81 | 5.50 | 3.08 | 5.78 |
| | F | 3.18 | 3.86 | 3.66 | 5.96 | 3.31 | 5.17 |
| $t_{1/2}$ (h) | M | - | - | 0.30 | 0.37 | 0.26 | - |
| | F | 0.27 | 0.21 | 0.33 | 0.22 | 0.34 | 0.40 |
| Cl (L·kg$^{-1}$·h$^{-1}$) | M | - | - | 0.58 | 0.75 | 0.73 | - |
| | F | 1.06 | - | 0.93 | 0.86 | 0.94 | 0.54 |
| V (L·kg$^{-1}$) | M | - | - | 0.25 | 0.40 | 0.28 | - |
| | F | 0.41 | - | 0.45 | 0.28 | 0.46 | 0.32 |

SD = single dose, MD = multiple dose
M = male, F = female
* : Org 48302 kinetic parameters were normalized to the Org 48302 doses
** : AUC under the dosing interval ($AUC_{(0-24)}$) was estimated by $AUC_{inf}$
- : could not be determined

Org 48302: Though data are not numerically reliable, there does appear to be generally similar $t_{1/2}$, clearance values, and volume of distribution as noted for Org 25969 presented in the previous table (the latter reliable data). Org 48302 kinetics appear to indicate a short half-life, rapid clearance and restricted volume of distribution as seen with Org 25969 and which would be considered typical with a gamma-cyclodextrin. In addition, the value of Org 48302 exposure parameters appeared to be comparable with those obtained in a single dose TK study in rats with batch AE, as shown in section 2.6.4.3. Absorption and PK/TK studies.

**Mortality**
No mortality during the study at any dose.

**Clinical signs**
There were no notable treatment-related signs in any group during the treatment- or recovery phase.

**Body weights**
Absolute and Relative (to start) body weight was transiently and statistically significantly reduced in HD♂ during SW1&2 though the degree of reduction (≤ 3-4% vs. placebo) is considered by the Sponsor to be not toxicologically significant and represents the transient reduction in food consumption noted in this group. *Reviewer's comment: Agree.* Body weight was not different between groups during the post-dosing recovery period.

**Food consumption**
Transient reduction in food intake was noted in females during first week of dosing in the MD♀. Food consumption was lower in HD♂ throughout the dosing period and for HD♀ during the last week of dosing only. Food consumption levels were not different between treated and controls during the recovery period.

**Ophthalmoscopy**
There were no notable treatment-related ophthalmologic observations. Occasional findings were not dose-dependent, consistent between groups, or observed in both genders and were described by Sponsor as being common incidental observations in the rat. *Reviewer's comment: Agree.*

**Hematology**
No treatment-related findings in RBC parameters were noted at LD or MD in males. In HD♂ and in females of all treated groups evidence of an adverse hematologic effect was noted on red cell parameters. This was characterized by ↓RBC (≤ -3% in males, ≤ -6% in females) and HCT values (≤ -1% in males and ≤ -3% in females) compared with controls animals at the end of the treatment period which did not fully recovery in males (-6% RBC, -3% HCT, both SSD) though some recovery noted in females (-3% RBC, -1%, both N.S.S.). These findings were supported by observation of ↑reticulocyte counts in HD♂ (+28%) and in LD♀ (+37%), MD♀ (+24%) & HD♀ (+95%). Reticulocyte counts were still elevated at the end of the 8-week recovery period (HD♂ +63%, HD♀ +27% compared with controls). Partially reflective of these findings (i.e. ↑reticulocyte levels), increased MCV and MCH values were noted in MD♂ and HD♂ and in HD♀ which did not demonstrate recovery. Increased RDW was noted of HD♀, though only after the recovery period.

Alterations in WBCs which appear to be treatment-related were an increase in neutrophils in HD♂ (absolute and relative, +17% and 77%, respectively) and in MD♂ (relatively only, +28%) at the end of the treatment period with full recovery. A decrease in lymphocytes (relative only) was noted in ≥MD♂ at the end of the treatment period with full recovery. A decreased mean absolute monocyte count (-27%) which

was not statistically significant was noted for HD♀ at the end of treatment which demonstrated no difference from placebo females at the end of the recovery period.

Mildly decreased PTT (-2%) was noted in HD♂ at the end of the treatment period. No statistically significant differences found in females.

Mildly decreased platelets (absolute) were observed in HD♂ (-4% vs. controls) at the end of the treatment period with evidence of recovery during the treatment-free period. No observations noted in females.

**Clinical chemistry**

No changes in clinical chemistry parameters were noted in males or females at the LD (30 mg/kg/day). Few treatment-related changes were noted at higher doses: ↓cholesterol was noted in MD♂ (-31%, SSD) and HD♂ (-27%, NSS) with full recovery; ↑albumin values (+4% SSD) was noted in HD♂ causing an ↑total protein and Albumin/Globulin ratio in this group with full recovery. No treatment-related electrolyte changes after treatment period, ↓sodium in males and ↓chloride values in females at the end of the recovery period not of toxicologic significance. *Reviewer's comment: The toxicologic significance of these changes are considered minor and non-adverse.*

**Urinalysis**

No changes in UA parameters were observed in males or females at the LD (30 mg/kg/day). Increased volume and decreased osmolality was observed in HD♀ at the end of treatment period with full recovery. Possible decreased mean creatinine for HD♀ at the end of the treatment period with full recovery. Mildly decreased mean glucose at end of treatment. Decreased mean NAG at the end of treatment which was not confirmed by NAG/creatinine ratio but which was not different from controls by the end of the recovery period. Increased mean pH in MD♀ and HD♀ without changes in males at the end of the treatment period. At the end of the recovery period pH values were lower in HD♂ and HD♀ compared with controls.

**Organ weights**

There were no treatment-related changes in organ weight. Sporadic values were statistically significantly different from control but demonstrated no dose-dependency, consistency between genders, and were of minor deviation. These changes are considered incidental findings by the Sponsor. *Reviewer's comment: Agree.*

**Gross pathology**

There were no notable treatment-related macroscopic observations.

**Histopathology**

Microscopic examination of tissues identified several target organs, consistent with previous studies in this species as shown in the table below:

| Target Organ/ Finding | Study Day | Incidence/ Severity | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 30 | 120 | 500 | 0 | 30 | 120 | 500 |
| **Lungs** | | | | | | | | | | |
| ↑foamy macrophages | SD29 | Total | 1 | 1 | 1 | 10 | 0 | 1 | 0 | 10 |
| | | *Min* | *1* | *1* | | | | *1* | | *4* |
| | | *Slight* | | | *1* | *4* | | | | *5* |
| | | *Mod* | | | | *5* | | | | *1* |
| | | *Marked* | | | | *1* | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Kidneys** | | | | | | | | | | |
| Vacuolated cortical tubular cells | SD29 | Total | 0 | 0 | 6 | 8 | 0 | 0 | 1 | 9 |
| | | *Min* | | | *5* | *2* | | | *1* | *2* |
| | | *Slight* | | | *1* | *5* | | | | *6* |
| | | *Mod* | | | | *1* | | | | *1* |
| **Urinary Bladder** | | | | | | | | | | |
| Foamy cytoplasm, umbrella cells | SD29 | Total | 0 | 0 | 10 | 10 | 0 | 1 | 8 | 9 |
| | | *Min* | | | *2* | *3* | | *1* | *2* | *1* |
| | | *Slight* | | | *4* | *5* | | | *5* | *8* |
| | | *Mod* | | | *4* | *2* | | | *1* | |

***Lungs***: A clear increase in foamy macrophages was noted in the HD♂ and HD♀ (10/10 animals in both cases) of minimal to marked severity. Periodic-Acid Schiff (PAS) staining revealed weak positive staining.

Recovery HD♂ did not demonstrate this finding and 1/10 Recovery HD♀ had a minimal finding of ↑foamy macrophages. Therefore this finding appears to show almost complete recovery with an 8-week treatment free period.

***Kidneys***: Vacuoles were noted in the cells of the cortical tubules of the kidneys of ≥MD♂ and ≥MD♀ with severity ranging from minimal to moderate. PAS staining of cortical tubular cells in the HD groups did not appear to indicate a stronger staining than found in control groups.

Recovery HD♂ and HD♀ demonstrated a lower incidence and lower severity of this finding (2/10 in both genders; minimal to slight) after an 8-week treatment-free period. This finding therefore appears to be partially resolved at the time of the recovery sacrifice.

***Urinary bladder***: Umbrella cells of the urothelium were noted to have a foamy cytoplasm in ≥MD♂ though in females this was observed at all doses (≥LD♀), noting that the LD♀ had a single incidence of minimal severity. As a single incidence of minimal to slight foamy cytoplasm was noted in umbrella cells of 1/10 control males and females at the end of the recovery period, the LD finding at the end of treatment is not considered to represent an effect of drug. Therefore, in females a treatment-related effect is considered to have occurred at ≥MD♀.

Recovery HD♂ and HD♀ demonstrated <u>no recovery</u> with an 8-week treatment-free period; 10/10 HD♂ and HD♀ demonstrated foamy cytoplasm in umbrella cells of minimal to moderate severity. However, 1/10 control males and females demonstrated foamy cytoplasm in this tissue (minimal or slight in severity).

Therefore, the NOAEL for histopathologic findings was the LD (30 mg/kg/day) and with the exception of findings in the urinary bladder all microscopic findings were in the process of reversing with 8-weeks treatment-free period.

## Dog studies

In dogs, one 2-wk study and one 4-wk study were conducted to assess the toxicity profile of Org 25969. In the 2-wk study, animals were administered with 0, 2, 8, and 30 mg/kg Org 25969 and no adverse findings were noted in physical examination, body weight and food consumption measurement, ECG assay, clinical pathology assays; histopathology was not conducted in this study. However, dosing levels of this study resulted in systemic exposure levels lower than those in human after administration with the proposed dosing levels seeking for approval; therefore, the results of the study can not be used to support the safety of the proposed dosages in human. In contrast, the 4-wk study with appropriate examinations including histopathology, dosing levels and recovery period was considered to be the pivotal study to support the proposed dosing levels in

human. This study was reviewed by Dr. Adam Wasserman and the review report was inserted in the following.

### STUDY TITLE: A 4-WEEK INTRAVENOUS TOXICITY AND TOXICOKINETIC STUDY WITH ORG 25969 IN MALE AND FEMALE BEAGLE DOGS

**Study purpose:**
From Sponsor's report (p. 15); the purpose was to assess:

- the sub-acute toxicity profile of Org 25969 in male and female beagle dogs upon repeated intravenous administration for 4 weeks;
- the reversibility of toxicity, if any, at the high dose during a 2-week drug-free recovery period;
- to determine the concentrations of Org 25969 in plasma samples from male and female beagle dogs after a single intravenous dose and after multiple daily intravenous dosing for approximately 4 weeks;
- if feasible, the toxicokinetic parameters of Org 25969 associated with the plasma concentrations, and, if feasible, to relate them to toxicological findings.

**Key study findings**:

- Intravenous administration of Org 25969 0, 25, 80, or 250 mg/kg/day to beagle dogs for 4 weeks resulted in minimal systemic toxicity. The only notable finding which appeared to be treatment related was a reduction in red blood cell parameters (RBC, HCT, HGB ↓17% vs. controls) at the 250 mg/kg/day dose level in male dogs at the end of 4-week treatment which was not associated with a compensatory increase in reticulocytes and which appeared to return to control or pre-dosing levels with a 2-week treatment-free recovery period. Female dogs did not demonstrate a meaningful change in red cell parameters.
- Although a 17% decrease in red cell parameters is considered to represent a potential adverse effect level in males, this reduction was not sufficient to elicit a compensatory reticulocyte elevation and was reversible. Therefore, in agreement with the Sponsor, the NOAEL is identified as 250 mg/kg/day.
- Toxicokinetics were conducted for the study but were found to be conducted outside of GLP and the resulting data in error; therefore, no direct TK can be ascribed from this study but the Sponsor considers the data from a 3-week PK study in the dog with identical dose levels to be informative.

**Study no.**: Document ID; NL0043937
**Volume #, and page #**:
**Conducting laboratory and location**: N.V. Organon, Department of TDD, Nistelrooisebaan 3, NL-5374 RD Schaijk, The Netherlands; Organon S.A., Department of TDD 22 rue Henri Goudier, 63203 Riom, Cedex, France (TK and Bioanalysis);
(b) (4)

**Date of study initiation**: May, 2002 ; Final report, March 2003
**GLP compliance**: Yes
**QA report**: yes (X) no ( )

**Drug, lot #, and % purity**: Org 25969, Lot AE, Batch U; 83.08% purity ([redacted] (b) (4) % total impurities); out of specification for mOsmol/kg (spec 280-330 mOsmol/kg; result 555 mOsmol/kg)

## METHODS

### *Test System*

**Species/strain**: Beagle Dog ([redacted] (b) (4), supplier)
**Age**: Not specified
**Weight**: Males 6.7-8.6 kg; females 5.4 – 7.1 kg

### *Study Design*

| Group No. | Group size and sex | | Dose | Treatment |
|---|---|---|---|---|
| | Female | Male | ($mg \cdot kg^{-1}$ per day) | |
| 1 | 5 | 5 | 0 | Vehicle |
| 2 | 4 | 4 | 25 | Org 25969 (low-dose, LD) |
| 3 | 4 | 4 | 80* | Org 25969 (mid-dose, MD) |
| 4 | 6 | 6 | 250 | Org 25969 (high-dose, HD) |

* Due to a technical error, the males of group 3 received a dose of 208 $mg \cdot kg^{-1}$ instead of 80 $mg \cdot kg^{-1}$ on the first day of dosing.

**Doses**: The MFD of 250 mg/kg was the highest dose administered; 0, 25, or 80 mg/kg/day were given as control, low, and mid-dose. Note: 80 mg/kg/day animals received 208 mg/kg on SD1 in error.

**Route, formulation, volume, and infusion rate**: Administered IV (vena cephalica; left forelimb usual, occasionally right forelimb), test article dissolved in saline (0.9% NaCl in water) and was vehicle, 2.5 mL/kg; 10 ml/min. Dosing adjusted once weekly based on BW. Concentrations formulated were 0, 10, 32 and 100 mg/mL.

**Number/sex/group or time point (main study)**: 4 dogs/sex/group

**Satellite groups used for toxicokinetics or recovery**: 1 dog/sex/control; 2 dogs/sex/HD

**Unique study design, husbandry or methodology issues**:

**Observations and times**

| Observational Parameter | Methods |
|---|---|
| Toxicokinetics | Blood samples taken for TK prior to dosing and at 2, 5, 15, 30, 60 minutes and 3, and 7 hr after dosing. Results evaluated by the original validation method and a partial validation method (for higher plasma concentrations than the original validation plan) – the latter for the MD (80 mg/kg/day) and HD (250 mg/kg/day). - **Note**: TK analysis was excluded from GLP due to subsequent re-analysis and determination that the STD and QC samples were "not integrated in a justified way" (p. 9; NL0042414; Amendment #2). |
| Clinical Observations | Mortality reported if occurred. Clinical signs noted during in-life period and animals wee "regularly observed for behavioral and physical abnormalities (no observation times described). Individual signs reported per group. Veterinary exam once during pre-dosing and dosing period as well as shortly after arrival. |
| Body Weights | 1X/week during pre-dosing period, D1, and 1X/week during dosing and post-dosing period. |

| | |
|---|---|
| Food Consumption | Evaluated at 1 hr post-feeding and again prior to next supply of rations. (Methods not described). Water consumption monitored without quantitative assessment. |
| Ophthalmology | Evaluated prior to start of dosing and at end of dosing period, the latter only in Controls and HD. Bilateral eval. Individual signs reported per group and per sex. |
| ECG | Standard bipolar leads I, II, III and augmented unipolar leads aVR, aVL, and aVF made in dogs during predosing period and approximately 10 minutes and 24 hr after the second treatment and at the end of the dosing period with same intervals. Analyzed for HR and conduction disturbances. Reported per group per sex. |
| Clinical Pathology | Standard evaluations of RBCs, WBCs, coags, blood chemistry, UA. Data obtained at pre-dosing and prior to sacrifice. |
| Terminal Procedures<br>Gross Pathology<br>Organ Weights<br>Histopathology | Macroscopic evaluation, organ weights (adrenals, kidneys, pituitary, testes, brain, liver, prostate, thymus, epididymides, ovaries, thyroid, heart, pancreas, spleen, uterus). Histopath from tissues in following table: |

| | | |
|---|---|---|
| Skeletal muscle | Diaphragm | Sternum |
| Femur | Heart | Aorta |
| Trachea | Lungs | Spleen |
| Lymph nodes | Bone marrow | Thymus |
| Tongue | Salivary glands | Pancreas |
| Liver | Gall bladder | Esophagus |
| Stomach | Duodenum | Jejunum |
| Ileum | Cecum | Colon |
| Rectum | Kidneys | Urinary bladder |
| Testes | Epididymides | Optic nerve |
| Adrenal glands | Prostate gland | Site of injection |
| Spinal cord | Skin | Mammary gland |
| Thyroid glands | Parathyroid glands | |
| Pituitary gland | Brain | |
| Peripheral nerve | Eye(s) | |

Path reports were peer-reviewed internally.

| | |
|---|---|
| Other Evaluations | |
| Statistics | Wilcoxin signed rank test, Covariance analysis (starting BW), Permutation test, Student t test. |

***Study Deviations/Impact***:

- The males of group 3 received a dose of 208 $mg \cdot kg^{-1}$ instead of 80 $mg \cdot kg^{-1}$ on the first day of dosing (technical error during preparation of the test article).
- Daily food ration given per dog (as calculated) was slightly lower than 200 g on several occasions.
- All citrated and heparin blood samples and all EDTA blood samples for bioanalysis were converted to plasma by centrifugation at room temperature in stead of centrifugation at 2 – 8 °C.
- No blood and urine sampling was performed on the recovery animals (omission).
- Esch E van was replaced by Dreef HC as responsible scientist for pathology.

**Additional deviation**: Toxicokinetic analysis was excluded from GLP report due to inappropriate integration of STD and QC samples, determined based on a re-analysis of data after report of findings from French Governmental inspection.

*Reviewer's comment:* Deviations do not appear to affect the conclusions or integrity of the study, though absence of direct TK data reduces the strength of the study; nevertheless, the Sponsor does have a study (3-week TK) which can be used as a bridge.

**RESULTS**

**Toxicokinetics**

See above, data is compromised by flawed standards and QC integration.

**Mortality**
No mortality.

**Clinical signs**
Occasional diarrhea in all groups, including controls. Changes at the site of injection were noted in two HD♀ animals on a single occasion. This consisted of a perivascularly thickened area several centimeters in length and slightly firm.

No clinical observations were noted in the single control or 2 HD recovery animals/sex at the end of the two-week period.

The Sponsor considers the change at the injection site to be due to inadvertent extravascular administration of partial dose due to the large volume necessary to achieve the HD. *Reviewer's comment: This seems plausible. Findings were not observed more than once and were not noted at the end of the recovery period.*

**Body weights**
No consistent effects on body weight were observed; mild body weight loss was noted in occasional controls and treated animals without an apparent dose-effect.

**Food consumption**
Although female dogs in treated groups demonstrated some reduction in food intake, in some instances seen generally throughout the dosing period, this was also observed in control animals and therefore this does not appear to be related to Org 25969.

**Ophthalmoscopy**
No treatment-related effects on ophthalmologic exam noted. Occasional observations of abnormalities were observed in these animals during the pre-dosing period and represent normal variations in this population.

**EKG**
Several findings were observed in the EKG read-outs in dogs. Occasional observations of 2° AV block in a single MD♀, prominent Q-waves in treated groups as well as controls, and other sporadic findings were not strongly dose-related and did not appear to correlate to the timing of treatment (some occurring before and weeks after dosing period had ended). QT prolongation was not observed.

**Hematology**
Reduced RBC, hematocrit and hemoglobin was observed in Org 25969-treated males compared with controls, this appeared biologically significant at the 250 mg/kg/day dose level and recovery was incomplete though the Sponsor notes the recovery data are comparable to the pre-dose values. Reticulocytes were not increased in males in response, however except for a mild increase noted in the recovery period. Female dogs did not demonstrate reductions in Red cell parameters at the end of the 4-week dosing period while reticulocytes were increased at dose levels ≥ 80 mg/kg/day and remained elevated in the 2-week post-dose recovery period.

| % Change from Controls | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Males (n=4/main study group) | | | | | Females (n=4/main study group) | | | | |
| Dose | 0 | 25 | 80 | 250 | 250 Recov (n=2) | 0 | 25 | 80 | 250 | 250 Recov (n=2) |
| RBC ($x10^{12}$/L) | 6.15 | -8 | +1 | **-17** | -12 | 6.33 | -3 | -9 | -4 | +3 |
| HCT | .403 | -6 | +1 | **-17** | -10 | .431 | -4 | -8 | -4 | +3 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| HGB (mmol/L) | 8.67 | -5 | +1 | -17 | -10 | 9.19 | -3 | -7 | -3 | +1 |
| Retic (abs; $x10^9$/L) | 91 | -13 | -7 | -21 | 0 | 88 | +2 | +23 | +31 | +61 |
| Retic (%) | 1.51 | -9 | -9 | -7 | +15 | 1.39 | +5 | +36 | +35 | +60 |

*Reviewer's comment: Although the increased reticulocytes may indicate a compensatory mechanism occurring in the female dog which is not observed in the males at the high dose, it is noted that reticulocyte levels are within the pre-dose levels observed in the female groups. Therefore, this apparent reticulocyte elevation is not considered biologically significant. Red cell parameters in the male dog appeared to return to pre-dosing or control levels; the single control animal used in the recovery group appears to be on the upper range of values and therefore artifactually makes the HD recovery animals appear to remain sub-normal.*

No treatment-related effects were noted on other hematologic parameters.

**Clinical chemistry**
No treatment-related effects on clinical chemistry parameters were noted.

**Urinalysis**
No treatment-related effects on urinalysis were noted.

**Organ weights**
No treatment-related effects on organ weights were noted.

**Gross pathology**
There were no treatment-related effects on gross examination. Hemorrhage was observed in the front leg of most control and Org 25969-treated animals and was considered to be the result of the intravenous administration procedure. *Reviewer's comment: This appears reasonable.*

**Histopathology**

Histopathologic findings were largely confined to the lung, liver, and kidney. No findings in the urinary bladder were reported. Incidence of findings is noted in the table below:

| **Incidence of Histopathologic Findings (treatment-period)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Males (N=4/group)** | | | | **Females (N=4/group)** | | | |
| ***Organ/Finding*** | **0** | **25** | **80** | **250** | **0** | **25** | **80** | **250** |
| **Lung** | | | | | | | | |
| Fibrosis | 1 | | | | | | | |
| Pronounced BALT | 1 | | | | | | | |
| Hemorrhage | | | 1 | | | | | |
| Emboli | | 1 | 1 | 1 | 2 | 1 | | 1 |
| Granulocytic cell infiltration | | 1 | | | | | | |
| Perivascular mononuclear cell aggregation | 2 | 1 | 1 | 3 | 1 | | | |
| Mononuclear cell aggregation | 1 | | | | | | 1 | |
| Foamy macrophages | | | | | 1 | 1 | 1 | |
| Pigmented macrophages | | 1 | | 1 | | | | |
| Granulomatous lesions | 1 | | | | | | | 1 |
| Submandibular salivary gland | | | | | | | | |
| Mononuclear cell infiltrates | | | | 1 | | 1 | 1 | 1 |
| **Liver** | | | | | | | | |
| Pigment accumulation in | | | | | | | | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Kupffer cells | | | | | | | | |
| **Kidneys** | | | | | | | | |
| ↑Tubular basophilia | | 1 | | 1 | | | | 1 |
| Tubular dilation | 1 | | | | | | | |
| Intratubular casts | | | | 1 | | | | |
| Protein-rich fluid-filled tubules | 1 | | | | | | | |
| Atrophic glomular tufts | | 2 | | | 2 | 1 | | |
| Mineralization | | 1 | | 1 | | | | |
| Mononuclear cell aggregation | | | | | | | | 1 |

No apparent treatment-related effects were noted. No treatment-related observations were noted in animals allowed a 2-weeks treatment-free period prior to sacrifice. *Reviewer's comment: Agree.*

### 2.6.6.4 Genetic toxicology

**In vitro Ames studies**

**1. Study title**: A bacterial reverse mutation test (Ames test) study with Org 25969 in Salmonella Typhimurium and Escherichia coli

**Key findings**: Org 25969 drug substance in this batch (batch L) did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: PCD 2359/SV057
Document ID: NL0027929

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD
Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands

**Date of study initiation**: 03/13/2001
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch L, 81.8%
*Note: analysis certificate was not attached with this study report; instead, batch analysis can be found with the NDA submission in the module 3.Quality.*

**Methods:**

Strains/species/cell line:
*Salmonella typhimurium*: TA98, TA100, TA1535, TA1537;
*E. Coli* : WP2 uvrA pKM101.

Metabolizing system: a mixture of the 88290 N/kg supernatant fraction (S9) of rat liver homogenate and cofactors was obtained from (b) (4) The capacity of the S9 batch (S9-41) to convert reference mutagens was demonstrated in each Ames test.

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

| Test compound | Dose per plate (µg) | | No. of plates | |
|---|---|---|---|---|
| | | | without S9-mix | with S9-mix |
| | Test compound | Active entity | | |
| Org 25969 | 0 * | 0 * | 3 | 3 |
| Org 25969 | 100 | 92 | 3 | 3 |
| Org 25969 | 250 | 230 | 3 | 3 |
| Org 25969 | 500 | 460 | 3 | 3 |
| Org 25969 | 1000 | 920 | 3 | 3 |
| Org 25969 | 2500 | 2300 | 3 | 3 |
| Org 25969 | 5000 | 4600 | 3 | 3 |
| 4-NQO | 0.3 | 0.3 | 3 | 0 |
| 4-NQO | 1 | 1 | 3 | 0 |
| 9-AC | 60 | 60 | 3 | 0 |
| $NaN_3$ | 1 | 1 | 3 | 0 |
| 2-AA | 2 | 2 | 0 | 3 |
| 2-AAF | 25 | 25 | 0 | 3 |
| BP | 5 | 5 | 0 | 3 |

a : 100 µL of ultrapure water was used.

- 4-NQO was tested at a concentration of 0.3 µg per plate in TA98 and TA100 and at 1 µg per plate in E. coli
- 9-AC was tested in TA1537
- $NaN_3$ was tested in TA1535
- 2-AA was tested in TA1535 and WP2 uvrA pKM101
- 2-AAF was tested in TA98 and TA100
- BP was tested in TA1537

Basis of dose selection: the sponsor has conducted a pilot Ames test. Org 25969 was tested in S. typhimurium strains TA98 and TA100 in the presence and absence of S9-mix at concentrations up to 1000 µg/ plate. There were no signs of cytotoxicity, precipitation, and no mutagenicity observed. Therefore, the sponsor chose the OECD protocol guideline recommended maximal test concentration (5000 µg/plate) as the highest concentration for the definitive test.

Negative controls: 100 µL aliquot of ultra pure water

Positive controls: The following reference mutagens were used as positive controls.

Without metabolic activation:

- 4-Nitroquinoline-N-oxide (4-NQO), 0.3 µg per plate for S. typhimurium strains TA98 and TA100 and 1 µg per plate for E. coli strain WP2 uvrA pKM101.
- Sodiumazide (NaN3), 1 µg per plate for S. typhimurium strain TA1535
- 9-Aminoacridine (9-AC), 60 µg per plate for S. typhimurium strain TA1537

With metabolic activation:

- 2-Acetylaminofluorene (2-AAF), 25 µg per plate for S. typhimurium strains TA98 and TA100.
- 2-Aminoanthracene (2-AA), 2 µg per plate in S. typhimurium strain TA1535 and E. coli strain WP2 uvrA pKM101.
- Benzo(a)pyrene (BP), 5 µg per plate in S. typhimurium strain TA1537.

Incubation and sampling times: The test was carried out in triplicate, with and without S9-mix. Before the experiment, a thawed sample of the stock culture was incubated in Oxoid Nutrient broth medium in a shaking waterbath at 37 °C overnight until an optical density of at least between 0.8 and 1.0 was reached. Then, 0.1 mL of this overnight culture plus 0.1 ml either the test article or the positive control article or the negative control article, with or without 0.5 mL S9-mix, was added to 3 mL molten soft agar at 45 °C, mixed and poured (plated) on Vogel-Bonner Minimal Agar plates. All plates were incubated in an incubator. The number of colonies was determined using an automatic

colony counter after incubation at 37 °C for approx. 48h. The mean number of revertants per plate was calculated for the control and test plates.

*Criteria for a valid assay:*
According to the sponsor, the assay was considered to be valid with the following criteria were met:

- the number of bacteria plated was higher than 5 x $10^7$ per plate
- mean number of revertants in the positive control group in the presence and the absence of S9-mix was more than twice the mean number of revertants in the corresponding negative control group

In addition, the following criteria were monitored:

- mean number of spontaneous revertants was within the normal control range in the absence (TA98: 19-42; TA100: 89-193; TA1535: 6-14; TA1537: 5-25 and WP2 uvrA pKM101: 65-174) and the presence of S9-mix (TA98: 23-50; TA100: 95-215; TA1535: 7- 17; TA1537: 6-25 and WP2 uvrA pKM101: 77-188)
- mean number of revertants in the positive control group in the presence of S9-mix exceeds the corresponding historical control value (control values: TA98: 588; TA100: 428; TA1535: 201; TA1537: 78 and WP2 uvrA pKM101: 319).

The sponsor indicated that "the latter two criteria were not absolute but serve as indicators, since historical data were not yet available for agar plates and top agars obtained from (b) (4)"

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if the following three criteria were met, in any of the strains in the presence or in the absence of S9-mix:

- a relationship between concentration and the number of revertant colonies was present
- the increase in the number of revertant colonies exceeds the upper limit of the normal control range
- a two-fold increase in the number of revertants was present as compared to the negative controls.

The sponsor further indicated that these criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle. The number of viable bacteria/plate was acceptable in all strains.

Study outcome:

Cytotoxic effect evidenced by inhibition of the background lawn was observed at concentrations of 2500 and 5000 µg/plate in E. coli in the absence and presence of S9-mix. Similar change was not seen in other strains. No precipitation was observed in any of the stains.

Revertant frequencies for all doses of drug substance (batch L), in all tester strains with and without S9, approximated or were less than control values. These results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The Sponsor summarized the results of the assay as shown in the following table.

**Table 9.1-1 Mean number of revertants per plate**

| Tester Strain | Treatment | Micrograms/plate | | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|---|
| | | Test compound | Active entity | mean | S.D. | mean | S.D. |
| TA98 | Org 25969 | 0 [a] | 0 [a] | 27 | 2.9 | 36 | 6.5 |
| TA98 | Org 25969 | 100 | 92 | 31 | 3.1 | 46 | 6.5 |
| TA98 | Org 25969 | 250 | 230 | 29 | 8.5 | 28 | 2.5 |
| TA98 | Org 25969 | 500 | 460 | 33 | 1.0 | 36 | 12.1 |
| TA98 | Org 25969 | 1000 | 920 | 31 | 4.2 | 33 | 4.6 |
| TA98 | Org 25969 | 2500 | 2300 | 31 | 5.7 | 34 | 9.7 |
| TA98 | Org 25969 | 5000 | 4600 | 35 | 15.6 | 35 | 2.6 |
| TA98 | 2-AAF | 25 | 25 | n.d. | n.d. | 1508 | 192 |
| TA98 | 4-NQO | 0.3 | 0.3 | 157 | 13.3 | n.d. | n.d. |
| TA100 | Org 25969 | 0 [a] | 0 [a] | 131 | 15.3 | 148 | 10.0 |
| TA100 | Org 25969 | 100 | 92 | 154 | 13.1 | 144 | 8.5 |
| TA100 | Org 25969 | 250 | 230 | 144 | 7.0 | 128 | 13.1 |
| TA100 | Org 25969 | 500 | 460 | 142 | 6.4 | 156 | 11.4 |
| TA100 | Org 25969 | 1000 | 920 | 136 | 17.6 | 162 | 15.4 |
| TA100 | Org 25969 | 2500 | 2300 | 133 | 18.1 | 153 | 0.0 |
| TA100 | Org 25969 | 5000 | 4600 | 130 | 11.1 | 136 | 14.6 |
| TA100 | 2-AAF | 25 | 25 | n.d. | n.d. | 728 | 41.5 |
| TA100 | 4-NQO | 0.3 | 0.3 | 1539 | 64.8 | n.d. | n.d. |
| TA1535 | Org 25969 | 0 [a] | 0 [a] | 17 | 6.6 | 15 | 5.0 |
| TA1535 | Org 25969 | 100 | 92 | 12 | 2.3 | 9 | 3.5 |
| TA1535 | Org 25969 | 250 | 230 | 9 | 2.9 | 10 | 0.6 |
| TA1535 | Org 25969 | 500 | 460 | 16 | 2.5 | 14 | 2.1 |
| TA1535 | Org 25969 | 1000 | 920 | 20 | 4.5 | 9 | 1.7 |
| TA1535 | Org 25969 | 2500 | 2300 | 16 | 1.0 | 19 | 2.6 |
| TA1535 | Org 25969 | 5000 | 4600 | 15 | 2.0 | 12 | 3.8 |
| TA1535 | 2-AA | 2 | 2 | n.d. | n.d. | 186 | 13.3 |
| TA1535 | $NaN_3$ | 1 | 1 | 331 | 7.6 | n.d. | n.d. |
| TA1537 | Org 25969 | 0 [a] | 0 [a] | 10 | 0.6 | 9 | 4.0 |
| TA1537 | Org 25969 | 100 | 92 | 10 | 3.0 | 8 | 2.9 |
| TA1537 | Org 25969 | 250 | 230 | 12 | 3.5 | 12 | 2.1 |
| TA1537 | Org 25969 | 500 | 460 | 16 | 2.3 | 17 | 5.0 |
| TA1537 | Org 25969 | 1000 | 920 | 10 | 1.5 | 11 | 2.5 |
| TA1537 | Org 25969 | 2500 | 2300 | 13 | 6.7 | 13 | 1.5 |
| TA1537 | Org 25969 | 5000 | 4600 | 9 | 1.0 | 14 | 4.0 |
| TA1537 | BP | 5 | 5 | n.d. | n.d. | 97 | 7.2 |
| TA1537 | 9-AC | 60 | 60 | 595 | 202 | n.d. | n.d. |
| E. coli | Org 25969 | 0 [a] | 0 [a] | 112 | 24.3 | 128 | 8.5 |
| E. coli | Org 25969 | 100 | 92 | 93 | 15.3 | 118 [c] | [c] |
| E. coli | Org 25969 | 250 | 230 | 96 | 10.4 | 106 | 11.8 |
| E. coli | Org 25969 | 500 | 460 | 112 | 6.1 | 114 | 27.1 |
| E. coli | Org 25969 | 1000 | 920 | 94 | 26.2 | 118 | 18.6 |
| E. coli | Org 25969 | 2500 | 2300 | 110 [b] | 9.6 | 119 [b] | 11.5 |
| E. coli | Org 25969 | 5000 | 4600 | [b] | -- | [b] | -- |
| E. coli | 2-AA | 2 | 2 | n.d. | n.d. | 2675 | 82.1 |
| E. coli | 4-NQO | 1 | 1 | 314 | 43.8 | n.d. | n.d. |

a : 100 µL of solvent was used
b : Signs of bacterial cell killing evidenced by the presence of his-/trp- colonies and/or thinning of the background lawn and/or a drop in the number of revertants, below the lower limit of the normal control range.
c : mean of two determinations. No standard deviation was calculated.
n.d. : Not determined.
S.D. : Standard Deviation

**2. Study title**: A bacterial reverse mutation test (Ames test) study with Org 25969 (Batch T) in Salmonella Typhimurium and Escherichia coli (including independent repeat)

**Key findings**: Org 25969 drug substance (batch L) did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 020401X/9718
Document ID: NL0042807

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 03/22/2002
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch T, 84.4%

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA1535, TA1537; *E. Coli* : WP2 uvrA pKM101.

Metabolizing system: Liver S9 fraction from rats was prepared from the livers of five young male SD rats which had received prior treatment with Phenobarbital and betanaphthoflavone. The efficacy of the S9 tissue fraction was previously checked in an Ames test and produced acceptable responses with indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using S. typhimurium tester strain TA100.

Doses used in definitive study: 0, 313, 625, 1250, 2500, 5000 µg/plate

Basis of dose selection: the sponsor has conducted a pilot Ames test for dose-selection. Org 25969 was tested in the all five tester strains in the presence and absence of S9-mix with the concentrations of 0, 50.0, 158, 500, 1580, 5000 µg/plate. There were no signs of cytotoxicity and mutagenesis observed. Therefore, the sponsor chose the OECD recommended concentration (5000 µg/plate) as the highest concentration for the definitive studies.

Negative controls: physiological saline supplied by the sponsor

Positive controls: positive control treatment used solutions prepared as following extracted from the sponsor's report:

Sodium azide (b) (4) batch 221999 1081) in distilled water.
9-Aminoacridine (b) (4) batch 12058-A) in DMSO.
2-Nitrofluorene (b) (4) batch 12532) in DMSO.
2-Aminoanthracene (b) (4) batch 58F-3462) in DMSO.
Methylmethanesulphonate (MMS) (b) (4) batch 359316/153696) in distilled water.
Sterile distilled water (b) (4) batch 01I1302).
DMSO (b) (4) batch 429076/1 30502).

The concentrations of these mutagens were shown in the following table as extracted from the sponsor's report.

| *Tester strain* | *Absence of S9* | *Presence of S9* |
|---|---|---|
| TA1535 | sodium azide<br>1 µg/plate | 2-aminoanthracene<br>1 µg/plate |
| TA100 | sodium azide<br>1 µg/plate | 2-aminoanthracene<br>1 µg/plate (2 µg/plate) |
| TA1537 | 9-amino-acridine<br>50 µg/plate | 2-aminoanthracene<br>1 µg/plate |
| TA98 | 2-nitrofluorene<br>2 µg/plate | 2-aminoanthracene<br>1 µg/plate (2 µg/plate) |
| WP2 *uvr*A | methylmethanesulphonate<br>500 µg/plate | 2-aminoanthracene<br>10 µg/plate (20 µg/plate) |

Incubation and sampling times: The test was carried out in triplicate, with and without S9-mix. The first experiment was performed using a plate-incorporation method. The components of the assay were added to molten overlay agar and vortexed. The mixture was then poured onto the surface of a minimal medium agar plate, and allowed to solidify prior to incubation. The overlay mixture was composed as follows as extracted from the sponsor's report:

| | | | |
|---|---|---|---|
| (i) | Overlay agar (held at 45°C) | 2 | ml |
| (ii) | Test or control item solution | 0.2 | ml |
| | Positive item solution or its solvent | 0.1 | ml |
| (iii) | S9 mix or phosphate buffer (pH 7.4, 0.1 M) | 0.5 | ml |
| (iv) | Bacterial suspension | 0.1 | ml |

The second experiment was performed using a pre-incubation method. The components as shown in the following (extracted from the sponsor's report) were added to an empty test tube. The incubate was vortexed and placed at 37 °C for 30 minutes. Two ml of overlay agar was then added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

| | | | |
|---|---|---|---|
| (i) | Bacterial suspension | 0.1 | ml |
| (ii) | Test or control item solution | 0.2 | ml |
| | Positive item solution or its solvent | 0.05 | ml |
| (iii) | S9 mix or phosphate buffer (pH 7.4, 0.1 M) | 0.5 | ml |

The prepared plates were inverted and incubated for approximately 72 hours at 37 °C, and the number of revertant colonies on each plate was counted. The mean and standard

error of the mean for each test point was calculated, and statistical analysis was conducted.

*Criteria for a valid assay:* not specified by the sponsor. Tables of historical control values of the negative and positive controls in the absence or presence of S9 were attached in this study report.

*Criteria for a positive response:* According to the sponsor, a test compound was considered positive (mutagenic) if the following three criteria were met, in any of the strains in the presence or in the absence of S9-mix:

- a relationship between concentration and the number of revertant colonies was present
- a two-fold increase in the number of revertants was present as compared to the negative controls.

The sponsor further indicated that these criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle.

Study outcome:
There were 2 experiments performed; No signs of toxicity were observed at any dose-level in any tester strain in the absence or presence of S9 metabolic activation. Revertant frequencies for all doses of drug substance (batch T), in all tester strains with and without S9, approximated or were less than control values. These results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The results of this study was not shown in this review.

**3. Study title**: Org 25969 batch V: reverse mutation test in five histidine-requiring strains of Salmonella Typhimurium

**Key findings**: Org 25969 in this batch (batch V) did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 020400X/082/100
Document ID: NL0042157

**Volume #, and page #**: eCTD

**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 06/07/2002
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch V, 83.3% (77% on the basis of active entitiy)

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA102, TA1535, and TA1537

Metabolizing system: mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male SD rats induced with Aroclor 1254 and obtained from (b) (4). Each batch of S9 was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-O-dealkylase activities).

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

| Experiment | S-9 | Concentration of treatment solution (mg/mL) | Final concentration (µg/plate) |
|---|---|---|---|
| Range-Finder Experiment and Mutation Experiment 1 | – and + | 0.016 | 1.6 |
| | | 0.080 | 8 |
| | | 0.40 | 40 |
| | | 2.00 | 200 |
| | | 10.00 | 1000 |
| | | 50.00 | 5000 |
| Mutation Experiment 2 | – and + | 1.5625 | 156.25 |
| | | 3.125 | 312.5 |
| | | 6.250 | 625 |
| | | 12.50 | 1250 |
| | | 25.00 | 2500 |
| | | 50.00 | 5000 |

Basis of dose selection: the sponsor has conducted toxicity range-finder experiment in strain TA100 in the presence and absence of S9-mix at concentrations as shown above. No evidence of toxicity as would normally be manifest by a thinning of the background bacterial lawn and/or a marked decrease in revertant numbers was observed following any of the treatments. Therefore, the sponsor chose the OECD recommended maximal test concentration (5000 µg/plate) as the highest concentration to test.

Negative controls: purified water

Positive controls: The positive control chemicals were supplied and used as tabulated below as extracted from the sponsor's report.

| Chemical | Source | Stock * concentration (µg/mL) | Final concentration (µg/plate) | Use Strain(s) | S-9 |
|---|---|---|---|---|---|
| 2-nitrofluorene (2NF) | (b) (4) | 50 | 5.0 | TA98 | – |
| Sodium azide ($NaN_3$) | | 20 | 2.0 | TA100, TA1535 | – |
| 9-aminoacridine (AAC) | | 500 | 50.0 | TA1537 | – |
| Glutaraldehyde (GLU) | | 250 | 25.0 | TA102 | – |
| Benzo[a]pyrene (B[a]P) | | 100** | 10.0 | TA98 | + |
| 2-aminoanthracene (AAN) | | 50** | 5.0 | TA100, TA1535, TA1537 | + |
| | | 200** | 20.0 | TA102 | + |

* With the exception of $NaN_3$ and GLU, which were prepared in water, all stock solutions were prepared in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO). All stock solutions were stored in aliquots at 1-10°C in the dark, with the exception of B[a]P which was stored in aliquots at –80°C in the dark.

** For Experiment 2 pre-incubation treatments, stock solutions of these positive control compounds were twice the concentration stated. This enabled the volume additions to be reduced to 0.05 mL (thus avoiding solvent-induced toxicity) and achievement of the final concentrations per plate as detailed above.

Incubation and sampling times: Two definitive experiments were conducted in triplicate to test the mutagenicity of Org 25969 batch V. The bacteria have been checked strain characteristics of histidine dependence, *uvrB* character, *rfa* character and resistance to ampicillin (TA98 and TA100) or ampicillin plus tetracycline (TA102). Negative (solvent) controls were included in each assay, in quintuplicate without and with S-9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S-9. The activity of the S-9 mix used in each experiment was confirmed by AAN or B[a]P treatments of the strains in the presence of S-9. For all assays, bacteria were cultured for 10 hours at 37±1°C in nutrient broth in a shaking incubator before treatment. Two hours after this pre-culture, 0.1 ml bacterial culture, 0.1 ml test article solution or culture, 0.5 ml 10% S-9 mix or buffer solution were added into a 2.5 ml molten agar at 46±1°C, followed by rapid mixing and then pouring on to volgel-Bonner E agar plates for incubation at 37±1 °C in the dark for 72 hours. As the mean negative (solvent) control counts for strain TA102 in the absence of S-9 in Experiment 1 exceeded the historical data range, these data were invalidated (Experiment 1 mutagenicity data for strain TA100 were provided by the Range-Finder Experiment treatments) and strain TA102 treatments were repeated in parallel with Experiment 2. As the results of the first experiment were negative, treatments in the presence of S-9 in Experiment 2 included a pre-incubation step. Quantities of test article or control solution, bacteria and S-9 mix detailed above, were mixed together and incubated for 1 hour at 37±1°C, with shaking, before the addition of 2.5 mL molten agar at 46±1°C. After incubation, plates were examined for evidence of toxicity to the background lawn, and counted for revertants using a Seescan Colony counter. The m-statistic was calculated to check that the data were Poisson distributed, and Dunnett's test was used to compare the counts of each dose

with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

*Criteria for a valid assay:*
According to the sponsor, the assay was considered to be valid if the following criteria were met:
1. The mean negative control counts fell within the normal ranges as defined in the attached table of historical negative control values.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S-9 preparation (table of histological positive control values were also attached in this study report)
3. No more than 5% of the plates were lost through contamination or some other unforeseen event.

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if statistically significant ($p < 0.01$) and dose-related increase of revertants observed and the positive response described were reproducible.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle. The number of viable bacteria/plate was acceptable in all strains.

Study outcome:
No evidence of toxicity was observed in any tester strain at any dose levels in both experiment 1 and 2.

Revertant frequencies for all doses of drug substance (batch V), in all tester strains with and without S9, approximated or were less than control values. These results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The Sponsor summarized the results of the assay as shown in the following table. It should be noted that although statistically significant increases in revertant numbers were observed following treatments at some individual concentration levels in strains TA1535 and TA102 in the presence of S-9 in Experiment 1 when the data were analyzed at the 1% level using Dunnett's test, these increases were small in magnitude and not dose-dependent, and were not seen following Experiment 2 pre-incubation treatments with strains TA1535 and TA102, therefore, these changes were not considered mutagenic effect of the test article.

**Org 25969 batch V: Summary of mean revertant colonies (-S-9) - Experiment 1**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| WATER | 100 µl | 25 ± 5 (M) | 97 ± 8 | 20 ± 11 | 9 ± 5 | 323 ± 70 |
| Org 25969 batch V | 1.6 | 29 ± 1 | 95 ± 13 | 22 ± 5 | 17 ± 6 | 368 ± 19 |
| | 8 | 21 ± 2 | 102 ± 2 | 20 ± 2 | 11 ± 3 | 394 ± 43 |
| | 40 | 20 ± 3 | 108 ± 12 | 19 ± 6 | 7 ± 5 | 349 ± 26 |
| | 200 | 21 ± 7 | 88 ± 7 | 20 ± 4 | 13 ± 3 | 426 ± 13 |
| | 1000 | 17 ± 2 | 94 ± 9 | 13 ± 6 | 13 ± 4 | 349 ± 11 |
| | 5000 | 23 ± 7 | 89 ± 12 | 18 ± 3 | 7 ± 4 | 340 ± 137 |
| Positive controls | Compound | 2NF | $NaN_3$ | $NaN_3$ | AAC | GLU |
| | Dose Level | 5 µg | 2 µg | 2 µg | 50 µg | 25 µg |
| | Mean ± SD | 659 ± 22 | 435 ± 11 | 583 ± 30 | 149 ± 36 | 681 ± 139 |

**Org 25969 batch V: Summary of mean revertant colonies (+S-9) - Experiment 1**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| WATER | 100 µl | 27 ± 4 | 93 ± 12 | 18 ± 2 | 16 ± 6 | 286 ± 19 |
| Org 25969 batch V | 1.6 | 32 ± 5 | 100 ± 14 | 23 ± 6 | 15 ± 4 | 336 ± 6 |
| | 8 | 31 ± 3 | 102 ± 6 | 15 ± 2 | 14 ± 2 | 325 ± 20 |
| | 40 | 30 ± 4 | 99 ± 6 | 31 ± 3 | 12 ± 2 | 289 ± 16 |
| | 200 | 38 ± 17 | 97 ± 6 | 25 ± 8 | 11 ± 4 | 294 ± 5 |
| | 1000 | 30 ± 3 | 95 ± 11 | 24 ± 7 | 19 ± 6 | 348 ± 51 |
| | 5000 | 37 ± 5 | 103 ± 10 | 22 ± 5 | 16 ± 2 | 281 ± 16 |
| Positive controls | Compound | B[a]P | AAN | AAN | AAN | AAN |
| | Dose Level | 10 µg | 5 µg | 5 µg | 5 µg | 20 µg |
| | Mean ± SD | 243 ± 18 | 805 ± 132 | 241 ± 15 | 188 ± 22 | 1711 ± 311 |

**Org 25969 batch V: Summary of mean revertant colonies (-S-9) - Experiment 2**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| WATER | 100 µl | 22 ± 4 | 110 ± 9 | 19 ± 4 | 7 ± 3 | 363 ± 27 |
| Org 25969 batch V | 156.25 | 23 ± 2 | 95 ± 5 | 12 ± 9 | 11 ± 5 | 349 ± 32 |
| | 312.5 | 22 ± 9 | 111 ± 6 | 23 ± 4 | 11 ± 1 | 379 ± 28 |
| | 625 | 21 ± 3 | 104 ± 26 | 17 ± 4 | 11 ± 4 | 156 ± 33 |
| | 1250 | 15 ± 2 | 86 ± 14 | 16 ± 4 | 7 ± 2 | 384 ± 14 |
| | 2500 | 17 ± 1 | 111 ± 12 | 16 ± 5 | 11 ± 0 | 330 ± 22 |
| | 5000 | 16 ± 7 | 97 ± 5 | 23 ± 9 | 9 ± 4 | 340 ± 31 |
| Positive controls | Compound | 2NF | $NaN_3$ | $NaN_3$ | AAC | GLU |
| | Dose Level | 5 µg | 2 µg | 2 µg | 50 µg | 25 µg |
| | Mean ± SD | 1025 ± 78 | 668 ± 26 | 448 ± 64 | 135 ± 32 | 571 ± 388 |

**Org 25969 batch V: Summary of mean revertant colonies (+S-9) - Experiment 2**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| WATER | 100 µl | 35 ± 7 | 115 ± 5 | 23 ± 4 | 17 ± 3 | 339 ± 14 |
| Org 25969 batch V | 156.25 | 33 ± 6 | 131 ± 3 | 21 ± 11 | 13 ± 5 | 328 ± 9 |
| | 312.5 | 29 ± 2 | 127 ± 11 | 22 ± 7 | 15 ± 4 | 365 ± 4 |
| | 625 | 25 ± 7 | 113 ± 10 | 21 ± 1 | 15 ± 5 | 378 ± 11 |
| | 1250 | 31 ± 5 | 132 ± 10 | 24 ± 4 | 17 ± 6 | 381 ± 27 |
| | 2500 | 29 ± 4 | 121 ± 7 | 18 ± 3 | 17 ± 3 | 344 ± 8 |
| | 5000 | 23 ± 8 | 119 ± 9 | 22 ± 4 | 17 ± 1 | 344 ± 41 |
| Positive controls | Compound | B[a]P | AAN | AAN | AAN | AAN |
| | Dose Level | 10 µg | 5 µg | 5 µg | 5 µg | 20 µg |
| | Mean ± SD | 349 ± 17 | 976 ± 86 | 186 ± 32 | 112 ± 18 | 1287 ± 255 |

**4. Study title**: Drug product of Org 25969 batch AE: reverse mutation test in five histidine-requiring strains of Salmonella Typhimurium

**Key findings**: Org 25969 in this batch (batch AE) did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 082/138
Document ID: NL0059995

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 07/26/2004
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch AE, 90.0%*
* The purity is based on the batch analysis report; the certificate of analysis attached to this report did not show the purity of Org 25969.

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA102, TA1535, and TA1537

Metabolizing system: mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male SD rats induced with Aroclor 1254 and obtained from (b) (4) Each batch of S9 was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-O-dealkylase activities).

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

| Experiment | S-9 | Concentration of treatment solution (mg/mL) | Final concentration (µg/plate) |
|---|---|---|---|
| Range-finder Experiment and Mutation Experiment 1 | – and + | 1.5625 | 156.25 |
| | | 3.125 | 312.5 |
| | | 6.25 | 625 |
| | | 12.50 | 1250 |
| | | 25.00 | 2500 |
| | | 50.00 | 5000 |
| Mutation Experiment 2 | – and + | 5.00 | 500 |
| | | 10.00 | 1000 |
| | | 20.00 | 2000 |
| | | 30.00 | 3000 |
| | | 40.00 | 4000 |
| | | 50.00 | 5000 |

Basis of dose selection: the sponsor has conducted toxicity range-finder experiment in strain TA100 in the presence and absence of S9-mix at concentrations as shown above. No evidence of toxicity as would normally be manifest by a thinning of the background bacterial lawn and/or a marked decrease in revertant numbers was observed following any of the treatments. Therefore, the sponsor chose the OECD recommended maximal test concentration (5000 µg/plate) as the highest concentration to test.

Negative controls: purified water

Positive controls: The positive control chemicals were supplied and used as tabulated below as extracted from the sponsor's report.

| Chemical | Source | Stock * concentration (µg/mL) | Final concentration (µg/plate) | Use Strain(s) | S-9 |
|---|---|---|---|---|---|
| 2-nitrofluorene (2NF) | (b) (4) | 50 | 5.0 | TA98 | – |
| Sodium azide ($NaN_3$) | | 20 | 2.0 | TA100, TA1535 | – |
| 9-aminoacridine (AAC) | | 500 | 50.0 | TA1537 | – |
| Glutaraldehyde (GLU) | | 250 | 25.0 | TA102 | – |
| Benzo[a]pyrene (B[a]P) | | 100** | 10.0 | TA98 | + |
| 2-aminoanthracene (AAN) | | 50** | 5.0 | TA100, TA1535, TA1537 | + |
| | | 200** | 20.0 | TA102 | + |

* With the exception of $NaN_3$ and GLU, which were prepared in water, all stock solutions were prepared in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO). All stock solutions were stored in aliquots at 1-10°C in the dark, with the exception of B[a]P which was stored in aliquots at –80°C in the dark.

** For Experiment 2 pre-incubation treatments, stock solutions of these positive control compounds were twice the concentration stated. This enabled the volume additions to be reduced to 0.05 mL (thus avoiding solvent-induced toxicity) and achievement of the final concentrations per plate as detailed above.

Incubation and sampling times: Two definitive experiments were conducted in triplicate to test the mutagenicity of Org 25969 batch AE in the absence and presence of S9. The bacteria have been checked strain characteristics of histidine dependence, *uvrB* character,

*rfa* character and resistance to ampicillin (TA98 and TA100) or ampicillin plus tetracycline (TA102). Negative (solvent) were included in quintuplicate without and with S-9 mix. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S-9. The activity of the S-9 mix used in each experiment was confirmed by AAN or B[a]P treatments of the strains in the presence of S-9. For all assays, bacteria were cultured for 10 hours at 37±1°C in nutrient broth in a shaking incubator before treatment. Two hours after this pre-culture, 0.1 ml bacterial culture, 0.1 ml test article solution or culture, 0.5 ml 10% S-9 mix or buffer solution were added into a 2.5 ml molten agar at 46±1°C, followed by rapid mixing and then pouring on to volgel-Bonner E agar plates for incubation at 37±1 °C in the dark for 72 hours. Experiment 1 mutagenicity data for strain TA100 were provided by the rangefinder experiment treatments. As the results of the first experiment were negative, treatments in the presence of S-9 in Experiment 2 included a pre-incubation step. Quantities of test article or control solution, bacteria and S-9 mix detailed above, were mixed together and incubated for 1 hour at 37±1°C, with shaking, before the addition of 2.5 mL molten agar at 46±1°C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure in hope of increasing the range of mutagenic chemicals that could be detected in the assay.

After 72 hour incubation, plates were examined for evidence of toxicity to the background lawn, and counted for revertants using a colony counter. The m-statistic was calculated to check that the data were Poisson distributed, and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

*Criteria for a valid assay:*
According to the sponsor, the assay was considered to be valid if the following criteria were met:
1. The mean negative control counts fell within the normal ranges as defined in the attached table of historical negative control values.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S-9 preparation (table of histological positive control values were also attached in this study report)
3. No more than 5% of the plates were lost through contamination or some other unforeseen event.

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if statistically significant ($p<0.01$) and dose-related increase of revertants observed and the positive response described were reproducible.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle.

Study outcome:
No evidence of toxicity was observed in any tester strain at any dose levels in both experiment 1 and 2.

No statistically significant, dose-related and reproducible increases in revertant numbers were observed following any strain treatments in the absence or presence of metabolic activation following treatment of the test article (batch AE). These results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The Sponsor summarized the results of the assay as shown in the following table.

**Drug product of Org25969 Batch AE: summary of mean revertant colonies (-S-9) Experiment 1**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 25 ± 7 | 146 ± 11 | 16 ± 4 | 8 ± 3 | 451 ± 34 |
| Drug product of Org25969 Batch AE | 156.25 | 26 ± 5 | 144 ± 6 | 17 ± 7 | 8 ± 4 | 463 ± 22 |
| | 312.5 | 32 ± 6 | 144 ± 17 | 20 ± 4 | 7 ± 3 | 461 ± 14 |
| | 625 | 30 ± 5 | 124 ± 19 | 16 ± 3 | 8 ± 2 | 434 ± 66 |
| | 1250 | 22 ± 2 | 147 ± 32 | 14 ± 6 | 7 ± 2 | 476 ± 60 |
| | 2500 | 29 ± 3 | 126 ± 8 | 19 ± 2 | 6 ± 3 | 440 ± 2 |
| | 5000 | 26 ± 4 | 128 ± 10 | 14 ± 3 | 8 ± 4 | 422 ± 60 |
| Positive controls | Compound | 2NF | $NaN_3$ | $NaN_3$ | AAC | GLU |
| | Dose Level | 5 µg | 2 µg | 2 µg | 50 µg | 25 µg |
| | Mean ± SD | 1246 ± 68 | 681 ± 16 | 799 ± 23 | 117 ± 23 | 723 ± 34 |

**Drug product of Org25969 Batch AE: summary of mean revertant colonies (+S-9) - Experiment 1**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 28 ± 5 | 161 ± 32 | 11 ± 3 | 13 ± 1 | 246 ± 13 |
| Drug product of Org25969 Batch AE | 156.25 | 34 ± 5 | 154 ± 11 | 14 ± 3 | 12 ± 6 | 269 ± 29 |
| | 312.5 | 31 ± 7 | 156 ± 6 | 16 ± 1 | 12 ± 5 | 269 ± 26 |
| | 625 | 34 ± 4 | 163 ± 16 | 19 ± 7 | 13 ± 2 | 285 ± 15 |
| | 1250 | 37 ± 6 | 154 ± 15 | 19 ± 1 | 8 ± 2 | 295 ± 29 |
| | 2500 | 31 ± 5 | 144 ± 16 | 16 ± 8 | 15 ± 7 | 268 ± 12 |
| | 5000 | 34 ± 4 | 146 ± 12 | 19 ± 4 | 9 ± 3 | 170 ± 37 (S) |
| Positive controls | Compound | B[a]P | AAN | AAN | AAN | AAN |
| | Dose Level | 10 µg | 5 µg | 5 µg | 5 µg | 20 µg |
| | Mean ± SD | 182 ± 32 | 2244 ± 84 | 232 ± 18 | 222 ± 33 | 1223 ± 143 |

**Drug product of Org25969 Batch AE: summary of mean revertant colonies (-S-9) - Experiment 2**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 27 ± 2 | 134 ± 10 | 19 ± 6 | 8 ± 3 | 327 ± 12 |
| Drug product of Org25969 Batch AE | 500 | 22 ± 8 | 112 ± 6 | 16 ± 2 | 5 ± 2 | 329 ± 18 |
| | 1000 | 27 ± 1 | 115 ± 6 | 23 ± 13 | 8 ± 2 | 344 ± 22 |
| | 2000 | 20 ± 8 | 139 ± 7 | 18 ± 5 | 8 ± 2 | 383 ± 39 |
| | 3000 | 27 ± 2 | 119 ± 3 | 17 ± 2 | 11 ± 2 | 337 ± 34 |
| | 4000 | 31 ± 8 | 126 ± 8 | 19 ± 5 | 7 ± 2 | 361 ± 11 |
| | 5000 | 27 ± 6 | 127 ± 9 | 18 ± 2 | 8 ± 3 | 345 ± 31 |
| Positive controls | Compound | 2NF | $NaN_3$ | $NaN_3$ | AAC | GLU |
| | Dose Level | 5 µg | 2 µg | 2 µg | 50 µg | 25 µg |
| | Mean ± SD | 697 ± 151 | 582 ± 35 | 559 ± 113 | 122 ± 69 | 497 ± 11 |

**Drug product of Org25969 Batch AE: summary of mean revertant colonies (+S-9) - Experiment 2**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 36 ± 6 | 126 ± 15 | 22 ± 3 | 16 ± 1 | 214 ± 7 |
| Drug product of Org25969 Batch AE | 500 | 33 ± 7 | 124 ± 12 | 22 ± 3 | 12 ± 2 | 196 ± 16 |
| | 1000 | 33 ± 6 | 144 ± 18 | 19 ± 6 | 8 ± 6 | 278 ± 24 |
| | 2000 | 34 ± 4 | 133 ± 19 | 15 ± 2 | 11 ± 1 | 290 ± 16 |
| | 3000 | 37 ± 1 | 133 ± 6 | 18 ± 2 | 14 ± 2 | 248 ± 52 |
| | 4000 | 37 ± 8 | 142 ± 17 | 23 ± 5 | 13 ± 3 | 289 ± 22 |
| | 5000 | 41 ± 9 | 149 ± 3 | 18 ± 2 | 12 ± 4 | 264 ± 65 |
| Positive controls | Compound | B[a]P | AAN | AAN | AAN | AAN |
| | Dose Level | 10 µg | 5 µg | 5 µg | 5 µg | 20 µg |
| | Mean ± SD | 358 ± 24 | 811 ± 104 | 210 ± 36 | 78 ± 15 | 956 ± 157 |

**5. Study title**: A bacterial reverse mutation test (Ames test) study with Org 25969 (Batch AM) in Salmonella Typhimurium and Escherichia coli with independent repeat

**Key findings**: Org 25969 in this batch (batch AM) did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 060062S
Document ID: NL00005824

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD
Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands

**Date of study initiation**: 03/16/2006
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch AM, 92.3%
*Note: analysis certificate was not attached with this study report; instead, batch analysis can be found in NDA submission in Module 3.Quality.*

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA1535, TA1537; *E. Coli* : WP2 uvrA pKM101.

Metabolizing system: a mixture of the 88290 N/kg supernatant fractions (S9) of rat liver homogenate and cofactors was used. The S9 was prepared from livers of male rats in which induction of drug metabolizing enzymes was provoked by previous treatment with polychlorinated biphenyl's (Aroclor 1254) (b) (4) . The capacity of the S9 batch (S9-41) to convert reference mutagens was demonstrated in each Ames test.

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

| Test item | Dose per plate | | No. of plates | |
|---|---|---|---|---|
| | Experiment 1 060062S(1) | Experiment 2 060062S(2) | | |
| | (µg) | (µg) | Without S9-mix | With S9-mix |
| Vehicle | [a] | [a] | 3 | 3 |
| Org 25969 | 156.25 | 500 | 3 | 3 |
| Org 25969 | 312.5 | 1000 | 3 | 3 |
| Org 25969 | 625 | 2000 | 3 | 3 |
| Org 25969 | 1250 | 3000 | 3 | 3 |
| Org 25969 | 2500 | 4000 | 3 | 3 |
| Org 25969 | 5000 | 5000 | 3 | 3 |
| 4-NQO | 0.3 | 0.3 | 3 | 0 |
| 4-NQO | 1 | 1 | 3 | 0 |
| 9-AC | 60 | 60 | 3 | 0 |
| $NaN_3$ | 1 | 1 | 3 | 0 |
| 2-AA | 2 | 2 | 0 | 3 |
| 2-AAF | 25 | 25 | 0 | 3 |
| BP | 5 | 5 | 0 | 3 |

[a] 100 µL Milli-Q water was used

Basis of dose selection: not indicated by the sponsor. However, based on the results of the studies previously conducted, the concentrations selected in the definitive study was considered adequate.

Negative controls: milli-Q water

Positive controls: The following reference mutagens were used as positive controls (extracted from the sponsor's report).

Without metabolic activation:

- 4-Nitroquinoline-N-oxide (4-NQO), 0.3 µg per plate for S. typhimurium strains TA98 and TA100 and 1 µg per plate for E. coli strain WP2 uvrA pKM101.
- Sodiumazide ($NaN_3$), 1 µg per plate for S. typhimurium strain TA1535.
- 9-Aminoacridine (9-AC), 60 µg per plate for S. typhimurium strain TA1537.

With metabolic activation:

- 2-Acetylaminofluorene (2-AAF), 25 µg per plate for S. typhimurium strains TA98 and TA100.
- 2-Aminoanthracene (2-AA), 2 µg per plate in S. typhimurium strain TA1535 and E. coli strain WP2 uvrA pKM101.
- Benzo(a)pyrene (BP), 5 µg per plate in S. typhimurium strain TA1537.

Incubation and sampling times: The test was carried out in triplicate, with and without S9-mix in both experiment 1 and experiment 2. Before the experiment, a thawed sample of the stock culture was incubated in Oxoid Nutrient broth medium in a shaking water bath at 37 °C overnight until an optical density of at least between 0.8 and 1.0 was reached. Then, 0.1 mL of this overnight culture plus 0.1 ml either the test article or the positive control article or the negative control article, with or without 0.5 mL S9-mix, was added to 3 mL molten soft agar at 45 °C, mixed and poured out (plated) on Vogel-Bonner Minimal Agar plates. All plates were incubated in an incubator. The number of colonies was determined using an automatic colony counter after incubation at 37 °C for approximate 48h. The mean number of revertants per plate was calculated for the control and test plates.

*Criteria for a valid assay:*

According to the sponsor, the assay was considered to be valid with the following criteria were met:

- the number of bacteria plated was higher than 5 x $10^7$ per plate
- mean number of revertants in the positive control group in the presence and the absence of S9-mix was more than twice the mean number of revertants in the corresponding negative control group
- the maximum concentration was 5000 µg per plate, or was within the toxic range, or was a precipitating concentration.
- mean number of spontaneous revertants was within the normal control range in the absence (TA98: 7-23; TA100: 61-124; TA1535: 2-17; TA1537: 1-10 and WP2 uvrA pKM101: 37-152) and the presence of S9-mix (TA98: 6-32; TA100: 52-122; TA1535: 5-14; TA1537: 1-12 and WP2 uvrA pKM101: 10-179)
- mean number of revertants in the positive control group in the presence of S9-mix exceeds the corresponding historical control value (control values in the absence: TA98: 57; TA100: 592; TA1535: 185; TA1537: 203 and WP2 uvrA pKM101: 271 and in the presence of S9-mix: TA98: 604; TA100: 221; TA1535: 118; TA1537: 52 and WP2 uvrA pKM101: 233)

The sponsor further indicated that the latter two criteria were not absolute but served as indicators.

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if the following three criteria were met, in any of the strains in the presence or in the absence of S9-mix:

- a relationship between concentration and the number of revertant colonies was present
- the increase in the number of revertant colonies exceeds the upper limit of the normal control range
- a two-fold increase in the number of revertants was present as compared to the negative controls.

The sponsor further indicated that these criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle.

Study outcome:
There were no signs of toxicity and precipitation in any strains tested at any dosing level in both experiment 1 and experiment 2.

Revertant frequencies for all doses of drug substance (batch AM), in all tester strains with and without S9, approximated or were less than control values. These results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The Sponsor summarized the results of the assay as shown in the following table.

**Experiment 1**

| Tester Strain | Treatment | Micrograms/ plate | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|
| | | | Mean | S.D. | Mean | S.D. |
| TA98 | Ultrapure water | 0 | 13 | 4.7 | 19 | 4.4 |
| TA98 | Org 25969 | 156.25 | 13 | 3.5 | 23 | 6.5 |
| TA98 | Org 25969 | 312.5 | 11 | 2.5 | 17 | 2.0 |
| TA98 | Org 25969 | 625 | 14 | 2.6 | 19 | 7.0 |
| TA98 | Org 25969 | 1250 | 17 | 3.2 | 18 | 4.0 |
| TA98 | Org 25969 | 2500 | 13 | 2.0 | 20 | 4.0 |
| TA98 | Org 25969 | 5000 | 16 | 4.0 | 23 | 3.5 |
| TA98 | 4-NQO | 0.3 | 121 | 13.1 | n.d. | n.d. |
| TA98 | 2-AAF | 25 | n.d. | n.d. | 437 | 14.6 |
| TA100 | Ultrapure water | 0 | 93 | 9.5 | 108 | 2.6 |
| TA100 | Org 25969 | 156.25 | 79 | 8.7 | 98 | 11.0 |
| TA100 | Org 25969 | 312.5 | 94 | 12.5 | 109 | 8.5 |
| TA100 | Org 25969 | 625 | 106 | 6.6 | 101 | 7.9 |
| TA100 | Org 25969 | 1250 | 91 | 6.0 | 94 | 6.4 |
| TA100 | Org 25969 | 2500 | 90 | 4.6 | 107 | 14.2 |
| TA100 | Org 25969 | 5000 | 72 | 3.8 | 86 | 14.4 |
| TA100 | 4-NQO | 0.3 | 1020 | 39.5 | n.d. | n.d. |
| TA100 | 2-AAF | 25 | n.d. | n.d. | 240 | 19.5 |
| TA1535 | Ultrapure water | 0 | 16 | 5.7 | 17 | 7.5 |
| TA1535 | Org 25969 | 156.25 | 13 | 0.6 | 21 | 5.3 |
| TA1535 | Org 25969 | 312.5 | 10 | 0.6 | 13 | 1.0 |
| TA1535 | Org 25969 | 625 | 11 | 4.9 | 17 | 2.6 |
| TA1535 | Org 25969 | 1250 | 11 | 5.7 | 19 | 1.5 |
| TA1535 | Org 25969 | 2500 | 17 | 3.8 | 14 | 4.9 |
| TA1535 | Org 25969 | 5000 | 9 | 3.6 | 16 | 3.1 |
| TA1535 | NaN3 | 1 | 406 | 25.4 | n.d. | n.d. |
| TA1535 | 2-AA | 2 | n.d. | n.d. | 272 | 35.1 |
| TA1537 | Ultrapure water | 0 | 8 | 2.3 | 7 | 1.2 |
| TA1537 | Org 25969 | 156.25 | 7 | 1.2 | 12 | 3.5 |
| TA1537 | Org 25969 | 312.5 | 10 | 3.8 | 10 | 1.0 |
| TA1537 | Org 25969 | 625 | 9 | 0.6 | 10 | 1.0 |
| TA1537 | Org 25969 | 1250 | 5 | 5.1 | 11 | 2.5 |
| TA1537 | Org 25969 | 2500 | 7 | 0.0 | 10 | 3.5 |
| TA1537 | Org 25969 | 5000 | 7 | 1.7 | 6 | 3.0 |
| TA1537 | 9-AC | 60 | 119 | 56.3 | n.d. | n.d. |
| TA1537 | BP | 5 | n.d. | n.d. | 85 | 9.9 |
| E. coli | Ultrapure water | 0 | 63 | 10.2 | 73 | 6.1 |
| E. coli | Org 25969 | 156.25 | 62 | 22.7 | 78 | 3.0 |
| E. coli | Org 25969 | 312.5 | 62 | 11.4 | 82 | 10.2 |
| E. coli | Org 25969 | 625 | 65 | 8.4 | 66 | 32.0 |
| E. coli | Org 25969 | 1250 | 71 | 2.3 | 89 | 9.6 |
| E. coli | Org 25969 | 2500 | 58 | 7.8 | 86 | 16.3 |
| E. coli | Org 25969 | 5000 | 66 | 10.5 | 77 | 12.1 |
| E. coli | 4-NQO | 1 | 1081 | 137 | n.d. | n.d. |
| E. coli | 2-AA | 2 | n.d. | n.d. | 468 | 17.1 |

**Experiment 2**

| Tester Strain | Treatment | Micrograms/ plate | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|
| | | | Mean | S.D. | Mean | S.D. |
| TA98 | Ultrapure water | 0 | 11 | 2.5 | 31 | 2.1 |
| TA98 | Org 25969 | 500 | 19 | 10.5 | 31 | 7.1 |
| TA98 | Org 25969 | 1000 | 15 | 3.6 | 31 | 5.1 |
| TA98 | Org 25969 | 2000 | 13 | 4.6 | 27 | 5.3 |
| TA98 | Org 25969 | 3000 | 12 | 3.6 | 30 | 5.6 |
| TA98 | Org 25969 | 4000 | 18 | 0.6 | 40 | 9.5 |
| TA98 | Org 25969 | 5000 | 14 | 5.0 | 29 | 7.6 |
| TA98 | 4-NQO | 0.3 | 129 | 17.3 | n.d. | n.d. |
| TA98 | 2-AAF | 25 | n.d. | n.d. | 717 | 77.8 |
| TA100 | Ultrapure water | 0 | 106 | 1.0 | 111 | 13.5 |
| TA100 | Org 25969 | 500 | 99 | 5.7 | 116 | 14.2 |
| TA100 | Org 25969 | 1000 | 93 | 9.2 | 113 | 4.2 |
| TA100 | Org 25969 | 2000 | 93 | 11.6 | 111 | 7.1 |
| TA100 | Org 25969 | 3000 | 89 | 6.9 | 114 | 11.9 |
| TA100 | Org 25969 | 4000 | 105 | 6.0 | 97 | 5.7 |
| TA100 | Org 25969 | 5000 | 103 | 4.2 | 118 | 10.1 |
| TA100 | 4-NQO | 0.3 | 1026 | 86.6 | n.d. | n.d. |
| TA100 | 2-AAF | 25 | n.d. | n.d. | 302 | 42.8 |
| TA1535 | Ultrapure water | 0 | 13 | 1.7 | 13 | 1.5 |
| TA1535 | Org 25969 | 500 | 9 | 3.8 | 12 | 2.1 |
| TA1535 | Org 25969 | 1000 | 13 | 2.5 | 14 | 2.5 |
| TA1535 | Org 25969 | 2000 | 11 | 6.2 | 10 | 4.4 |
| TA1535 | Org 25969 | 3000 | 16 | 2.5 | 11 | 3.6 |
| TA1535 | Org 25969 | 4000 | 13 | 9.5 | 16 | 5.2 |
| TA1535 | Org 25969 | 5000 | 15 | 2.1 | 12 | 2.3 |
| TA1535 | NaN3 | 1 | 512 | 37.3 | n.d. | n.d. |
| TA1535 | 2-AA | 2 | n.d. | n.d. | 40 | 8.4 |
| TA1537 | Ultrapure water | 0 | 8 | 2.1 | 8 | 3.1 |
| TA1537 | Org 25969 | 500 | 9 | 1.2 | 7 | 5.0 |
| TA1537 | Org 25969 | 1000 | 8 | 4.0 | 8 | 3.0 |
| TA1537 | Org 25969 | 2000 | 6 | 0.6 | 12 | 5.1 |
| TA1537 | Org 25969 | 3000 | 10 | 5.2 | 9 | 2.1 |
| TA1537 | Org 25969 | 4000 | 10 | 3.2 | 12 | 7.9 |
| TA1537 | Org 25969 | 5000 | 8 | 0.6 | 7 | 3.0 |
| TA1537 | 9-AC | 60 | 165 | 49.0 | n.d. | n.d. |
| TA1537 | BP | 5 | n.d. | n.d. | 145 | 15.1 |
| E. coli | Ultrapure water | 0 | 63 | 5.0 | 77 | 10.0 |
| E. coli | Org 25969 | 500 | 82 | 9.5 | 72 | 8.1 |
| E. coli | Org 25969 | 1000 | 64 | 10.2 | 73 | 15.5 |
| E. coli | Org 25969 | 2000 | 75 | 15.1 | 72 | 7.8 |
| E. coli | Org 25969 | 3000 | 65 | 3.8 | 68 | 15.6 |
| E. coli | Org 25969 | 4000 | 54 | 29.5 | 69 | 10.3 |
| E. coli | Org 25969 | 5000 | 69 | 9.0 | 64 | 3.8 |
| E. coli | 4-NQO | 1 | 1263 | 55.7 | n.d. | n.d. |
| E. coli | 2-AA | 2 | n.d. | n.d. | 672 | 78.8 |

**6. Study title**: Org 25969 batch AJ (impurity enriched): reverse mutation test in five histidine-requiring strains of Salmonella Typhimurium

**Key findings**: Org 25969 with impurities in this batch (batch AJ) did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 082/142
Document ID: NL0060035

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 07/26/2004
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: mixture of Org 25969, Org 48302, (b) (4) impurities, Batch AJ, content of active entity of Org 25969 (11.4%) and Org 48302 (15.5%), content of total (b) (4)

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA102, TA1535, and TA1537

Metabolizing system: The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male SD rats induced with Aroclor 1254 and obtained from (b) (4). Each batch of S9 was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-O-dealkylase activities).

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

| Experiment | S-9 | Concentration of treatment solution (mg/mL) | Final concentration *(µg/plate) |
|---|---|---|---|
| Range-finder Experiment and Mutation Experiment 1 | − and + | 0.016 | 1.6 |
| | | 0.08 | 8 |
| | | 0.40 | 40 |
| | | 2.00 | 200 |
| | | 10.00 | 1000 |
| | | 50.00 | 5000 |
| Mutation Experiment 2 | − and + | 1.5625 | 156.25 |
| | | 3.125 | 312.5 |
| | | 6.25 | 625 |
| | | 12.50 | 1250 |
| | | 25.00 | 2500 |
| | | 50.00 | 5000 |

* final concentration refers to the concentration of all cyclodextrins including Org 25969

Basis of dose selection: the sponsor has conducted toxicity range-finder experiment in strain TA100 in the presence and absence of S9-mix at concentrations as shown above. No evidence of toxicity as would normally be manifest by a thinning of the background bacterial lawn and/or a marked decrease in revertant numbers was observed following any of the treatments. Therefore, the sponsor chose the OECD recommended maximal test concentration (5000 µg/plate) as the highest concentration to test.

Negative controls: purified water

Positive controls: The positive control chemicals were supplied and used as tabulated below as extracted from the sponsor's report.

| Chemical | Source | Stock # concentration (µg/mL) | Final concentration (µg/plate) | Use Strain(s) | S-9 |
|---|---|---|---|---|---|
| | (b) (4) | | | | |
| 2-nitrofluorene (2NF) | | 50 | 5.0 | TA98 | − |
| Sodium azide ($NaN_3$) | | 20 | 2.0 | TA100, TA1535 | − |
| 9-aminoacridine (AAC) | | 500 | 50.0 | TA1537 | − |
| Glutaraldehyde (GLU) | | 250 | 25.0 | TA102 | − |
| Benzo[a]pyrene (B[a]P) | | 100* | 10.0 | TA98 | + |
| 2-aminoanthracene (AAN) | | 50* | 5.0 | TA100, TA1535, TA1537 | + |
| | | 200* | 20.0 | TA102 | + |

\# With the exception of $NaN_3$ and GLU, which were prepared in water, all stock solutions were prepared in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO). All stock solutions were stored in aliquots at 1-10°C in the dark, with the exception of B[a]P which was stored in aliquots at –80°C in the dark.

* For Experiment 2 pre-incubation treatments, stock solutions of these positive control compounds were twice the concentration stated. This enabled the volume additions to be reduced to 0.05 mL (thus avoiding solvent-induced toxicity) whilst maintaining the final concentrations per plate detailed above.

Incubation and sampling times: Two definitive experiments were conducted in triplicate to test the mutagenicity of Org 25969 batch AJ (impurity enriched) in the absence and presence of S9. The bacteria have been checked strain characteristics of histidine dependence, *uvrB* character, *rfa* character and resistance to ampicillin (TA98 and TA100) or ampicillin plus tetracycline (TA102). Negative (solvent) were included in quintuplicate without and with S-9 mix. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S-9. The activity of the S-9 mix used in each experiment was confirmed by AAN or B[a]P treatments of the strains in the presence of S-9. For all assays, bacteria were cultured for 10 hours at 37±1°C in nutrient broth in a shaking incubator before treatment. Two hours after this pre-culture, 0.1 ml bacterial culture, 0.1 ml test article solution or culture, 0.5 ml 10% S-9 mix or buffer solution were added into a 2.5 ml molten agar at 46±1°C, followed by rapid mixing and then pouring on to volgel-Bonner E agar plates for incubation at 37±1 °C in the dark for 72 hours. Experiment 1 mutagenicity data for strain TA100 were provided

by the rangefinder experiment treatments. As the results of the first experiment were negative, treatments in the presence of S-9 in Experiment 2 included a pre-incubation step. Quantities of test article or control solution, bacteria and S-9 mix detailed above, were mixed together and incubated for 1 hour at 37±1°C, with shaking, before the addition of 2.5 mL molten agar at 46±1°C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure in hope of increasing the range of mutagenic chemicals that could be detected in the assay.

After 72 hour incubation, plates were examined for evidence of toxicity to the background lawn, and counted for revertants using colony counter. The m-statistic analysis was calculated to check that the data were Poisson distributed, and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

*Criteria for a valid assay:*
According to the sponsor, the assay was considered to be valid if the following criteria were met:
1. The mean negative control counts fell within the normal ranges as defined in the attached table of historical negative control values.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S-9 preparation (table of histological positive control values were also attached in this study report)
3. No more than 5% of the plates were lost through contamination or some other unforeseen event.

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if statistically significant ($p < 0.01$) and dose-related increase of revertants observed and the positive response described were reproducible.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle.

Study outcome:
No evidence of toxicity was observed in any tester strain at any dose levels in both experiment 1 and 2.

No statistically significant, dose-related and reproducible increases in revertant numbers were observed following any strain treatments in the absence or presence of metabolic activation following treatment of the test article (batch AJ). These results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The Sponsor summarized the results of the assay as shown in the following table.

**Org 25969 batch AJ (impurity enriched): summary of mean revertant colonies (-S-9) - Experiment 1**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 24 ± 5 | 116 ± 10 | 20 ± 4 | 7 ± 4 | 314 ± 14 |
| Org 25969 batch AJ (impurity enriched) | 1.6 | 24 ± 8 | 126 ± 13 | 20 ± 7 | 8 ± 2 | 341 ± 13 |
| | 8 | 28 ± 4 | 116 ± 25 | 14 ± 5 | 8 ± 6 | 352 ± 9 |
| | 40 | 30 ± 1 | 130 ± 12 | 21 ± 5 | 8 ± 1 | 341 ± 40 |
| | 200 | 27 ± 5 | 98 ± 22 | 20 ± 8 | 7 ± 4 | 346 ± 33 |
| | 1000 | 33 ± 5 | 139 ± 19 | 16 ± 5 | 8 ± 4 | 330 ± 20 |
| | 5000 | 33 ± 4 | 144 ± 28 | 16 ± 8 | 7 ± 2 | 319 ± 12 |
| Positive controls | Compound | 2NF | $NaN_3$ | $NaN_3$ | AAC | GLU |
| | Dose Level | 5 µg | 2 µg | 2 µg | 50 µg | 25 µg |
| | Mean ± SD | 758 ± 117 | 528 ± 90 | 663 ± 15 | 58 ± 10 | 559 ± 13 |

**Org 25969 batch AJ (impurity enriched): summary of mean revertant colonies (+S-9) - Experiment 1**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 34 ± 10 | 122 ± 9 | 14 ± 4 | 9 ± 5 | 263 ± 45 |
| Org 25969 batch AJ (impurity enriched) | 1.6 | 32 ± 8 | 128 ± 14 | 19 ± 7 | 10 ± 3 | 241 ± 27 |
| | 8 | 42 ± 6 | 136 ± 3 | 16 ± 5 | 16 ± 7 | 242 ± 17 |
| | 40 | 30 ± 4 | 128 ± 6 | 16 ± 2 | 14 ± 6 | 263 ± 5 |
| | 200 | 35 ± 3 | 159 ± 39 | 19 ± 5 | 13 ± 3 | 251 ± 29 |
| | 1000 | 39 ± 2 | 134 ± 21 | 20 ± 1 | 15 ± 3 | 247 ± 35 |
| | 5000 | 26 ± 3 | 135 ± 7 | 23 ± 1 | 11 ± 1 | 220 ± 10 |
| Positive controls | Compound | B[a]P | AAN | AAN | AAN | AAN |
| | Dose Level | 10 µg | 5 µg | 5 µg | 5 µg | 20 µg |
| | Mean ± SD | 352 ± 24 | 1139 ± 175 | 258 ± 26 | 103 ± 23 | 1199 ± 144 |

**Org 25969 batch AJ (impurity enriched): summary of mean revertant colonies (-S-9) - Experiment 2**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 26 ± 6 | 138 ± 11 | 19 ± 5 | 15 ± 3 | 368 ± 19 |
| Org 25969 batch AJ (impurity enriched) | 156.25 | 25 ± 7 | 126 ± 29 | 26 ± 6 | 11 ± 2 | 334 ± 21 |
| | 312.5 | 32 ± 2 | 163 ± 19 | 25 ± 7 | 15 ± 3 | 366 ± 9 |
| | 625 | 28 ± 5 | 164 ± 18 | 24 ± 3 | 15 ± 3 | 382 ± 10 |
| | 1250 | 30 ± 13 | 148 ± 9 | 30 ± 6 | 11 ± 3 | 380 ± 9 |
| | 2500 | 20 ± 6 | 153 ± 9 | 26 ± 6 | 12 ± 6 | 396 ± 32 |
| | 5000 | 31 ± 4 | 168 ± 22 | 28 ± 3 | 10 ± 1 | 395 ± 2 |
| Positive controls | Compound | 2NF | $NaN_3$ | $NaN_3$ | AAC | GLU |
| | Dose Level | 5 µg | 2 µg | 2 µg | 50 µg | 25 µg |
| | Mean ± SD | 645 ± 71 | 572 ± 28 | 754 ± 38 | 55 ± 16 | 844 ± 21 |

**Org 25969 batch AJ (impurity enriched): summary of mean revertant colonies (+S-9) - Experiment 2**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 31 ± 4 | 145 ± 16 | 22 ± 4 | 13 ± 5 | 403 ± 30 |
| Org 25969 batch AJ (impurity enriched) | 156.25 | 33 ± 17 | 143 ± 32 | 20 ± 4 | 10 ± 6 | 358 ± 46 |
| | 312.5 | 27 ± 5 | 171 ± 20 | 21 ± 2 | 9 ± 3 | 420 ± 72 |
| | 625 | 28 ± 6 | 166 ± 19 | 21 ± 4 | 11 ± 5 | 329 ± 52 |
| | 1250 | 37 ± 2 | 156 ± 19 | 18 ± 3 | 9 ± 6 | 364 ± 67 |
| | 2500 | 31 ± 3 | 167 ± 7 | 18 ± 2 | 7 ± 5 | 356 ± 29 |
| | 5000 | 26 ± 7 | 141 ± 13 | 24 ± 4 | 11 ± 8 | 333 ± 46 |
| Positive controls | Compound | B[a]P | AAN | AAN | AAN | AAN |
| | Dose Level | 10 µg | 5 µg | 5 µg | 5 µg | 20 µg |
| | Mean ± SD | 324 ± 25 | 1804 ± 208 | 269 ± 34 | 148 ± 47 | 1371 ± 71 |

**7. Study title**: A bacterial reverse mutation test (Ames test) study with Org 25969 drug product containing degradation products in Salmonella Typhimurium and Escherichia coli (with independent repeat)

**Key findings**: The degraded Org 25969 batch containing high levels of certain impurities or degradants did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 0401855
Document ID: NL0056306

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD
Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands

**Date of study initiation**: 07/22/2004
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**:
The test item (degraded drug product) was supplied as a solution in water for injection with a concentration of 93.21 mg/mL. The drug product was prepared from drug substance batches U, W and Z and consisted of 43.77, 9.03 and 45.49 mg of batch U, W and Z, respectively per mL. The drug product produced was stored for approximately 2 to 2.5 months at 60 °C to allow for the formation of degradation products. Prior to this experiment the impurity profile of the degraded drug product was determined and after this experiment the impurity profile was determined again.

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA1535, TA1537; *E. Coli* : WP2 uvrA pKM101.

Metabolizing system: a mixture of the 88290 N/kg supernatant fraction (S9) of rat liver homogenate and cofactors was obtained from (b) (4) The capacity of the S9 batch (S9-41) to convert reference mutagens was demonstrated in each Ames test.

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

*Experiment 1:*

| Test item | Dose per plate (µg) | No. of plates Without S9-mix | with S9-mix |
|---|---|---|---|
| Org 25969 | 0 [a] | 3 | 3 |
| Org 25969 | 111 | 3 | 3 |
| Org 25969 | 333 | 3 | 3 |
| Org 25969 | 1000 | 3 | 3 |
| Org 25969 | 2500 | 3 | 3 |
| Org 25969 | 5000 | 3 | 3 |
| 4-NQO | 0.3 | 3 | 0 |
| 4-NQO | 1 | 3 | 0 |
| 9-AC | 60 | 3 | 0 |
| $NaN_3$ | 1 | 3 | 0 |
| 2-AA | 2 | 0 | 3 |
| 2-AAF | 25 | 0 | 3 |
| BP | 5 | 0 | 3 |

*Experiment 2 (independent repeat):*

| Test item | Dose per plate (µg) | No. of plates Without S9-mix | with S9-mix |
|---|---|---|---|
| Org 25969 | 0 [a] | 3 | 3 |
| Org 25969 | 375 | 3 | 3 |
| Org 25969 | 750 | 3 | 3 |
| Org 25969 | 1500 | 3 | 3 |
| Org 25969 | 3000 | 3 | 3 |
| Org 25969 | 5000 | 3 | 3 |
| 4-NQO | 0.3 | 3 | 0 |
| 4-NQO | 1 | 3 | 0 |
| 9-AC | 60 | 3 | 0 |
| $NaN_3$ | 1 | 3 | 0 |
| 2-AA | 2 | 0 | 3 |
| 2-AAF | 25 | 0 | 3 |
| BP | 5 | 0 | 3 |

[a] 100 µL ultrapure water was used.

Basis of dose selection: the sponsor did not conduct any pilot test. However, based on results of the previous studies, the dose selection as shown above appeared to be adequate.

Negative controls: 100 µL aliquot of ultra pure water

Positive controls: The following reference mutagens were used as positive controls.

Without metabolic activation:

- 4-Nitroquinoline-N-oxide (4-NQO), 0.3 µg per plate for S. typhimurium strains TA98 and TA100 and 1 µg per plate for E. coli strain WP2 uvrA pKM101.
- Sodiumazide (NaN3), 1 µg per plate for S. typhimurium strain TA1535
- 9-Aminoacridine (9-AC), 60 µg per plate for S. typhimurium strain TA1537

With metabolic activation:

- 2-Acetylaminofluorene (2-AAF), 25 µg per plate for S. typhimurium strains TA98 and TA100.
- 2-Aminoanthracene (2-AA), 2 µg per plate in S. typhimurium strain TA1535 and E. coli strain WP2 uvrA pKM101.
- Benzo(a)pyrene (BP), 5 µg per plate in S. typhimurium strain TA1537.

Incubation and sampling times: The test was carried out in triplicate, with and without S9-mix in both experiment 1 and experiment 2. Before the experiment, a thawed sample of the stock culture was incubated in Oxoid Nutrient broth medium in a shaking water-bath at 37 °C overnight until an optical density of at least between 0.8 and 1.0 was reached. Then, 0.1 mL of this overnight culture plus 0.1 ml either the test article or the positive control article or the negative control article, with or without 0.5 mL S9-mix, was added to 3 mL molten soft agar at 45 °C, mixed and poured out (plated) on Vogel-Bonner Minimal Agar plates. All plates were incubated in an incubator. Plates were inspected for contamination, presence of precipitate, inhibition of background lawn, and presence of $His^-$ or $Trp^-$ colonies, and the number of colonies was determined using an automatic colony counter after incubation at 37 °C for approx. 48h. The mean number of revertants per plate was calculated for the control and test plates.

*Criteria for a valid assay:*

According to the sponsor, the assay was considered to be valid with the following criteria were met:

- the number of bacteria plated was higher than 5 x $10^7$ per plate
- mean number of revertants in the positive control group in the presence and the absence of S9-mix was more than twice the mean number of revertants in the corresponding negative control group
- the maximum concentration was 5000 µg per plate, or was within the toxic range, or was a precipitating concentration.
- mean number of spontaneous revertants was within the normal control range in the absence (TA98: 7-23; TA100: 61-124; TA1535: 2-17; TA1537: 1-10 and WP2 uvrA pKM101: 37-152) and the presence of S9-mix (TA98: 6-32; TA100: 52-122; TA1535: 5-14; TA1537: 1-12 and WP2 uvrA pKM101: 10-179)
- mean number of revertants in the positive control group in the presence of S9-mix exceeds the corresponding historical control value (control values in the absence: TA98: 57; TA100: 592; TA1535: 185; TA1537: 203 and WP2 uvrA pKM101: 271 and in the presence of S9-mix: TA98: 604; TA100: 221; TA1535: 118; TA1537: 52 and WP2 uvrA pKM101: 233)

The sponsor further indicated that the latter two criteria were not absolute but served as indicators.

*Criteria for a positive response:*

According to the sponsor, a test compound was considered positive (mutagenic) if the following three criteria were met, in any of the strains in the presence or in the absence of S9-mix:

- a relationship between concentration and the number of revertant colonies was present
- the increase in the number of revertant colonies exceeds the upper limit of the normal control range
- a two-fold increase in the number of revertants was present as compared to the negative controls.

The sponsor further indicated that these criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle. The number of viable bacteria/plate was acceptable in all strains.

Study outcome:
There were no signs of toxicity and precipitation in any tester strain at any doses of test article treatment in both experiment 1 and 2.

Revertant frequencies for all doses of test article, in all tester strains with and without S9, approximated or were less than control values. These results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The Sponsor summarized the results of the assay as shown in the following table.

**Table 9.1-1 Mean number of revertants per plate (experiment 1)**

Ames mean data

Experiment No.: 040185S(1)

| Tester Strain | Treatment | Micrograms/ plate | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|
| | | | Mean | S.D. | Mean | S.D. |
| TA98 | Ultrapure water | 0 | 23 | 1.7 | 33 | 7.5 |
| TA98 | Org 25969 | 111 | 17 | 0.6 | 13 | 4.2 |
| TA98 | Org 25969 | 333 | 12 | 1.5 | 25 | 4.7 |
| TA98 | Org 25969 | 1000 | 19 | 3.1 | 26 | 4.0 |
| TA98 | Org 25969 | 2500 | 20 | 2.6 | 15 | 2.1 |
| TA98 | Org 25969 | 5000 | 16 | 4.0 | 21 | 5.5 |
| TA98 | 4-NQO | 0.3 | 164 | 2.3 | n.d. | n.d. |
| TA98 | 2-AAF | 25 | n.d. | n.d. | 1328 | 38.1 |
| TA100 | Ultrapure water | 0 | 88 | 3.1 | 104 | 13.5 |
| TA100 | Org 25969 | 111 | 105 | 13.2 | 121 | 16.7 |
| TA100 | Org 25969 | 333 | 125 | 6.6 | 112 | 7.5 |
| TA100 | Org 25969 | 1000 | 111 | 13.1 | 117 | 14.6 |
| TA100 | Org 25969 | 2500 | 119 | 6.0 | 100 | 4.6 |
| TA100 | Org 25969 | 5000 | 112 | 3.5 | 114 | 13.6 |
| TA100 | 4-NQO | 0.3 | 1104 | 30.6 | n.d. | n.d. |
| TA100 | 2-AAF | 25 | n.d. | n.d. | 589 | 42.4 |
| TA1535 | Ultrapure water | 0 | 12 | 3.1 | 10 | 5.7 |
| TA1535 | Org 25969 | 111 | 17 | 2.9 | 12 | 2.1 |
| TA1535 | Org 25969 | 333 | 14 | 1.0 | 5 | 1.5 |
| TA1535 | Org 25969 | 1000 | 17 | 3.8 | 9 | 1.2 |
| TA1535 | Org 25969 | 2500 | 17 | 8.0 | 6 | 1.2 |
| TA1535 | Org 25969 | 5000 | 12 | 2.6 | 7 | 3.5 |
| TA1535 | NaN3 | 1 | 352 | 22.3 | n.d. | n.d. |
| TA1535 | 2-AA | 2 | n.d. | n.d. | 187 | 9.1 |
| TA1537 | Ultrapure water | 0 | 6 | 3.1 | 7 | 2.6 |
| TA1537 | Org 25969 | 111 | 5 | 2.0 | 6 | 2.5 |
| TA1537 | Org 25969 | 333 | 6 | 1.7 | 4 | 1.2 |
| TA1537 | Org 25969 | 1000 | 5 | 0.6 | 8 | 2.5 |
| TA1537 | Org 25969 | 2500 | 6 | 2.5 | 6 | 3.2 |
| TA1537 | Org 25969 | 5000 | 6 | 0.6 | 5 | 2.6 |
| TA1537 | 9-AC | 60 | 1991 | 362 | n.d. | n.d. |
| TA1537 | BP | 5 | n.d. | n.d. | 203 | 20.6 |
| E. coli | Ultrapure water | 0 | 139 | 22.6 | 154 | 10.7 |
| E. coli | Org 25969 | 111 | 151 | 13.3 | 161 | 13.7 |
| E. coli | Org 25969 | 333 | 136 | 12.0 | 145 | 19.8 |
| E. coli | Org 25969 | 1000 | 142 | 5.5 | 143 | 10.7 |
| E. coli | Org 25969 | 2500 | 132 | 4.6 | 122 | 7.6 |
| E. coli | Org 25969 | 5000 | 143 | 15.0 | 157 | 13.3 |
| E. coli | 4-NQO | 1 | 896 | 34.5 | n.d. | n.d. |
| E. coli | 2-AA | 2 | n.d. | n.d. | 459 | 45.6 |

**Table 9.1-2 Mean number of revertants per plate (experiment 2; independent repeat)**

Ames mean data

Experiment No.: 040185S(2)

| Tester Strain | Treatment | Micrograms/ plate | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|
| | | | Mean | S.D. | Mean | S.D. |
| TA98 | Ultrapure water | 0 | 15 | 4.6 | 15 | 4.0 |
| TA98 | Org 25969 | 375 | 13 | 2.0 | 13 | 4.0 |
| TA98 | Org 25969 | 750 | 18 | 5.0 | 14 | 7.2 |
| TA98 | Org 25969 | 1500 | 13 | 4.7 | 15 | 4.6 |
| TA98 | Org 25969 | 3000 | 11 | 8.0 | 14 | 2.5 |
| TA98 | Org 25969 | 5000 | 10 | 1.7 | 19 | 0.6 |
| TA98 | 4-NQO | 0.3 | 182 | 13.0 | n.d. | n.d. |
| TA98 | 2-AAF | 25 | n.d. | n.d. | 1090 | 63.3 |
| TA100 | Ultrapure water | 0 | 103 | 8.0 | 76 | 9.5 |
| TA100 | Org 25969 | 375 | 80 | 4.5 | 74 | 4.9 |
| TA100 | Org 25969 | 750 | 91 | 3.1 | 81 | 18.6 |
| TA100 | Org 25969 | 1500 | 87 | 6.8 | 78 | 6.1 |
| TA100 | Org 25969 | 3000 | 63 | 6.2 | 68 | 19.6 |
| TA100 | Org 25969 | 5000 | 73 | 10.6 | 76 | 9.7 |
| TA100 | 4-NQO | 0.3 | 1337 | 57.1 | n.d. | n.d. |
| TA100 | 2-AAF | 25 | n.d. | n.d. | 333 | 19.5 |
| TA1535 | Ultrapure water | 0 | 10 | 1.2 | 8 | 2.3 |
| TA1535 | Org 25969 | 375 | 8 | 0.6 | 8 | 0.6 |
| TA1535 | Org 25969 | 750 | 6 | 1.5 | 7 | 0.6 |
| TA1535 | Org 25969 | 1500 | 7 | 2.1 | 6 | 2.1 |
| TA1535 | Org 25969 | 3000 | 12 | 3.5 | 9 | 4.2 |
| TA1535 | Org 25969 | 5000 | 7 | 2.6 | 7 | 2.5 |
| TA1535 | NaN3 | 1 | 343 | 29.6 | n.d. | n.d. |
| TA1535 | 2-AA | 2 | n.d. | n.d. | 196 | 66.9 |
| TA1537 | Ultrapure water | 0 | 5 | 4.2 | 6 | 3.5 |
| TA1537 | Org 25969 | 375 | 5 | 1.7 | 7 | 1.2 |
| TA1537 | Org 25969 | 750 | 5 | 2.5 | 8 | 2.0 |
| TA1537 | Org 25969 | 1500 | 3 | 1.0 | 7 | 1.5 |
| TA1537 | Org 25969 | 3000 | 4 | 0.6 | 6 | 2.1 |
| TA1537 | Org 25969 | 5000 | 4 | 1.0 | 8 | 0.6 |
| TA1537 | 9-AC | 60 | 1392 | 43.1 | n.d. | n.d. |
| TA1537 | BP | 5 | n.d. | n.d. | 221 | 20.0 |
| E. coli | Ultrapure water | 0 | 187 | 9.8 | 170 | 3.1 |
| E. coli | Org 25969 | 375 | 172 | 21.5 | 172 | 23.1 |
| E. coli | Org 25969 | 750 | 173 | 27.2 | 190 | 13.1 |
| E. coli | Org 25969 | 1500 | 171 | 17.2 | 178 | 13.1 |
| E. coli | Org 25969 | 3000 | 172 | 7.5 | 180 | 8.5 |
| E. coli | Org 25969 | 5000 | 178 | 15.3 | 187 | 15.9 |
| E. coli | 4-NQO | 1 | 1053 | 38.2 | n.d. | n.d. |
| E. coli | 2-AA | 2 | n.d. | n.d. | 331 | 30.5 |

**8. Study title**: Org 48302 batch B: reverse mutation test in five histidine-requiring strains of Salmonella Typhimurium

**Key findings**: Org 48302 in this batch did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 082/140
Document ID: NL0060048

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 07/26/2004
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 48302, Batch B, 87.3%

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA102, TA1535, and TA1537

Metabolizing system: The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male SD rats induced with Aroclor 1254 and obtained from (b) (4). Each batch of S9 was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-O-dealkylase activities).

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

| Experiment | S-9 | Concentration of treatment solution (mg/mL) | Final concentration (µg/plate) |
|---|---|---|---|
| Range-finder Experiment and Mutation Experiment 1 | – and + | 0.016 | 1.6 |
| | | 0.08 | 8 |
| | | 0.40 | 40 |
| | | 2.00 | 200 |
| | | 10.00 | 1000 |
| | | 50.00 | 5000 |
| Mutation Experiment 2 | – and + | 0.032768[b] | 3.2768[b] |
| | | 0.08192[b] | 8.192[b] |
| | | 0.2048[b] | 20.48[b] |
| | | 0.512[ab] | 51.2[ab] |
| | | 1.28[ab] | 128[ab] |
| | | 3.20[ab] | 320[ab] |
| | | 8.00[ab] | 800[ab] |
| | | 20.00[ab] | 2000[ab] |
| | | 50.00[ab] | 5000[ab] |

a Concentrations used for all strains in the presence of S-9 and strains TA98, TA100, TA1535 and TA1537 in the absence of S-9
b Concentrations used for strain TA102 in the absence of S-9

Basis of dose selection: the sponsor has conducted toxicity range-finder experiment in strain TA100 in the presence and absence of S9-mix at concentrations as shown above. No evidence of toxicity as would normally be manifest by a thinning of the background bacterial lawn and/or a marked decrease in revertant numbers was observed following any of the treatments. Therefore, the sponsor chose the OECD recommended maximal test concentration (5000 µg/plate) as the highest concentration to test.

Negative controls: purified water

Positive controls: The positive control chemicals were supplied and used as tabulated below as extracted from the sponsor's report.

| Chemical | Source | Stock * concentration (µg/mL) | Final concentration (µg/plate) | Use Strain(s) | S-9 |
|---|---|---|---|---|---|
| | (b) (4) | | | | |
| 2-nitrofluorene (2NF) | | 50 | 5.0 | TA98 | – |
| Sodium azide ($NaN_3$) | | 20 | 2.0 | TA100, TA1535 | – |
| 9-aminoacridine (AAC) | | 500 | 50.0 | TA1537 | – |
| Glutaraldehyde (GLU) | | 250 | 25.0 | TA102 | – |
| Benzo[a]pyrene (B[a]P) | | 100** | 10.0 | TA98 | + |
| 2-aminoanthracene (AAN) | | 50** | 5.0, 10.0** | TA100, TA1535, TA1537 | + |
| | | 200** | 20.0, 40.0** | TA102 | + |

* With the exception of $NaN_3$ and GLU, which were prepared in water, all stock solutions were prepared in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO). All stock solutions were stored in aliquots at 1-10°C in the dark, with the exception of B[a]P which was stored in aliquots at –80°C in the dark.

** For Experiment 2 pre-incubation treatments, stock solutions of positive control compounds were twice the concentration stated. In error volume additions remained at 0.1 mL and the final concentrations per plate are detailed above (refer to minor deviations Appendix 8)

Incubation and sampling times: Two definitive experiments were conducted in triplicate to test the mutagenicity of Org 48302 batch B (impurity enriched) in the absence and presence of S9. The bacteria have been checked strain characteristics of histidine dependence, *uvrB* character, *rfa* character and resistance to ampicillin (TA98 and TA100) or ampicillin plus tetracycline (TA102). Negative (solvent) were included in quintuplicate without and with S-9 mix. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S-9. The activity of the S-9 mix used in each experiment was confirmed by AAN or B[a]P treatments of the strains in the presence of S-9. For all assays, bacteria were cultured for 10 hours at 37±1°C in nutrient broth in a shaking incubator before treatment. Two hours after this pre-culture, 0.1 ml bacterial culture, 0.1 ml test article solution or culture, 0.5 ml 10% S-9 mix or buffer solution were added into a 2.5 ml molten agar at 46±1°C, followed by rapid mixing and then pouring on to volgel-Bonner E agar plates for incubation at 37±1 °C in the dark for 72 hours. As the results of the first experiment were negative, treatments in the presence of S-9 in Experiment 2 included a pre-incubation step. Quantities of test article or control solution, bacteria and S-9 mix detailed above, were mixed together and

incubated for 1 hour at 37±1°C, with shaking, before the addition of 2.5 mL molten agar at 46±1°C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure in hope of increasing the range of mutagenic chemicals that could be detected in the assay.

After 72 hour incubation, plates were examined for evidence of toxicity to the background lawn, and counted for revertants using a Seescan Colony counter. The m-statistic was calculated to check that the data were Poisson distributed, and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

*Criteria for a valid assay:*
According to the sponsor, the assay was considered to be valid if the following criteria were met:
1. The mean negative control counts fell within the normal ranges as defined in the attached table of historical negative control values.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S-9 preparation (table of histological positive control values were also attached in this study report)
3. No more than 5% of the plates were lost through contamination or some other unforeseen event.

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if statistically significant ($p<0.01$) and dose-related increase of revertants observed and the positive response described were reproducible.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle.

Study outcome:
No evidence of toxicity was observed in any tester strain at any dose levels in both experiment 1 and 2 except a slight thinning of the background bacterial lawn was observed at the highest test dose in strain TA102 in the presence of S-9.

No statistically significant, dose-related and reproducible increases in revertant numbers were observed following any strain treatments in the absence or presence of metabolic activation following treatment of the test article (batch AJ). These results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The Sponsor summarized the results of the assay as shown in the following table. It should be noted that a statistically significant increase in revertants was observed following Experiment 1 treatments of TA102 in the absence of S-9 when the data were analyzed at the $p = 0.01$ level using the Dunnett's test. However, this increase occurred

at a low dose level, was small in magnitude, and was not reproduced in Experiment 2. Therefore this increase in revertants was not considered to treatment-related.

**Org 48302 Batch B: summary of mean revertant colonies (-S-9) - Experiment 1**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 29 ± 5 | 129 ± 11 | 20 ± 5 | 10 ± 5 | 313 ± 18 |
| Org 48302 Batch B | 1.6 | 30 ± 5 | 124 ± 11 | 24 ± 4 | 12 ± 2 | 307 ± 9 |
| | 8 | 23 ± 7 | 121 ± 16 | 16 ± 3 | 15 ± 5 | 447 ± 84 |
| | 40 | 20 ± 3 | 116 ± 11 | 22 ± 3 | 10 ± 2 | 318 ± 6 |
| | 200 | 27 ± 2 | 118 ± 11 | 17 ± 2 | 6 ± 2 | 340 ± 20 |
| | 1000 | 26 ± 1 | 132 ± 4 | 22 ± 9 | 8 ± 1 | 406 ± 130 |
| | 5000 | 24 ± 8 | 124 ± 6 | 28 ± 5 | 9 ± 0 | 341 ± 11 |
| Positive controls | Compound | 2NF | $NaN_3$ | $NaN_3$ | AAC | GLU |
| | Dose Level | 5 µg | 2 µg | 2 µg | 50 µg | 25 µg |
| | Mean ± SD | 729 ± 112 | 460 ± 55 | 457 ± 85 | 68 ± 24 | 506 ± 22 |

**Org 48302 Batch B: summary of mean revertant colonies (+S-9) - Experiment 1**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 25 ± 4 | 113 ± 17 | 16 ± 4 | 10 ± 4 | 171 ± 29 |
| Org 48302 Batch B | 1.6 | 26 ± 10 | 100 ± 6 | 17 ± 4 | 12 ± 5 | 158 ± 24 |
| | 8 | 35 ± 8 | 140 ± 27 | 13 ± 3 | 14 ± 1 | 210 ± 18 |
| | 40 | 34 ± 4 | 119 ± 9 | 17 ± 4 | 11 ± 4 | 186 ± 9 |
| | 200 | 29 ± 7 | 102 ± 22 | 14 ± 2 | 14 ± 2 | 196 ± 13 |
| | 1000 | 31 ± 3 | 119 ± 26 | 18 ± 1 | 10 ± 6 | 176 ± 16 |
| | 5000 | 28 ± 4 | 102 ± 3 | 18 ± 3 | 11 ± 3 | 122 ± 15 (S) |
| Positive controls | Compound | B[a]P | AAN | AAN | AAN | AAN |
| | Dose Level | 10 µg | 5 µg | 5 µg | 5 µg | 20 µg |
| | Mean ± SD | 334 ± 34 | 1050 ± 191 | 273 ± 29 | 150 ± 28 | 1094 ± 230 |

**Org 48302 Batch B: summary of mean revertant colonies (-S-9) - Experiment 2**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| WATER | 100 µl | 17 ± 4 | 151 ± 19 | 19 ± 4 | 6 ± 3 | 290 ± 18 |
| Org 48302 Batch B | 3.2768 | NT | NT | NT | NT | 263 ± 17 |
| | 8.192 | NT | NT | NT | NT | 281 ± 24 |
| | 20.48 | NT | NT | NT | NT | 268 ± 6 |
| | 51.2 | 16 ± 1 | 157 ± 17 | 24 ± 7 | 5 ± 3 | 292 ± 11 |
| | 128 | 29 ± 4 | 129 ± 22 | 23 ± 6 | 6 ± 2 | 275 ± 14 |
| | 320 | 21 ± 4 | 168 ± 10 | 23 ± 9 | 6 ± 2 | 277 ± 15 |
| | 800 | 23 ± 5 | 159 ± 17 | 17 ± 6 | 7 ± 1 | 296 ± 18 |
| | 2000 | 16 ± 7 | 154 ± 10 | 28 ± 5 | 10 ± 3 | 275 ± 17 |
| | 5000 | 14 ± 4 | 171 ± 6 | 19 ± 3 | 8 ± 2 | 277 ± 7 |
| Positive controls | Compound | 2NF | $NaN_3$ | $NaN_3$ | AAC | GLU |
| | Dose Level | 5 µg | 2 µg | 2 µg | 50 µg | 25 µg |
| | Mean ± SD | 364 ± 29 | 443 ± 26 | 467 ± 31 | 48 ± 8 | 578 ± 42 |

**Org 48302 Batch B: summary of mean revertant colonies (+S-9) - Experiment 2**

| Substance | Dose Level µg/plate | TA98 | TA100 | TA1535 | TA1537 | TA102 |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Water | 100 µl | 30 ± 4 | 166 ± 23 | 29 ± 5 | 12 ± 2 | 276 ± 12 |
| Org 48302 Batch B | 51.2 | 29 ± 10 | 179 ± 14 | 30 ± 7 | 15 ± 5 | 282 ± 29 |
| | 128 | 35 ± 1 | 180 ± 15 | 35 ± 2 | 15 ± 6 | 299 ± 10 |
| | 320 | 36 ± 6 | 171 ± 12 | 33 ± 9 | 12 ± 4 | 285 ± 75 |
| | 800 | 32 ± 4 | 187 ± 35 | 29 ± 2 | 11 ± 3 | 313 ± 12 |
| | 2000 | 34 ± 1 | 188 ± 49 | 29 ± 3 | 10 ± 8 | 291 ± 10 |
| | 5000 | 36 ± 5 | 175 ± 37 | 32 ± 13 | 12 ± 3 | 272 ± 40 |
| Positive controls | Compound | B[a]P | AAN | AAN | AAN | AAN |
| | Dose Level | 20 µg | 10 µg | 10 µg | 10 µg | 40 µg |
| | Mean ± SD | 260 ± 17 | 1227 ± 124 | 266 ± 12 | 111 ± 39 | 1331 ± 23 |

**9. Study title**: A bacterial reverse mutation test (Ames test) study with [redacted] (b) (4) in Salmonella Typhimurium and Escherichia coli

**Key findings**: [redacted] (b) (4) with impurities in this batch did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 030074S
Document ID: NL0046279

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD
Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands

**Date of study initiation**: 02/18/2003
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: [redacted] (b) (4), Batch A, 62.0%

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA1535, TA1537; *E. Coli* : WP2 uvrA pKM101.

Metabolizing system: a mixture of the 88290 N/kg supernatant fraction (S9) of rat liver homogenate and cofactors was obtained from [redacted] (b) (4) The capacity of the S9 batch (S9-41) to convert reference mutagens was demonstrated in each Ames test.

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

| Test item | Dose per plate (µg) | No. of plates Without S9-mix | with S9-mix |
|---|---|---|---|
| Org [redacted] (b) (4) | 0 [a] | [redacted] (b) (4) | |
| Org | 12.3 [b] | | |
| Org | 37.0 [b] | | |
| Org | 111 | | |
| Org | 333 | | |
| Org | 1000 | | |
| Org | 2500 | | |
| Org | 5000 | | |
| 4-NQO | 0.3 | 3 | 0 |
| 4-NQO | 1 | 3 | 0 |
| 9-AC | 60 | 3 | 0 |
| $NaN_3$ | 1 | 3 | 0 |
| 2-AA | 2 | 0 | 3 |
| 2-AAF | 25 | 0 | 3 |
| BP | 5 | 0 | 3 |

a 100 µL ultrapure water was used.
b concentrations tested only in TA100 and E. coli
- 4-NQO (4-Nitroquinoline-N-Oxide) was tested at a concentration of 0.3 µg per plate in TA98 and TA100 and at 1 µg per plate in E. coli
- 9-AC ( 9-Aminoacridine) was tested in TA1537
- $NaN_3$ (Sodiumazide) was tested in TA1535
- 2-AA (2- Aminoanthracene) was tested in TA1535 and E. coli
- 2-AAF (2-Acetylaminofluorene) was tested in TA98 and TA100
- BP (Benzo(a)pyrene) was tested in TA1537

Basis of dose selection: A dose-range-finding study in the absence and presence of S9-mix using strains TA100 and WP2 uvrA pKM101 was executed by the sponsor to assess the bacterial killing activity of the compound. Strains TA100 and WP2 uvrA pKM101 were plated for his+ and trp+ revertants, respectively, in triplicate. Concentrations of the test article used were 4.12, 13.3, 37.0, 111, 333, 1000, 2500, and 5000 µg per plate. There were no signs of cytotoxicity, and precipitation observed. Therefore, the sponsor chose the OECD recommended maximal test concentration (5000 µg/plate) as the highest concentration to test.

Negative controls: 100 µL aliquot of ultra pure water

Positive controls: The following reference mutagens were used as positive controls.

Without metabolic activation:

- 4-Nitroquinoline-N-oxide (4-NQO), 0.3 µg per plate for S. typhimurium strains TA98 and TA100 and 1 µg per plate for E. coli strain WP2 uvrA pKM101.
- Sodiumazide (NaN3), 1 µg per plate for S. typhimurium strain TA1535
- 9-Aminoacridine (9-AC), 60 µg per plate for S. typhimurium strain TA1537

With metabolic activation:

- 2-Acetylaminofluorene (2-AAF), 25 µg per plate for S. typhimurium strains TA98 and TA100.
- 2-Aminoanthracene (2-AA), 2 µg per plate in S. typhimurium strain TA1535 and E. coli strain WP2 uvrA pKM101.
- Benzo(a)pyrene (BP), 5 µg per plate in S. typhimurium strain TA1537.

Incubation and sampling times: The test was carried out in triplicate, with and without S9-mix. Before the experiment, a thawed sample of the stock culture was incubated in Oxoid Nutrient broth medium in a shaking water bath at 37 °C overnight until an optical density of at least between 0.8 and 1.0 was reached. Then, 0.1 mL of this overnight culture ($1 \times 10^6$) plus 0.1 ml either the test article or the positive control article or the negative control article, with or without 0.5 mL S9-mix, was added to 3 mL molten soft agar at 45 °C, mixed and poured out (plated) on Vogel-Bonner Minimal Agar plates. All plates were incubated in an incubator. The number of colonies was determined using an automatic colony counter after incubation at 37 °C for approx. 48h. After incubation, the plates were inspected for contamination, presence of precipitate, inhibition of the background lawn, and the presence of his- and trp- colonies, and then the mean number of revertants per plate was calculated for the control and test plates. It should be noted the results from the dose-range-finding study were used in the definitive study and assays of TA100 and WP2 E. coli were not conducted again.

*Criteria for a valid assay:*

According to the sponsor, the assay was considered to be valid with the following criteria were met:

- the number of bacteria plated was higher than 5 x $10^7$ per plate
- mean number of revertants in the positive control group in the presence and the absence of S9-mix was more than twice the mean number of revertants in the corresponding negative control group

In addition, the following criteria were monitored:

- mean number of spontaneous revertants was within the normal control range in the absence (TA98: 19-42; TA100: 89-193; TA1535: 6-14; TA1537: 5-25 and WP2 uvrA pKM101: 65-174) and the presence of S9-mix (TA98: 23-50; TA100: 95-215; TA1535: 7- 17; TA1537: 6-25 and WP2 uvrA pKM101: 77-188)
- mean number of revertants in the positive control group in the presence of S9-mix exceeds the corresponding historical control value (control values: TA98: 588; TA100: 428; TA1535: 201; TA1537: 78 and WP2 uvrA pKM101: 319).

The sponsor indicated that "the latter two criteria were not absolute but serve as indicators.

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if the following three criteria were met, in any of the strains in the presence or in the absence of S9-mix:

- a relationship between concentration and the number of revertant colonies was present
- the increase in the number of revertant colonies exceeds the upper limit of the normal control range
- a two-fold increase in the number of revertants was present as compared to the negative controls.

The sponsor further indicated that these criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle. The number of viable bacteria/plate was acceptable in all strains.

Study outcome:
Cytotoxic effect evidenced by inhibition of the background lawn was observed at concentrations of 2500 and 5000 µg/plate in E. coli in the absence and presence of S9-mix. Similar change was not seen in other strains. No precipitation was observed in any of the stains.

Revertant frequencies for all doses of drug substance (b) (4)), in all tester strains with and without S9, approximated or were less than control values. These results

indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system. The Sponsor summarized the results of the assay as shown in the following table.

Table 9.1-1 Mean number of revertants per plate (main study + DRF)

| Tester Strain | Treatment | Micrograms/ Plate | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|
| | | | Mean | S.D. | Mean | S.D. |
| TA98 | (b) (4) | 0 * | (b) (4) | | | |
| TA98 | | 111 | | | | |
| TA98 | | 333 | | | | |
| TA98 | | 1000 | | | | |
| TA98 | | 2500 | | | | |
| TA98 | | 5000 | | | | |
| TA98 | 2-AAF | 25 | n.d. | n.d. | 1072 | 20.8 |
| TA98 | (b) (4) | 0.3 | 120 | 13.0 | n.d. | n.d. |
| TA100 | | 0 * | (b) (4) | | | |
| TA100 | | 4.12 | | | | |
| TA100 | | 12.3 | | | | |
| TA100 | | 37.0 | | | | |
| TA100 | | 111 | | | | |
| TA100 | | 333 | | | | |
| TA100 | | 1000 | | | | |
| TA100 | | 2500 | | | | |
| TA100 | | 5000 | | | | |
| TA100 | 2-AAF | 25 | n.d. | n.d. | 448 | 11.1 |
| TA100 | 4-NQO | 0.3 | 1000 | 39.5 | n.d. | n.d. |
| TA1535 | (b) (4) | 0 * | (b) (4) | | | |
| TA1535 | | 111 | | | | |
| TA1535 | | 333 | | | | |
| TA1535 | | 1000 | | | | |
| TA1535 | | 2500 | | | | |
| TA1535 | | 5000 | | | | |
| TA1535 | 2-AA | 2 | n.d. | n.d. | 223 | 5.3 |
| TA1535 | NaN3 | 1 | 263 | 5.0 | n.d. | n.d. |
| TA1537 | (b) (4) | 0 * | (b) (4) | | | |
| TA1537 | | 111 | | | | |
| TA1537 | | 333 | | | | |
| TA1537 | | 1000 | | | | |
| TA1537 | | 2500 | | | | |
| TA1537 | | 5000 | | | | |
| TA1537 | BP | 5 | n.d. | n.d. | 207 | 43.7 |
| TA1537 | 9-AC | 80 | 482 | 9.8 | n.d. | n.d. |
| E. coli | (b) (4) | 0 * | (b) (4) | | | |
| E. coli | | 4.12 | | | | |
| E. coli | | 12.3 | | | | |
| E. coli | | 37.0 | | | | |
| E. coli | | 111 | | | | |
| E. coli | | 333 | | | | |
| E. coli | | 1000 | | | | |
| E. coli | | 2500 | | | | |
| E. coli | | 5000 | | | | |
| E. coli | 2-AA | 2 | n.d. | n.d. | 423 | 12.5 |
| E. coli | 4-NQO | 1 | 753 | 79.6 | n.d. | n.d. |

**10. Study title**: A second bacterial reverse mutation test (Ames test) study with (b) (4) in Salmonella Typhimurium and Escherichia coli
*Note: this study is an independent repeat of study 9.*

**Key findings**: (b) (4) with impurities in this batch did not cause a positive increase in the mean number of revertants per plate in any test strain at the concentration up to 5000 µg/plate in the presence or absence of rat liver S9.

**Study no.**: 030188
Document ID: NL0050642

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD
Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands

**Date of study initiation**: 05/01/2003
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: (b) (4), Batch A, 62.0%

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA1535, TA1537; *E. Coli* : WP2 uvrA pKM101.

Metabolizing system: a mixture of the 88290 N/kg supernatant fraction (S9) of rat liver homogenate and cofactors was obtained from (b) (4) The capacity of the S9 batch (S9-41) to convert reference mutagens was demonstrated in each Ames test.

Doses used in definitive study: as shown in the following table extracted from the sponsor's report

The strains were plated for revertants according to following table:

Experiment 1:

| Test item | Dose per plate (µg) | No. of plates Without S9-mix | with S9-mix |
|---|---|---|---|
| (b) (4) | 0 [a] | | (b) (4) |
| | 375 | | |
| | 750 | | |
| | 1500 | | |
| | 3000 | | |
| | 5000 | | |
| 4-NQO | 0.3 | 3 | 0 |
| 4-NQO | 1 | 3 | 0 |
| 9-AC | 60 | 3 | 0 |
| $NaN_3$ | 1 | 3 | 0 |
| 2-AA | 2 | 0 | 3 |
| 2-AAF | 25 | 0 | 3 |
| BP | 5 | 0 | 3 |

a 100 µL ultrapure water was used.
- 4-NQO (4-Nitroquinoline-N-Oxide) was tested at a concentration of 0.3 µg per plate in TA98 and TA100 and at 1 µg per plate in E. coli
- 9-AC ( 9-Aminoacridine) was tested in TA1537
- $NaN_3$ (Sodiumazide) was tested in TA1535
- 2-AA (2- Aminoanthracene) was tested in TA1535 and E. coli
- 2-AAF (2-Acetylaminofluorene) was tested in TA98 and TA100
- BP (Benzo(a)pyrene) was tested in TA1537

Experiment 2: TA1535 with or without filtration / TA1535 including a pre-incubation step:

| Test item | Dose per plate (µg) | No. of plates [b] Without S9-mix | with S9-mix |
|---|---|---|---|
| (b) (4) | 0 [a] | | (b) (4) |
| | 375 | | |
| | 750 | | |
| | 1500 | | |
| | 3000 | | |
| | 5000 | | |
| $NaN_3$ | 1 | 3 | 0 |
| 2-AA | 2 | 0 | 3 |

a 100 µL ultrapure water was used.
b per experiment (i.e. with or without filtration / including a pre-incubation step)

Basis of dose selection: Dose-range-finding study was not conducted in this study, but the dose selection in the definitive study is considered adequate based on the results of previous studies.

Negative controls: 100 µL aliquot of ultra pure water

Positive controls: The following reference mutagens were used as positive controls.

Without metabolic activation:

- 4-Nitroquinoline-N-oxide (4-NQO), 0.3 µg per plate for S. typhimurium strains TA98 and TA100 and 1 µg per plate for E. coli strain WP2 uvrA pKM101.
- Sodiumazide (NaN3), 1 µg per plate for S. typhimurium strain TA1535
- 9-Aminoacridine (9-AC), 60 µg per plate for S. typhimurium strain TA1537

With metabolic activation:

- 2-Acetylaminofluorene (2-AAF), 25 µg per plate for S. typhimurium strains TA98 and TA100.
- 2-Aminoanthracene (2-AA), 2 µg per plate in S. typhimurium strain TA1535 and E. coli strain WP2 uvrA pKM101.
- Benzo(a)pyrene (BP), 5 µg per plate in S. typhimurium strain TA1537.

Incubation and sampling times: The test was carried out in triplicate, with and without S9-mix. Before the experiment, a thawed sample of the stock culture was incubated in Oxoid Nutrient broth medium in a shaking waterbath at 37 °C overnight until an optical density of at least between 0.8 and 1.0 was reached. Then, 0.1 mL of this overnight culture ($1 \times 10^6$) plus 0.1 ml either the test article or the positive control article or the negative control article, with or without 0.5 mL S9-mix, was added to 3 mL molten soft agar at 45 °C, mixed and poured out (plated) on Vogel-Bonner Minimal Agar plates. All plates were incubated in an incubator. The number of colonies was determined using an automatic colony counter after incubation at 37 °C for approx. 48h. After incubation, the plates were inspected for contamination, presence of precipitate, inhibition of the background lawn, and the presence of his- and trp- colonies, and then the mean number of revertants per plate was calculated for the control and test plates. The incubations of Salmonella typhimurium strains TA98 and TA100 with Org (b) (4) in the presence of S9-mix were repeated because the positive control for TA98 and TA100 (2-AAF) did not induce a mutagenic response. Only the last obtained results are reported. The incubations with Salmonella typhimurium TA1535 in the absence and presence of S9-mix were also repeated. The experiment was performed with and without sterilization of the stock solution with Org (b) (4) by filtration over a Millipore filters (0.45 µm). In addition incubations with Org (b) (4) in TA1535 in the absence and presence of S9-mix including a pre-incubation step (60 min at 37 °C) were performed to clarify inconclusive results obtained in experiment 1.

*Criteria for a valid assay:*
According to the sponsor, the assay was considered to be valid with the following criteria were met:

- the number of bacteria plated was higher than $5 \times 10^7$ per plate
- mean number of revertants in the positive control group in the presence and the absence of S9-mix was more than twice the mean number of revertants in the corresponding negative control group

In addition, the following criteria were monitored:

- mean number of spontaneous revertants was within the normal control range in the absence (TA98: 19-42; TA100: 89-193; TA1535: 6-14; TA1537: 5-25 and WP2 uvrA pKM101: 65-174) and the presence of S9-mix (TA98: 23-50; TA100: 95-215; TA1535: 7- 17; TA1537: 6-25 and WP2 uvrA pKM101: 77-188)
- mean number of revertants in the positive control group in the presence of S9-mix exceeds the corresponding historical control value (control values: TA98: 588; TA100: 428; TA1535: 201; TA1537: 78 and WP2 uvrA pKM101: 319).

The sponsor indicated that "the latter two criteria were not absolute but serve as indicators.

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if the following three criteria were met, in any of the strains in the presence or in the absence of S9-mix:

- a relationship between concentration and the number of revertant colonies was present
- the increase in the number of revertant colonies exceeds the upper limit of the normal control range
- a two-fold increase in the number of revertants was present as compared to the negative controls.

The sponsor further indicated that these criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle. The number of viable bacteria/plate was acceptable in all strains.

Study outcome:
No signs of toxicity and precipitation were observed in any tester strains at any dose levels

(b) (4) did not induce a concentration-related increase in the number of revertants per plate in the S. typhimurium strains TA98, TA100 and TA1537 and in the E. coli strain, in the presence or the absence of S9-mix. In TA1535 2-3 fold increase in the mean number of revertants was observed at 5000 µg/plate. This value (18 ± 8.9) is slightly exceeded the upper limit of the normal control range for this strain (7-17 according to the sponsor). The results of this study were shown below as extracted from the sponsor's report.

**Table 10.1-1 Mean number of revertants per plate**

| Tester Strain | Treatment | Micrograms/ Plate | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|
| | | | Mean | S.D. | Mean | S.D. |
| TA98 | (b) (4) | 0 * | (b) (4) | | | (b) (4) |
| TA98 | | 375 | | | | |
| TA98 | | 750 | | | | |
| TA98 | | 1500 | | | | |
| TA98 | | 3000 | | | | |
| TA98 | | 5000 | | | | |
| TA98 | 4-NQO | 0.3 | 156 | 21.1 | n.d. | - |
| TA98 | 2-AA | 25 | n.d. | - | 1203 | 3.1 |
| TA100 | (b) (4) | 0 * | (b) (4) | | | (b) (4) |
| TA100 | | 375 | | | | |
| TA100 | | 750 | | | | |
| TA100 | | 1500 | | | | |
| TA100 | | 3000 | | | | |
| TA100 | | 5000 | | | | |
| TA100 | 4-NQO | 0.3 | 1311 | 78.4 | n.d. | - |
| TA100 | 2-AA | 25 | n.d. | - | 667 | 60.7 |
| TA1535 | (b) (4) | 0 * | (b) (4) | | | (b) (4) |
| TA1535 | | 375 | | | | |
| TA1535 | | 750 | | | | |
| TA1535 | | 1500 | | | | |
| TA1535 | | 3000 | | | | |
| TA1535 | | 5000 | | | | |
| TA1535 | $NaN_3$ | 1 | 359 | 8.1 | n.d. | - |
| TA1535 | 2-AA | 2 | n.d. | - | 329 | 45.1 |
| TA1537 | (b) (4) | 0 * | (b) (4) | | | (b) (4) |
| TA1537 | | 375 | | | | |
| TA1537 | | 750 | | | | |
| TA1537 | | 1500 | | | | |
| TA1537 | | 3000 | | | | |
| TA1537 | | 5000 | | | | |
| TA1537 | 9-AC | 60 | 843 | 138 | n.d. | - |
| TA1537 | BP | 5 | n.d. | - | 219 | 48.4 |
| E. coli | (b) (4) | 0 * | (b) (4) | | | (b) (4) |
| E. coli | | 375 | | | | |
| E. coli | | 750 | | | | |
| E. coli | | 1500 | | | | |
| E. coli | | 3000 | | | | |
| E. coli | | 5000 | | | | |
| E. coli | 4-NQO | 1 | 1199 | 47.8 | n.d. | - |
| E. coli | 2-AA | 2 | n.d. | - | 508 | 29.7 |

A secondary study was conducted on strain TA1535 to confirm these changes. The results were shown below as extracted from the sponsor's report indicating that (b) (4) did not increase the number of revertants in this strain.

**Table 10.2-1 Mean number of revertants per plate without filtration**

| Tester Strain | Treatment | Micrograms/ Plate | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|
| | | | Mean | S.D. | Mean | S.D. |
| TA1535 | (b) (4) | 0 * | (b) (4) | | | |
| TA1535 | | 375 | | | | |
| TA1535 | | 750 | | | | |
| TA1535 | | 1500 | | | | |
| TA1535 | | 3000 | | | | |
| TA1535 | | 5000 | | | | |
| TA1535 | $NaN_3$ | 1 | 346 | 19.9 | n.d. | - |
| TA1535 | 2-AA | 2 | n.d. | - | 227 | 10.5 |

* : 100 µL ultrapure water was used
n.d. : Not determined
S.D. : Standard deviation

**Table 10.2-2 Mean number of revertants per plate with filtration**

| Tester Strain | Treatment | Micrograms/ Plate | No. of revertants per plate without S9-mix | | No. of revertants per plate with S9-mix | |
|---|---|---|---|---|---|---|
| | | | Mean | S.D. | Mean | S.D. |
| TA1535 | (b) (4) | 0 * | (b) (4) | | | |
| TA1535 | | 375 | | | | |
| TA1535 | | 750 | | | | |
| TA1535 | | 1500 | | | | |
| TA1535 | | 3000 | | | | |
| TA1535 | | 5000 | | | | |
| TA1535 | $NaN_3$ | 1 | 346 | 19.9 | n.d. | - |
| TA1535 | 2-AA | 2 | n.d. | - | 227 | 10.5 |

Taken these together, these results indicate a negative response both in the presence and absence of a rat liver S9 metabolizing system in all the tester strains.

**11. Study title**: Evaluation of the mutagenic activity of bromocyclodextin in Salmonella Typhimurium reverse mutation assay and the Escherichia Coli reverse mutation assay (with independent repeat)

**Key findings**: Bromocyclodextin in this batch did not cause a positive increase in the mean number of revertants per plate in any test strain in the presence or absence of rat liver S9 at a level exhibiting limited solubility (3330 µg/plate) under the test condition of this study.

**Study no.**: 418962/070624
Document ID: NL00036687

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 10/01/2004
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )

**Drug, lot #, and % purity**: bromocyclodextin, Batch EMZR03K 1A, 94.5%

**Methods:**

Strains/species/cell line: *Salmonella typhimurium*: TA98, TA100, TA1535, TA1537; *E. Coli* : WP2 uvrA pKM101.

Metabolizing system: Rat liver microsomal enzymes (S9) were prepared in this CRO lab. Before use, all S9-batches were characterized with the metabolic activation requiring positive control benzo[a]pyrene in tester strain TA98 at the concentration of 5 µg/plate.

Doses used in definitive study:

Experiment 1: This study only used 3 strains: TA1535, TA1537 and TA98. The dose ranges selected were 10, 33, 100, 333, 1000, and 3330 µg/plate in the absence or presence of S9. Results from the dose-range finding studies in strains of TA100 and WP2 were reported as a part of experiment 1 and dosing range was 3, 10, 33, 100, 333, 1000, 3330, 5000 µg/plate in the absence or presence of S9.

Experiment 2: The following dose range was selected as extracted from the sponsor's report.

TA1535, TA1537, TA98 and $WP_2$uvrA:
With and without S9-mix: 33, 100, 333, 1000 and 3330 µg a.i./plate
TA1537:
Without S9-mix: 10, 33, 100, 333, 1000 and 3330 µg a.i./plate
With S9-mix: 33, 100, 333, 1000 and 3330 µg a.i./plate

Basis of dose selection: Dose-range-finding study was conducted in the tester strains TA100 and WP2 uvrA with concentrations of 3, 10, 33, 100, 333, 1000, 3330, and 5000 µg/plate in the absence and presence of S9. The test substance precipitated in the top agar at concentrations of 333 µg/plate and above and at concentration of 3330 and 5000 µg/plate on the plates at the start and at the end of the incubation period. The highest concentration of Broomcyclodextrine was the level exhibiting limited solubility (3330 µg/plate).

Negative controls: The vehicle of the test article, DMSO

Positive controls: The following reference mutagens were used as positive controls.

Without metabolic activation (-S9-mix):

| Strain | Chemical | Concentration/plate | Solvent |
|---|---|---|---|
| TA1535 | sodium azide (SA) | 5 µg | Saline |
| | (b) (4) | | |
| TA1537 | 9-aminoacridine (9AC) | 60 µg | Milli-Q water |
| | (b) (4) | | |
| TA98 | 2-nitrofluorene (NF) (b) (4) | 10 µg | DMSO |
| TA100 | methylmethanesulfonate (MMS) ( (b) (4) | 650 µg | DMSO |
| $WP_2uvrA$ | 4-nitroquinoline N-oxide (4-NQO) | 10 µg | DMSO |

With metabolic activation (+S9-mix):
The positive control substance used for all tester strains was 2-aminoanthracene (2AA) (b) (4)
The following doses were used:

| Strain | Concentration/plate | Amount of S9-mix | Solvent |
|---|---|---|---|
| TA1535 | 1 µg | 5 and 10% | DMSO |
| TA1537 | 2.5 µg | 5% | DMSO |
| TA1537 | 5 µg | 10% | DMSO |
| TA98 | 1 µg | 5 and 10% | DMSO |
| TA100 | 1 µg | 5% | DMSO |
| TA100 | 2.5 µg | 10% | DMSO |
| $WP_2uvrA$ | 10 µg | 5 and 10% | DMSO |

Incubation and sampling times: The test was carried out in triplicate, with and without S9-mix. Before the experiment, a thawed sample of the stock culture was incubated in Oxoid Nutrient broth medium in a shaking waterbath at 37 °C until an optical density of 1.0 at 700 nm ($1 \times 10^9$ cells/ml) was reached. Then, 0.1 mL of bacterial culture plus 0.1 ml either the test article or the positive control article or the negative control article, with or without 0.5 mL S9-mix, was added to 3 mL molten soft agar at 45 °C, mixed and poured out (plated) on Vogel-Bonner Minimal Agar plates. All plates were incubated in an incubator at 37 °C for approx. 48h. After incubation, the plates were inspected for contamination, presence of precipitate, inhibition of the background lawn, and the presence of his- and trp- colonies, and then the number of colonies was determined using an automatic colony counter after incubation. The mean number of revertants per plate was calculated for the control and test plates.

*Criteria for a valid assay:*

According to the sponsor, the assay was considered to be valid with the following criteria (extracted from the sponsor's report) were met:

a) The negative control data (number of spontaneous revertants per plate) should be within the laboratory historical range for each tester strain.

| Strain | | Minimum value | Maximum value | Mean | ± | 3 x S.D. |
|---|---|---|---|---|---|---|
| TA1535 | - S9-mix | 3 | 24 | 11 | ± | 11 |
| | + S9-mix | 3 | 28 | 10 | ± | 11 |
| TA1537 | - S9-mix | 3 | 18 | 6 | ± | 8 |
| | + S9-mix | 3 | 20 | 7 | ± | 9 |
| TA98 | - S9-mix | 12 | 41 | 18 | ± | 14 |
| | + S9-mix | 12 | 49 | 22 | ± | 18 |
| TA100 | - S9-mix | 61 | 195 | 132 | ± | 69 |
| | + S9-mix | 59 | 188 | 116 | ± | 80 |
| $WP_2uvrA$ | - S9-mix | 4 | 32 | 13 | ± | 15 |
| | + S9-mix | 4 | 31 | 13 | ± | 15 |

b) The positive control chemicals should produce responses in all tester strains, which are within the laboratory historical range documented for each positive control substance. Furthermore, the mean plate count should be at least three times the concurrent vehicle control group mean.

| Strain | | Minimum value | Maximum value | Mean | ± | 3 x S.D. |
|---|---|---|---|---|---|---|
| TA1535 | - S9-mix | 90 | 1801 | 644 | ± | 894 |
| | + S9-mix | 58 | 1015 | 157 | ± | 267 |
| TA1537 | - S9-mix | 77 | 1384 | 350 | ± | 600 |
| | + S9-mix | 58 | 1009 | 242 | ± | 487 |
| TA98 | - S9-mix | 101 | 1794 | 528 | ± | 858 |
| | + S9-mix | 147 | 1591 | 578 | ± | 777 |
| TA100 | - S9-mix | 320 | 2099 | 940 | ± | 599 |
| | + S9-mix | 197 | 2227 | 787 | ± | 984 |
| $WP_2uvrA$ | - S9-mix | 64 | 1406 | 700 | ± | 793 |
| | + S9-mix | 56 | 1053 | 200 | ± | 405 |

c) The selected dose range should include a clearly toxic concentration or should exhibit limited solubility as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/plate.

*Criteria for a positive response:*
According to the sponsor, a test compound was considered positive (mutagenic) if the following criteria (extracted from the sponsor's report) were met, in any of the strains in the presence or in the absence of S9-mix:

a) It induces at least a 2-fold, dose related increase in the number of revertants with respect to the number induced by the solvent control in any of the tester strains, either with or without metabolic activation. However, any mean plate count of less than 20 is considered to be not biologically relevant.
b) The positive response should be reproducible in at least one independently repeated experiment.

The preceding criteria were not absolute and other modifying factors might enter into the final evaluation decision.

**Results**

Study validity: The assay appears to be valid. Appropriate replicate numbers, positive controls demonstrated clear increases in tester strain revertants while the negative control was within or close to the historical range for the tester strains with this vehicle.

Study outcome:
No signs of toxicity and precipitation were observed in any tester strains at any dose levels. Precipitation was observed in the top agar at concentrations of 333 µg/plate and above and at concentration of 3330 and/or 5000 µg/plate on the plates at the start and at the end of the incubation period in all the experiments

Broomcyclodextrine did not induce increase in the number of revertants per plate in the S. typhimurium strains TA98, TA100, TA1535, and TA1537 and in the E. coli strain, in

the presence or the absence of S9-mix at the concentrations tested under the test condition. The sponsor has summarized the results and the summarized table was shown below.

**Experiment 1**

| Dose (µg a.i./plate) | Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain | | | | |
|---|---|---|---|---|---|
| | TA1535 | TA1537 | TA98 | TA100 | $WP_2uvrA$ |
| | Without S9-mix | | | | |
| positive control | 1485 ± 65 | 169 ± 42 | 1017 ± 104 | 858 ± 52 | 492 ± 37 |
| solvent control | 10 ± 3 | 8 ± 2 | 15 ± 2 | 137 ± 10 | 9 ± 2 |
| 3 | | | | 130 ± 5 | 10 ± 2 |
| 10 | 11 ± 3 | 4 ± 3 | 16 ± 4 | 125 ± 10 | 14 ± 3 |
| 33 | 15 ± 6 | 5 ± 2 | 16 ± 3 | 110 ± 7 | 11 ± 4 |
| 100 | 12 ± 3 | 4 ± 1 | 15 ± 2 | 125 ± 6 | 10 ± 4 |
| 333 | 8 ± 5 | 3 ± 1 | 14 ± 4 | 126 ± 5 | 14 ± 3 |
| 1000 | 10 ± 6 | 0 ± 1 | 16 ± 4 | 69 ± 20 | 8 ± 1 |
| 3330 [SP] | 2 ± 1 | 0 ± 0 | 10 ± 3 | 30 ± 17 | 8 ± 0 |
| 5000 [SP] | | | | 10 ± 2 | 14 ± 3 |
| | With S9-mix[1] | | | | |
| positive control | 171 ± 21 | 166 ± 32 | 500 ± 35 | 761 ± 23 | 343 ± 123 |
| solvent control | 13 ± 3 | 6 ± 4 | 19 ± 6 | 133 ± 24 | 13 ± 2 |
| 3 | | | | 135 ± 18 | 12 ± 4 |
| 10 | | | | 136 ± 2 | 12 ± 4 |
| 33 | 10 ± 4 | 6 ± 1 | 19 ± 1 | 115 ± 8 | 15 ± 2 |
| 100 | 8 ± 3 | 6 ± 2 | 18 ± 3 | 114 ± 12 | 15 ± 5 |
| 333 | 11 ± 6 | 5 ± 2 | 14 ± 1 | 124 ± 11 | 11 ± 5 |
| 1000 | 12 ± 8 | 5 ± 2 | 19 ± 7 | 112 ± 11 | 10 ± 1 |
| 3330 [SP] | 11 ± 2 | 3 ± 0 | 15 ± 3 | 117 ± 11 | 9 ± 2 |
| 5000 [SP] | | | | 105 ± 5 | 15 ± 1 |

**Experiment 2**

| Dose (µg/plate) | Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain | | | | |
|---|---|---|---|---|---|
| | TA1535 | TA1537 | TA98 | TA100 | $WP_2uvrA$ |
| | Without S9-mix | | | | |
| positive control | 431 ± 144 | 121 ± 27 | 457 ± 26 | 582 ± 8 | 439 ± 35 |
| solvent control | 10 ± 1 | 3 ± 1 | 20 ± 3 | 110 ± 6 | 12 ± 5 |
| 10 | | 5 ± 1 | | | |
| 33 | 12 ± 3 | 4 ± 2 | 19 ± 2 | 125 ± 7 | 8 ± 3 |
| 100 | 12 ± 4 | 4 ± 2 | 23 ± 6 | 127 ± 5 | 10 ± 3 |
| 333 | 11 ± 3 | 4 ± 2 | 19 ± 8 | 116 ± 14 | 10 ± 1 |
| 1000 | 11 ± 3 | 3 ± 1 | 15 ± 3 | 107 ± 10 | 12 ± 2 |
| 3330 [SP] | 8 ± 2 | 1 ± 1 | 13 ± 6 | 77 ± 22 | 9 ± 5 |
| | With S9-mix[1] | | | | |
| positive control | 108 ± 13 | 276 ± 107 | 312 ± 33 | 671 ± 115 | 107 ± 12 |
| solvent control | 14 ± 3 | 3 ± 1 | 34 ± 4 | 87 ± 18 | 13 ± 6 |
| 33 | 9 ± 2 | 4 ± 0 | 28 ± 6 | 97 ± 13 | 11 ± 3 |
| 100 | 12 ± 6 | 3 ± 1 | 30 ± 9 | 110 ± 16 | 11 ± 1 |
| 333 | 10 ± 2 | 6 ± 3 | 26 ± 3 | 90 ± 3 | 8 ± 0 |
| 1000 | 11 ± 4 | 5 ± 2 | 19 ± 2 | 94 ± 8 | 11 ± 1 |
| 3330 [SP] | 10 ± 4 | 4 ± 2 | 17 ± 4 | 106 ± 5 | 9 ± 3 |

## In Vitro Chromosomal Aberration Assays

**1. Study title**: A chromosomal aberrations test study with Org 25969 in human peripheral blood lymphocytes in vitro

**Key findings**: Org 25969 in this batch (batch L) is negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration up to 5000 µg/ml.

Document ID: NL0028990
**Study no.**: PCD 2261/SU180

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands

**Date of study initiation**: 01/17/2001
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: Org 25969 and impurities, Batch L, (b) (4) %
*Note: analysis certificate was not attached with this study report; instead, batch analysis can be found in NDA submission at Module 3.Quality.*

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of healthy non-smoking male donors

Metabolizing system: To simulate mammalian liver drug metabolism, a mixture of the supernatant fraction of rat liver homogenate and cofactors was used. The S9 was commercially obtained from (b) (4) and was tested regularly for their ability to activate reference mutagens.

Doses used in definitive study: based on the results from the dose-range finding test, the following concentrations were used in the main study.

- In the absence of S9-mix:
  - 3h exposure/24h harvest:
    100, 200, 500, 1000, 2000, 5000 µg·mL$^{-1}$
    (active entity: 92, 184, 460, 920, 1840 and 4600 µg·mL$^{-1}$)
  - 24h exposure/24h harvest:
    100, 200, 500, 1000, 2000, 5000 µg·mL$^{-1}$
    (active entity: 92, 184, 460, 920, 1840 and 4600 µg·mL$^{-1}$)
- In the presence of S9-mix:
  - 3h exposure/24h harvest:
    100, 200, 500, 1000, 2000, 5000 µg·mL$^{-1}$
    (active entity: 92, 184, 460, 920, 1840 and 4600 µg·mL$^{-1}$)

Basis of dose selection: A dose-range-finding study was conducted at concentrations of 10, 30, 100, 300, and 1000 µg/ml with the absence and presence of S9. There were no sign of toxicity observed at a concentration up to 1000 µg/ml. Therefore, the sponsor

used the OECD recommended maximal concentration (5000 µg/ml) as the highest dose in the definitive study as shown above.

Negative and vehicle controls: The solvent for the test article, ultrapure water, was used as negative control.

Positive controls: The following mutagens were used as positive controls (extracted from the sponsor's report).

Without metabolic activation :

- 4-Nitroquinoline-1-oxide (4-NQO)
  - Dose levels : 2.50 and 5.00 $\mu g.mL^{-1}$ culture medium
  - Solvent : DMSO
  - Stock concentrations : 0.250 and 0.500 $mg.mL^{-1}$ DMSO

With metabolic activation :

- Cyclophosphamide (CP)
  - Dose levels : 12.5 and 25 $\mu g.mL^{-1}$ culture medium
  - Solvent : Hanks Balanced Salt Solution (HBSS)
  - Stock concentrations : 1.25 and 2.50 $\mu g.mL^{-1}$ HBSS

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Two days prior to exposure 0.4 mL whole blood in 4.5 mL growth medium was inoculated per tube. Lymphocytes were stimulated to divide in the culture medium with serum, supplemented with phytohemagglutinin 1% (v/v) for 48h at 37 °C. Cells were then cultured in a $CO_2$ incubator with 5% ± 0.5% $CO_2$, relative humidity > 80% and a temperature of 37 °C ± 1 °C for 3 hours with the treatment of test articles, negative, or positive controls in the absence or presence of S9. Cells were harvested at approximate 24 hours from the initiation of treatment. Additional set of Cells in the absence of S9-mix were exposed continuously for approximately 24h were harvested at the end of the exposure period. During the last three hours before harvest, cell division was arrested by addition of the spindle inhibitor colchicine at a final concentration of 1 µg/mL. Thereafter cell cultures were centrifuged for 10 minutes at 1991 N/kg and the supernatant was removed. Cells in the remaining cell pellet were swollen by a 15 minutes treatment with hypotonic 0.075 mol/L KCl at 37 °C. Cells were fixed by adding an equal volume of cold, freshly prepared, methanol /acetic acid mixture (3:1 v/v). Fixed cells were dropped on wet slides which were immersed for 24h in 96% ethanol. Slides were then dried and stained for 20 min in 5% (v/v) Giemsa stain and mounted with coverslips. One hundred/culture suitable metaphase cells (44-46 chromosomes/cell) were examined microscopically and analyzed for chromosome aberrations. Cells with more than 46 chromosomes observed during examination were noted and recorded separately. Definitions of chromosome aberrations were shown in the following extracted from the sponsor's report.

**10.4 DEFINITIONS OF CHROMOSOME ABERRATIONS**

| Aberration | Description |
|---|---|
| Chromatid gap | A non-staining region in a chromatid smaller than the width of a chromatid. Minimal misalignment may be present. |
| Chromosome gap | A non-staining region in both chromatids smaller than the width of the chromatids. Minimal misalignment may be present. |
| Chromatid break | A non-staining region in a chromatid and a distinct misalignment or dislocation must be present. |
| Chromosome break | A non-staining region in both chromatids and a distinct misalignment or dislocation must be present. |
| Minute | An acentric fragment from a chromatid, smaller than the width of a single chromatid, usually circular. |
| Double minutes | Two acentric fragments from a chromosome usually circular, smaller than the width of a single chromatid. |
| Dicentric chromosome | A chromosome containing two centromeres. |
| Ring chromosome | A ring structure with a distinct lumen. |
| Exchange figure | An exchange between two or more chromosomes resulting in the formation of a tri- or more-armed configuration. |
| Multiple aberrations | A metaphase containing seven or more of the above mentioned aberrations (excluding chromatid and chromosome gaps). |
| Polyploidy | A chromosome number more than 68 chromosomes and being a multiple of the normal chromosome set. |
| Endoreduplication | A form of polyploidy in which the homologous chromosomes are aligned. |
| Aneuploidy | Metaphases with one or more chromosomes in addition to the normal diploid number (47-68). |

Mitotic index was evaluated from the negative and vehicle control, and a range of concentrations. Percent polyploidy and endoreduplication were also analyzed by evaluating at least 100 metaphases. The number and types of chromosome aberrations per culture were recorded and the total number of chromosome aberrations of both replicate cultures (including and excluding gaps) was calculated. Fisher's exact test ($p<0.05$) was used for statistical analysis.

*Criteria for a valid assay:*
A valid test is one in which the negative control groups are within the historical range for solvent controls and the percentage of chromosomal aberrations in the positive control groups are statistically increased relative to the solvent control excluding gaps (*note: The Sponsor did not provide a table with historical control data for negative/vehicle and positive control substances*).

*Criteria for a positive response:*
A test article was considered positive for inducing chromosomal aberrations if the following criteria (extracted from the sponsor's report) were met.

- If a statistically significant concentration-related increase in the number of cells with chromosome aberrations (excluding gaps) was present,
- if at this concentration(s) the number of cells with aberrations excluding gaps exceeded the normal range, and
- if a statistically significant increase in the number of cells with aberrations was observed in the absence of a clear concentration-response relationship, but results were reproducible in an independently repeated experiment.

The sponsor further indicated that the criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index and degree of confluency are appropriate methods to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control produced no cells with chromosomal aberrations and the positive controls used produced chromosomal aberrations in a percentage of cells that were within the range of the historical dataset for positive controls and was statistically significantly increased over the negative solvent control.

Study outcome:
There were no signs of toxicity represented by RMI at a concentration up to 5000 µg/ml. The results of the chromosome aberration assay in this study did not elicit dose-dependent and statistically significant increase in chromatid and chromosome aberrations, which indicate a negative response in human peripheral lymphocytes both in the presence and absence of a rat liver S9 metabolizing system. Therefore, Org25969 with its impurities in batch L is not considered to be clastogenic. The data summary extracted from the sponsor's report was shown below.

**Table 10.3-1 Aberrations. 3h exposure / 24h harvest - without metabolic activation**

| | Org 25969 [a] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [d] 1000 µg·mL⁻¹ (920 µg·mL⁻¹) | | | Org 25969 [b] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [e] 2000 µg·mL⁻¹ (1840 µg·mL⁻¹) | | | Org 25969 [c] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [f] 5000 µg·mL⁻¹ (4600 µg·mL⁻¹) | | | 4-NQO [g] 2.50 µg·mL⁻¹ (2.50 µg·mL⁻¹) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Relative Metaphase Index | 100 | | | 82 | | | 100 | | | 79 | | | 100 | | | 98 | | | ne | | |
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| No. of cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of cells with aberrations incl. Gaps | 8 | 7 | 15 | 4 | 5 | 9 | 16 | 18 | 34 | 10 | 12 | 22 | 11 | 12 | 23 | 4 | 5 | 9 | 20 | 22 | 42 |
| No. of cells with aberrations excl. gaps | 7 | 3 | 10 | 1 | 1 | 2 | 10 | 10 | 20 | 8 | 4 | 12 | 9 | 5 | 14 | 2 | 2 | 4 | 16 | 16 | 32 |
| **Chromatid aberrations** | | | | | | | | | | | | | | | | | | | | | |
| gap | 1 | 4 | 5 | 3 | 4 | 7 | 10 | 7 | 17 | 4 | 9 | 13 | 3 | 6 | 9 | 3 | 4 | 7 | 3 | 8 | 11 |
| break | 2 | 3 | 5 | 1 | 1 | 2 | 5 | 9 | 14 | 5 | 3 | 8 | 4 | 3 | 7 | 1 | 2 | 3 | 5 | 14 | 19 |
| minutes | | | | | | | | | | | | | | | | | | | | | |
| exchanges | 2 | | 2 | | | | | | | | | | | | | | | | 9 | 3 | 12 |
| **Chromosome aberrations** | | | | | | | | | | | | | | | | | | | | | |
| gap | | 2 | 2 | | | | | 1 | 1 | | | | | 1 | 1 | | | | 2 | | 2 |
| break | 3 | | 3 | | | | 5 | 2 | 7 | 3 | 1 | 4 | 4 | 1 | 5 | 1 | | 1 | 12 | 3 | 15 |
| dicentric | | | | | | | | | | | | | 1 | 1 | 2 | | | | 1 | | 1 |
| ring | | | | | | | | | | | | | | | | | | | | | |
| double minutes | | | | | | | | | | | | | | | | | | | | | |
| others | | | | | | | | | | | | | | | | | | | [1] | | [1] |
| total aberrations incl. Gaps | 8 | 9 | 17 | 4 | 5 | 9 | 20 | 19 | 39 | 12 | 13 | 25 | 12 | 12 | 24 | 5 | 6 | 11 | 32 | 35 | 67 |
| total aberrations excl. gaps | 7 | 3 | 10 | 1 | 1 | 2 | 10 | 11 | 21 | 8 | 4 | 12 | 9 | 5 | 14 | 2 | 2 | 4 | 27 | 27 | 54 |
| **Numerical aberrations** | | | | | | | | | | | | | | | | | | | | | |
| endoreduplication | | | | | 1 | 1 | 1 | | 1 | | | | 1 | | 1 | | | | | | |
| polyploidy | 1 | 1 | 2 | | | | | | | | | | | | | | 1 | 1 | 2 | | 2 |
| aneuploidy | 1 | 1 | 2 | | 2 | 2 | 3 | | 3 | | 1 | 1 | 2 | 5 | 7 | | 2 | 2 | | | |

**Table 10.3-2 Aberrations. 24h exposure / 24h harvest - without metabolic activation**

| | Org 25969 [a] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [d] 500 µg·mL⁻¹ (460 µg·mL⁻¹) | | | Org 25969 [h] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [e] 2000 µg·mL⁻¹ (1840 µg·mL⁻¹) | | | Org 25969 [c] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [f] 5000 µg·mL⁻¹ (4600 µg·mL⁻¹) | | | 4-NQO [g] 2.50 µg·mL⁻¹ (2.50 µg·mL⁻¹) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Relative Metaphase Index | 100 | | | 94 | | | 100 | | | 112 | | | 100 | | | 90 | | | ne | | |
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| No. of cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of cells with aberrations incl. Gaps | 7 | 4 | 11 | 2 | 7 | 9 | 8 | 9 | 17 | 7 | 7 | 14 | 3 | 12 | 15 | 7 | 8 | 15 | 20 | 22 | 42 |
| No. of cells with aberrations excl. gaps | 3 | 1 | 4 | 1 | 2 | 3 | 5 | 6 | 11 | 4 | 5 | 9 | 2 | 6 | 8 | 3 | 5 | 8 | 16 | 16 | 32 |
| **Chromatid aberrations** | | | | | | | | | | | | | | | | | | | | | |
| gap | 4 | 3 | 7 | | 4 | 4 | 1 | 2 | 3 | 4 | 2 | 6 | 1 | 6 | 7 | 2 | 4 | 6 | 3 | 8 | 11 |
| break | 1 | | 1 | 1 | 1 | 2 | 2 | 2 | 4 | | 5 | 5 | | 4 | 4 | | 5 | 5 | 5 | 14 | 19 |
| minutes | | | | | | | | | | | | | | | | | | | | | |
| exchanges | | | | | | | | | | | | | | 1 | 1 | | | | 9 | 3 | 12 |
| **Chromosome aberrations** | | | | | | | | | | | | | | | | | | | | | |
| gap | 1 | | 1 | 1 | 1 | 2 | 2 | 1 | 3 | | 1 | 1 | | | | 2 | | 2 | 2 | | 2 |
| break | 2 | 1 | 3 | | 1 | 1 | 3 | 5 | 8 | 4 | 2 | 6 | 2 | 3 | 5 | 3 | | 3 | 12 | 3 | 15 |
| dicentric | | | | | | | | | | 1 | | 1 | | | | | | | 1 | | 1 |
| ring | | | | | | | | | | | | | | | | | | | | | |
| double minutes | | | | | | | | | | | | | | | | | | | | | |
| others | [2] | | [2] | | | | | | | | | | | | | | | | [1] | | [1] |
| total aberrations incl. Gaps | 8 | 4 | 12 | 2 | 7 | 9 | 8 | 10 | 18 | 9 | 10 | 19 | 3 | 14 | 17 | 7 | 9 | 16 | 32 | 35 | 67 |
| total aberrations excl. gaps | 3 | 1 | 4 | 1 | 2 | 3 | 5 | 7 | 12 | 5 | 7 | 12 | 2 | 8 | 10 | 3 | 5 | 8 | 27 | 27 | 54 |
| **Numerical aberrations** | | | | | | | | | | | | | | | | | | | | | |
| endoreduplication | | 1 | 1 | | | | | | | | 1 | 1 | | 1 | 1 | 1 | 1 | 2 | | | |
| polyploidy | | 2 | 2 | 1 | 4 | 5 | | | | 1 | | 1 | | | | 1 | | 1 | 2 | | 2 |
| aneuploidy | | | | | | | | | | | | | | | | | | | | | |

Table 10.3-3 Aberrations. 3h exposure / 24h harvest - with metabolic activation

| (Active entity) | Org 25969 [a] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [d] 1000 µg·mL⁻¹ (920 µg·mL⁻¹) | | | Org 25969 [b] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [e] 2000 µg·mL⁻¹ (1840 µg·mL⁻¹) | | | Org 25969 [c] 0 µg·mL⁻¹ (0 µg·mL⁻¹) | | | Org 25969 [f] 5000 µg·mL⁻¹ (4600 µg·mL⁻¹) | | | CP 12.5 µg·mL⁻¹ (12.5 µg·mL⁻¹) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Relative Metaphase Index** | **100** | | | **86** | | | **100** | | | **82** | | | **100** | | | **98** | | | **ne** | | |
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| No. of cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 50 | 50 | 100 |
| No. of cells with aberrations incl. Gaps | 7 | 8 | 15 | 8 | 4 | 12 | 3 | 4 | 7 | 4 | 3 | 7 | 7 | 4 | 11 | 6 | 9 | 15 | 20 | 28 | 48 |
| No. of cells with aberrations excl. gaps | 6 | 3 | 9 | 5 | 1 | 6 | 0 | 2 | 2 | 1 | 1 | 2 | 3 | 2 | 5 | 4 | 4 | 8 | 18 | 24 | 42 |
| **Chromatid aberrations** | | | | | | | | | | | | | | | | | | | | | |
| gap | 2 | 5 | 7 | 3 | 5 | 8 | 2 | 2 | 4 | 2 | 2 | 4 | 4 | 2 | 6 | 2 | 5 | 7 | 2 | 10 | 12 |
| break | 4 | 2 | 6 | 4 | 1 | 5 | | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 3 | 1 | 4 | 5 | 6 | 10 | 16 |
| minutes | | | | | | | | | | | | | | | | | | | | | |
| exchanges | | 1 | 1 | 1 | | 1 | | 1 | 1 | | | | | | | | | | 4 | 4 | 8 |
| **Chromosome aberrations** | | | | | | | | | | | | | | | | | | | | | |
| gap | | | | | | | 1 | | 1 | 1 | | 1 | | | | | | | 1 | 2 | 3 |
| break | 2 | | 2 | | | | | | | | | | 1 | | 1 | 3 | | 3 | 13 | 11 | 24 |
| dicentric | | | | | | | | | | | | | 1 | | 1 | | | | 1 | | 1 |
| ring | | | | | | | | | | | | | | | | | | | | | |
| double minutes | | | | | | | | | | | | | | | | | | | | | |
| others | | | | | | | | | | | | | (2 | (2 | 2(2 | | | | | (1 | (1 |
| total aberrations incl. Gaps | 8 | 8 | 16 | 9 | 6 | 15 | 3 | 4 | 7 | 4 | 3 | 7 | 7 | 4 | 11 | 6 | 9 | 15 | 27 | 44 | 71 |
| total aberrations excl. gaps | 6 | 3 | 9 | 6 | 1 | 7 | 0 | 2 | 2 | 1 | 1 | 2 | 3 | 2 | 5 | 4 | 4 | 8 | 24 | 32 | 56 |
| **Numerical aberrations** | | | | | | | | | | | | | | | | | | | | | |
| endoreduplication | 1 | | 1 | | 1 | 1 | | | | | | | | | | 2 | 1 | 3 | | | |
| polyploidy | | | | | | | | | | 1 | | 1 | | | | | | | | | |
| aneuploidy | 3 | | 3 | 1 | | 1 | 1 | 1 | 2 | 2 | 2 | 4 | | | | 1 | 1 | 2 | | | |

a : 50 µL of ultrapure water was used
b : 100 µL of ultrapure water was used
c : 250 µL of ultrapure water was used
d : 50 µL test compound was used
e : 100 µL test compound was used
f : 250 µL test compound was used

(1: multiaberrant cell
(2: pulverised chromosome (is not included in the total amount of aberrations)
ne: not evaluated

Normal control range cells with aberrations per 100 cells. Excl gaps: 0-5 (using 50 µL of solvent)

**2. Study title**: Org 25969: induction of chromosomal aberrations in cultured human peripheral blood lymphocytes

**Key findings**: Org 25969 in this batch (batch V) is negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration up to 5000 µg/ml.

Document ID: NL0042249
**Study no.**: 020398X/082/101

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 06/13/2002
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: Org 25969 and impurities, Batch V, (b) (4) %

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of 3 healthy non-smoking female volunteers.

Metabolizing system: The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from (b) (4). Each batch was

checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities).

Doses used in definitive study: Based on the results from the dose-range finding test, the following concentrations were used in the 2 experiments of the main study.

| Experiment 1 Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | Hours treatment + hours recovery 3+17 -S-9 | 3+17 +S-9 |
|---|---|---|---|
| 3.754 | 375.4 | ✓ | ✓ |
| 5.006 | 500.6 | ✓ | ✓ |
| 6.674 | 667.4 | ✓ | ✓ |
| 8.899 | 889.9 | ✓ | ✓ |
| 11.87 | 1187 | ✓ | ✓ |
| 15.82 | 1582 | ✓ | ✓ |
| 21.09 | 2109 | ✓ | ✓ |
| 28.13 | 2813 | ✓ | ✓ |
| 37.50 | 3750 | ✓ | ✓ |
| 50.00 | 5000 | ✓ | ✓ |

✓ Indicates concentration tested

| Experiment 2 Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | Hours treatment + hours recovery 20+0 -S-9 | 3+17 +S-9 |
|---|---|---|---|
| 6.711 | 671.1 | ✓ | |
| 8.389 | 838.9 | ✓ | |
| 10.49 | 1049 | ✓ | |
| 13.11 | 1311 | ✓ | |
| 16.39 | 1639 | ✓ | |
| 20.48 | 2048 | ✓ | |
| 25.60 | 2560 | ✓ | ✓ |
| 32.00 | 3200 | ✓ | ✓ |
| 40.00 | 4000 | ✓ | ✓ |
| 50.00 | 5000 | ✓ | ✓ |

✓ Indicates concentration tested

Basis of dose selection: A dose-range-finding study was not conducted. The sponsor used the OECD recommended maximal concentration (5000 µg/ml) as the highest dose in the definitive study as shown above.

Negative and vehicle controls: sterile purified water

Positive controls: The positive control chemicals were dissolved in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) immediately prior to use, as shown in the follows (extracted from the sponsor's report):

| Chemical | Supplier | Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | S-9 |
|---|---|---|---|---|
| 4-Nitroquinoline 1-oxide (NQO) | (b) (4) | 0.125 | 1.25 | - |
| | | 0.250 | 2.50 | - |
| | | 0.500 | 5.00 | - |
| Cyclophosphamide (CPA) | | 0.3125 | 3.125 | + |
| | | 0.625 | 6.25 | + |
| | | 1.25 | 12.5 | + |

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL heparinised blood

into Hepes-buffered RPMI medium containing 20% (v/v) fetal calf serum and 50 µg/mL gentamycin. Phytohaemagglutinin was included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37°C and rocked continuously. Before the treatment, 0.5 ml S-9 mix or KCl was added. One set of quadruplicate cultures (A, B, C and D) for each of the treatment regimes was then treated with the solvent and one set of duplicate cultures with the test article (1 mL per culture). Additional duplicate cultures for treatments in the absence and presence of S-9 were treated with 0.1 mL of the positive control chemicals. The culture volume was adjusted to 10 ml; all cultures were then incubated at 37ºC. In experiment 1, cells were treated for 3 hours and harvested 17 hours after the termination of treatment. In experiment 2, similar experiment regimen was applied except that additional set of cells were treated for 20 hours and harvested immediately after treatment. Approximately 2 hours prior to harvest, colchicine was added to give a final concentration of approximately 1 µg/mL to arrest dividing cells in metaphase.

At the defined sampling time cultures were centrifuged at approximately 300 x 'g' for 10 minutes; the supernatant was carefully removed and cells were resuspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37 °C for 15 minutes to allow cell swelling to occur. In Experiment 2, 9 mL of KCl was added to all cultures in error. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension. This procedure was repeated several times (centrifuging at approximately 1250 x g, 2-3 minutes) until the cell pellets were clean.

Lymphocytes were kept in fixative in the refrigerator at least overnight for proper fixation. Then, cells were pelleted and re-suspended in a minimal amount of fresh fixative so as to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean microscope slides. Slides were flamed, as necessary, to improve metaphase spreading. After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with cover-slips. Slides were examined for mitotic index to determine the cytotoxic effect of the test article, and selected dosing groups were used for aberration assay. Slides from the selected treatments and from solvent and positive controls were coded using randomly generated letters. One hundred metaphases from each code were analyzed for chromosome aberrations. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with more than 46 chromosomes, i.e. polyploid, endoreduplicated, and hyperdiploid cells, was not considered to be chromosome aberration and recorded separately.

Classification of structural aberrations was based on the scheme described by ISCN as shown below extracted from the sponsor’s report. After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as 1) cells with structural aberrations including gaps; 2) cells with structural aberrations

excluding gaps; 3) polyploid, endoreduplicated or hyperdiploid cells. The totals for category 2 in negative control cultures were compared with the laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test article treated cultures was also compared with normal ranges. The statistical significance of any data set derived from Fisher's exact test ($p < 0.05$) was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. The proportions of cells in categories 1 and 3 were also examined but were as a reference for aberration.

**Abbreviations and classification of observations**

| | | |
|---|---|---|
| abs | = | aberrations |
| rep | = | replicate |
| tot | = | total |

**Gaps**

| | | |
|---|---|---|
| csg | = | chromosome gap |
| ctg | = | chromatid gap |

**Chromosome deletions**

| | | |
|---|---|---|
| del | = | chromosome deletion |
| d min | = | double minute |
| f | = | isolocus fragment |

**Chromosome exchanges**

| | | |
|---|---|---|
| t | = | interchange between chromosomes (e.g. reciprocal translocation) |
| inv | = | chromosome intrachange (e.g. pericentric inversion) |
| dic | = | dicentric |
| dic+f | = | dicentric with accompanying fragment |
| acr | = | acentric ring |
| r+f | = | centric ring with accompanying fragment |
| r | = | centric ring |

**Chromatid deletions**

| | | |
|---|---|---|
| del | = | chromatid deletion |
| min | = | single minute |

**Chromatid exchanges**

| | | |
|---|---|---|
| qr | = | interchange between chromatids of different chromosomes (e.g. quadriradial) |
| cx | = | obligate complex interchange |
| e | = | chromatid intrachange |
| tr/tr+f | = | isochromatid/chromatid interchange (triradial)/with accompanying fragment |
| su | = | intra-arm intrachange with sister union of broken ends |
| nud | = | intra-arm intrachange with non-union of broken ends distally |
| nup | = | intra-arm intrachange with non-union of broken ends proximally |

**Other structural aberrations**

| | | |
|---|---|---|
| pvz | = | pulverised cell |
| mabs | = | multiple aberrations (greater than 7 aberrations per cell or too many aberrations to permit accurate analysis) |

**Numerical aberrations**

| | | |
|---|---|---|
| E | = | endoreduplicated |
| H | = | hyperdiploid (47-68 chromosomes) |
| P | = | polyploid (greater than 68 chromosomes) |

*Criteria for a valid assay:*

According to the sponsor, an assay is considered to be valid if the following criteria are met:

1. The binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures.
2. The proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range (*note: The Sponsor has provided a table with historical control data for negative/vehicle control substances*).
3. At least 160 cells out of an intended 200 are analyzable at each dose level.

4. The positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations.

*Criteria for a positive response:*
A test article is considered as positive in this assay if the following criteria were met:

1. The proportions of cells with structural aberrations at one or more concentration exceed the normal range in both replicate cultures.
2. A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at these doses.

Increased incidence of cells with gaps or increased proportions of cells with structural aberrations not exceeding the normal range, or occurring only at very high or very toxic concentrations are likely to be concluded as "equivocal". Evidence of a dose-related effect is considered useful but not essential in the evaluation of a positive result. Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in negative control cultures. Their appearance is therefore considered to be of particular biological significance.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index is appropriate methods to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control produced no cells with chromosomal aberrations and the positive controls used produced chromosomal aberrations in a percentage of cells that were within the range of the historical dataset for positive controls and was statistically significantly increased over the negative solvent control.

Study outcome:
There was only slight concentration-independent mitotic index inhibition observed at the highest 3 concentrations in the absence and presence of S9 as compared to control in both experiment 1 and 2. There were no changes in mitotic index in other doses in both experiments. The cells of highest 3 concentrations in both experiments were thus the groups for chromosome aberration.

Treatment of cultures with Org 25969 batch V in the absence and presence of S-9 (both experiments) resulted in frequencies of cells with structural aberrations, which were similar to those observed in concurrent vehicle controls for all concentrations analyzed. The aberrant cell frequency of all Org 25969 treated cultures fell within historical negative control (normal) ranges. Therefore, Org25969 with its impurities in batch V is not considered to be clastogenic. The data summary was shown below (extracted from the sponsor's report).

**3 hour treatment -S-9, 17 hour recovery (3+17), Experiment 1**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 2 | 1 | 9.1 |
| | B | 100 | 2 | 2 | 8.6 |
| | C | ND | ND | ND | 11.1 |
| | D | ND | ND | ND | 10.0 |
| | Totals | 200 | 4 | 3 | (9.7) |
| 2813 | A | 100 | 0 | 0 | 9.5 |
| | B | 100 | 4 | 1 | 8.9 |
| | Totals | 200 | 4 | 1 | (9.2) |
| 3750 | A | 100 | 1 | 1 | 12.6 |
| | B | 100 | 1 | 1 | 8.3 |
| | Totals | 200 | 2 | 2 | (10.5) |
| 5000 | A | 100 | 1 | 0 | 9.1 |
| | B | 100 | 2 | 2 | 9.2 |
| | Totals | 200 | 3 | 2 | (9.2) |
| NQO, 2.50 | A | 100 | 10 | 8 | |
| | B | 100 | 11 | 10 | |
| | Totals | 200 | 21 | 18* | |

**3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 1**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 1 | 1 | 10.4 |
| | B | 100 | 1 | 1 | 9.8 |
| | C | ND | ND | ND | 8.7 |
| | D | ND | ND | ND | 11.3 |
| | Totals | 200 | 2 | 2 | (10.1) |
| 2813 | A | 100 | 3 | 1 | 8.2 |
| | B | 100 | 4 | 3 | 11.4 |
| | Totals | 200 | 7 | 4 | (9.8) |
| 3750 | A | 100 | 2 | 2 | 9.5 |
| | B | 100 | 1 | 0 | 8.8 |
| | Totals | 200 | 3 | 2 | (9.2) |
| 5000 | A | 100 | 0 | 0 | 11.5 |
| | B | 100 | 2 | 1 | 9.4 |
| | Totals | 200 | 2 | 1 | (10.5) |
| CPA, 6.25 | A | 100 | 31 | 28 | |
| | B | 100 | 26 | 23 | |
| | Totals | 200 | 57 | 51* | |

**20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 2**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 2 | 2 | 12.4 |
| | B | 100 | 1 | 0 | 9.4 |
| | C | ND | ND | ND | 10.5 |
| | D | ND | ND | ND | 11.1 |
| | Totals | 200 | 3 | 2 | (10.9) |
| 3200 | A | 100 | 0 | 0 | 11.0 |
| | B | 100 | 3 | 2 | 10.6 |
| | Totals | 200 | 3 | 2 | (10.8) |
| 4000 | A | 100 | 2 | 1 | 10.3 |
| | B | 100 | 2 | 2 | 8.7 |
| | Totals | 200 | 4 | 3 | (9.5) |
| 5000 | A | 100 | 0 | 0 | 10.4 |
| | B | 100 | 1 | 1 | 9.5 |
| | Totals | 200 | 1 | 1 | (10.0) |
| NQO, 2.50 | A | 100 | 11 | 8 | |
| | B | 100 | 7 | 5 | |
| | Totals | 200 | 18 | 13* | |

**3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 2**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 1 | 0 | 14.2 |
| | B | 100 | 1 | 0 | 11.8 |
| | C | ND | ND | ND | 10.3 |
| | D | ND | ND | ND | 12.5 |
| | Totals | 200 | 2 | 0 | (12.2) |
| 3200 | A | 100 | 1 | 1 | 11.8 |
| | B | 100 | 2 | 1 | 12.2 |
| | Totals | 200 | 3 | 2 | (12.0) |
| 4000 | A | 100 | 0 | 0 | 9.5 |
| | B | 100 | 2 | 2 | 10.6 |
| | Totals | 200 | 2 | 2 | (10.1) |
| 5000 | A | 100 | 0 | 0 | 12.5 |
| | B | 100 | 2 | 1 | 13.2 |
| | Totals | 200 | 2 | 1 | (12.9) |
| CPA, 6.25 | A | 100 | 50 | 46 | |
| | B | 100 | 35 | 33 | |
| | Totals | 200 | 85 | 79* | |

**3. Study title**: Org 25969: induction of chromosomal aberrations in cultured human peripheral blood lymphocytes

**Key findings**: Org 25969 with its impurities in this batch (batch T) is negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration up to 5000 µg/ml.

Document ID: NL0043003
**Study no.**: 020399X/9717

**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 07/24/2002
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch T, 84.4%

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of 1 healthy non-smoking male volunteer.

Metabolizing system: Liver S9 fraction from rats was prepared from the livers of five young male SD rats which had received prior treatment with Phenobarbital and betanaphthoflavone. The efficacy of the S9 tissue fraction was previously checked in an Ames test and produced acceptable responses with indirect mutagens 2-aminoanthracene and benzo(a)pyrene, using S. typhimurium tester strain TA100.

Doses used in definitive study: For both experiments, 39.1, 78.1, 156, 313, 625, 1250, 2500, 5000 µg/ml were used both in the absence and presence of S9 metabolism.

Basis of dose selection: A dose-range-finding study was not conducted. The sponsor used the OECD recommended maximal concentration (5000 µg/ml) as the highest dose in the definitive study as shown above.

Negative and vehicle controls: untreated cells was used as negative control and physiological saline was used as vehicle control.

Positive controls: The positive control chemicals Mitomycin-C (-S9) and Cyclophosphamide (+S9) were prepared in injectable grade distilled water.

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Whole blood cultures were

established by placing 0.5 mL heparinised blood into 4.5 ml RPMI medium with Phytohaemagglutinin. Blood cultures were incubated for approximately 48 hours at 37°C. For test article treatment, diluted test article in the absence or presence of S9 mix was added to the culture tube with cell culture media. The culture volume was 10 ml; all cultures were then incubated at 37ºC. In experiment 1, cells were treated for 3 hours and harvested 24 hours after the initiation of treatment. Three hours before cell harvest, Colemid was added to inhibit cell division. In experiment 2, the assay was conducted according to the following scheme:

| | Absence of S9<br>24 hour sampling | Presence of S9<br>24 hour sampling |
|---|---|---|
| 0 hrs | Commence treatment | Commence treatment |
| +3 hrs | - | Remove treatment medium, wash and add recovery medium |
| +21 hrs | Add colcemid | Add colcemid |
| +24 hrs | Harvest, prepare slides | Harvest, prepare slides |

| | Absence of S9<br>44 hour sampling | Presence of S9<br>44 hour sampling |
|---|---|---|
| 0 hrs | Commence treatment | Commence treatment |
| +3 hrs | - | Remove treatment medium, wash and add recovery medium |
| +41 hrs | Add colcemid | Add colcemid |
| +44 hrs | Harvest, prepare slides | Harvest, prepare slides |

After incubation, cell were then resuspended in hypotonic solution, and subsequently fixed in methanol/acetic acid fixative. Cell suspension was mounted on slides and stained with 35 Giemsa in tap water. Mitotic index was examined to select appropriate dose-levels for aberrant cell scoring. The slides of selected doses are randomly assigned code numbers. Only metaphases containing 44-46 chromosomes are scored for aberrations. From 100 eligible metaphases per culture, the number of chromosomes, the specific types and numbers of aberration are recorded. Statistical analysis was performed using Fisher's exact test on both including and excluding gaps from the aberration counts.

*Criteria for a valid assay:*
Not specifically described by the sponsor, however, the sponsor has provided the historical value of negative and positive controls.

*Criteria for a positive response:*
A test article is considered as positive in this assay if the following criteria were met:
1. Chromosome aberration increases over the concurrent controls

2. Increases over historical controls
3. Reproducibility with the replicate cultures. Where 2 experiments are performed, increase must be observed in both experiments

The sponsor also indicated that biological significance such as dose-relationship, the type of aberration will be considered.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index is appropriate method to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control did not produce chromosomal aberrations exceeding the historical control and the positive controls used produced chromosomal aberrations in a percentage of cells that were within the range of the historical dataset for positive controls and was statistically significantly increased over the negative solvent control.

Study outcome:
In the first experiment, there was no treatment-related toxicity observed at a concentration up to 5000 µg/ml. In the second experiment, mitotic index was reduced at the 3 highest dose levels; but the magnitude of decrease was < 50%. Based on this, the sponsor has chosen the 3 highest dosing levels to analyze chromosome aberration.

Treatment of cultures with Org 25969 batch T in the absence and presence of S-9 (both experiments) resulted in frequencies of cells with structural aberrations, which were similar to those observed in concurrent vehicle controls for all concentrations analyzed. The aberrant cell frequency of all Org 25969 treated cultures fell within historical negative control (normal) ranges. Therefore, Org25969 with its impurities in batch V is not considered to be clastogenic.

**4. Study title**: Org 25969 batch AE drug product: induction of chromosomal aberrations in cultured human peripheral blood lymphocytes

**Key findings**: Org 25969 in this batch (batch AE) is negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration up to 5000 µg/ml.

Document ID: NL0060033
**Study no.**: 082/139

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 07/27/2004
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch AE, 90.0%

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of 3 healthy non-smoking female volunteers.

Metabolizing system: The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from [redacted] (b) (4). Each batch was checked by the manufacturer for sterility, protein content, ability to convert known pro-mutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities).

Doses used in definitive study: Based on the results from the dose-range finding test, the following concentrations were used in the 2 experiments of the main study.

| Experiment 1 Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | Hours treatment + hours recovery 3+17 -S-9 | 3+17 +S-9 |
|---|---|---|---|
| 2.500 | 250.0 | ✓ | ✓ |
| 5.000 | 500.0 | ✓ | ✓ |
| 10.00 | 1000 | ✓ | ✓ |
| 20.00 | 2000 | ✓ | ✓ |
| 30.00 | 3000 | ✓ | ✓ |
| 40.00 | 4000 | ✓ | ✓ |
| 50.00 | 5000 | ✓ | ✓ |

• Indicates concentration tested

| Experiment 2 Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | Hours treatment + hours recovery 20+0 -S-9 | 3+17 +S-9 |
|---|---|---|---|
| 2.500 | 250.0 | ✓ | ✓ |
| 5.000 | 500.0 | ✓ | ✓ |
| 10.00 | 1000 | ✓ | ✓ |
| 20.00 | 2000 | ✓ | ✓ |
| 30.00 | 3000 | ✓ | ✓ |
| 40.00 | 4000 | ✓ | ✓ |
| 50.00 | 5000 | ✓ | ✓ |

• Indicates concentration tested

Basis of dose selection: A dose-range-finding study was not conducted. The sponsor used the OECD recommended maximal concentration (5000 µg/ml) as the highest dose in the definitive study as shown above based on the results of previous studies.

Negative and vehicle controls: sterile purified water

Positive controls: The positive control chemicals were dissolved in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) immediately prior to use, as shown in the following (extracted from the sponsor's report):

| Chemical | Supplier | Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | S-9 |
|---|---|---|---|---|
| | (b) (4) | | | |
| 4-Nitroquinoline 1-oxide (NQO) | | 0.125 | 1.25 | - |
| | | 0.250 | 2.50 | - |
| | | 0.500 | 5.00 | - |
| Cyclophosphamide (CPA) | | 0.3125 | 3.125 | + |
| | | 0.625 | 6.25 | + |
| | | 1.25 | 12.5 | + |

Cells treated with 5.00 µg NQO/mL (Experiments 1 and 2) and 12.5 and 6.25 µg CPA/mL (Experiments 1 and 2, respectively) gave satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage according to the sponsor. These were selected for analysis.

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL heparinised blood into Hepes-buffered RPMI medium containing 20% (v/v) fetal calf serum and 50 µg/mL gentamycin. Phytohaemagglutinin was included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37°C and rocked continuously. Before the treatment, 0.5 ml S-9 mix or KCl was added. One set of quadruplicate cultures (A, B, C and D) for each of the treatment regimes was then treated with the solvent and one set of duplicate cultures with the test article (1 mL per culture). Additional duplicate cultures for treatments in the absence and presence of S-9 were treated with 0.1 mL of the positive control chemicals. The culture volume was adjusted to 10 ml; all cultures were then incubated at 37ºC. In experiment 1, cells were treated for 3 hours and harvested 17 hours after the termination of treatment. In experiment 2, similar experiment regimen was applied in the presence of S9, but cells were treated for 20 hours and harvested immediately after treatment in the absence of S9. Approximately 2 hours prior to harvest, colchicine was added (final concentration of approximately 1 µg/mL) to arrest dividing cells in metaphase.

At the defined sampling time cultures were centrifuged at approximately 300 x g for 10 minutes; the supernatant was removed and cells were re-suspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37 °C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension. This procedure was repeated several times (centrifuging at approximately 1250 x g, 2-3 minutes) until the cell pellets were clean.

Lymphocytes were kept in fixative in the refrigerator at least overnight for proper fixation. Then, cells were pelleted and re-suspended in a minimal amount of fresh fixative so as to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean microscope slides. Slides were flamed, as necessary, to improve metaphase spreading. After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with cover-slips. Slides were examined for mitotic index to determine the cytotoxic effect of the test article, and selected dosing groups were used for aberration assay. Slides from the selected treatments and from solvent and positive controls were coded using randomly generated letters. One hundred metaphases from each code were analyzed for chromosome aberrations. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with more than 46 chromosomes, i.e. polyploid, endoreduplicated, and hyperdiploid cells, was not considered to be chromosome aberration and recorded separately.

Classification of structural aberrations was based on the scheme described by ISCN as shown right extracted from the sponsor's report. After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as 1) cells with structural aberrations including gaps; 2) cells with structural aberrations excluding gaps; 3) polyploid, endoreduplicated or hyperdiploid cells. The totals for category 2 in negative control cultures were compared with the laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test article treated cultures was also compared with normal ranges. The statistical significance of any data set derived from Fisher's exact test ($p < 0.05$) was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. The proportions of cells in categories 1 and 3 were also examined but were as a reference for aberration.

**Abbreviations and classification of observations**

| | | |
|---|---|---|
| abs | = | aberrations |
| rep | = | replicate |
| tot | = | total |
| **Gaps** | | |
| csg | = | chromosome gap |
| ctg | = | chromatid gap |
| **Chromosome deletions** | | |
| del | = | chromosome deletion |
| d.min | = | double minute |
| f | = | isolocus fragment |
| **Chromosome exchanges** | | |
| t | = | interchange between chromosomes (e.g. reciprocal translocation) |
| inv | = | chromosome intrachange (e.g. pericentric inversion) |
| dic | = | dicentric |
| dic+f | = | dicentric with accompanying fragment |
| acr | = | acentric ring |
| r+f | = | centric ring with accompanying fragment |
| r | = | centric ring |
| **Chromatid deletions** | | |
| del | = | chromatid deletion |
| min | = | single minute |
| **Chromatid exchanges** | | |
| qr | = | interchange between chromatids of different chromosomes (e.g. quadriradial) |
| cx | = | obligate complex interchange |
| e | = | chromatid intrachange |
| tr/tr+f | = | isochromatid/chromatid interchange (triradial)/with accompanying fragment |
| su | = | intra-arm intrachange with sister union of broken ends |
| nud | = | intra-arm intrachange with non-union of broken ends distally |
| nup | = | intra-arm intrachange with non-union of broken ends proximally |
| **Other structural aberrations** | | |
| pvz | = | pulverised cell |
| mabs | = | multiple aberrations (greater than 7 aberrations per cell or too many aberrations to permit accurate analysis) |
| **Numerical aberrations** | | |
| E | = | endoreduplicated |
| H | = | hyperdiploid (47-68 chromosomes) |
| P | = | polyploid (greater than 68 chromosomes) |

*Criteria for a valid assay:*
According to the sponsor, an assay is considered to be valid if the following criteria are met:
1. The binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures.
2. The proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range (*note: The Sponsor has provided a table with historical control data for negative/vehicle control substances*).
3. At least 160 cells out of an intended 200 are analyzable at each dose level.
4. The positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations.

*Criteria for a positive response:*
A test article is considered as positive in this assay if the following criteria were met:
1. The proportions of cells with structural aberrations at one or more concentration exceed the normal range in both replicate cultures.
2. A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at these doses.

Increased incidence of cells with gaps or increased proportions of cells with structural aberrations not exceeding the normal range, or occurring only at very high or very toxic concentrations are likely to be concluded as "equivocal". Evidence of a dose-related effect is considered useful but not essential in the evaluation of a positive result. Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in negative control cultures. Their appearance is therefore considered to be of particular biological significance.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index is appropriate methods to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control and the positive controls used produced chromosomal aberrations in a percentage of cells that were within the range of the historical dataset for negative and positive controls, and positive control was statistically significantly increased over the negative solvent control.

Study outcome:
There was only slight mitotic index inhibition observed at the highest concentrations in the absence and presence of S9 as compared to control in both experiment 1 and 2. There were no changes in mitotic index in other doses in both experiments. The cells of highest 3 concentrations in both experiments (3000, 4000, 5000 µg/ml) were thus used for chromosome aberration analysis.

Treatment of cultures with Org 25969 batch AE in the absence and presence of S-9 (both experiments) resulted in frequencies of cells with structural aberrations that were similar to those observed in concurrent vehicle controls for all concentrations analyzed. Numbers of aberrant cells (excluding gaps) in all except one of treated cultures fell within historical negative control ranges. In Experiment 1, one culture exposed to 5000 µg/ml of the test article in the absence of S9 mix, exhibited frequencies of chromosome aberrations that exceeded the historical negative control 95% reference rang value (0-4) but still within the observed range (0-7). Since similar results were not observed in the other culture in the same experiment and the two cultures in experiment 2, this appeared to be an isolated instance. Therefore, Org25969 with its impurities in batch V is not considered to be clastogenic. The data summary was shown below (extracted from the sponsor's report).

**3-hour treatment -S-9, 17-hour recovery (3+17), Experiment 1**
**Donor sex: male**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 2 | 0 | 12.1 |
| | B | 100 | 0 | 0 | 13.2 |
| | C | ND | ND | ND | 13.3 |
| | D | ND | ND | ND | 13.9 |
| | Totals | 200 | 2 | 0 | (13.1) |
| 3000 | A | 100 | 2 | 1 | 14.3 |
| | B | 100 | 1 | 0 | 13.9 |
| | Totals | 200 | 3 | 1 | (14.1) |
| 4000 | A | 99 | 0 | 0 | 10.3 |
| | B | 100 | 0 | 0 | 12.2 |
| | Totals | 199 | 0 | 0 | (11.3) |
| 5000 | A | 100 | 0 | 0 | 8.9 |
| | B | 100 | **6** | **5** | 11.4 |
| | Totals | 200 | 6 | 5 | (10.2) |
| NQO, 5.0 | A | 100 | **26** | **23** | |
| | B | 100 | **17** | **14** | |
| | Totals | 200 | 43 | 37[a] | |

**3-hour treatment +S-9, 17-hour recovery (3+17), Experiment 1**
**Donor sex: male**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 99 | 0 | 0 | 14.0 |
| | B | 100 | 0 | 0 | 14.8 |
| | C | ND | ND | ND | 12.8 |
| | D | ND | ND | ND | 13.9 |
| | Totals | 199 | 0 | 0 | (13.9) |
| 3000 | A | 100 | 1 | 0 | 13.6 |
| | B | 100 | 0 | 0 | 14.9 |
| | Totals | 200 | 1 | 0 | (14.3) |
| 4000 | A | 100 | 0 | 0 | 11.4 |
| | B | 100 | 0 | 0 | 13.2 |
| | Totals | 200 | 0 | 0 | (12.3) |
| 5000 | A | 100 | 0 | 0 | 10.5 |
| | B | 100 | 0 | 0 | 13.2 |
| | Totals | 200 | 0 | 0 | (11.9) |
| CPA, 12.5 | A | 100 | **43** | **34** | |
| | B | 91 | **35** | **33** | |
| | Totals | 191 | 78 | 67[a] | |

**20-hour treatment -S-9, 0-hour recovery (20+0), Experiment 2**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 1 | 1 | 10.4 |
| | B | 100 | 1 | 1 | 11.3 |
| | C | ND | ND | ND | 11.9 |
| | D | ND | ND | ND | 10.9 |
| | Totals | 200 | 2 | 2 | (11.1) |
| 3000 | A | 100 | 0 | 0 | 12.1 |
| | B | 100 | 1 | 1 | 12.4 |
| | Totals | 200 | 1 | 1 | (12.3) |
| 4000 | A | 100 | 0 | 0 | 11.4 |
| | B | 100 | 0 | 0 | 10.9 |
| | Totals | 200 | 0 | 0 | (11.2) |
| 5000 | A | 100 | 1 | 0 | 10.0 |
| | B | 100 | 0 | 0 | 9.7 |
| | Totals | 200 | 1 | 0 | (9.9) |
| NQO, 5.0 | A | 87 | **29** | **26** | |
| | B | 100 | **29** | **28** | |
| | Totals | 187 | 58 | 54[a] | |

**3-hour treatment +S-9, 17-hour recovery (3+17), Experiment 2**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 0 | 0 | 11.1 |
| | B | 100 | 1 | 0 | 10.7 |
| | C | ND | ND | ND | 12.1 |
| | D | ND | ND | ND | 11.1 |
| | Totals | 200 | 1 | 0 | (11.3) |
| 3000 | A | 100 | 0 | 0 | 11.8 |
| | B | 100 | 1 | 0 | 10.9 |
| | Totals | 200 | 1 | 0 | (11.4) |
| 4000 | A | 100 | 0 | 0 | 11.1 |
| | B | 100 | 0 | 0 | 11.9 |
| | Totals | 200 | 0 | 0 | (11.5) |
| 5000 | A | 100 | 3 | 1 | 10.1 |
| | B | 100 | 0 | 0 | 11.7 |
| | Totals | 200 | 3 | 1 | (10.9) |
| CPA, 6.25 | A | 100 | **40** | **38** | |
| | B | 56 | **20** | **20** | |
| | Totals | 156 | 60 | 58[a] | |

**5. Study title**: Org 25969 batch drug product AJ (impurity enriched): induction of chromosomal aberrations in cultured human peripheral blood lymphocytes

**Key findings**: Org 25969 with its impurities in this batch (batch AJ) is negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration up to 5000 µg/ml or its limit of cytotoxicity under the test condition of this study.

**Study no.**: 082/143
Document ID: NL0060039

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 07/27/2004
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: mixture of Org 25969, Org 48302, and other impurities, Batch AJ, content of active entity of Org 25969 (11.4%) and Org 48302 (15.5%), content of total (b) (4)

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of 3 healthy non-smoking female volunteers.

Metabolizing system: The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from (b) (4). Each batch was checked by the manufacturer for sterility, protein content, ability to convert known pro-mutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities).

Doses used in definitive study: Based on the results from the dose-range finding test, the following concentrations were used in the 2 experiments of the main study.

| Experiment 1 Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | Hours treatment + hours recovery 3+17 –S-9 | 3+17 +S-9 |
|---|---|---|---|
| 0.625 | 62.50 | ✓ | ✓ |
| 1.250 | 125.0 | ✓ | ✓ |
| 2.500 | 250.0 | ✓ | ✓ |
| 5.000 | 500.0 | ✓ | ✓ |
| 10.00 | 1000 | ✓ | ✓ |
| 20.00 | 2000 | ✓ | ✓ |
| 30.00 | 3000 | ✓ | ✓ |
| 40.00 | 4000 | ✓ | ✓ |
| 50.00 | 5000 | ✓ | ✓ |

Indicates concentration tested

| Experiment 2 Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | Hours treatment + hours recovery 20+0 –S-9 | 3+17 +S-9 |
|---|---|---|---|
| 0.625 | 62.50 | ✓ | ✓ |
| 1.250 | 125.0 | ✓ | ✓ |
| 2.500 | 250.0 | ✓ | ✓ |
| 5.000 | 500.0 | ✓ | ✓ |
| 10.00 | 1000 | ✓ | ✓ |
| 20.00 | 2000 | ✓ | ✓ |
| 30.00 | 3000 | ✓ | ✓ |
| 40.00 | 4000 | ✓ | ✓ |
| 50.00 | 5000 | ✓ | ✓ |

Indicates concentration tested

Basis of dose selection: A dose-range-finding study was not conducted. The sponsor used the OECD recommended maximal concentration (5000 µg/ml) as the highest dose in the definitive study as shown above based on solubility and results of previous studies. During the preliminary solubility assessment, DMSO was found to be unsuitable as a solvent owing to poor solubility. Dilution in purified water into culture medium at a final concentration of approximately 5020 µg/mL and 3061 µg/mL resulted in slight visible precipitate, whilst a final concentration of 1530 µg/mL did not result in visible precipitate.

Negative and vehicle controls: sterile purified water

Positive controls: The positive control chemicals were dissolved in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) immediately prior to use, as shown in the following (extracted from the sponsor's report):

| Chemical | Supplier | Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | S-9 |
|---|---|---|---|---|
| | (b) (4) | | | |
| 4-Nitroquinoline 1-oxide (NQO) | | 0.125 | 1.25 | - |
| | | 0.250 | 2.50 | - |
| | | 0.500 | 5.00 | - |
| Cyclophosphamide (CPA) | | 0.3125 | 3.125 | + |
| | | 0.625 | 6.25 | + |
| | | 1.25 | 12.5 | + |

Cells treated with 5.00 µg NQO/mL (Experiments 1 and 2) and 12.5 and 6.25 µg CPA/mL (Experiments 1 and 2, respectively) gave satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage according to the sponsor. These were selected for analysis.

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL heparinised blood

into Hepes-buffered RPMI medium containing 20% (v/v) fetal calf serum and 50 µg/mL gentamycin. Phytohaemagglutinin was included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37°C and rocked continuously. Before the treatment, 0.5 ml S-9 mix or KCl was added. One set of quadruplicate cultures (A, B, C and D) for each of the treatment regimes was then treated with the solvent and one set of duplicate cultures with the test article (1 mL per culture). Additional duplicate cultures for treatments in the absence and presence of S-9 were treated with 0.1 mL of the positive control chemicals. The culture volume was adjusted to 10 ml; all cultures were then incubated at 37ºC. In experiment 1, cells were treated for 3 hours and harvested 17 hours after the termination of treatment. In experiment 2, similar experiment regimen was applied in the presence of S9, but cells were treated for 20 hours and harvested immediately after treatment in the absence of S9. Approximately 2 hours prior to harvest, colchicine was added (final concentration of approximately 1 µg/mL) to arrest dividing cells in metaphase.

At the defined sampling time cultures were centrifuged at approximately 300 x g for 10 minutes; the supernatant was removed and cells were re-suspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37 °C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension. This procedure was repeated several times (centrifuging at approximately 1250 x g, 2-3 minutes) until the cell pellets were clean.

Lymphocytes were kept in fixative in the refrigerator at least overnight for proper fixation. Then, cells were pelleted and re-suspended in a minimal amount of fresh fixative so as to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean microscope slides. Slides were flamed, as necessary, to improve metaphase spreading. After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with cover-slips. Slides were examined for mitotic index to determine the cytotoxic effect of the test article, and selected dosing groups were used for aberration assay. Slides from the selected treatments and from solvent and positive controls were coded using randomly generated letters. One hundred metaphases from each code were analyzed for chromosome aberrations. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with more than 46 chromosomes, i.e. polyploid, endoreduplicated, and hyperdiploid cells, was not considered to be chromosome aberration and recorded separately.

Classification of structural aberrations was based on the scheme described by ISCN as shown below extracted from the sponsor's report. After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as 1) cells with structural aberrations including gaps; 2) cells with structural aberrations excluding gaps; 3) polyploid, endoreduplicated or hyperdiploid cells. The totals for

category 2 in negative control cultures were compared with the laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test article treated cultures was also compared with normal ranges. The statistical significance of any data set derived from Fisher's exact test ($p < 0.05$) was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. The proportions of cells in categories 1 and 3 were also examined but were as a reference for aberration.

**Abbreviations and classification of observations**

| | | |
|---|---|---|
| abs | = | aberrations |
| rep | = | replicate |
| tot | = | total |

**Gaps**

| | | |
|---|---|---|
| csg | = | chromosome gap |
| ctg | = | chromatid gap |

**Chromosome deletions**

| | | |
|---|---|---|
| del | = | chromosome deletion |
| d min | = | double minute |
| f | = | isolocus fragment |

**Chromosome exchanges**

| | | |
|---|---|---|
| t | = | interchange between chromosomes (e.g. reciprocal translocation) |
| inv | = | chromosome intrachange (e.g. pericentric inversion) |
| dic | = | dicentric |
| dic+f | = | dicentric with accompanying fragment |
| acr | = | acentric ring |
| r+f | = | centric ring with accompanying fragment |
| r | = | centric ring |

**Chromatid deletions**

| | | |
|---|---|---|
| del | = | chromatid deletion |
| min | = | single minute |

**Chromatid exchanges**

| | | |
|---|---|---|
| qr | = | interchange between chromatids of different chromosomes (e.g. quadriradial) |
| cx | = | obligate complex interchange |
| e | = | chromatid intrachange |
| tr/tr+f | = | isochromatid/chromatid interchange (triradial)/with accompanying fragment |
| su | = | intra-arm intrachange with sister union of broken ends |
| nud | = | intra-arm intrachange with non-union of broken ends distally |
| nup | = | intra-arm intrachange with non-union of broken ends proximally |

**Other structural aberrations**

| | | |
|---|---|---|
| pvz | = | pulverised cell |
| mabs | = | multiple aberrations (greater than 7 aberrations per cell or too many aberrations to permit accurate analysis) |

**Numerical aberrations**

| | | |
|---|---|---|
| E | = | endoreduplicated |
| H | = | hyperdiploid (47-68 chromosomes) |
| P | = | polyploid (greater than 68 chromosomes) |

*Criteria for a valid assay:*

According to the sponsor, an assay is considered to be valid if the following criteria are met:

1. The binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures.
2. The proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range (*note: The Sponsor has provided a table with historical control data for negative/vehicle control substances*).
3. At least 160 cells out of an intended 200 are analyzable at each dose level.
4. The positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations.

*Criteria for a positive response:*
A test article is considered as positive in this assay if the following criteria were met:
1. The proportions of cells with structural aberrations at one or more concentration exceed the normal range in both replicate cultures.
2. A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at these doses.

Increased incidence of cells with gaps or increased proportions of cells with structural aberrations not exceeding the normal range, or occurring only at very high or very toxic concentrations are likely to be concluded as "equivocal". Evidence of a dose-related effect is considered useful but not essential in the evaluation of a positive result. Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in negative control cultures. Their appearance is therefore considered to be of particular biological significance.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index is appropriate methods to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control and the positive controls used produced chromosomal aberrations in a percentage of cells that were within the range of the historical dataset for negative and positive controls, and positive control was statistically significantly increased over the negative solvent control.

Study outcome:
The results of mitotic index determinations for the treatments in Experiment 1 were as follows (extracted from the sponsor's report):

| Treatment (µg/mL) | Mitotic index (%) 3+17 hours, -S-9 A/C | B/D | MIH* | 3+17 hours, +S-9 A/C | B/D | MIH* |
|---|---|---|---|---|---|---|
| Solvent | 6.7/5.6 | 6.2/5.8 | - | 7.9/7.3 | 7.9/7.5 | - |
| 62.50 | NS | NS | - | 7.6 | 7.8 | 0 |
| 125.0 | NS | NS | - | 7.2 | 7.2 | 6 |
| 250.0 | NS | NS | - | 5.5 | 7.1 | 18 |
| 500.0 | 5.8 | 6.4 | 0 | 5.0 | 5.2 | 33 |
| 1000 | 5.7 | 5.5 | 8 | 4.5 | 4.4 | 42 |
| 2000 | 4.4 | 5.1 | 22 | 4.4 | 5.2 | 37 |
| 3000 | 5.5 | 4.7 | 16 | 5.2 | 5.1 | 33 |
| 4000 | 4.6 | 5.2 | 19 | 5.2 | 5.0 | 33 |
| 5000 | 4.8 | 4.4 | 24 | 5.1 | 4.8 | 35 |

NS = not scored
*Mitotic inhibition (%) = [1 - (mean $MI_T$/mean $MI_C$)] x 100%
(where T = treatment and C = negative control)
(Slides from solvent control cultures C and D scored for mitotic index only)
A/C, B/D refers to the number of cultures treated (four [A, B, C, D] for vehicle controls and two [A, B] for test article and positive controls)

Therefore, 500, 3000, and 5000 µg/ml was selected for analysis in the absence of S9, while 62.5, 250, and 5000 µg/ml was selected in the presence of S9 in this experiment.

In experiment 2, mitotic index inhibition after test article treatment was shown below as extracted from the sponsor's report, and doses selected for analysis were 62.5, 500, and 3000 µg/ml in the absence of S9 and 250, 2000, 5000 in the presence of S9.

| Treatment (µg/mL) | Mitotic index (%) 20+0 hours, -S-9 A/C | B/D | MIH* | 3+17 hours, +S-9 A/C | B/D | MIH* |
|---|---|---|---|---|---|---|
| Solvent | 9.8/10.7 | 10.4/8.4 | - | 9.1/9.9 | 10.6/10.2 | - |
| 62.50 | 8.4 | 9.3 | 10 | 9.9 | 12.6 | 0 |
| 125.0 | 7.5 | 7.3 | 25 | 10.0 | 13.9 | 0 |
| 250.0 | 7.3 | 7.6 | 24 | 10.1 | 10.9 | 0 |
| 500.0 | 7.7 | 7.0 | 25 | 10.7 | 7.2 | 10 |
| 1000 | 5.3 | 7.2 | 36 | 6.8 | 6.3 | 34 |
| 2000 | 4.6 | 4.0 | 56 | 6.9 | 7.5 | 28 |
| 3000 | 4.8 | 4.2 | 54 | 9.9 | 9.5 | 3 |
| 4000 | 2.7 | 3.1 | 70 | 7.4 | 7.3 | 26 |
| 5000 | 3.2 | 2.4 | 72 | 8.6 | 8.4 | 15 |

*Mitotic inhibition (%) = [1 - (mean MIT/mean MIC)] x 100%
(where T = treatment and C = negative control)
(Slides from solvent control cultures C and D scored for mitotic index only).
A/C, B/D refers to the number of cultures treated (four [A, B, C, D] for vehicle controls and two [A, B] for test article and positive controls)

In Experiment 1 there were more cells showing chromosome aberrations in cultures exposed to Org 25969 drug substance batch AJ (impurity enriched) than in the concurrent vehicle controls since there were no aberrations in the negative control in this experiment, but the aberrant cell frequency of all cultures exposed to Org 25969 drug substance batch AJ (impurity enriched) fell within historical negative control (normal) ranges. In Experiment 2, treatment of cultures with Org 25969 drug substance batch AJ (impurity enriched) in the absence and presence of S-9 resulted in frequencies of cells with structural aberrations that were similar to those in concurrent negative controls. Therefore, impurity enriched batch AJ is not considered to be clastogenic. The data summary was shown below (extracted from the sponsor's report).

**3 hour treatment -S-9, 17 hour recovery (3+17), Experiment 1**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 0 | 0 | 6.7 |
| | B | 100 | 0 | 0 | 6.2 |
| | C | ND | ND | ND | 5.6 |
| | D | ND | ND | ND | 5.8 |
| | Totals | 200 | 0 | 0 | (6.1) |
| 500.0 | A | 100 | 4 | 3 | 5.8 |
| | B | 100 | 1 | 1 | 6.4 |
| | Totals | 200 | 5 | 4 | (6.1) |
| 3000 | A | 100 | 0 | 0 | 5.5 |
| | B | 100 | 1 | 1 | 4.7 |
| | Totals | 200 | 1 | 1 | (5.1) |
| 5000 | A | 100 | 0 | 0 | 4.8 |
| | B | 100 | 5 | 4 | 4.4 |
| | Totals | 200 | 5 | 4 | (4.6) |
| NQO, 5.0 | A | 100 | 18 | 18 | |
| | B | 100 | 12 | 11 | |
| | Totals | 200 | 30 | 29[a] | |

**3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 1**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 0 | 0 | 7.9 |
| | B | 100 | 0 | 0 | 7.9 |
| | C | ND | ND | ND | 7.3 |
| | D | ND | ND | ND | 7.5 |
| | Totals | 200 | 0 | 0 | (7.7) |
| 62.50 | A | 100 | 0 | 0 | 7.6 |
| | B | 100 | 0 | 0 | 7.8 |
| | Totals | 200 | 0 | 0 | (7.7) |
| 250.0 | A | 100 | 2 | 2 | 5.5 |
| | B | 100 | 2 | 2 | 7.1 |
| | Totals | 200 | 4 | 4 | (6.3) |
| 5000 | A | 100 | 5 | 4 | 5.1 |
| | B | 100 | 2 | 1 | 4.8 |
| | Totals | 200 | 7 | 5 | (5.0) |
| CPA, 6.25 | A | 100 | 33 | 30 | |
| | B | 100 | 27 | 24 | |
| | Totals | 200 | 60 | 54[a] | |

**20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 2**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 0 | 0 | 9.8 |
| | B | 100 | 0 | 0 | 10.4 |
| | C | ND | ND | ND | 10.7 |
| | D | ND | ND | ND | 8.4 |
| | Totals | 200 | 0 | 0 | (9.8) |
| 62.50 | A | 100 | 0 | 0 | 8.4 |
| | B | 100 | 0 | 0 | 9.3 |
| | Totals | 200 | 0 | 0 | (8.9) |
| 500.0 | A | 100 | 1 | 1 | 7.7 |
| | B | 100 | 2 | 1 | 7.0 |
| | Totals | 200 | 3 | 2 | (7.4) |
| 3000 | A | 100 | 1 | 0 | 4.8 |
| | B | 100 | 0 | 0 | 4.2 |
| | Totals | 200 | 1 | 0 | (4.5) |
| NQO, 5.0 | A | 99 | **52** | **47** | |
| | B | 100 | **53** | **46** | |
| | Totals | 199 | 105 | 93[a] | |

**3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 2**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 0 | 0 | 9.1 |
| | B | 100 | 0 | 0 | 10.6 |
| | C | ND | ND | ND | 9.9 |
| | D | ND | ND | ND | 10.2 |
| | Totals | 200 | 0 | 0 | (10.0) |
| 250.0 | A | 100 | 1 | 0 | 10.1 |
| | B | 100 | 0 | 0 | 10.9 |
| | Totals | 200 | 1 | 0 | (10.5) |
| 2000 | A | 100 | 1 | 0 | 6.9 |
| | B | 100 | 2 | 1 | 7.5 |
| | Totals | 200 | 3 | 1 | (7.2) |
| 5000 | A | 100 | 1 | 1 | 8.6 |
| | B | 100 | 3 | 3 | 8.4 |
| | Totals | 200 | 4 | 4 | (8.5) |
| CPA, 12.5 | A | 55 | **22** | **21** | |
| | B | 66 | **29** | **28** | |
| | Totals | 121 | 51 | 49[a] | |

**6. Study title**: A chromosome aberration test with Org 25969 batch drug product containing degradation products in cultured human peripheral blood lymphocytes in vitro (with independent repeat)

**Key findings**: Org 25969 with its impurities and degradation products are negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration up to 5000 µg/ml.

Document ID: NL0060000
**Study no.**: 040184S

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands

**Date of study initiation**: 07/15/2004
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**:
The test item (degraded drug product) was supplied as a solution in water for injection with a concentration of 93.21 mg/mL. The drug product was prepared from drug substance batches U, W and Z and consisted of 43.77, 9.03 and 45.49 mg of batch U, W and Z, respectively per mL. The drug product produced was stored for approximately 2 to 2.5 months at 60 °C to allow for the formation of degradation products. Prior to this experiment the impurity profile of the degraded drug product was determined and after this experiment the impurity profile will be determined again.

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of healthy non-smoking male donors

Metabolizing system: To simulate mammalian liver drug metabolism, a mixture of the supernatant fraction of rat liver homogenate and cofactors was used. The S9 was commercially obtained from [redacted] (b) (4), and was tested regularly for their ability to activate reference mutagens.

Doses used in definitive study: The following concentrations were used in the main study.

*Experiment 1:*
In the absence of S9-mix
3h/24h 250, 500, 1000, 2500 and 5000 $\mu g \cdot mL^{-1}$
In the presence of S9-mix
3h/24h 250, 500, 1000, 2500 and 5000 $\mu g \cdot mL^{-1}$

*Experiment 2 (independent repeat):*
In the absence of S9-mix
24h/24h 988, 1481, 2222, 3333 and 5000 $\mu g \cdot mL^{-1}$
In the presence of S9-mix
3h/24h 988, 1481, 2222, 3333 and 5000 $\mu g \cdot mL^{-1}$

Basis of dose selection: A dose-range-finding study was not conducted by the sponsor. The dose selection in the definitive study was based on results of previous studies as shown above.

Negative and vehicle controls: The solvent for the test article, ultrapure water, was used as negative control.

Positive controls: The following mutagens were used as positive controls (extracted from the sponsor's report).

Without metabolic activation :
- 4-Nitroquinoline-1-oxide (4-NQO)
  - Dose levels : 2.50 and 5.00 $\mu g.mL^{-1}$ culture medium
  - Solvent : DMSO
  - Stock concentrations : 0.250 and 0.500 $mg.mL^{-1}$ DMSO

With metabolic activation :
- Cyclophosphamide (CP)
  - Dose levels : 12.5 and 25 $\mu g.mL^{-1}$ culture medium
  - Solvent : Hanks Balanced Salt Solution (HBSS)
  - Stock concentrations : 1.25 and 2.50 $\mu g.mL^{-1}$ HBSS

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Two days prior to exposure whole blood in growth medium was inoculated. Lymphocytes were stimulated to divide in the culture medium with serum, supplemented with phytohemagglutinin 1% (v/v) for 48h at 37 °C. Cells were then cultured in a $CO_2$ incubator with 5% ± 0.5% $CO_2$, relative

humidity > 80% and a temperature of 37 °C ± 1 °C for 3 hours with the treatment of test articles, negative, or positive controls in the absence or presence of S9. Cells were harvested at approximate 24 hours from the initiation of treatment. Additional set of Cells in the absence of S9-mix were exposed continuously for approximately 24h were harvested at the end of the exposure period. During the last three hours before harvest, cell division was arrested by addition of the spindle inhibitor colchicine at a final concentration of 1 µg/mL. Thereafter cell cultures were centrifuged for 10 minutes at 1991 N/kg and the supernatant was removed. Cells in the remaining cell pellet were swollen by a 15 minutes treatment with hypotonic 0.075 mol/L KCl at 37 °C. Cells were fixed by adding an equal volume of cold, freshly prepared, methanol /acetic acid mixture (3:1 v/v). Fixed cells were dropped on wet slides which were immersed for 24h in 96% ethanol. Slides were then dried and stained for 20 min in 5% (v/v) Giemsa stain and mounted with coverslips. One hundred/culture suitable metaphase cells (44-46 chromosomes/cell) were examined microscopically and analyzed for chromosome aberrations. Cells with more than 46 chromosomes observed during examination were recorded separately. Definitions of chromosome aberrations were shown in the following extracted from the sponsor's report.

**10.4 DEFINITIONS OF CHROMOSOME ABERRATIONS**

| Aberration | Description |
|---|---|
| Chromatid gap | A non-staining region in a chromatid smaller than the width of a chromatid. Minimal misalignment may be present. |
| Chromosome gap | A non-staining region in both chromatids smaller than the width of the chromatids. Minimal misalignment may be present. |
| Chromatid break | A non-staining region in a chromatid and a distinct misalignment or dislocation must be present. |
| Chromosome break | A non-staining region in both chromatids and a distinct misalignment or dislocation must be present. |
| Minute | An acentric fragment from a chromatid, smaller than the width of a single chromatid, usually circular. |
| Double minutes | Two acentric fragments from a chromosome usually circular, smaller than the width of a single chromatid. |
| Dicentric chromosome | A chromosome containing two centromeres. |
| Ring chromosome | A ring structure with a distinct lumen. |
| Exchange figure | An exchange between two or more chromosomes resulting in the formation of a tri- or more-armed configuration. |
| Multiple aberrations | A metaphase containing seven or more of the above mentioned aberrations (excluding chromatid and chromosome gaps). |
| Polyploidy | A chromosome number more than 68 chromosomes and being a multiple of the normal chromosome set. |
| Endoreduplication | A form of polyploidy in which the homologous chromosomes are aligned. |
| Aneuploidy | Metaphases with one or more chromosomes in addition to the normal diploid number (47-68). |

Mitotic index was evaluated from the negative and vehicle control, and a range of concentrations. Percent polyploidy and endoreduplication were also analyzed by evaluating at least 100 metaphases. The number and types of chromosome aberrations per culture were recorded and the total number of chromosome aberrations of both replicate cultures (including and excluding gaps) was calculated. Fisher's exact test ($p<0.05$) was used for statistical analysis.

*Criteria for a valid assay:*
A valid test is one in which the negative control groups are within the historical range for solvent controls and the percentage of chromosomal aberrations in the positive control groups are statistically increased relative to the solvent control excluding gaps (*note: The Sponsor did not provide a table with historical control data for negative/vehicle and positive control substances*).

*Criteria for a positive response:*
A test article was considered positive for inducing chromosomal aberrations if the following criteria (extracted from the sponsor's report) were met.

- If a statistically significant concentration-related increase in the number of cells with chromosome aberrations (excluding gaps) was present,
- if at this concentration(s) the number of cells with aberrations excluding gaps exceeded the normal range, and
- if a statistically significant increase in the number of cells with aberrations was observed in the absence of a clear concentration-response relationship, but results were reproducible in an independently repeated experiment.

The sponsor further indicated that the criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index are appropriate methods to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control the positive controls used produced chromosomal aberrations in a percentage of cells that were within the range of the historical dataset, respectively, and the aberration after positive control treatment was statistically significantly increased over the negative solvent control.

Study outcome:
There was no dose-dependent decrease in relative mitotic index observed in any of the experiment. The sponsor has chosen the 3 highest dosing levels to be used for analysis in the absence and presence of S9.

The results of the chromosome aberration assay in this study did not elicit dose-dependent and statistically significant increase in chromatid and chromosome aberrations, which indicate a negative response in human peripheral lymphocytes both in the presence and absence of a rat liver S9 metabolizing system. Therefore, Org25969 with its impurities and degradation product are not considered to be clastogenic. The data summary extracted from the sponsor's report was shown below.

Table 10.3-1 3h exposure / 24h harvest – without metabolic activation (Exp. 1)

| | Org 25969* 0 µg·mL⁻¹ | | | Org 25969 1000 µg·mL⁻¹ | | | Org 25969 2500 µg·mL⁻¹ | | | Org 25969 5000 µg·mL⁻¹ | | | 4-NQO** 2.50 µg·mL⁻¹ | | | 4-NQO** 5.00 µg·mL⁻¹ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Relative Metaphase Index** | 100 | | | 89 | | | 72 | | | 65 | | | ne | | | ne | | |
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| No. of cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 43[a] | 41[b] | 84 |
| No. of cells with aberrations incl. gaps | 1 | 2 | 3 | 5 | 1 | 6 | 3 | 2 | 5 | 3 | 1 | 4 | 6 | 7 | 13 | 4 | 6 | 10 |
| No. of cells with aberrations excl. gaps | 0 | 2 | 2 | 3 | 1 | 4 | 2 | 1 | 3 | 3 | 1 | 4 | 4 | 6 | 10 | 4 | 5 | 9 |
| **Chromatid aberrations** | | | | | | | | | | | | | | | | | | |
| gap | 1 | 0 | 1 | 1 | 0 | 1 | 2 | 1 | 3 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| break | 0 | 1 | 1 | 2 | 2 | 4 | 1 | 0 | 1 | 2 | 1 | 3 | 2 | 3 | 5 | 2 | 2 | 4 |
| minutes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| exchanges | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 4 | 4 | 1 | 1 | 2 |
| **Chromosome aberrations** | | | | | | | | | | | | | | | | | | |
| gap | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 1 | 1 | 2 | 0 | 1 | 1 |
| break | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 2 | 2 | 2 | 0 | 2 | 2 | 1 | 3 | 1 | 2 | 3 |
| dicentric | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ring | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| double minutes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| others | 1[c] | 0 | 1[c] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1[c] | 0 | 1[c] | 0 | 0 | 0 |
| total aberrations incl. gaps | 1 | 2 | 3 | 5 | 2 | 7 | 5 | 4 | 9 | 4 | 1 | 5 | 6 | 9 | 15 | 5 | 6 | 11 |
| total aberrations excl. gaps | 0 | 2 | 2 | 3 | 2 | 5 | 2 | 2 | 4 | 4 | 1 | 5 | 4 | 8 | 12 | 4 | 5 | 9 |
| **Numerical aberrations** | | | | | | | | | | | | | | | | | | |
| endoreduplication | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| polyploidy | 1 | 1 | 2 | 0 | 0 | 0 | 2 | 1 | 3 | 2 | 0 | 2 | 1 | 0 | 1 | 0 | 0 | 0 |
| aneuploidy | 0 | 1 | 1 | 3 | 1 | 4 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 2 |

Table 10.3-2 3h exposure / 24h harvest – with metabolic activation (Exp. 1)

| | Org 25969* 0 µg·mL⁻¹ | | | Org 25969 1000 µg·mL⁻¹ | | | Org 25969 2500 µg·mL⁻¹ | | | Org 25969 5000 µg·mL⁻¹ | | | CP 12.50 µg·mL⁻¹ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Relative Metaphase Index** | 100 | | | 105 | | | 75 | | | 87 | | | ne | | |
| Culture | B | C | B+C | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| No. of cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of cells with aberrations incl. gaps | 6 | 3 | 9 | 4 | 5 | 9 | 7 | 2 | 9 | 7 | 5 | 12 | 18 | 10 | 28 |
| No. of cells with aberrations excl. gaps | 4 | 3 | 7 | 3 | 4 | 7 | 5 | 1 | 6 | 4 | 3 | 7 | 10 | 8 | 18 |
| **Chromatid aberrations** | | | | | | | | | | | | | | | |
| gap | 3 | 0 | 3 | 0 | 1 | 1 | 1 | 0 | 1 | 2 | 2 | 4 | 7 | 2 | 9 |
| break | 3 | 4 | 7 | 0 | 2 | 2 | 4 | 1 | 5 | 1 | 1 | 2 | 7 | 2 | 9 |
| minutes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| exchanges | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 3 | 3 |
| **Chromosome aberrations** | | | | | | | | | | | | | | | |
| gap | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 0 | 2 |
| break | 1 | 0 | 1 | 2 | 2 | 4 | 2 | 0 | 2 | 2 | 3 | 5 | 5 | 6 | 11 |
| dicentric | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ring | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| double minutes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| others | 0 | 0 | 0 | 0 | 1[a] | 1[a] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| total aberrations incl. gaps | 7 | 4 | 11 | 4 | 5 | 9 | 8 | 2 | 10 | 7 | 7 | 14 | 21 | 13 | 34 |
| total aberrations excl. gaps | 4 | 4 | 8 | 3 | 4 | 7 | 6 | 1 | 7 | 4 | 4 | 8 | 12 | 11 | 23 |
| **Numerical aberrations** | | | | | | | | | | | | | | | |
| endoreduplication | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| polyploidy | 0 | 0 | 0 | 2 | 0 | 2 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| aneuploidy | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 3 | 0 | 3 | 1 | 3 | 4 |

Table 10.3-3 24h exposure / 24h harvest – without metabolic activation (Exp. 2)

| | Org 25969* 0 µg·mL⁻¹ | | | Org 25969 2222 µg·mL⁻¹ | | | Org 25969 3333 µg·mL⁻¹ | | | Org 25969 5000 µg·mL⁻¹ | | | 4-NQO 2.50 µg·mL⁻¹ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Relative Metaphase Index** | 100 | | | 147 | | | 139 | | | 113 | | | ne | | |
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| No. of cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of cells with aberrations incl. gaps | 1 | 3 | 4 | 0 | 1 | 1 | 2 | 1 | 3 | 0 | 1 | 1 | 17 | 14 | 31 |
| No. of cells with aberrations excl. gaps | 0 | 2 | 2 | 0 | 1 | 1 | 1 | 1 | 2 | 0 | 1 | 1 | 16 | 12 | 28 |
| **Chromatid aberrations** | | | | | | | | | | | | | | | |
| gap | 1 | 1 | 2 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| break | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 5 | 7 | 12 |
| minutes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| exchanges | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 18 | 10 | 28 |
| **Chromosome aberrations** | | | | | | | | | | | | | | | |
| gap | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| break | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 5 | 4 | 9 |
| dicentric | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ring | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| double minutes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| others | 0 | 1[a] | 1[a] | 0 | 0 | 0 | 0 | 1[a] | 1[a] | 0 | 0 | 0 | 2[b] 1[a] | 0 | 2[b] 1[a] |
| total aberrations incl. gaps | 1 | 3 | 4 | 0 | 1 | 1 | 2 | 1 | 3 | 0 | 1 | 1 | 43 | 23 | 66 |
| total aberrations excl. gaps | 0 | 2 | 2 | 0 | 1 | 1 | 1 | 1 | 2 | 0 | 1 | 1 | 42 | 21 | 63 |
| **Numerical aberrations** | | | | | | | | | | | | | | | |
| endoreduplication | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 2 | 0 | 2 |
| polyploidy | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| aneuploidy | 0 | 0 | 0 | 0 | 2 | 2 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |

Table 10.3-4 3h exposure / 24h harvest – with metabolic activation (Exp. 2)

| | Org 25969* 0 µg·mL⁻¹ | | | Org 25969 2222 µg·mL⁻¹ | | | Org 25969 3333 µg·mL⁻¹ | | | Org 25969 5000 µg·mL⁻¹ | | | CP 12.50 µg·mL⁻¹ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Relative Metaphase Index** | 100 | | | 120 | | | 110 | | | 125 | | | ne | | |
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| No. of cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 50 | 50 | 100 |
| No. of cells with aberrations incl. gaps | 2 | 2 | 4 | 2 | 7 | 9 | 7 | 1 | 8 | 4 | 4 | 8 | 17 | 18 | 35 |
| No. of cells with aberrations excl. gaps | 1 | 1 | 2 | 0 | 1 | 1 | 4 | 0 | 4 | 2 | 2 | 4 | 15 | 14 | 29 |
| **Chromatid aberrations** | | | | | | | | | | | | | | | |
| gap | 1 | 1 | 2 | 2 | 6 | 8 | 2 | 0 | 2 | 2 | 2 | 4 | 5 | 4 | 9 |
| break | 0 | 1 | 1 | 0 | 1 | 1 | 3 | 0 | 3 | 2 | 2 | 4 | 12 | 10 | 22 |
| minutes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| exchanges | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| **Chromosome aberrations** | | | | | | | | | | | | | | | |
| gap | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 | 0 | 0 | 4 | 3 | 7 |
| break | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 5 | 3 | 8 |
| dicentric | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ring | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| double minutes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| others | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| total aberrations incl. gaps | 2 | 2 | 4 | 2 | 8 | 10 | 7 | 1 | 8 | 4 | 4 | 8 | 26 | 23 | 49 |
| total aberrations excl. gaps | 1 | 1 | 2 | 0 | 1 | 1 | 4 | 0 | 4 | 2 | 2 | 4 | 17 | 16 | 33 |
| **Numerical aberrations** | | | | | | | | | | | | | | | |
| endoreduplication | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| polyploidy | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 |
| aneuploidy | 2 | 3 | 5 | 2 | 0 | 2 | 0 | 1 | 1 | 2 | 0 | 2 | 3 | 2 | 5 |

**7. Study title**: Org 48302: induction of chromosomal aberrations in cultured human peripheral blood lymphocytes

**Key findings**: Org 48302 with its impurities in this batch is negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration up to 5000 µg/ml or its limit of cytotoxicity under the test condition of this study.

Document ID: NL0060042
**Study no.**: 082/141

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: [redacted] (b) (4)

**Date of study initiation**: 07/27/2004
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: Org 48302, Batch B, 87.3%

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of 3 healthy non-smoking female volunteers.

Metabolizing system: The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from [redacted] (b) (4) Each batch was checked by the manufacturer for sterility, protein content, ability to convert known pro-mutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities).

Doses used in definitive study: The following concentrations were used in the 2 experiments of the main study.

| Experiment 1 Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | Hours treatment + hours recovery 3+17 -S-9 | 3+17 +S-9 |
|---|---|---|---|
| 0.625 | 62.50 | ✓ | ✓ |
| 1.250 | 125.0 | ✓ | ✓ |
| 2.500 | 250.0 | ✓ | ✓ |
| 5.000 | 500.0 | ✓ | ✓ |
| 10.00 | 1000 | ✓ | ✓ |
| 20.00 | 2000 | ✓ | ✓ |
| 30.00 | 3000 | ✓ | ✓ |
| 40.00 | 4000 | ✓ | ✓ |
| 50.00 | 5000 | ✓ | ✓ |

Indicates concentration tested

| Experiment 2 Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | Hours treatment + hours recovery 20+0 -S-9 | 3+17 +S-9 |
|---|---|---|---|
| 1.250 | 125.0 | ✓ | ✓ |
| 2.500 | 250.0 | ✓ | ✓ |
| 5.000 | 500.0 | ✓ | ✓ |
| 10.00 | 1000 | ✓ | ✓ |
| 20.00 | 2000 | ✓ | ✓ |
| 30.00 | 3000 | ✓ | ✓ |
| 40.00 | 4000 | ✓ | ✓ |
| 50.00 | 5000 | ✓ | ✓ |

Indicates concentration tested

Basis of dose selection: The sponsor used the OECD recommended maximal concentration (5000 µg/ml) as the highest dose in the definitive study as shown above based on solubility. During the preliminary solubility assessment, DMSO was found to be unsuitable as a solvent owing to poor solubility. Dilution in purified water into culture

medium at a final concentration of approximately 6919 µg/mL did not result in visible precipitation.

Negative and vehicle controls: sterile purified water

Positive controls: The positive control chemicals were dissolved in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) immediately prior to use, as shown in the following (extracted from the sponsor's report):

| Chemical | Supplier | Concentration of treatment solution (mg/mL) | Final concentration (µg/mL) | S-9 |
|---|---|---|---|---|
| | (b) (4) | | | |
| 4-Nitroquinoline 1-oxide (NQO) | | 0.125 | 1.25 | - |
| | | 0.250 | 2.50 | - |
| | | 0.500 | 5.00 | - |
| Cyclophosphamide (CPA) | | 0.3125 | 3.125 | + |
| | | 0.625 | 6.25 | + |
| | | 1.25 | 12.5 | + |

Cells treated with 5.00 µg NQO/mL (Experiments 1 and 2) and 12.5 and 6.25 µg CPA/mL (Experiments 1 and 2, respectively) gave satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage according to the sponsor. These were selected for analysis.

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.4 mL heparinised blood into Hepes-buffered RPMI medium containing 20% (v/v) fetal calf serum and 50 µg/mL gentamycin. Phytohaemagglutinin was included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37°C and rocked continuously. Before the treatment, 0.5 ml S-9 mix or KCl was added. One set of quadruplicate cultures (A, B, C and D) for each of the treatment regimes was then treated with the solvent and one set of duplicate cultures with the test article (1 mL per culture). Additional duplicate cultures for treatments in the absence and presence of S-9 were treated with 0.1 mL of the positive control chemicals. The culture volume was adjusted to 10 ml; all cultures were then incubated at 37ºC. In experiment 1, cells were treated for 3 hours and harvested 17 hours after the termination of treatment. In experiment 2, similar experiment regimen was applied in the presence of S9, but cells were treated for 20 hours and harvested immediately after treatment in the absence of S9. Approximately 2 hours prior to harvest, colchicine was added (final concentration of approximately 1 µg/mL) to arrest dividing cells in metaphase.

At the defined sampling time cultures were centrifuged at approximately 300 x g for 10 minutes; the supernatant was removed and cells were re-suspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37 °C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension.

This procedure was repeated several times (centrifuging at approximately 1250 x g, 2-3 minutes) until the cell pellets were clean.

Lymphocytes were kept in fixative in the refrigerator at least overnight for proper fixation. Then, cells were pelleted and re-suspended in a minimal amount of fresh fixative so as to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean microscope slides. Slides were flamed, as necessary, to improve metaphase spreading. After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with cover-slips. Slides were examined for mitotic index to determine the cytotoxic effect of the test article, and selected dosing groups were used for aberration assay. Slides from the selected treatments and from solvent and positive controls were coded using randomly generated letters. One hundred metaphases from each code were analyzed for chromosome aberrations. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with more than 46 chromosomes, i.e. polyploid, endoreduplicated, and hyperdiploid cells, was not considered to be chromosome aberration and recorded separately.

Classification of structural aberrations was based on the scheme described by ISCN. After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as 1) cells with structural aberrations including gaps; 2) cells with structural aberrations excluding gaps; 3) polyploid, endoreduplicated or hyperdiploid cells. The totals for category 2 in negative control cultures were compared with the laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test article treated cultures was also compared with normal ranges. The statistical significance of any data set derived from Fisher's exact test ($p < 0.05$) was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. The proportions of cells in categories 1 and 3 were also examined but were as a reference for aberration.

*Criteria for a valid assay:*
According to the sponsor, an assay is considered to be valid if the following criteria are met:
1. The binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures.
2. The proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range (*note: The Sponsor has provided a table with historical control data for negative/vehicle control substances*).
3. At least 160 cells out of an intended 200 are analyzable at each dose level.
4. The positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations.

*Criteria for a positive response:*
A test article is considered as positive in this assay if the following criteria were met:

1. The proportions of cells with structural aberrations at one or more concentration exceed the normal range in both replicate cultures.
2. A statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at these doses.

Increased incidence of cells with gaps or increased proportions of cells with structural aberrations not exceeding the normal range, or occurring only at very high or very toxic concentrations are likely to be concluded as "equivocal". Evidence of a dose-related effect is considered useful but not essential in the evaluation of a positive result. Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in negative control cultures. Their appearance is therefore considered to be of particular biological significance.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index is appropriate methods to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control and the positive controls used produced chromosomal aberrations in a percentage of cells that were within the range of the historical dataset for negative and positive controls, and positive control was statistically significantly increased over the negative solvent control.

Study outcome:
In both experiments, mitotic index determination found minor mitotic index inhibition in a dose-independent manner (<15%). Therefore, the 3 highest dose levels (3000, 4000, 5000 μg/ml) were choose for analysis.

Treatment of cultures with Org 48302 in the absence and presence of S-9 in both experiments resulted in frequencies of cells with structural aberrations that were similar to those in concurrent negative controls and numbers of aberrant cells (excluding gaps) in all Org 48302 treated cultures fell within historical negative control ranges (Appendix 5). Therefore, impurity enriched batch AJ is not considered to be clastogenic. The data summary was shown below (extracted from the sponsor's report).

**3 hour treatment -S-9, 17 hour recovery (3+17), Experiment 1**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 0 | 0 | 11.6 |
| | B | 100 | 1 | 1 | 11.6 |
| | C | ND | ND | ND | 11.5 |
| | D | ND | ND | ND | 12.3 |
| | Totals | 200 | 1 | 1 | (11.8) |
| 3000 | A | 100 | 0 | 0 | 10.5 |
| | B | 100 | 0 | 0 | 10.0 |
| | Totals | 200 | 0 | 0 | (10.3) |
| 4000 | A | 100 | 0 | 0 | 10.5 |
| | B | 100 | 1 | 1 | 11.5 |
| | Totals | 200 | 1 | 1 | (11.0) |
| 5000 | A | 100 | 2 | 2 | 9.3 |
| | B | 100 | 1 | 1 | 10.6 |
| | Totals | 200 | 3 | 3 | (10.0) |
| NQO, 2.5 | A | 100 | **8** | **6** | |
| | B | 100 | **15** | **13** | |
| | Totals | 200 | 23 | 19[a] | |

**3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 1**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 0 | 0 | 8.8 |
| | B | 100 | 0 | 0 | 11.9 |
| | C | ND | ND | ND | 11.0 |
| | D | ND | ND | ND | 10.7 |
| | Totals | 200 | 0 | 0 | (10.6) |
| 3000 | A | 100 | 0 | 0 | 9.0 |
| | B | 100 | 1 | 1 | 8.4 |
| | Totals | 200 | 1 | 1 | (8.7) |
| 4000 | A | 100 | 1 | 1 | 10.2 |
| | B | 100 | 0 | 0 | 8.8 |
| | Totals | 200 | 1 | 1 | (9.5) |
| 5000 | A | 100 | 1 | 1 | 8.6 |
| | B | 100 | 1 | 1 | 9.4 |
| | Totals | 200 | 2 | 2 | (9.0) |
| CPA, 6.25 | A | 100 | **27** | **24** | |
| | B | 100 | **30** | **23** | |
| | Totals | 200 | 57 | 47[a] | |

**20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 2**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 100 | 0 | 0 | 4.4 |
| | B | 100 | 0 | 0 | 7.5 |
| | C | ND | ND | ND | 5.7 |
| | D | ND | ND | ND | 9.3 |
| | Totals | 200 | 0 | 0 | (6.7) |
| 3000 | A | 100 | 0 | 0 | 6.5 |
| | B | 100 | 0 | 0 | 8.4 |
| | Totals | 200 | 0 | 0 | (7.5) |
| 4000 | A | 100 | 0 | 0 | 5.4 |
| | B | 100 | 0 | 0 | 6.5 |
| | Totals | 200 | 0 | 0 | (6.0) |
| 5000 | A | 100 | 0 | 0 | 5.7 |
| | B | 100 | 1 | 1 | 7.5 |
| | Totals | 200 | 1 | 1 | (6.6) |
| NQO, 5.0 | A | 54 | **19** | **17** | |
| | B | 71 | **19** | **18** | |
| | Totals | 125 | 38 | 35[a] | |

**3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 2**
**Donor sex: female**

| Treatment (µg/mL) | Replicate | Cells Scored | Cells with Aberrations Including Gaps | Cells with Aberrations Excluding Gaps | Mitotic Index (mean) |
|---|---|---|---|---|---|
| Solvent | A | 51 | 0 | 0 | 9.9 |
| | B | 100 | 0 | 0 | 9.5 |
| | C | 100 | 1 | 1 | 6.8 |
| | D | 100 | 0 | 0 | 7.9 |
| | Totals | 351 | 1 | 1 | (8.5) |
| 3000 | A | 100 | 0 | 0 | 9.2 |
| | B | 100 | 0 | 0 | 8.8 |
| | Totals | 200 | 0 | 0 | (9.0) |
| 4000 | A | 100 | 0 | 0 | 7.2 |
| | B | 100 | 1 | 1 | 8.0 |
| | Totals | 200 | 1 | 1 | (7.6) |
| 5000 | A | 100 | 0 | 0 | 10.6 |
| | B | 100 | 0 | 0 | 9.1 |
| | Totals | 200 | 0 | 0 | (9.9) |
| CPA, 12.5 | A | 100 | **10** | **10** | |
| | B | 85 | **6** | **6** | |
| | Totals | 185 | 16 | 16[a] | |

**8. Study title**: Evaluation of the ability of (b) (4) to induce chromosome aberrations in cultured peripheral human lymphocytes

**Key findings**: (b) (4) with is negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration up to 5000 µg/ml.

Document ID: NL0047277
**Study no.**: 030069X/377944

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 02/18/2003
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: (b) (4), Batch A, (b) (4)%

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of healthy non-smoking male donors

Metabolizing system: Rat liver microsomal enzymes (S9) were prepared in (b) (4).

Doses used in definitive study:
1000, 333, 5000 µg/ml were used in the two experiments of the main study in the presence or absence of S9.

Basis of dose selection: A dose-range-finding study was conducted by the sponsor with the concentration of 100, 333, 1000, 3330, 5000 µg/ml (b) (4). There was no sign of cytotoxic effect indicated by decrease of mitotic index. Therefore, the highest dosing level was selected to be 5000 µg/ml in the definitive study.

Negative and vehicle controls: F10 medium buffered with 20 mM HEPES

Positive controls: The following mutagens were used as positive controls (extracted from the sponsor's report).

Without metabolic activation (-S9-mix):
Mitomycin C (MMC-C; CAS no. 50-07-7 (b) (4) was used as a direct acting mutagen at a final concentration of 0.5 µg/ml for a 3 h exposure period, 0.2 µg/ml for a 24 h exposure period and 0.1 µg/ml for a 48 h exposure period.

With metabolic activation (+S9-mix):
Cyclophosphamide (CP; CAS no. 50-18-0 (b) (4) was used as an indirect acting mutagen, requiring metabolic activation, at a final concentration of 15 µg/ml for a 3 h exposure period (24 h fixation time).

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Lymphocytes were stimulated to divide in the culture medium with F10 complete culture medium containing 20% foetal calf serum, L-glutamine, penicillin/streptomycin sodium bicarbonate, supplemented with phytohemagglutinin 1% (v/v) at 37 °C for 48 hours. Cells were then cultured in a $CO_2$

incubator with 5% ± 0.5% $CO_2$, relative humidity > 80% and a temperature of 37 °C ± 1 °C for 3 hours with the treatment of test articles, negative, or positive controls in the absence or presence of S9 in the first experiment. Cells were harvested at approximate 24 hours from the initiation of treatment. In the second experiment, same treatment duration was used for cells in the presence of S9 but harvested 48 hours after initiation of treatment. In the absence of S9, cells were treated in duplicate for 24 hours or 48 hours, and were harvested immediately. During the last 2.5 hours before harvest, cell division was arrested by addition of the spindle inhibitor colchicine at a concentration of 0.5 µg/mL. Thereafter cells were swollen by a 5 minutes treatment with hypotonic 0.56% KCl at 37 °C, and then fixed by adding methanol /acetic acid mixture (3:1 v/v). Fixed cells were dropped on wet slides which were immersed for 24h in 96% ethanol. Slides were then dried and stained for 10 - 30 min in 5% (v/v) Giemsa stain and mounted with coverslips. The mitotic index of each culture was determined by counting the number of metaphases per 1000 cells. At least 100/culture suitable metaphase cells (44-46 chromosomes/cell) were examined microscopically and analyzed for chromosome aberrations. Cells with more than 46 chromosomes observed during examination were recorded separately. Definitions of chromosome aberrations were shown in the following extracted from the sponsor's report. Chi-square test was used for statistically analysis.

| Aberration | Abbreviation | Description |
|---|---|---|
| Chromatid gap | g' | An achromatic lesion which appears as an unstained region in the chromatid arm, the size of which is equal to or smaller than the width of the chromatid and the apparently "broken" segments of the chromatid arm are in alignment. |
| Chromosome gap | g" | An achromatic lesion which appears as an unstained region in both chromatids at the same position, the size of which is equal to or smaller than the width of the chromatid and the apparently "broken" segments of the chromatids are in alignment. |
| Chromatid break | b' | An achromatic lesion in a chromatid arm, the size of which is larger than the width of the chromatid. The broken segments of the chromatid arm are aligned or unaligned. |
| Chromosome break | b" | An achromatic lesion in both chromatids at the same position, the size of which is larger than the width of the chromatid. The broken segments of the chromatids are aligned or unaligned. |
| Chromatid deletion | d' | Deleted material at the end of a chromatid arm. |
| Minute | m' | A single, usually circular, part of a chromatid lacking a centromere. |
| Double minutes | m" | Two, usually circular, parts of a chromatid lacking a centromere. |
| Dicentric chromosome | dic | A chromosome containing two centromeres. |
| Tricentric chromosome | tric | A chromosome containing three centromeres. |

*Criteria for a valid assay:*

A test will be considered to be valid if it met the following criteria according to the sponsor:

1) the number of chromosome aberrations found in the solvent control cultures should reasonably be within the laboratory historical control data range (0-6 with S9 and 0-5

without S9 aberrant cells per 100 metaphases with gaps excluded) (*note: The Sponsor did not provide a table with historical control data for negative/vehicle and positive control substances*).
2) The positive control substances should produce a statistically significant (Chi-square test, $p < 0.05$) increase in the number of cells with chromosome aberrations.
3) a homogeneous response between the replicate cultures is observed

*Criteria for a positive response:*
A test article was considered positive for inducing chromosomal aberrations if the following criteria were met according to the sponsor:
1) It induced a dose-related statistically significant (Chi-square test, $p < 0.05$) increase in the number of cells with chromosome aberrations.
2) A statistically significant increase in the frequencies of the number of cells with chromosome aberrations was observed in the absence of a clear dose-response relationship.

A test substance was considered negative in the chromosome aberration test if none of the tested concentrations induced a statistically significant (Chi-square test, $p < 0.05$) increase in the number of cells with chromosome aberrations.

The sponsor further indicated that the criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index is an appropriate method to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control and the positive controls used produced cells with chromosomal aberrations produced chromosomal aberrations in a percentage that were within the range of the historical dataset and aberrant cells with the treatment of the positive controls was statistically significantly increased over the negative solvent control.

Study outcome:
There was only minor decrease of mitotic index with 3-hour test article treatment in the absence and presence of S9 (<13%) in the experiment 1. In experiment2, the results of mitotic index determination were shown below as extracted from the sponsor's report.

**TABLE 5. Mitotic index of donor cultures treated with (b) (4) in the second cytogenetic assay**

Period of treatment: From: 12-03-2003 To: 14-03-2003

| (b) (4) concentration (µg/ml) | Number of metaphases per 1000 cells [a] Absolute | Percentage of control |
|---|---|---|
| Without metabolic activation (-S9-mix) | | |
| 24 h exposure time, 24 h fixation time | | |
| Control [b] | 38 - 44 | 100 |
| 1000 | 34 - 36 | 85 |
| 3330 | 31 - 25 | 68 |
| 5000 | 20 - 16 | 44 |
| MMC-C; 0.2 µg/ml | 10 - 10 | 24 |
| 48 h exposure time, 48 h fixation time | | |
| Control [b] | 28 - 32 | 100 |
| 1000 | 25 - 25 | 83 |
| 3330 | 26 - 30 | 93 |
| 5000 | 24 - 28 | 87 |
| MMC-C; 0.1 µg/ml | 11 - 10 | 35 |
| With metabolic activation (+S9-mix) | | |
| 3 h exposure time, 48 h fixation time | | |
| Control [b] | 26 - 33 | 100 |
| 1000 | 22 - 23 | 76 |
| 3330 | 17 - 13 | 51 |
| 5000 | 21 - 19 | 68 |
| CP; 15 µg/ml | 22 - 18 | – [c] |

In the first experiment, (b) (4) induced a statistically significant increase in the number of cells with chromosome aberration at 5000 µg/ml in the absence of S9, but this value was within the historical data range of the negative control. In the presence of S9, there was no increase of chromosome aberration at any dose tested as compared to negative control. In the experiment 2, no increase of chromosome aberration was observed at any dose levels in the absence and presence of S9. All these result indicated a negative response in human peripheral lymphocytes. Therefore, Org25969 with its impurities in batch L is not considered to be clastogenic. The data summary extracted from the sponsor's report was shown below.

**TABLE 3. Chromosome aberrations in donor cultures treated with (b) (4) in the absence of S9 mix in the first cytogenetic assay (3 h exposure time, 24 h fixation time)**

| Conc µg/ml | F10 HEPES (5.0% v/v) | | | 1000 µg/ml | | | 3330 µg/ml | | | 5000 µg/ml | | | MMC-C 0.5 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 90 | | | 90 | | | 89 | | | 63 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) | 1 | 0 | 1 | 1 | 3 | 4 | 1 | 1 | 2 | 4 | 4 | 8 *) | 12 | 10 | 22 ***) |
| No. of Cells with aberrations (- gaps) | 0 | 0 | 0 | 0 | 3 | 3 | 1 | 1 | 2 | 4 | 3 | 7 *) | 11 | 10 | 21 ***) |
| g' | | | | 1 | | | | | | | 1 | | 1 | | |
| g" | 1 | | | | | | | | | | | | | | |
| b' | | | | | 1 | | | | | 1 | | | 2 | 4 | |
| b" | | | | | 2 | | 2 | | | 2 | 3 | | 3 | 3 | |
| m' | | | | | | | | 1 | | | | | | | |
| m" | | | | | | | | | | | | | | | |
| exch. | | | | | | | | | | | | | 5 | 3 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | 1 | | | 1 | | |
| miss. | | | | | | | | | | | | | | | |
| total aberr (+ gaps) | 1 | 0 | | 1 | 3 | | 2 | 1 | | 4 | 4 | | 12 | 10 | |
| total aberr (- gaps) | 0 | 0 | | 0 | 3 | | 2 | 1 | | 4 | 3 | | 11 | 10 | |

**TABLE 4. Chromosome aberrations in donor cultures treated with (b) (4) in the presence of S9-mix in the first cytogenetic assay (3 h exposure time, 24 h fixation time)**

| Conc µg/ml | F10 HEPES (5.0% v/v) | | | 1000 µg/ml | | | 3330 µg/ml | | | 5000 µg/ml | | | CP 15 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 103 | | | 104 | | | 87 | | | 29 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) | 5 | 0 | 5 | 4 | 3 | 7 | 3 | 3 | 6 | 3 | 1 | 4 | 13 | 21 | 34 ***) |
| No. of Cells with aberrations (- gaps) | 5 | 0 | 5 | 4 | 3 | 7 | 1 | 3 | 4 | 3 | 1 | 4 | 11 | 21 | 32 ***) |
| g' | | | | | | | | | | | | | 3 | | |
| g" | | | | | | | 2 | | | | | | | | |
| b' | 2 | | | 1 | 2 | | 2 | 2 | | 1 | 1 | | 9 | 16 | |
| b" | 2 | | | 3 | 1 | | | 1 | | 2 | | | 3 | 5 | |
| m' | | | | | | | | | | | | | | | |
| m" | | | | | | | | | | | | | 2 | | |
| exch. | | | | | | | | | | | | | 1 | 5 | |
| dic | | | | | | | | | | | | | | | |
| d' | 2 | | | | | | | | | | | | | | |
| misc. | | | | | | | | | | | | | | | |
| total aberr (+ gaps) | 5 | 0 | | 4 | 3 | | 4 | 3 | | 3 | 1 | | 18 | 26 | |
| total aberr (- gaps) | 5 | 0 | | 4 | 3 | | 2 | 3 | | 3 | 1 | | 15 | 26 | |

TABLE 6. Chromosome aberrations in donor cultures treated with (b) (4) in the absence of S9-mix in the second cytogenetic assay (24 h exposure time, 24 h fixation time)

| Conc µg/ml | F10 HEPES (5.0% v/v) | | | 1000 µg/ml | | | 3330 µg/ml | | | 5000 µg/ml | | | MMC-C 0.2 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 85 | | | 68 | | | 44 | | | 24 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 55[1] | 155 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) | 1 | 0 | 1 | 1 | 1 | 2 | 2 | 3 | 5 | 3 | 3 | 6 | 28 | 36 | ***) 64 |
| No. of Cells with aberrations (- gaps) | 1 | 0 | 1 | 1 | 1 | 2 | 2 | 1 | 3 | 1 | 3 | 4 | 28 | 34 | ***) 62 |
| g' | | | | | | | 1 | | | 1 | | | 1 | 3 | |
| g" | | | | | | | | 2 | | 1 | | | 1 | 1 | |
| b' | 1 | | | | 1 | | 1 | 1 | | | | | 11 | 19 | |
| b" | | | | 1 | | | 1 | | | | 2 | | 13 | 17 | |
| m' | | | | | | | | | | | | | 1 | 3 | |
| m" | | | | | | | | | | | | | | | |
| exch. | | | | | | | | | | | | | 5 | 6 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | 1 | 1 | | | | |
| misc. | | | | | | | | | | | | | r | | |
| total aberr (+ gaps) | 1 | 0 | | 1 | 1 | | 3 | 3 | | 3 | 3 | | 33 | 49 | |
| total aberr (- gaps) | 1 | 0 | | 1 | 1 | | 2 | 1 | | 1 | 3 | | 31 | 45 | |

TABLE 7. Chromosome aberrations in donor cultures treated with (b) (4) in the absence of S9-mix in the second cytogenetic assay (48 h exposure time, 48 h fixation time)

| Conc µg/ml | F10 HEPES (5.0% v/v) | | | 1000 µg/ml | | | 3330 µg/ml | | | 5000 µg/ml | | | MMC-C 0.1 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 83 | | | 93 | | | 87 | | | 35 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 50 | 50 | 100 |
| No. of Cells with aberrations (+ gaps) | 1 | 3 | 4 | 1 | 2 | 3 | 3 | 2 | 5 | 2 | 1 | 3 | 35 | 31 | ***) 66 |
| No. of Cells with aberrations (- gaps) | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 1 | 2 | 34 | 30 | ***) 64 |
| g' | | 1 | | | | | 1 | | | 1 | | | 1 | 2 | |
| g" | | | | | | | 1 | | | | | | 2 | 4 | |
| b' | | 2 | | 1 | 1 | | | 1 | | 1 | 1 | | 17 | 8 | |
| b" | | | | | | | 1 | 1 | | | | | 13 | 23 | |
| m' | | | | | 1 | | | | | | | | 4 | 1 | |
| m" | | | | | | | | | | | | | 3 | | |
| exch. | | | | | | | | | | | | | 5 | 11 | |
| dic | | | | | | | | | | | | | | | |
| d' | 1 | | | | | | | | | | | | | | |
| misc. | | | | | | | | | | | | | p | | |
| total aberr (+ gaps) | 1 | 3 | | 1 | 2 | | 3 | 2 | | 2 | 1 | | 46 | 49 | |
| total aberr (- gaps) | 1 | 2 | | 1 | 2 | | 1 | 2 | | 1 | 1 | | 43 | 43 | |

TABLE 8. Chromosome aberrations in donor cultures treated with (b) (4) in the presence of S9-mix in the second cytogenetic assay (3 h exposure time, 48 h fixation time)

| Conc µg/ml | F10 HEPES (5.0% v/v) | | | 1000 µg/ml | | | 3330 µg/ml | | | 5000 µg/ml | | | CP 15 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 76 | | | 51 | | | 66 | | | - [1] | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) | 1 | 2 | 3 | 1 | 2 | 3 | 2 | 0 | 2 | 2 | 1 | 3 | 24 | 22 | ***) 46 |
| No. of Cells with aberrations (- gaps) | 1 | 1 | 2 | 1 | 2 | 3 | 1 | 0 | 1 | 1 | 1 | 2 | 21 | 20 | ***) 41 |
| g' | | | | | | | 1 | | | | | | 3 | 2 | |
| g" | | 1 | | | | | | | | 1 | | | 6 | 3 | |
| b' | 1 | 1 | | | | | 1 | | | 1 | 1 | | 11 | 10 | |
| b" | | | | | 2 | | | | | | | | 13 | 9 | |
| m' | | | | | | | | | | | | | 2 | 1 | |
| m" | | | | | | | | | | | | | 2 | | |
| exch. | | | | | | | | | | | | | 1 | 2 | |
| dic | | | | | | | | | | | | | 1 | | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | p | poly | | | | | | | | | | |
| total aberr (+ gaps) | 1 | 2 | | 1 | 2 | | 2 | 0 | | 2 | 1 | | 39 | 27 | |
| total aberr (- gaps) | 1 | 1 | | 1 | 2 | | 1 | 0 | | 1 | 1 | | 30 | 22 | |

**9. Study title**: Evaluation of the ability of bromocyclodextin to induce chromosome aberrations in cultured peripheral human lymphocytes (with independent repeat)

**Key findings**: bromocyclodextin is negative for inducing structural chromosomal aberrations in cultured peripheral human lymphocytes in the presence or absence of rat liver S9 at concentration to produce precipitation and greater than 50% decrease of mitotic index.

Document ID: INT00036688

**Study no.**: 030069X/377944

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 10/21/2004
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: Bromocyclodextin, Batch EMZR03K 1A, 94.5%

**Methods:**

Strains/species/cell line: Human peripheral lymphocytes obtained from whole blood of healthy non-smoking male donors

Metabolizing system: Rat liver microsomal enzymes (S9) were prepared in (b) (4).

Doses used in definitive study:
Experiment 1: 10, 33, 100 µg/ml broomcyclodextrine
Experiment 2: without S9: 10, 33, 66, 100, 166, and 333 µg/ml
With S9: 10, 33, 100 µg/ml

Basis of dose selection: A dose-range-finding study was conducted by the sponsor and the highest dose was determined by solubility of broomcyclodextrine. At a concentration of 100 µg/ml, broomcyclodextrine precipitated in the culture medium. In the dose-range-finding study, cells were treated for 3 hours and 24 or 48 hours with broomcyclodextrine with concentrations up to 100 µg/ml and 333 µg/ml respectively. There was no sign of cytotoxic effect indicated by decrease of mitotic index at these dosing levels while precipitation was observed at 100 µg/ml after 3 hour treatment. After 24 or 48 hour treatment, decrease in mitotic index and presence of precipitation were observed at both 100 and 333 µg/ml as shown below (extracted from the sponsor's report). The dosing levels were selected in the definitive study as shown above.

**Table 1** **Mitotic index of donor cultures treated with Broomcyclodextrine in the dose range finding test**

Period of treatment (24 h fixation time): From: 21-10-2004 To: 22-10-2004
Period of treatment (48 h fixation time): From: 27-10-2004 To: 29-10-2004

| Broomcyclodextrine concentration (µg a.i./ml) | Number of metaphases per 1000 cells | |
|---|---|---|
| | Absolute | Percentage of control |
| Without metabolic activation (-S9-mix) | | |
| 3 h exposure time, 24 h fixation time | | |
| Control [a] | 63 | 100 |
| 1 | 58 | 92 |
| 3 | 83 | 132 |
| 10 | 76 | 121 |
| 33 | 74 | 117 |
| 100 [b] | 64 | 102 |
| 24 h exposure time, 24 h fixation time | | |
| Control [a] | 39 | 100 |
| 1 | 49 | 126 |
| 3 | 41 | 105 |
| 10 | 38 | 97 |
| 33 | 44 | 113 |
| 100 [b] | 24 | 62 |
| 333 [b] | 13 | 33 |
| 48 h exposure time, 48 h fixation time | | |
| Control [a] | 47 | 100 |
| 1 | 54 | 115 |
| 3 | 46 | 98 |
| 10 | 51 | 109 |
| 33 | 48 | 102 |
| 100 [b] | 23 | 49 |
| 333 [b] | 11 | 23 |
| With metabolic activation (+S9-mix) | | |
| 3 h exposure time, 24 h fixation time | | |
| Control [a] | 54 | 100 |
| 1 | 72 | 133 |
| 3 | 45 | 83 |
| 10 | 61 | 113 |
| 33 | 52 | 96 |
| 100 [b] | 78 | 144 |

[a] Dimethyl sulfoxide
[b] Broomcyclodextrine precipitated in the culture medium.

Negative and vehicle controls: The vehicle of the test article, DMSO

Positive controls: The following mutagens were used as positive controls (extracted from the sponsor's report).

Without metabolic activation (-S9-mix):
Mitomycin C (MMC-C; CAS no. 50-07-7, (b) (4) was used as a direct acting mutagen at a final concentration of 0.5 µg/ml for a 3 h exposure period, 0.2 µg/ml for a 24 h exposure period and 0.1 µg/ml for a 48 h exposure period.

With metabolic activation (+S9-mix):
Cyclophosphamide (CP; CAS no. 50-18-0, (b) (4) was used as an indirect acting mutagen, requiring metabolic activation, at a final concentration of 15 µg/ml for a 3 h exposure period (24 h fixation time).

Incubation and sampling times: The test was carried out in duplicate (solvent cultures quadruplicate) in the presence and the absence of S9-mix. Lymphocytes were stimulated to divide in the culture medium with F10 complete culture medium containing 20% foetal calf serum, L-glutamine, penicillin/streptomycin sodium bicarbonate, supplemented with

phytohemagglutinin 1% (v/v) at 37 °C for 48 hours. Cells were then cultured in a $CO_2$ incubator with 5% ± 0.5% $CO_2$, relative humidity > 80% and a temperature of 37 °C ± 1 °C for 3 hours with the treatment of test articles, negative, or positive controls in the absence or presence of S9 in the first experiment. Cells were harvested at approximate 24 hours from the initiation of treatment. In the second experiment, same treatment duration was used for cells in the presence of S9 but harvested 48 hours after initiation of treatment. In the absence of S9, cells were treated in duplicate for 24 hours or 48 hours, and were harvested immediately. During the last 2.5 - 3 hours before harvest, cell division was arrested by addition of the spindle inhibitor colchicine at a concentration of 0.5 µg/mL. Thereafter cells were swollen by a 5 minutes treatment with hypotonic 0.56% KCl at 37 °C, and then fixed by adding methanol /acetic acid mixture (3:1 v/v). Fixed cells were dropped on wet slides which were immersed for 24h in 96% ethanol. Slides were then dried and stained for 10 - 30 min in 5% (v/v) Giemsa stain and mounted with coverslips. The mitotic index of each culture was determined by counting the number of metaphases per 1000 cells. At least 100/culture suitable metaphase cells (44-46 chromosomes/cell) were examined microscopically and analyzed for chromosome aberrations. Cells with more than 46 chromosomes observed during examination were recorded separately. Definitions of chromosome aberrations were shown in the following extracted from the sponsor's report. Chi-square test was used for statistically analysis.

| Aberration | Abbreviation | Description |
|---|---|---|
| Chromatid gap | g' | An achromatic lesion which appears as an unstained region in the chromatid arm, the size of which is equal to or smaller than the width of the chromatid and the apparently "broken" segments of the chromatid arm are in alignment. |
| Chromosome gap | g" | An achromatic lesion which appears as an unstained region in both chromatids at the same position, the size of which is equal to or smaller than the width of the chromatid and the apparently "broken" segments of the chromatids are in alignment. |
| Chromatid break | b' | An achromatic lesion in a chromatid arm, the size of which is larger than the width of the chromatid. The broken segments of the chromatid arm are aligned or unaligned. |
| Chromosome break | b" | An achromatic lesion in both chromatids at the same position, the size of which is larger than the width of the chromatid. The broken segments of the chromatids are aligned or unaligned. |
| Chromatid deletion | d' | Deleted material at the end of a chromatid arm. |
| Minute | m' | A single, usually circular, part of a chromatid lacking a centromere. |
| Double minutes | m" | Two, usually circular, parts of a chromatid lacking a centromere. |
| Dicentric chromosome | dic | A chromosome containing two centromeres. |
| Tricentric chromosome | tric | A chromosome containing three centromeres. |

*Criteria for a valid assay:*

A test will be considered to be valid if it met the following criteria according to the sponsor:

1) the number of chromosome aberrations found in the solvent control cultures should reasonably be within the laboratory historical control data range (0-6 with S9 and 0-5 without S9 aberrant cells per 100 metaphases with gaps excluded) (*note: The Sponsor provided a table with historical control data for negative/vehicle control substances*).
2) The positive control substances should produce a statistically significant (Chi-square test, $p < 0.05$) increase in the number of cells with chromosome aberrations.
3) a homogeneous response between the replicate cultures is observed

*Criteria for a positive response:*
A test article was considered positive for inducing chromosomal aberrations if the following criteria were met according to the sponsor:
1) It induced a dose-related statistically significant (Chi-square test, $p < 0.05$) increase in the number of cells with chromosome aberrations.
2) A statistically significant increase in the frequencies of the number of cells with chromosome aberrations was observed in the absence of a clear dose-response relationship.

A test substance was considered negative in the chromosome aberration test if none of the tested concentrations induced a statistically significant (Chi-square test, $p < 0.05$) increase in the number of cells with chromosome aberrations. The sponsor further indicated that the criteria were not absolute, but serve as indicators, because other factors may influence the evaluation of the data.

**Results**

Study validity: the study appears to be valid; appropriate replicates, counting methodology and criteria were used. Mitotic index is an appropriate method to establish an upper concentration limit when using peripheral blood lymphocytes or other cells in a suspension. The negative solvent control and the positive controls used produced cells with chromosomal aberrations produced chromosomal aberrations in a percentage that were within the range of the historical dataset and aberrant cells with the treatment of the positive controls was statistically significantly increased over the negative solvent control.

Study outcome:
Presence of precipitation was observed with 3-hour test article treatment at 100 µg/ml in the absence and presence of S9 in the experiment 1, and all the doses were selected for analysis. In experiment2, the results of mitotic index determination were shown below as extracted from the sponsor's report. For 24 and 48 hour treatment, the sponsor has chosen 66, 100, and 166 µg/ml dosed cells for analysis, and all the doses were analyzed for 3 hour treatment.

| Broomcyclodextrine concentration (µg a.i./ml) | Number of metaphases per 1000 cells [a] Absolute | Percentage of control |
|---|---|---|
| Without metabolic activation (-S9-mix) | | |
| 24 h exposure time, 24 h fixation time | | |
| Control [b] | 87 - 86 | 100 |
| 10 | 86 - 77 | 94 |
| 33 | 85 - 92 | 102 |
| 66 | 89 - 78 | 97 |
| 100 [c] | 63 - 52 | 66 |
| 166 [c] | 48 - 36 | 49 |
| 333 [c] | 23 - 19 | 24 |
| MMC-C; 0.2 µg/ml | 32 - 43 | 43 |
| 48 h exposure time, 48 h fixation time | | |
| Control [b] | 42 - 49 | 100 |
| 10 | 50 - 46 | 105 |
| 33 | 48 - 46 | 103 |
| 66 | 44 - 43 | 96 |
| 100 [c] | 24 - 24 | 53 |
| 166 [c] | 23 - 19 | 46 |
| 333 [c] | 7 - 14 | 23 |
| MMC-C; 0.1 µg/ml | 38 - 41 | 87 |
| With metabolic activation (+S9-mix) | | |
| 3 h exposure time, 48 h fixation time | | |
| Control [b] | 61 - 64 | 100 |
| 10 | 41 - 38 | 63 |
| 33 | 38 - 39 | 62 |
| 100 [c] | 40 - 43 | 66 |
| CP; 15 µg/ml | 42 - 41 | – [d] |
| CP; 10 µg/ml | 55 - 41 | – [d] |

[a] Duplicate cultures
[b] Dimethyl sulfoxide
[c] Broomcyclodextrine precipitated in the culture medium
[d] CP was fixed after 24 hours. Therefore, the mitotic index could not be calculated as percentage of control.

No statistically significant increase of chromosome aberration was observed at any dose levels in the absence and presence of S9, indicating a negative response in human peripheral lymphocytes. Therefore, Org25969 with its impurities in batch L is not considered to be clastogenic. The data summary extracted from the sponsor's report was shown below.

## Experiment 1

### without S9

| Conc µg/ml | DMSO (1.0% v/v) | | | 10 µg a.i./ml | | | 33 µg a.i./ml | | | 100 µg a.i./ml | | | MMC-C 0.5 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 82 | | | 82 | | | 86 | | | 74 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [a] | 0 | 0 | 0 | 2 | 0 | 2 | 2 | 0 | 2 | 3 | 2 | 5 | 18 | 17 | ***) 35 |
| No. of Cells with aberrations (- gaps) | 0 | 0 | 0 | 2 | 0 | 2 | 1 | 0 | 1 | 3 | 2 | 5 | 17 | 15 | ***) 32 |
| g' | | | | | | | 1 | | | | | | | 3 | |
| g" | | | | | | | | | | | | | 2 | | |
| b' | | | | 1 | | | | | | | | | 4 | 4 | |
| b" | | | | 1 | | | 1 | | | 2 | 1 | | 6 | 1 | |
| m' | | | | | | | | | | | | | | | |
| m" | | | | | | | | | | | | | | | |
| exch. | | | | | | | | | | | 1 | | 7 | 9 | |
| dic | | | | | | | | | | 1 | | | | 1 | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | poly | | | | | | | | | intra | |
| total aberr (+ gaps) | 0 | 0 | | 2 | 0 | | 2 | 0 | | 3 | 2 | | 19 | 19 | |
| total aberr (- gaps) | 0 | 0 | | 2 | 0 | | 1 | 0 | | 3 | 2 | | 17 | 16 | |

### with S9

| Conc µg/ml | DMSO (1.0% v/v) | | | 10 µg a.i./ml | | | 33 µg a.i./ml | | | 100 µg a.i./ml | | | CP 15 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 94 | | | 81 | | | 111 | | | 58 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [a] | 1 | 0 | 1 | 2 | 0 | 2 | 2 | 1 | 3 | 1 | 0 | 1 | 20 | 24 | ***) 44 |
| No. of Cells with aberrations (- gaps) | 1 | 0 | 1 | 2 | 0 | 2 | 1 | 1 | 2 | 1 | 0 | 1 | 19 | 21 | ***) 40 |
| g' | | | | | | | 1 | | | | | | 2 | 6 | |
| g" | | | | | | | | | | | | | | 1 | |
| b' | | | | 1 | | | | | | 1 | | | 10 | 16 | |
| b" | 2 | | | 1 | | | 1 | 1 | | 1 | | | 8 | 4 | |
| m' | | | | | | | | | | | | | | | |
| m" | | | | | | | | | | | | | | | |
| exch. | | | | | | | | | | | | | 9 | 7 | |
| dic | | | | | | | | | | | | | | 3 | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | | | | | | | | | | | |
| total aberr (+ gaps) | 2 | 0 | | 2 | 0 | | 2 | 1 | | 2 | 0 | | 29 | 37 | |
| total aberr (- gaps) | 2 | 0 | | 2 | 0 | | 1 | 1 | | 2 | 0 | | 27 | 30 | |

## Experiment 2

### without S9 (24 hour treatment)

| Conc µg/ml | DMSO (1.0% v/v) | | | 66 µg a.i./ml | | | 100 µg a.i./ml | | | 166 µg a.i./ml | | | MMC-C 0.2 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 97 | | | 66 | | | 49 | | | 43 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [a] | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 27 | 22 | ***) 49 |
| No. of Cells with aberrations (- gaps) | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 26 | 21 | ***) 47 |
| g' | | | | | | | | | | | | | 3 | 1 | |
| g" | | | | | | | | | | | | | | | |
| b' | | 1 | | | | | | | | | | | 10 | 5 | |
| b" | | | | | | | | | | | | | 6 | 4 | |
| m' | | | | | | | | | | | | | 1 | | |
| m" | | | | | | | | | | | | | 2 | | |
| exch. | | | | | | | | | | | | | 6 | 12 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | | | | poly | | | | | 2intra | 2intra | |
| total aberr (+ gaps) | 0 | 1 | | 0 | 0 | | 0 | 0 | | 0 | 0 | | 30 | 25 | |
| total aberr (- gaps) | 0 | 1 | | 0 | 0 | | 0 | 0 | | 0 | 0 | | 27 | 24 | |

### without S9 (48 hour treatment)

| Conc µg/ml | DMSO (1.0% v/v) | | | 66 µg a.i./ml | | | 100 µg a.i./ml | | | 166 µg a.i./ml | | | MMC-C 0.1 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 96 | | | 53 | | | 46 | | | 87 | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [b] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 38 | 43 | ***) 81 |
| No. of Cells with aberrations (- gaps) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 37 | 43 | ***) 80 |
| g' | | | | | | | | | | | | | 1 | | |
| g" | | | | | | | | | | | | | | | |
| b' | | | | | | | | | | | | | 18 | 29 | |
| b" | | | | | | | | 1 | | | | | 12 | 9 | |
| m' | | | | | | | | | | | | | | | |
| m" | | | | | | | | | | | | | 2 | 2 | |
| exch. | | | | | | | | | | | | | 12 | 18 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | | | | p | | | | | | intra | |
| total aberr (+ gaps) | 0 | 0 | | 0 | 0 | | 0 | 2 | | 0 | 0 | | 45 | 59 | |
| total aberr (- gaps) | 0 | 0 | | 0 | 0 | | 0 | 2 | | 0 | 0 | | 44 | 59 | |

| Conc µg/ml | DMSO (1.0% v/v) | | | 10 µg a.i./ml | | | 33 µg a.i./ml | | | 100 µg a.i./ml | | | CP 15 µg/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Culture | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B | A | B | A+B |
| Mitotic Index (%) | 100 | | | 83 | | | 82 | | | 88 | | | . [b] | | |
| No. of Cells scored | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 | 100 | 100 | 200 |
| No. of Cells with aberrations (+ gaps) [b] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 29 | 30 | ***) 59 |
| No. of Cells with aberrations (- gaps) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 28 | 30 | ***) 58 |
| g' | | | | | | | | | | | | | 2 | 2 | |
| g" | | | | | | | | | | | | | | | |
| b' | | | | | | | | | | 1 | | | 19 | 17 | |
| b" | | | | | | | | | | | | | 2 | 5 | |
| m' | | | | | | | | | | | | | 1 | 1 | |
| m" | | | | | | | | | | | | | 1 | 3 | |
| exch. | | | | | | | | | | | | | 7 | 11 | |
| dic | | | | | | | | | | | | | | | |
| d' | | | | | | | | | | | | | | | |
| misc. | | | | | | | | | | | | | | r | |
| total aberr (+ gaps) | 0 | 0 | | 0 | 0 | | 0 | 0 | | 1 | 0 | | 32 | 40 | |
| total aberr (- gaps) | 0 | 0 | | 0 | 0 | | 0 | 0 | | 1 | 0 | | 30 | 38 | |

←With S9 (3 hour treatment)

**In vivo micronucleus assays**

**Study title**: Micronucleus test in bone marrow cells of the mouse with Org 25969

**Key findings**: the in vivo micronucleus study in mice is considered negative at dose levels up to 2000 mg/kg.

**Study no.**: SV197/337387

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)
**Date of study initiation**: 10/26/2001
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: Org 25969 and impurities, Batch S, impurity of Org 25969 is (b) (4) %

**Methods:**

Strains/species/cell line: young adult male and female (6-8 wks old) NMRI BR mice rats of the SD IGS BR strain with body weight range of approximately 30.8 to 32.8 gram and 26.4 and 27.6 gram in males and females, respectively.

Doses used in definitive study: since in the dose range finding study no toxic effects were observed, a limit study was performed in which male and female mice were dosed twice with 887 mg Org 25959 (2000 mg drug substance)/kg body weight. The treatment scheme was shown below as extracted from the sponsor's report.

| Treatment | Dose (mg/kg body weight) | Sampling time (hours) | Group |
|---|---|---|---|
| Vehicle [1] | - | 24 | A |
| ORG 25969 [2] | 2 x 887 | 24, 48 | B, C |
| Cyclophosphamide | 50 | 48 | D |

[1] Physiological saline
[2] The animals treated with ORG 25969 were dosed twice within 2 hours.

Basis of dose selection: In a dose-range finding study 4 animals (2 males and 2 females) received two iv injection of 887 mg Org 25969 (2000 mg drug substance) per kg body weight within two hours, all animals showed no reaction to the treatment.

Negative controls: physiological saline

Positive controls: Cyclophosphamide (CP; CAS no. 50-18-0; (b) (4) dissolved in physiological saline dosed at 50 mg/kg

Methods: This is a limited micronucleus assay due to the lack of toxicity at dose of 2000 mg/kg. Mice (5/sex/group) were treated with the test article as well as the negative and

positive control with 2 consecutive doses with a 2 hour interval. Signs of toxicity were recorded during the study and the animals were sacrificed by cervical dislocation 24 or 48 hours after treatment of 25969. For control articles, animals treated with negative controls were sacrificed at 24-hour and positive control animals were sacrificed at 48-hour after treatment. After the sacrifice, both femurs were removed for marrow extraction from five animals in each group. For each animal, the marrow flushed from the bones was fetal calf serum. Bone marrow cell suspension was dropped on slides and fixed for 5 min in 100% methanol; two slides were prepared per animal. The slides were automatically stained using the "Wright-stain-procedure" in an "Ames" HEMA-tek slide stainer. The number of micronucleated polychromatic erythrocytes (PCE) was counted in 2000 polychromatic erythrocytes. The ratio polychromatic to normochromatic erythrocytes (MCE) was determined by counting and differentiated the first 1000 erythrocytes at the same time. Micronuclei were only counted in polychromatic erythrocytes.

*Criteria for a valid assay:* To be considered a valid test, the following conditions must be met: 1) the positive control substance induced a statistically significant (Wilcoxon Rank Sum Test, two-sided test at $P < 0.05$) increase in the frequency of micronucleated PCE in contrast to the negative vehicle control; 2) the incidence of micronucleated PCE in the negative control animals should be within the laboratory historical control data range (M: $1.2 \pm 3.7^0/_{00}$; F: $1.2 \pm 4.1^0/_{00}$).

*Criteria for a positive response:* A test article is considered to be positive in the micronucleus test if it induced a biologically relevant as well as a statistically significant (Wilcoxon Rank Sum Test; two-sided test at $P < 0.05$) increase in the increase in the frequency of micronucleated PCE in contrast to the negative control in the combined data for both sexes or in the data for male or female groups separately. A test article is considered to be positive in the micronucleus test if none of the tested concentrations or sampling time-point showed a statistically significant increase in the incidence of micronucleated PCE.

**Results**

Study validity: The study appears valid. Methodologies relating to replicate numbers, counting methods, criteria for a valid study and positive response as well as the dosing level were acceptable.

Study outcome: Animals showed no abnormality in any treatment group. The test article did not induce increase in micronucleated PCE following twice dosing of 887 mg Org 25969 (1000 drug substance)/kg. The result summary was shown below (extracted from the sponsor's report).

TABLE 2 MEAN NUMBER OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES PER 2000 POLYCHROMATIC ERYTHROCYTES AND RATIO OF POLYCHROMATIC/NORMOCHROMATIC ERYTHROCYTES.

| Group | Treatment | Dose (mg/kg body weight) | Sampling time (hours) | Number of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes (mean ± S.D.) (1) | Ratio polychromatic/ normochromatic erythrocytes (mean ± S.D.) (1) |
|---|---|---|---|---|---|
| | MALES | | | | |
| A | Solvent control | 0 | 24 | 0.6 ± 0.9 | 1.07 ± 0.25 |
| B | ORG 25969 | 2 x 887 | 24 | 0.8 ± 0.8 | 1.02 ± 0.17 |
| C | ORG 25969 | 2 x 887 | 48 | 0.2 ± 0.4 | 1.28 ± 0.21 |
| D | CP | 50 | 48 | 35.2 ± 12.6 (2) | 0.29 ± 0.13 |
| | FEMALES | | | | |
| A | Solvent control | 0 | 24 | 0.4 ± 0.5 | 1.04 ± 0.08 |
| B | ORG 25969 | 2 x 887 | 24 | 0.6 ± 1.3 | 0.84 ± 0.07 |
| C | ORG 25969 | 2 x 887 | 48 | 0.8 ± 1.1 | 1.34 ± 0.22 |
| D | CP | 50 | 48 | 16.0 ± 8.2 (2) | 0.43 ± 0.11 |

Solvent control = physiological saline
CP = Cyclophosphamide
(1) Five animals per treatment group
(2) Significantly different from corresponding control group (Wilcoxon Rank Sum Test, $P \leq 0.01$).

**2. Study title**: A micronucleus test in with Org 25969/Org 48302 in male and female Wistar rats

**Key findings**: the in vivo micronucleus study in mice is considered negative.

Document ID: NL0060385
**Study no.**: 040253S

**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands
**Date of study initiation**: 09/17/2004
**GLP compliance**: Yes
**QA reports**: yes ( X ) no ( )
**Drug, lot #, and % purity**: Org 25969/Org 48302 and impurities, Batch AE.

**Methods:**
*Note: This assay is a supplemental study of the 4-week rat study, details in study design and method can be found in the review of the study*

Strains/species/cell line: young adult male and female Wistar (HSD/Cpd: WU) rats.

Doses used in definitive study:

Grouping and dose regimen of animals of the 4-week toxicity test :

| Group | Size + Sex | | Treatment | Dose per administration ($mg \cdot kg^{-1}$) | Examination of slides (bone marrow smears) | |
|---|---|---|---|---|---|---|
| | M | F | | | M | F |
| 1 (placebo, PL1) | 20* | 20* | Org 25969 | 0 | 6 | 6 |
| 2 (low-dose, DS2) | 10 | 10 | Org 25969 | 30 | 0 | 0 |
| 3 (mid-dose, DS3) | 10 | 10 | Org 25969 | 120 | 0 | 0 |
| 4 (high-dose, DS4) | 20* | 20* | Org 25969 | 500 | 6 | 6 |
| 5 (positive control) | 6 | 6 | MMS | 50 | 6 | 6 |

* 10 animals per sex (Groups 1 and 4) were allocated to the post-dosing period.

Basis of dose selection: None

Negative controls: physiological saline

Positive controls: As shown below extracted from the sponsor's report.

| | |
|---|---|
| MMS | : Methylmethanesulfonate |
| Structural formula | : $CH_3$-O-$SO_2$-$CH_3$ |
| Supplier | : (b) (4) |
| Pharmaceutical form | : Sterile aqueous solution |
| Solvent | : Hanks Balanced Salt Solution (HBBS) |
| Supplier | : (b) (4) |
| Concentration | : 25 $mg \cdot mL^{-1}$ |

The positive control item was administered once, intraperitoneally, at a dose level of 50 $mg \cdot kg^{-1}$.

Methods: Bone marrow smears were made from the left femur of all males and females from each group sacrificed at the end of the dosing period. The left femur was freed from surrounding tissue and trimmed at a level where the marrow canal terminates. The marrow canal was flushed with 2 mL fetal calf serum from a syringe with a hypodermic needle. The marrow was collected in a centrifuge tube. Three slides were prepared per animal. The slides were stained by submersion in 0.24 mmol/L Acridine Orange solution in 1/15 mol/L Sorensen buffer for at least one minute. Bone marrow preparations of animals of the negative control group, the highest test-article dosing group and the MMS-treated group were examined microscopically. The number of micronucleated polychromatic erythrocytes (PCE) was counted in 2000 polychromatic erythrocytes. The ratio polychromatic to normochromatic erythrocytes (MCE) was determined by counting and differentiated the first 500 erythrocytes at the same time. Micronuclei were only counted in polychromatic erythrocytes.

*Criteria for a valid assay:* To be considered a valid test, the following conditions must be met: 1) The mean number of micronuclei in the negative control treated animals was within the normal control range (based on historical negative control values); 2) The positive control group (MMS) showed a positive response with respect to the number of micronuclei, exceeding the lower limit of the normal value of MMS-treated groups based on historical data of MMS-treated rats); 3) At least one dose level without signs of bone marrow cytotoxicity was examined.

*Criteria for a positive response:* A test article is considered to be positive in the micronucleus test if 1) a statistically significant increase (in comparison with the negative control group) in the number of MN-PCEs was present; 2) a relationship between the dose and the number of MN-PCEs per group was present; 3) the mean number of MN-PCEs exceeded the upper limit of the normal control range.

**Results**

Study validity: The study appears valid. Methodologies relating to replicate numbers, counting methods, criteria for a valid study and positive response as well as the dosing level were acceptable.

Study outcome: The test article did not induce increase in micronucleated PCE following treatment of 500 mg/kg drug substance containing Org 25969 and Org 48302 and impurities. The result summary was shown below (extracted from the sponsor's report).

**Table 9.1-1 Number of MNPCEs per 1000 PCEs determined in 2000 PCEs**

Male rats

| Group | Treatment | Dose ($mg \cdot kg^{-1}$) | Number of animals | Number of MNPCEs * (mean ± S.D.) |
|---|---|---|---|---|
| 1 | control | 0 | 6 | 2.1 ± 0.8 |
| 4 | Org 25969 | 500 | 6 | 2.3 ± 0.5 [a] |
| 5 | MMS | 50 | 6 | 17.2 ± 2.3 [b] |

a : value is not significantly increased from control value (p>0.05)
b : value is significantly increased from control value (p<0.001)
* Normal control range[13] : 0.452 – 3.212 MNPCEs
Lower limit of normal value of the positive control group[13] : 4.929 MNPCEs

Female rats

| Group | Treatment | Dose ($mg \cdot kg^{-1}$) | Number of animals | Number of MNPCEs * (mean ± S.D.) |
|---|---|---|---|---|
| 1 | control | 0 | 6 | 0.8 ± 0.5 |
| 4 | Org 25969 | 500 | 6 | 0.8 ± 0.5 [a] |
| 5 | MMS | 50 | 6 | 9.3 ± 4.3 [b] |

a : value is not significantly increased from control value (p>0.05)
b : value is significantly increased from control value (p<0.001)
* Normal control range[13] : 0.452 – 3.212 MNPCEs
Lower limit of normal value of the positive control group[13] : 4.929 MNPCEs

**Table 9.1-2 Ratio PCE/NCE determined in 500 erythrocytes**

Male rats

| Group | Treatment | Dose ($mg \cdot kg^{-1}$) | Number of animals | Ratio PCE/NCE * (mean ± S.D.) |
|---|---|---|---|---|
| 1 | control | 0 | 6 | 1.21 ± 0.45 |
| 4 | Org 25969 | 500 | 6 | 1.35 ± 0.45 |

* Normal control range[13] : 0.154 – 1.770 MNPCEs

Female rats

| Group | Treatment | Dose ($mg \cdot kg^{-1}$) | Number of animals | Ratio PCE/NCE * (mean ± S.D.) |
|---|---|---|---|---|
| 1 | control | 0 | 6 | 1.26 ± 0.83 |
| 4 | Org 25969 | 500 | 6 | 0.91 ± 0.45 |

* Normal control range[13] : 0.154 – 1.770 MNPCEs

### 2.6.6.5 Carcinogenicity

Carcinogenicity study is not required since the drug product is indicated for single treatment.

### 2.6.6.6 Reproductive and developmental toxicology

*Fertility and early embryonic development*

**Study title**: An intravenous fertility and early embryo-fetal development study with Org 25969 in Sprague Dawley rats

**Key study findings**:

- Slight decrease in body weight of female animals (about 10%) indicating maternal toxicity at 100 and 500 mg/kg.
- There were no change in mating index and fertility parameters including mating index, fertility index, pre-coital interval, live concepti, and gestational index in Org 25969 treated groups as compared to control.
- Macroscopic post-mortem examination found no Org 25969 treatment-related changes in male or female reproductive organs. Organ weight of these tissues in

Org 25969 treated groups were not significantly different from that of the control, Histopathological examination was not conducted.

- The NOAEL for fertility and early embryonic development is considered to be at 500 mg/kg for rat based on the results of this study.

Document ID: NL0043955
**Study no.**: 020210X
**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)
**Date of study initiation**: 08/09/2002
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch U, 78.8%

**Methods**
Doses: 0, 20, 100, and 500 mg/kg/day drug substances.

Study Design: Test article was given for 29 days before mating and during the mating period until sacrifice in males. In females, the animals were treated for 14 days prior to mating and during the mating period until sacrifice for un-mated females. Pregnant animals will be treated continuously during the pregnancy till day 5 post-coitum inclusive.

Species/strain: Sprague-Dawley rats/Crl CD® (SD) IGS Br

Number/sex/group: 22/sex/group rats were used as shown in the following table as extracted from the sponsor's report.

| Group | Number of animals | Dose-level (mg/kg/day) |
|---|---|---|
| 1 | 22 males<br>22 females | 0 |
| 2 | 22 males<br>22 females | 20 |
| 3 | 22 males<br>22 females | 100 |
| 4 | 22 males<br>22 females | 500 |

Route, formulation, volume, and infusion rate: IV bolus injection in the tail vein, 100 mg/ml drug solution diluted in 0.9% NaCl, 5 ml/kg/day

Satellite groups used for toxicokinetics: None, TK assay was not conducted in this study.

Parameters and endpoints evaluated:
*Clinical examinations:* morbidity and mortality, clinical signs, body weight, and food consumption in both male and females.

*Terminal examinations:* Each female was placed with the same male for 14 days unless mating occurred. The estrous cycle stage was determined from a fresh vaginal lavage stained with methylene blue each morning, from one week prior to the beginning of the treatment and during the mating period, until the female were mated. Mating was confirmed by checking the presence of a vaginal plug or of sperm in a vaginal lavage, and the day of confirmed mating was designated day 0 *post-coitum* (p.c.). The males were sacrificed after all females were mated. A macroscopic post-mortem examination of the thoracic and abdominal organs was performed on all animals; special attention was paid and weight was recorded on the reproductive organs (testes and epididymides). Females failed to mate were sacrificed no more than 7 days after the end of the mating period. The presence of possible implantation scars on the uterus was checked using the ammonium sulphide staining. The mated females were sacrificed on day 15 *post-coitum* and subjected to hysterectomy. The ovaries and uterus were examined to determine the number of corpora lutea, number and distribution of dead and live concepti (embryos), number and distribution of early and late resorptions, and number and distribution of implantation sites (or uterine scars). For all sacrificed females a macroscopic post-mortem examination of the principle thoracic and abdominal organs with particular attention paid to the reproductive organs was performed. Organ weight of ovaries was recorded. All the macroscopic lesions and selected tissues from male or female were sampled and kept preserved in 10% buffered formalin (testes and epididymides which were fixed in Bouin's fluid) including epididymides, seminal vesicles, vagina, ovaries, testes, prostate, uterus. However, microscopic examination was not conducted.

Data assessment: Data were expressed as group mean values ± standard deviation (body weight, food consumption, and mean number of corpora lutea, implantations, resorptions and concepti) or as proportions (pre-implantation loss, post-implantation loss, mating and fertility indices). The following calculations were performed for each group as extracted from the sponsor's report. ANOVA and Dunnett's test were used on mean values and the Fisher exact probability test was used on percentage values for statistical analysis.

$$\text{Mating index: } \frac{\text{Number of mated animals}}{\text{Number of paired animals}} \times 100$$

$$\text{Fertility index: } \frac{\text{Number of pregnant female partners}}{\text{Number of mated pairs}} \times 100$$

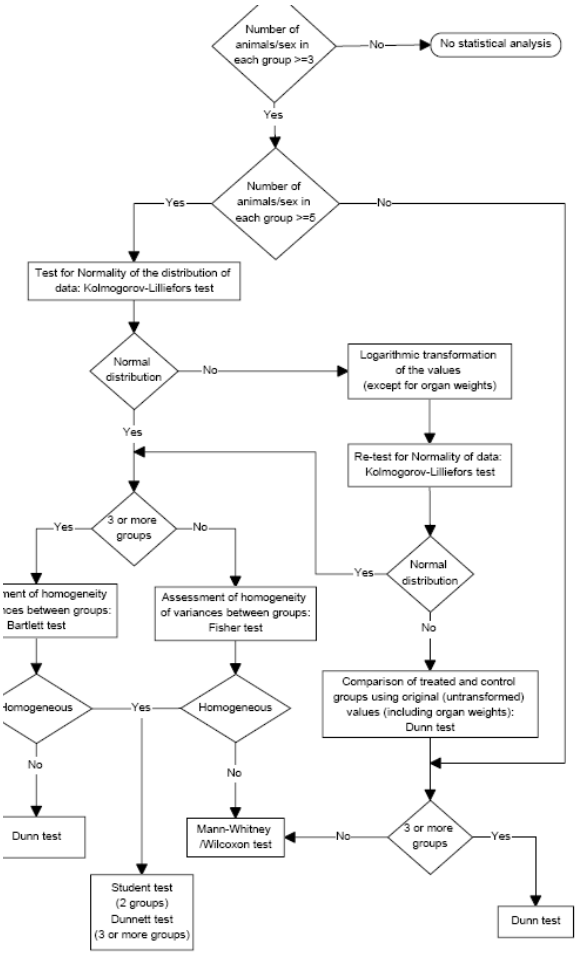
$$\text{Pre-implantation loss: } \frac{\text{Number of corpora lutea - Number of implantation sites}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Post-implantation loss: } \frac{\text{Number of implantation sites - Number of live concepti}}{\text{Number of implantation sites}} \times 100$$

$$\text{Pre-coital interval: } \text{Number of days of pairing before successful mating}$$

$$\text{Gestation index: } \frac{\text{Number of live concepti}}{\text{Number of pregnant females}}$$

ANOVA and Dunnett's test were used on mean values and the Fisher exact probability test was used on percentage values for statistical analysis. For organ weight statistical analysis, the following scheme was used (extracted from the sponsor's report)




**Results**

Mortality: Each animal was checked twice a day for mortality and signs of morbidity. No mortality was found in all groups.

Clinical signs: Animals were observed once a day for clinical signs. Piloerection and round back were noted in 2 females at 500 mg/kg from gestational days 4 or 6 until sacrifice, which were considered to be treatment-related. There were no other treatment-related effects observed during the study.

Body weight: The body weight of males was recorded twice a week during the treatment. At the end of the treatment, there was a minor decrease in body weight gain (approximately 10%) in both 100 and 500 mg/kg groups as compared to the control animals. The body weight of females was also recorded twice a week before mated.

During pregnancy, body weight of animals was recorded on day 0, 3, 6, 9, and 15 *post-coitum*. There was no change noted in any treated groups during pre-mating period. Minor decrease (approximately 10%) in body weight gain was observed in the animals of 500 mg/kg group during pregnancy.

Food consumption: Slight decrease (7%) was observed in males of 100 and 500 mg/kg; no change was noted in females at any dose.

Mating and Fertility parameters:
A summary of mating and fertility data was shown in the following as extracted from the sponsor's report. No effects were detected on mating and fertility index, pre-coital interval, estrous cycle, and gestational index after treatment at dose up to 500 mg/kg as compared to control.

| Dose-level (mg/kg/day) | 0 | 20 | 100 | 500 |
|---|---|---|---|---|
| Number of female in study | 22 | 22 | 22 | 22 |
| Mated females | 18(22)* | 21(22)* | 21(22)* | 19(22)* |
| Pregnant females | 18(22)* | 20(21)* | 21(22)* | 18(21)* |
| Non pregnant females | 0 | 1 | 0 | 1 |
| Female mating index (%) | 82(100)* | 95(100)* | 95(100)* | 86(100)* |
| Female fertility index (%) | 100 | 95 | 100 | 95 |
| Pre-coital interval (days) | 1.78 | 3.52 | 2.38 | 2.42 |
| Females with live concepti | 18 | 20 | 21 | 18 |
| Gestation index (%) | 100 | 100 | 100 | 100 |

*: pregnant females with no evidence of mating are taken into consideration for calculation of the mating and fertility indexes.

Necropsy:
There were no treatment-related change found in the weight of reproductive organs of male (testes and epididymides) and female (ovaries). Macroscopic post-mortem examination did not find treatment-related changes in the reproductive organs as well as other tissues in males and females. Histopathological examination was not conducted in this study.

Hysterectomy:
The results of hysterectomy showed no significant difference in the corpora lutea, implantation sites, and concepti between control and groups of test article treated, indicating there was no increased pre- and post-implantation loss as shown in the following (extracted from the sponsor's report).

| Dose (mg/kg/day) | | 0 | 20 | 100 | 500 |
|---|---|---|---|---|---|
| Pregnant Females Alive at Term | N | 18 | 20 | 21 | 18 |
| with all Dead Conceptii | N | 0 | 0 | 0 | 0 |
| with Live Conceptii | N | 18 | 20 | 21 | 18 |
| Corpora Lutea | TOTAL | 331 | 365 | 359 | 343 |
| No. per animal | MEAN | 18.4 d | 18.3 | 17.1 | 19.1 |
| | S.D. | 2.6 | 3.1 | 4.1 | 2.7 |
| Implantation Sites | TOTAL | 286 | 320 | 322 | 290 |
| No. per animal | MEAN | 15.9 d | 16.0 | 15.3 | 16.1 |
| | S.D. | 1.9 | 1.9 | 3.6 | 1.7 |
| Preimplantation Loss | TOTAL | 45 f | 45 | 37 | 53 |
| | % | 13.6 | 12.3 | 10.3 | 15.5 |
| Conceptii | N | 273 | 306 | 311 | 274 |
| No. per animal | MEAN | 15.2 d | 15.3 | 14.8 | 15.2 |
| | S.D. | 1.9 | 1.8 | 3.5 | 1.6 |
| Alive | % | 100.0 | 100.0 | 99.7 | 100.0 |
| Dead | % | 0.0 | 0.0 | 0.3 | 0.0 |
| Live Conceptii | N | 273 f | 306 | 310 | 274 |
| % of implantation sites | | 95.5 | 95.6 | 96.3 | 94.5 |
| No. per animal | MEAN | 15.2 d | 15.3 | 14.8 | 15.2 |
| | S.D. | 1.9 | 1.8 | 3.5 | 1.6 |
| Dead Conceptii | N | 0 f | 0 | 1 | 0 |
| % of implantation sites | | 0.0 | 0.0 | 0.3 | 0.0 |
| No. per animal | MEAN | 0.0 d | 0.0 | 0.0 | 0.0 |
| | S.D. | 0.0 | 0.0 | 0.2 | 0.0 |
| Postimplantation Loss | TOTAL | 13 f | 14 | 12 | 16 |
| % of implantation sites | | 4.5 | 4.4 | 3.7 | 5.5 |
| No. per animal | MEAN | 0.7 d | 0.7 | 0.6 | 0.9 |
| | S.D. | 0.7 | 0.7 | 0.7 | 0.8 |

Statistical key: d=ANOVA + Dunnett-test f=Fishers exact test

*Embryofetal development*

**Study title**: An intravenous embryo-fetal development with Org 25969 in Sprague Dawley rats

**Key study findings**:

- Pregnant rats were treated with Org 25969 (batch U) at 0, 20, 100, and 500 mg/kg from GD6-17.
- Minimal maternal toxicity was observed at 100 and 500 mg/kg represented by minimal decrease in body weight gain (approximately 7%) during the first-week treatment (GD 6-9) and decrease in food consumption (approximately 7%) during the treatment period (GD6-18).
- There were no treatment-related changes in pre- and post-implantation loss, and live fetus numbers in hysterectomy assay.
- There were no treatment-related changes in offspring except increased dilated renal pelvis in animals of 500 mg/kg (6.5%) as compared to control (0.7%) - examination was not conducted at 30 and 150 mg/kg). However, this change was not considered to be adverse since this change is considered a variation but not abnormality and the recorded value (6.5%) was within the historical range (0-8.5%; mean = 4.9%).

- TK analysis was not conducted in this study, but data from PK/TK analysis in general toxicity and PK studies in this species is considered to be appropriate to estimate the systemic exposure in pregnant rats.
- Overall, the primary change was minor decrease in body weight in maternal animals which is monitorable and considered to be of little toxicology significance.

**Study no.**: 020211X
Document ID: 0043173
**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)
**Date of study initiation**: 07/08/2002
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969 and impurities, Batch U, impurity of 25969 is (b) (4) %

**Methods**

Doses: 0, 20, 100, and 500 mg/kg/day drug substances.

Study Design: Test article was given form day 6 to day 17 *post-coitum* (GD6-17) inclusive

Species/strain: mated female Sprague-Dawley rats/Crl CD® (SD) IGS Br of 10-12 weeks old with a mean body weight of 258 g (195 – 340 g)

Number/sex/group: 22 mated female rats/ group were used as shown in the following table (extracted from the sponsor's report).

| Group | Number of mated females | Dose-level (mg/kg/day) | Animal numbers |
|---|---|---|---|
| 1 | 22 | 0 | A23551 to A23572 |
| 2 | 22 | 20 | A23573 to A23594 |
| 3 | 22 | 100 | A23595 to A23616 |
| 4 | 22 | 500* | A23617 to A23638 |

*: 500 mg/kg/day is the maximum feasible dose based on a solution of 100 mg/mL and a maximum dosing volume (for intravenous bolus route) of 5 mL/kg.

Route, formulation, volume, and infusion rate: IV bolus injection in the tail vein, 100 mg/ml drug solution diluted in 0.9% NaCl, 5 ml/kg/day

Satellite groups used for toxicokinetics: none. TK analysis has been conducted in single- and repeat-dose general toxicity and toxicokinetics studies. These studies demonstrated that systemic Org 25969 exposure was increased in proportion to the increase of dosing level and there was no accumulation observed after 4-week treatment. Therefore, TK

data from general toxicity and toxicokinetics studies will be informative to estimate the systemic exposure in pregnant rats.

Parameters and endpoints evaluated:

*Clinical examinations:* morbidity and mortality, clinical signs, body weight, and food consumption.

*Terminal examinations of dams:* On day 20 *post-coitum*, all the females were sacrificed. The weight of the gravid uterus was recorded for each pregnant female (with at least one live fetus) at hysterectomy. The weight of ovaries of all females was also recorded. The ovaries and uterus were examined to determine the number of corpora lutea, number and distribution of dead and live fetuses, number and distribution of early and late resorptions, and the number and distribution of implantation sites uterine scars). After hysterectomy, macroscopic post-mortem examination of the principle thoracic and abdominal organs, and a gross evaluation of placentas was also performed. The macroscopic lesions from all dams were collected and preserved in 10% buffered formalin.

Fetuses were examined for body weight, sex ratio, and external alterations including all visible structures, surfaces and orifices of all groups. In addition, skeletal and soft tissue malformation and variation were examined at control and high dose group; animals of low-dose and mid-dose group were not examined, as described by the sponsor (extracted from the sponsor's report). The sex of each live fetus was determined at the time of evisceration (after fixation in alcohol) or at the time of the Wilson sectioning.

2.6.3 Soft tissue examination

Approximately half of the live fetuses per litter of all groups was fixed with Harrisson's fluid. A detailed soft tissue examination was performed in fetuses of the control and high-dose groups, using a free-hand serial sectioning technique (Wilson technique) which included the observation of all the organs and structures of the head, neck, thorax and abdomen.

2.6.4 Skeletal examination

The remaining live fetuses of all groups were fixed with ethyl alcohol and eviscerated. The skeleton of all fetuses was stained with alizarin red S (modified Dawson technique) and a detailed examination of the skeleton was performed in fetuses of the control and high-dose groups. This examination included the observation of all the bone structures of the head, spine, rib cage, pelvis and limbs in order to assess the degree of ossification.

Data assessment: Data were expressed as group mean values ± standard deviation (maternal body weight, food consumption, fetal body weight, and number of corpora lutea, implantations, fetuses, and resorption) or as proportions (pre-implantation loss, post-implantation loss, fetal findings). The following calculations were performed for each group as extracted from the sponsor's report. ANOVA and Dunnett's test were used on mean values and the Fisher exact probability test was used on percentage values for statistical analysis.

The pre-implantation loss: $\frac{\text{Number of corpora lutea - Number of implantation sites}}{\text{Number of corpora lutea}} \times 100$

The post-implantation loss: $\frac{\text{Number of implantation sites - Number of live fetuses}}{\text{Number of implantation sites}} \times 100$

Fetal or litter incidence: $\frac{\text{Number of fetuses or litters with a particular finding}}{\text{Total number of fetuses or litters examined}} \times 100$

Mean proportion of affected fetuses: $\frac{\text{Sum of proportion of fetuses affected in each litter}}{\text{Total number of litters examined}}$

**Results**

Mortality (dams): There was no death in any group

Clinical signs (dams): Blood in vagina was observed in 2, 3, 2, and 7 animals of control, 20, 100, and 500 mg/kg, which was not considered to be treatment-related by the sponsor. The sponsor indicated that it might be the consequence of the restraining device disturbing the pregnant animals (*Reviewer's comment:* These changes were not seen in the segment 1 and segment 3 rat studies, indicating no relevance to Org 25969 treatment). There were no other clinical signs observed. In addition, there was no abortion or total resorption in any group.

Body weight (dams): there was a minimal decrease in body weight gain during the first week of the treatment (GD6-9) (7%) at 100 and 500 mg/kg. No remarkable treatment-related change in body weight in animals of drug-treated observed as compared to control during the second weight of treatment.

Food consumption (dams): minimal decrease in food consumption was observed during the treatment period (GD6-18: around 7%) at 100 and 500 mg/kg as compared to control. There was no change in food consumption at 30 mg/kg. This change was generally correlated with the minimal body weight gain increase at these treated groups.

Terminal and necroscopic evaluations of dams: there were no treatment-related findings observed in drug-treated groups as compared to control. In addition, there were no changes in organ weight of ovaries and uterus between control and treated groups. There were 2 animals (non-pregnant) with dilation and serous content in uterine horn at 100 mg/kg; there were similar changes in pregnant animals of other groups including control. This change is not likely to be treatment-related. The sponsor indicated this is common in rats of this strain and age.

Hysterectomy:
The results of hysterectomy was summarized by the sponsor and the table was shown in the below (extracted from the sponsor's report), indicating no increased pre- and post-implantation loss, decrease in live fetus numbers. It should be noted significant increase

in pre-implantation loss in 100 mg/kg was observed as compared to control (19.1% vs. 12.9%). However, similar change was not seen at 20 and 500 mg/kg; this change, therefore, is not likely to be treatment-related.

| Dose (mg/kg/day) | | 0 | 20 | 100 | 500 |
|---|---|---|---|---|---|
| Pregnant Females Alive at Term | N | 22 | 21 | 19 | 20 |
| with Total Resorptions | N | 0 | 0 | 0 | 0 |
| with all Dead Fetuses | N | 0 | 0 | 0 | 0 |
| with Live Fetuses | N | 22 | 21 | 19 | 20 |
| Corpora Lutea | TOTAL | 334 | 308 | 293 | 294 |
| No. per animal | MEAN | 15.2 d | 14.7 | 15.4 | 14.7 |
| | S.D. | 2.6 | 1.8 | 2.9 | 2.3 |
| Implantation Sites | TOTAL | 291 | 274 | 237 | 261 |
| No. per animal | MEAN | 13.2 d | 13.0 | 12.5 | 13.1 |
| | S.D. | 2.0 | 1.7 | 4.1 | 1.9 |
| Preimplantation Loss | TOTAL | 43 f | 34 | 56* | 33 |
| | % | 12.9 | 11.0 | 19.1 | 11.2 |
| Fetuses | N | 282 | 264 | 228 | 253 |
| No. per animal | MEAN | 12.8 d | 12.6 | 12.0 | 12.6 |
| | S.D. | 2.1 | 2.2 | 4.2 | 2.0 |
| Alive | % | 100.0 | 100.0 | 100.0 | 100.0 |
| Dead | % | 0.0 | 0.0 | 0.0 | 0.0 |
| Live Fetuses | N | 282 f | 264 | 228 | 253 |
| % of implantation sites | | 96.9 | 96.4 | 96.2 | 96.9 |
| No. per animal | MEAN | 12.8 d | 12.6 | 12.0 | 12.6 |
| | S.D. | 2.1 | 2.2 | 4.2 | 2.0 |
| Dead Fetuses | N | 0 f | 0 | 0 | 0 |
| % of implantation sites | | 0.0 | 0.0 | 0.0 | 0.0 |
| No. per animal | MEAN | 0.0 | 0.0 | 0.0 | 0.0 |
| | S.D. | 0.0 | 0.0 | 0.0 | 0.0 |

Statistical key: d=ANOVA + Dunnett-test f=Fishers exact test * = p<0.05

Offspring:
There was no change in fetal body weight, and no remarkable difference in sex ratio in drug-treated groups as compared to control. There was no treatment-related external and skeletal malformation and variation observed in drug-treated group as compared to control. In addition, there was no soft tissue malformation observed in any dose group.

The results indicated increased incidence of dilated renal pelvis in animals of 500 mg/kg dosing group (6.5%) as compared to control (0.7%). The sponsor indicated that the value of variation (6.5%) at 500 mg/kg was still in the range of historical control data (0 – 8.5%; mean = 4.9%) and examination in mid and low dose groups were not examined. This change, thus, is not considered to be adverse.

**Study title**: An intravenous embryo-fetal development study, including toxicokinetics, with Org 25969 in New Zealand white rabbits

**Key study findings**:

- Animals were treated with Org 25969 (batch U) at 0, 20, 65, and 200 mg/kg/day during organogenesis from GD 6-18.
- Dose-dependent decrease in body weight gain in dam animals were observed during the treatment period at 20 (↓5.4%), 65 (↓16%) and 200 mg/kg (↓31%), likely due to decreased food consumption. This change was reversible as

represented by compensatory increase in body weight gain during treatment free period from GD 19 to 29.

- Macroscopic examination in dams indicated changes in gonads (conjunctive tissue, periovarian region: serous cysts) in 1/20 animals of all drug-treated groups, but not in control. This change was not dose-dependent, and was reportedly commonly recorded in animals of this strain at this age, indicating incidental change.
- Decrease in offspring body weight was observed in all groups of animals with approximately 5%, 10%, and 14% at 20, 65, and 200 mg/kg, likely due to maternal toxicity.
- Skeletal examination showed increased skeletal variation including increased percentage of incomplete ossification of 1st to 4th sternebra and 6th sternebra, and unossified 1st metacarpal at 200 mg/kg (examination was not conducted at 20 and 65 mg/kg), likely to be at least partly due to the lower fetal weight in this group. These changes are within historical control value range except the incomplete ossification of 1st and 4th sternebra (12.6% vs. 1.4 – 11% in historical control). Overall, these skeletal variations are considered to be of no major toxicological significance.
- Skeletal examination also showed thoracic hemivertebra and misshapen thoracic vertiebra, misaligned sternebra, and fused rib in one fetus at high-dose group, which was not seen in the control. This change is considered to be incidental since similar changes were reported in normal animals of historical control.
- Overall, the major change was decrease in body weight on both maternal and offspring animals, which is monitorable and limited toxicology significance. The NOAEL in maternal and offspring animals is considered to be at 200 mg/kg.

**Study no.**: 020226X
Document No.: NL0043447
**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)
**Date of study initiation**: 07/29/2002
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969 and impurities, Batch U, impurity of 25969 is (b) (4) %

**Methods**

Doses: 0, 20, 65, and 200 mg/kg/day drug substances.

Study Design: Test article was given during the organogenesis from day 6 to day 18 *post-coitum* inclusive (GD 6-18), and dams were sacrificed on day 29 post-coitum for examinations. On Day 14 post-coitum (GD 14), blood samples were collected from 10 animals of each treated group for toxicokinetics assay.

Species/strain: mated female rabbits/KBL New Zealand White of approximately 18-20 weeks old with mean body weight of 3554 g (range: 3155 g to 4080 g)

Number/sex/group: 20/sex/group rats were used as shown in the following table (extracted from the sponsor's report).

| Group | Number of mated females | Dose-level (mg/kg/day) | Animal numbers |
|---|---|---|---|
| 1 | 20 | 0 | A30001 to A30020 |
| 2 | 20 | 20 | A30021 to A30040 |
| 3 | 20 | 65 | A30041 to A30060 |
| 4 | 20 | 200* | A30061 to A30080 |

*: maximum injectable dose-level by bolus injection (100 mg/mL and 2 mL/kg IV).

Route, formulation, volume, and infusion rate: IV bolus injection in the ear vein, 100 mg/ml drug solution diluted in 0.9% NaCl, 2 ml/kg/day.

Satellite groups used for toxicokinetics: None. Ten animals of each treated group were selected for TK analysis.

Parameters and endpoints evaluated:

Toxicokinetics assay: On day 14 post-coitum, approximately 1 ml blood samples were collected from 10 animals to determine plasma levels of Org 259696 after dosing at the following time-points:

- 5 females at 0 (pre-dose), 0.16, 0.75 and 3 hours post-dosing,
- 5 females at 0.083, 0.33, 1.5 and 6 hours post-dosing.

*Clinical examinations:* morbidity and mortality, clinical signs, body weight, and food consumption.

*Terminal examinations:* On day 29 *post-coitum*, all the females were sacrificed. The weight of the gravid uterus was recorded for each pregnant female (with at least one live fetus) at hysterectomy. The weight of ovaries was also recorded. The ovaries and uterus were examined to determine the number of corpora lutea, number and distribution of dead and live fetuses, number and distribution of early and late resorptions, and the number and distribution of implantation sites uterine scars). After hysterectomy, macroscopic post-mortem examination of the principle thoracic and abdominal organs, and a gross evaluation of placentas was also performed. The macroscopic lesions from all dams were collected and preserved in 10% buffered formalin.

Fetuses were examined for body weight, sex ratio, and external alterations including all visible structures, surfaces and orifices of all groups. Skeletal and soft tissue malformation and variation were examined. All fetuses were subjected to fresh dissection of the soft tissue, which included the observation of all the organs and structures of the neck, thorax and abdomen. The heads of half of the fetuses were removed and fixed in Harrisson's fluid. Evaluation of the brain, eyes, nasal passages, and

tongue was performed in the control and the high dose-groups. The brains of the other half of the fetuses were fixed in Bouin's fluid, and then horizontally sectioned and examined for abnormalities. The carcasses of the fetuses were fixed with ethyl alcohol. Examination of the skeleton was conducted in the control and the high dose group after staining with alizarin red S including the observation of all the bone structures of the head, spine, rib cage, pelvis and limbs.

Data assessment: Data were expressed as group mean values ± standard deviation (maternal body weight, food consumption, fetal body weight, and number of corpora lutea, implantations, fetuses, and resorptions) or as proportions (pre-implantation loss, post-implantation loss, fetal findings). The following calculations were performed for each group as extracted from the sponsor's report. ANOVA and Dunnett's test were used on mean values and the Fisher exact probability test was used on percentage values for statistical analysis.

The pre-implantation loss: $\frac{\text{Number of corpora lutea - Number of implantation sites}}{\text{Number of corpora lutea}} \times 100$

The post-implantation loss: $\frac{\text{Number of implantation sites - Number of live fetuses}}{\text{Number of implantation sites}} \times 100$

Fetal or litter incidence: $\frac{\text{Number of fetuses or litters with a particular finding}}{\text{Total number of fetuses or litters examined}} \times 100$

Mean proportion of affected fetuses: $\frac{\text{Sum of proportion of fetuses affected in each litter}}{\text{Total number of litters examined}}$

## Results

Toxicokinetics: The results were summarized and shown below (extracted from the sponsor's report):

| Dose-level (mg/kg/day) | 20<br>Org 25969 | 65<br>Org 25969 | 200<br>Org 25969 |
|---|---|---|---|
| Day of sampling | 14 p.c. | 14 p.c. | 14 p.c. |
| $AUC_{0.08-6}$ (µg.h/mL) | 85.9 | 362.3 | 1238.4 |
| $AUC_{0-6}$ (µg.h/mL) | 96.5 | 395.3 | 1367.2 |
| AUC to-∞ (µg.h/mL) | 100.5 | 403.7 | 1400.8 |
| $AUC_{0.08b-6}$/dose | 4.29 | 5.57 | 6.19 |
| $AUC_{0-t}$/dose | 4.82 | 6.08 | 6.84 |
| $AUC_{0-\infty}$/dose | 5.02 | 6.21 | 7.0 |
| $C_0$*(µg/mL) | 117 | 325.8 | 1583.8 |
| $C_{0.08}$ (µg/mL) | 137 ± 17 | 479 ± 73 | 1519 ± 231 |
| $C_0$*/dose | 5.85 | 5.01 | 7.92 |
| $C_{0.08}$/dose | 6.85 | 7.37 | 7.59 |
| $T_{1/2}$ (h) | 0.650 | 1.107 | 1.140 |
| Clearance (L/min) | 0.0033 | 0.0027 | 0.0024 |
| Vz (L) | 0.1867 | 0.2572 | 0.2348 |

*p.c.: post-coitum.*

Both $C_{max}$ and AUC level increased in a dose-proportional manner. Similarly, terminal half-life, clearance and the volume of distribution parameters were in the same order for each test treated group and thus were independent of dose-level increase.

Mortality (dams): There was no death in any group

Clinical signs (dams): No remarkable treatment-related clinical signs observed. There was no abortion or total resorption in any group.

Body weight (dams): Decrease in body weight gain was observed in all treated groups as compared to control during treatment period (day 6-19 *post-coitum*) as shown in the following table. A compensatory increase in body weight gain was observed during the treatment-free period in a reverse dose-dependent manner, indicating reversibility of this change.

| Body Weight Gain | Control (g) | 20 mg/kg | 65 mg/kg | 200 mg/kg |
|---|---|---|---|---|
| Day 6-19 | 190 | 180 (↓5.3%) | 159 (↓16%) | 132 (↓31%) |
| Day 19-29 | 138 | 160 (↑16%) | 153 (↑11%) | 147 (↑6.5%) |
| Day 6-29 | 328 | 340 (↑3.7%) | 312 (↓4.9%) | 279 (↓15%) |

Food consumption (dams): There was a decrease in food consumption at the high dose group during the treatment period (14%) especially during the last 3 days of treatment (day 15-19: ↑30%). Slight decrease was also observed at 65 mg/kg (10%) during treatment. Food consumption decrease was partially recovered at 200 mg/kg. This change was correlated with decreased body weight gain in these groups.

Terminal and necroscopic evaluations of dams: Macroscopic examination indicated changes in gonads (conjunctive tissue, periovarian region: serous cysts) in 1/20 animals of all drug-treated groups, but not in control. This change was not dose-dependent; the animals at low-dose and high-dose group with this change were non-pregnant, but the animal at mid-dose group was pregnant. The sponsor indicated this finding was commonly recorded in animals of this strain at this age. The animal with this change in high dose group had decreased ovary weight (50%) as compared to animals of the same group as well as other groups. There were no other macroscopic findings reported in control and drug-treated groups. In addition, there were no significant difference in the mean organ weight of ovaries and uterus between control and treated groups.

Hysterectomy:
The results of hysterectomy was summarized by the sponsor and the table was shown in the below (extracted from the sponsor's report), indicating no increased pre- and post-implantation loss, decrease in live fetus numbers.

| Dose (mg/kg/day) | | 0 | 20 | 65 | 200 |
|---|---|---|---|---|---|
| Pregnant Females Alive at Term | N | 19 | 19 | 20 | 19 |
| with Total Resorptions | N | 0 | 0 | 0 | 0 |
| with all Dead Fetuses | N | 0 | 0 | 0 | 0 |
| with Live Fetuses | N | 19 | 19 | 20 | 19 |
| Corpora Lutea | TOTAL | 193 | 201 | 236 | 227 |
| No. per animal | MEAN | 10.2 d | 10.6 | 11.8 | 11.9 |
| | S.D. | 2.6 | 2.7 | 3.6 | 2.0 |
| Implantation Sites | TOTAL | 164 | 177 | 213 | 199 |
| No. per animal | MEAN | 8.6 d | 9.3 | 10.6 | 10.5 |
| | S.D. | 2.8 | 2.2 | 4.0 | 2.6 |
| Preimplantation Loss | TOTAL | 29 f | 24 | 23 | 28 |
| | % | 15.0 | 11.9 | 9.7 | 12.3 |
| Fetuses | N | 158 | 172 | 205 | 191 |
| No. per animal | MEAN | 8.3 d | 9.1 | 10.3 | 10.1 |
| | S.D. | 2.5 | 2.1 | 3.5 | 2.4 |
| Alive | % | 100.0 | 100.0 | 100.0 | 100.0 |
| Dead | % | 0.0 | 0.0 | 0.0 | 0.0 |
| Live Fetuses | N | 158 f | 172 | 205 | 191 |
| % of implantation sites | | 96.3 | 97.2 | 96.2 | 96.0 |
| No. per animal | MEAN | 8.3 d | 9.1 | 10.3 | 10.1 |
| | S.D. | 2.5 | 2.1 | 3.5 | 2.4 |
| Dead Fetuses | N | 0 f | 0 | 0 | 0 |
| % of implantation sites | | 0.0 | 0.0 | 0.0 | 0.0 |
| No. per animal | MEAN | 0.0 | 0.0 | 0.0 | 0.0 |
| | S.D. | 0.0 | 0.0 | 0.0 | 0.0 |

Statistical key: d=ANOVA + Dunnett-test f=Fishers exact test

Offspring:

The sex ratio was similar in all groups and close to 50% (range 44-56%). Dose-dependent decrease in body weight was observed after drug administration as shown below.

| Fetus body weight | control | 20 mg/kg | 65 mg/kg | 200 mg/kg |
|---|---|---|---|---|
| M&F | 39.0 | 36.9 (↓5.4%) | 35.5 (↓9.5%) | 33.6 (↓14%)* |
| M | 39.2 | 37.2 (↓5.1%) | 35.3 (↓10%)* | 34.2 (↓13%)* |
| F | 39.2 | 36.5 (↓6.9%) | 36.1 (↓7.9%) | 33.8 (↓14%)* |

*: $p<0.05$;

There was no treatment-related external and soft tissue malformation and variation. Skeletal examination found thoracic hemivertebra and misshapen thoracic vertiebra, misaligned sternebra, and fused rib in one fetus at high-dose group, which was not seen in the control. However, due to the low incidence and spontaneous incidence in normal animals, this finding shall not be considered to be treatment-related. Statistically significant increase in the fetal incidence of the variations observed in the high-dose group as compared to control and was shown in the following (extracted from the sponsor's report).

| Dose-level (mg/kg/day) | 0 | 200 |
|---|---|---|
| - *Incomplete ossification of 1st to 4th sternebra* | | |
| . Fetal incidence (%) | 1.9<br>*Historical control data: 1.4 to 11%* | 12.6*** |
| -*Incomplete ossification of 6th sternebra* | | |
| . Fetal incidence (%) | 17.1<br>*Historical control data: 8.8 to 49%* | 29.3** |
| - *Unossified 1st metacarpal* | | |
| . Fetal incidence (%) | 4.4<br>*Historical control data: 1.5 to 12.1%* | 9.9 |

**: p<0.01
***: p<0.001

The sponsor indicated that these changes "represented a delay in ossification which was probably in part secondary to the lower fetal weight recorded at this dose-level as a consequence of the decrease in maternal body weight." This reviewer agreed.

*Perinatal and postnatal development*

**1. Study title**: Study for effects on pre- and postnatal development including maternal function in the Sprague Dawley rat

**Key study findings**:

- Pregnant female animals were treated with Org 25969 at 0, 30, 120, and 500 mg/kg from GD6 to PND21 which covered the organogenesis to weaning.
- $F_0$ female rats did not show remarkable treatment-related changes in in-life observations during the treatment-period and macroscopic necropsy observation after termination of treatment.
- Mating and fertility of F0 female was not affected during the treatment such as duration of gestation, number of implantation sites and implantation loss.
- Postnatal loss of $F_1$ pups appeared to be increased during PND0-4 in the high-dose (500 mg/kg) and mid-dose group (120 mg/kg).
    - Postnatal loss during day 0 to 4 post partum was found to be increased in the high dose group compared to control (19.2% vs. 10.4%), but not in mid-dose group (11.6%). Total litter loss during GD0-4 was noted for 2, 2, and 3 dams in groups 1, 3, and 4, respectively. However, in group 3, two dams had extremely small little size (No. 46: 4; No. 51: 2) as compared to others; the implantation sites of these two animals were 18 and 13, respectively. Therefore, cannibalization was likely to occur in these two dams immediately after birth before the first litter check. In addition, there was one litter loss in group 2 and group 3 before the first litter check after delivery, which is most likely due to cannibalization since body weight of these two animals were comparable with others. These two animals were not included for reproduction analysis. Taken all these in consideration, there appeared to be increased postnatal loss in group 3 (120 mg/kg) and group 4 (500 mg/kg).
    - Histopathological examination of heart, lungs, liver, and kidneys of the F1 pups of dead or partially cannibalized did not identify the mechanism of postnatal loss during PND0-4.
- There were no remarkable abnormalities observed in the surviving $F_1$ pups during lactation period. In addition, there were no remarkable treatment-related changes

in F1 surviving animals in physical development, behavior evaluation, necropsy, and reproduction evaluation.

- Overall, the primary change was postnatal $F_1$ pup loss during PND0-4. The mechanism was unknown. The sponsor indicated that the postnatal loss during PND0-4 may be due to treatment-related increase in cannibalization. This reviewer agreed this possibility shall not be excluded; however, additional follow-up studies may be needed.
- The NOAEL for postnatal development was considered at 30 mg/kg.

**Study no.**: 853724
Document ID: 0058898
**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4) *
* The histopathological evaluation was conducted in N.V. Organon, Department of TDD, NL 5340 BH Oss/The Netherlands
**Date of study initiation**: 05/19/2004
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch W (batch TX018), 87.5%

**Methods**
Doses: 0, 30, 120, 500 mg/kg drug substance

Species/strain: mated female Sprague Dawley rat /Ico: OFA-SD of approximately 10 weeks age with body weight of 224-289 g

Number/sex/group: 22/group for groups 1-4, 5 in group 5, as shown below (extracted from the sponsor's report).

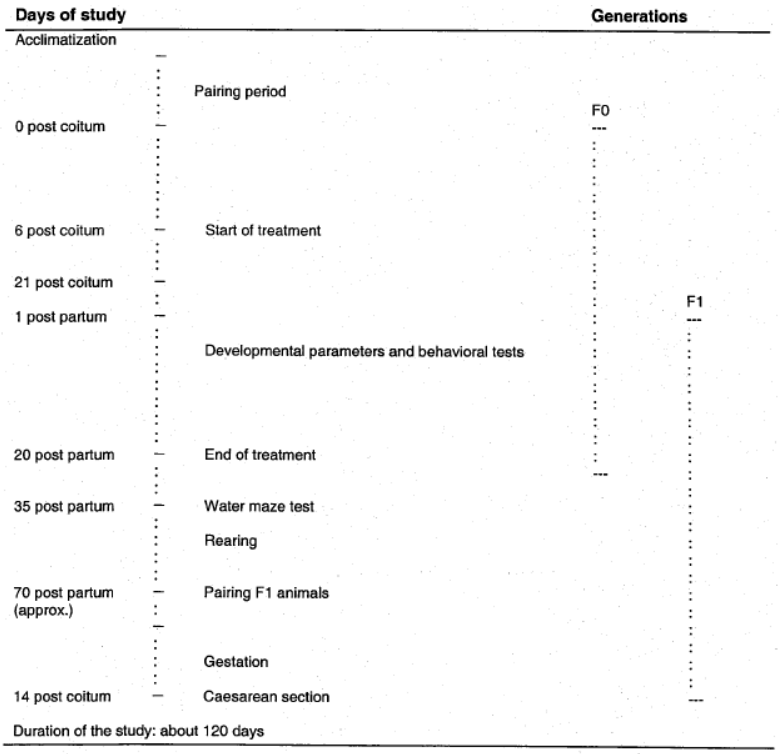
| Group | Female Numbers | Dose mg/kg body weight |
|---|---|---|
| 1 | 1 - 22 | 0 (vehicle control) |
| 2 | 23 - 44 | 30 |
| 3 | 45 - 66 | 120 |
| 4 | 67 - 88 | 500 |
| 5 | 89 - 93 | 0 (vehicle control) |

Route, formulation, volume, and infusion rate: intravenously injection, 100 mg/ml drug substance diluted with 0.9% NaCl solution, 5 ml/kg, 2.5 ml/min

Satellite groups used for toxicokinetics: none

Study design: The study schedule was summarized in the following (extracted from the sponsor's report). The test article was administered intravenously once daily from day 6 post coitum (GD6) through to day 21 post partum (PND21) for groups 1 -4 at 0, 30, 120, and 500 mg/kg, respectively. Parameters and endpoints of F0 and F1 generation were examined. Group 5 consisted of 5 pregnant females. Pups of these females were used as age- and gender-matched controls for pups of group 2 – 4 which died or had to be

euthanized during the first 5 days post partum. These controls were deemed necessary for histopathological evaluation of deceased pups, since organs of very young animals are difficult to evaluate without age-matched controls.




Parameters and endpoints evaluated: All animals were checked twice daily for mortalities and clinical signs/symptoms. On days 0 – 4 post partum (PND0-4), clinical signs and mortalities were recorded 3 times daily. Body weight and food consumption were also examined. All dams (F0) were allowed to give birth and rear there litters (F1 pups) up to day 21 post partum. The offspring was examined after completion of delivery and through the lactation period. If birth did not occur on the expected date, the female was sacrificed and examined about 3 days later. If no implantation sites were evident, the uterus was placed in an aqueous solution of ammonium sulphide to accentuate possible hemorrhagic areas of implantation sites. The behavior of the dams during parturition was observed. The duration of gestation was calculated. Nesting and nursing behavior of the dams were observed daily.

For F1 offspring, observations including sex of pups and number of newborns with gross abnormalities, number of missing (cannibalized) or dead pups, number of live and dead pups after parturition and on days 1, 2, 3, 4, 7, 14, and 21 of lactation. Abnormal findings in the pups (daily), individual pup weights and mean body weights by sex on days 0, 1, 4, 7 14, 28, and 35 post partum were recorded. In addition, developmental parameters were also monitored for all F1 pups, including pinna unfolding, incisor eruption, onset of coat development, eye opening, testes descent, cleavage of balano-preputial gland, vaginal opening. The following behavior tests were performed: righting

reflex, photophobotaxis, cliff avoidance, palmar grasp ability, negative geotaxis, exploratory locomotor pattern, direct papillary reflex, hearing ability, water maze test.

F1 animals were paired at about 10 weeks old, and F1 females were sacrificed on day 14 post coitum and necropsy reproduction data and any macroscopic abnormalities were recorded.

Termination and necropsy:
Animals which survived to scheduled necropsy were sacrificed, and necropsied with macroscopic abnormalities recorded as detailed in the following. Organs/tissues with gross lesions of the F0, F1 parent and F1 pups with malformations or died during the study were fixed in neutral phosphate buffered 4% formaldehyde solution for possible pathological examination.

- F0 parental females were sacrificed after weaning of the offspring on day 21 post partum. F0 parental females of group 5 were sacrificed after their pups had been sacrificed.
- The uteri of all F0 females were placed in an aqueous solution of ammonium sulphide[4] to accentuate possible haemorrhagic areas of implantation sites.
- F1 parental females were sacrificed on day 14 post coitum and the reproduction data were recorded. If no implantation sites were evident, the uterus was placed in an aqueous solution of ammonium sulphide[4] to accentuate possible haemorrhagic areas of implantation sites.
- F1 offspring were sacrificed when the developmental parameters and behavioral tests except the water maze test had been completed (i.e. around day 35 post partum).
- F1 males were sacrificed after the F1 females.
- F0 and F1 parental females that died during the study were also examined as stated above.
- F1 offspring that died during the study were examined externally.
- F1 stillbirths and live offspring from groups 1 - 4 which died or had to be killed between days 0 and 5 post partum were fixed and preserved in neutral phosphate buffered 4% formaldehyde solution for histopathological examination. Before immersion in the fixative the thorax and abdominal cavity were opened to facilitate fixation of the lungs and kidneys, respectively. Furthermore, for every dead pup in groups 2 - 4, two pups of the same sex and age (taken from two different litters) from group 5 were killed and fixed in the same way to serve as control pups for possible further histopathological examinations. The remaining pups from group 5 were killed and discarded without further examination after the last litters from groups 1 - 4 had reached at least day 5 post partum.

Statistical methods: Dunnett t-test was used for variables with normal distribution, and Steel (many-one t-test) test was applied for data without normal distribution. Fisher's exact test was used if the variables could be dichotomized without loss of information.

**Results**

$F_0$ in-life:

Mortality: none

Clinical signs: unremarkable

Body weight and food consumption during gestation and lactation period: unremarkable

Mating/Fertility: The results summarized below (extracted from the sponsor's report) indicated that there was no change in pregnancy rate with drug treatment. All pregnant animals give birth. However, cannibalization and litter loss were observed in some animals; these findings were not dose-dependent. It should be noted that whole litter loss was observed in all groups including control with 2, 1, 3, 3 litter losses in control, 30, 150, and 500 mg/kg after birth.

| Group<br>Dose (mg/kg) | 1<br>(0) | 2<br>(30) | 3<br>(120) | 4<br>(500) | 5<br>(0) |
|---|---|---|---|---|---|
| Female numbers | 1 - 22 | 23 - 44 | 45 - 66 | 67 - 88 | 89 - 93 |
| Number of mated females | 22 | 22 | 22 | 22 | 5 |
| Non pregnant (A) | 1 | 0 | 1 | 2 | 0 |
| Total pregnant * | 21 | 22 | 21 | 20 | 5 |
| Gave birth | 21 | 22 | 21 | 20 | 5 |
| Gave birth to confirmed living pups (B) | 21 | 21 | 20 | 20 | 5 |
| With live pups at day 21 post partum ** (C) | 19 | 21 | 18 | 17 | --- |

* = Used for calculation of group mean values for food consumption and body weight gain during gestation

** = Used for calculation of group mean values for food consumption and body weight gain during lactation

(A) Female Nos. 9 (group 1), 52 (group 3), 70 (group 4) and 78 (group 4) were not pregnant

(B) Female Nos. 42 (group 2) and 57 (group 3) gave birth but cannibalized their pups shortly after or during littering. Since viability, number and sex of these pups could not be determined, these females could not be included into calculation of breeding data.

(C) Female Nos. 8 (group 1), 18 (group 1), 46 (group 3), 58 (group 3), 67 (group 4), 68 (group 4) and 71 (group 4) lost the whole litter.

$F_0$ necropsy: not remarkable

$F_0$ Reproduction data: the results were summarized below as extracted from the sponsor's report. There were no difference in duration of gestation, and number of implantation sites in treated groups as compared to control. Post-implantation loss was increased in the mid-dose group as compared to control (14.1% vs. 6.2%) mainly due to low number of pups in 2 animals in this group. Further investigation indicated that this was caused by postnatal loss before first little check due to cannibalization. Postnatal loss during day 0 to 4 post partum was found to be increased in the high dose group compared to control (19.2% vs. 10.4%), but not in group 3 (11.6%). Total litter loss during GD0-4 was noted for 2, 2, and 3 dams in groups 1, 3, and 4, respectively. In group 3, two dams had extremely small little size (No. 46: 4; No. 51: 2) as compared to others; the implantation sites of these two animals were 18 and 13, respectively. Therefore, cannibalization was likely to occur before the first litter check. In addition, there was one litter loss in group 2 and group 3 before the first litter check after delivery, which is most likely due to cannibalization since body weight of these two animals were comparable with others. These two animals were not included for reproduction analysis. Taken all these in consideration, there appeared to be slightly increased postnatal loss in group 3 (120 mg/kg) and group 4 (200 mg/kg).

| | GROUP 1 0 MG/KG | GROUP 2 30 MG/KG | GROUP 3 120 MG/KG | GROUP 4 500 MG/KG |
|---|---|---|---|---|
| LITTERS | | | | |
| TOTAL | 21 | 21 | 20 | 20 |
| DURATION OF GESTATION | | | | |
| MEAN (+) | 21.4 | 21.3 | 21.4 | 21.2 |
| ST.DEV | 0.51 | 0.46 | 0.50 | 0.37 |
| N | 21 | 21 | 20 | 20 |
| IMPLANTATIONS | | | | |
| TOTAL | 310 | 312 | 320 | 313 |
| MEAN (+) | 14.8 | 14.9 | 16.0 | 15.7 |
| ST.DEV | 2.51 | 2.06 | 3.11 | 2.60 |
| N | 21 | 21 | 20 | 20 |
| POST IMPLANTATION LOSS | | | | |
| % OF IMPLANTATIONS | 6.8 | 6.1 | 14.1 | 8.6 |
| LITTERS AFFECTED (#) | 12 | 10 | 13 | 14 |
| TOTAL (#) | 21 | 19 | 45 ## | 27 |
| MEAN (+) | 1.0 | 0.9 | 2.3 | 1.4 |
| ST.DEV | 1.14 | 1.45 | 3.48 | 1.50 |
| N | 21 | 21 | 20 | 20 |
| DEAD PUPS AT FIRST LITTER CHECK | | | | |
| LITTERS AFFECTED (#) | 0 | 0 | 0 | 0 |
| TOTAL | 0 | 0 | 0 | 0 |
| MEAN (+) | 0.0 | 0.0 | 0.0 | 0.0 |
| ST.DEV | 0.00 | 0.00 | 0.00 | 0.00 |
| N | 21 | 21 | 20 | 20 |
| LIVING PUPS AT FIRST LITTER CHECK | | | | |
| % OF MALES / FEMALES (#) | 53 / 47 | 46 / 54 | 50 / 50 | 53 / 47 |
| TOTAL | 289 | 293 | 275 | 286 |
| MEAN (+) | 13.8 | 14.0 | 13.8 | 14.3 |
| ST.DEV | 2.57 | 2.69 | 4.60 | 2.99 |
| N | 21 | 21 | 20 | 20 |
| POSTNATAL LOSS DAYS 0 - 4 P.P. | | | | |
| % OF LIVING PUPS | 10.4 | 0.3 | 11.6 | 19.2 |
| LITTERS AFFECTED (#) | 4 | 1 | 10 # | 7 |
| TOTAL (#) | 30 | 1 ## | 32 | 55 ## |
| MEAN (+) | 1.4 | 0.0 | 1.6 | 2.8 |
| ST.DEV | 4.20 | 0.22 | 3.20 | 6.00 |
| N | 21 | 21 | 20 | 20 |
| LIVING PUPS DAY 4 P.P. | | | | |
| TOTAL | 259 | 292 | 243 | 231 |
| MEAN (+) | 12.3 | 13.9 | 12.2 | 11.6 |
| ST.DEV | 4.87 | 2.70 | 5.40 | 5.64 |
| N | 21 | 21 | 20 | 20 |
| BREEDING LOSS DAYS 5 - 21 P.P. | | | | |
| % OF LIVING PUPS AT DAY 4 P.P. | 0.8 | 2.4 | 2.5 | 1.3 |
| LITTERS AFFECTED (#) | 2 | 5 | 4 | 2 |
| TOTAL (#) | 2 | 7 | 6 | 3 |
| MEAN (+) | 0.1 | 0.3 | 0.3 | 0.2 |
| ST.DEV | 0.30 | 0.66 | 0.66 | 0.49 |
| N | 21 | 21 | 20 | 20 |
| LIVING PUPS DAY 21 P.P. | | | | |
| % OF MALES / FEMALES (#) | 53 / 47 | 46 / 54 | 51 / 49 | 56 / 44 |
| TOTAL | 257 | 285 | 237 | 228 |
| MEAN (+) | 12.2 | 13.6 | 11.9 | 11.4 |
| ST.DEV | 4.83 | 2.52 | 5.24 | 5.56 |
| N | 21 | 21 | 20 | 20 |

+ : Steel-test significant at 5% level
# / ## : Fisher's Exact Test significant at 5% (#) or 1% (##) level

Histopathological examination of pups of dead or partially cannibalized:

F1 pups that died between day 0 and 4 post partum (PND0-4) and corresponding age-matched control pups from group 5 were processed for histopathological examination of heart, lung, liver and kidneys. The following findings were reported:

1. Minimal to slight eosinophilic material in 1/10 lungs examined in the pups of group 1 (placebo treated group), in **13/16** lungs examined in the pups of group 3 (120 mg/kg), in **7/14** lungs examined in the pups of group 4 (500 mg/kg) and in 1/36 lungs that were examined in the pups of group 5 (placebo treated group; corresponding age-matched control pups).
2. Slight to marked glycogen in 2/9 livers examined in pups of group 1 (placebo treated group), 2/15 livers examined in pups of group 3 (120 mg/kg), 14/15 livers examined in pups of group 4 (500 mg/kg) and 13/38 livers examined in pups of group 5 (placebo treated group; corresponding age-matched control pups).

There were no other abnormal changes observed in these dead fetuses. According to the sponsor, the histopathologic changes of minimal to eosinophilic material observed in the fetal lungs are not considered to be drug-related because:

- The severity of the effect was only minimal to slight;
- There was no dose-response relationship;
- The lesions were mainly observed in fetuses that were found dead and were already subject to post-mortem changes;
- Eosinophilic material in the alveoli (alveolar edema) is often present in the lungs of normal rats dying during the course of a study as a part of agonal or shock-like state preceding death;
- No other changes in the lungs were observed.

(*Reviewer's comment:* these arguments are generally reasonable and this reviewer agreed that the pup death during PND0-4 was not likely to be due to changes in the lungs; therefore, the reason of pup death was not clear based on the results of this study).

$F_1$ physical development: There were no treatment-related changes observed in external examination at first litter check after parturition, body weight and sex ratios, and developmental indices such as pinna unfolding, incisor eruption, onset of coat development, opening of the eye, testes descent and vaginal opening.

$F_1$ behavioral evaluation: There were no treatment-related changes observed.

$F_1$ necropsy: not remarkable

$F_1$ reproduction: There were no treatment-related changes observed

$F_2$ findings: F2 generation was not delivered.

**2. Study title**: An investigational peri- and postnatal development study with Org 25969 in the Sprague Dawley rat

The objective of this GLP-compliant study were to examine whether the duration of dosing plays a role in the occurrence of Org 25969 induced increased neonatal mortality and/or pathophysiology of this effect, the most vulnerable period during gestation and/or early lactation for inducing neonatal mortality, and the potential cause of neonatal death.

A dose of 500 mg/kg Org 25969 (batch AE) was administered intravenously to pregnant Sprague Dawley rats for different periods during the pregnancy and lactation as shown in the following (extracted from the sponsor's report). PL1 (control) group is to determine the spontaneous neonatal mortality/late fetal death rate, and PL5 (control) group is to provide age and sex matched control pups as reference material for histopathological examination of organs of diseased pups in the treatment group.

| Group | Group size and sex | | Dose | Dosing period | Treatment |
|---|---|---|---|---|---|
| | Female | Male | ($mg \cdot kg^{-1}$) | | |
| PL1 | 22 | - | 0 | gestation day 6 - lactation day 4 | 0.9% NaCl |
| DS2 | 22 | - | 500 | gestation day 6 – lactation day 4 | Org 25969 |
| DS3 | 22 | - | 500 | gestation day 6 – gestation day 10 | Org 25969 |
| DS4 | 22 | - | 500 | gestation day 11 –gestation day 15 | Org 25969 |
| PL5 | 5 | - | 0 | gestation day 6 – lactation day 4 | 0.9% NaCl |
| DS6 | 24 | - | 500 | gestation day 16 – gestation day 20 | Org 25969 |
| DS7 | 23 | - | 500 | gestation day 21 – lactation day 4 | Org 25969 |
| DS8 | 23 | - | 500 | gestation day 6 – gestation day 20 | Org 25969 |

During pregnancy, the animals were observed for mortality and morbidity. Body weight and food consumption were also recorded. At the end of pregnancy all animals, except the animals in DS8, were allowed to litter. The number of live and dead fetuses and litter weight was recorded. Neonatal mortality and neonatal survival were calculated. Dams were watched for pup cannibalism or neglecting during and after parturition. Fetuses that were partly cannibalized, found dead or stillborn were examined macroscopically, and heart, liver, brain, kidneys, urinary bladder and lungs were examined microscopically; findings from these animals were compared to those in age-matched control pups (PL5). Females in DS8 were selected for cesarian section on day 21 of pregnancy, at which the number and distribution of implantation points, live and dead fetuses, ovary weight and number of corpora lutea was recorded. Post-implantation loss was calculated.

There were no treatment-related changes observed in parameters examined except decreased neonatal survival rate at DS6 group within 24 hours after delivery as compared to control (78.7% vs. 99.6%). In contrast, this change was not observed in drug-treated groups with other treatment-intervals in groups of DS2, DS3, DS4, and DS7. This change was likely to be due to cannibalism. There were 5/21 females in DS6, 1/21 females in DS4 and 1/19 placebo females observed cannibalizing the majority their litters. In addition, 1/21 females in DS2, 1/18 females in DS3, 4/21 females in DS4, 2/21 females in DS6 and 2/18 females in DS7 were likely to have cannibalized one or more pups, based on the number of missing (not live and not dead) pups at pup count. There were no treatment-related macroscopic and microscopic findings in fetuses that were dead or partly cannibalized as compared to age-matched control. Fetuses obtained by cesarian section from DS8 group showed good viability and a normal distribution of live and dead fetuses, which may indicate no intrinsic abnormalities within the fetuses that causes the dams to start cannibalizing their young. The mechanism of increased cannibalism in DS6 group was not clear. The sponsor indicated that increased cannibalism in this group was likely to be due to stress since these animals had not been customized completely to the dosing procedure; drug treatment may play also play a role on this change. However, this can not explain the findings in the regular peri- and postnatal study. The fact that group DS2 (500 mg/kg treatment during GD6-LD4 which

mimicked the highest treatment regimen in the regular prenatal and postnatal development study) did not show an increased neonatal mortality in this study indicates that postnatal loss of F1 pups may be incidental in the regular prenatal and postnatal developmental study.

**3. Study title**: A second investigational peri- and postnatal development study with Org 25969 in the Sprague Dawley rat

The purpose of this study was to investigate the possible cause of the increased incidence of cannibalism in animals with Org 25969 administration. The sponsor has examined whether in-utero exposure of pups induces maternal cannibalism in untreated foster-dams and/or ex-utero exposure of pups induces maternal cannibalism in untreated foster dams. Mated females were treated with placebo and drug substance (Batch AM) as indicated in the table below (extracted from the sponsor's report).

| Group | Group size and sex | | Treatment |
|---|---|---|---|
| | Female | Male* | |
| PL1 | 22 | - | Untreated dams for ex-utero placebo treated pups |
| DS2 | 24 | - | Untreated dams for ex-utero Org 25969 treated pups |
| DS3 | 23 | - | Biological dams for in-utero exposed Org 25969 pups |
| DS4 | 23 | - | Untreated Fosterdams for in-utero exposed Org 25969 pups |
| PL5 | 22 | - | Biological dams for in-utero exposed placebo pups |
| PL6 | 22 | - | Untreated Fosterdams for in-utero exposed placebo pups |

*Males were used for mating only.

**The rationales for the groups are indicated below:**
**PL1:** control group to determine spontaneous incidence of cannibalism.
**DS2:** to establish the presence and incidence of cannibalism of pups, that were ex-utero exposed to 0.5 $mg \cdot kg^{-1}$ Org 25969
**DS3:** this group comprises dams that were treated with 500 $mg \cdot kg^{-1}$ Org 25969 in order to deliver pups that were in-utero exposed to Org 25969
**DS4:** to establish the presence and incidence of cannibalism of crossfostered pups, that were in-utero exposed to Org 25969
**PL5:** this group comprised dams that were treated with placebo (0.9% saline) in order to deliver pups that were in-utero exposed to placebo
**PL6:** to establish the presence and incidence of cannibalism of crossfostered pups, that were in-utero exposed to placebo.

PL1 and DS2 received no treatment during pregnancy and were allowed to litter. After littering the pups of the dams in PL1 were swabbed with 0.9% saline and the pups of the dams in DS2 were swabbed with 0.5 mg/ml Org 25969 until they were wet using a swab stick. In both groups the number of pups was counted from littering up to day four after delivery. DS3 and PL5 received a daily intravenous dose of 500 mg/kg (DS3) or saline (PL5) from GD6 – 21. At GD21, the animals in DS3 and PL5 were sacrificed and pups were delivered by cesarean section, and cross fostered to untreated foster dams in DS4 and PL6. The animals in DS4 and PL6 did not receive any treatment during pregnancy. After littering, their own litters were removed and replaced by a foster litter from DS3 and PL5, respectively. Hence, the animals in DS4 received in utero Org 25969 exposed foster pups from the Org 25969 treated dams in DS3 and the animals in PL6 received foster pups from the PL5 dams that were treated with 0.9% saline during pregnancy. In both DS4 and PL6 the number of pups was counted from cross fostering up to day four after cross fostering.

Neonatal survival was comparable in the litters in which the pups were swabbed with 0.5 mg/kg Org 25969 (DS2) as compared to the litters in which the pups were swabbed with saline (PL1) (95.8% and 92.1%, respectively). In-utero exposure of Org 25969 appeared to cause a slightly higher neonatal loss as compared to control. In DS4, 9/18 females showed (partial) litter loss. One female (4018) showed complete litter loss (14 pups) and two other females (4010 and 4012) lost 15 and 9 pups, respectively. In these litters all pups had been cannibalized. In addition, 4 females (4026, 4024, 4034 and 4046) lost 3 pups of which only one pup of female 4024 was found dead and the rest had been cannibalized. Finally one female (4048) lost two pups that had been cannibalized and one female (4022) lost one pup that was found dead. In PL6, 9/18 females showed litter loss. Two females (6018 and 6024) lost three pups which were cannibalized. Four females (6020, 6032, 6034 and 6036) lost two pups, of which only one pup of 6036 was found dead and the others had been cannibalized. Three females (6002, 6026 and 6016) lost one pup each that were cannibalized. Although the number of litters that showed neonatal mortality is comparable in the group of dams fostering in utero Org 25969 exposed pups and the group of dams fostering in utero placebo exposed pups, the number of pups that died in each litter was higher in the group fostering in utero Org 25969 exposed pups, resulting in a neonatal survival of 85.1% for the placebo pups (PL6) and 79.4% for the exposed pups (DS4).

*Developmental studies in juvenile animals*

**1. Study title**: Org 25969: single dose toxicity study in the neonatal/juvenile rat by subcutaneous and intravenous administration

**Key study findings**:

- Juvenile rats (16/sex/group) at age of 7 days, 14 days, and 21 days were treated with single-dose 0 (0.9% saline), 120, and 500 mg/kg Org 25969 (batch AE).
- A single dose of ORG 25969 at doses up to 500 mg/kg on Days 7, 14 or 21 of age appeared to be well tolerated. There were no remarkable clinical signs, mortalities, changes in body weight and parameters of clinical pathology were considered to be treatment-related.
- Reversible minimal to slight tubular vacuolation in kidney were observed primarily at 500 mg/kg in animals of age 7 days and 14 days after dosing. Similar changes were occasionally seen at 120 mg/kg.
- Minimal disruption of the enamel epithelium with presence of amorphous material was observed in the incisors of one female rat treated of age 21 days at 500 mg/kg on day 14 after dosing.
- NOAEL is 120 mg/kg.

**Document ID:** INT00038388
**Study no.**: 062852
**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)

**Date of study initiation**: 3/02/2006

**GLP compliance**: Yes
**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Org 25969, Batch AE (TZ021-1-2), 97.9%

**Methods**
Doses: 0 (0.9% saline), 120, and 500 mg/kg of active entity including Org 25969, Org 48302 and impurities

Species/strain: HsdRCC Han:Wist rat at age of day 7, day 14, and day 21.

Number/sex/group: 16/sex/group at each age-point

Route, formulation, volume, and infusion rate: SC injection for rats at age of day 7 and 14 and IV for rats at age of day 21 in a volume of 5 ml/kg

Satellite groups used for toxicokinetics: none

Study design: The study design was shown below as extracted from the sponsor's report. Half of the animals were sacrificed 24 hours after dosing, and half were sacrificed after 2 week recovery.

| Group | Treatment | Dose (mg/kg) # | Day of treatment/ route | Number of juvenile rats $ | |
|---|---|---|---|---|---|
| | | | | Male | Female |
| 1 | Control | 0 | 7/SC | 16 | 16 |
| 2 | ORG 25969 | 120 | 7/SC | 16 | 16 |
| 3 | ORG 25969 | 500 | 7/SC | 16 | 16 |
| 4 | Control | 0 | 14/SC | 16 | 16 |
| 5 | ORG 25969 | 120 | 14/SC | 16 | 16 |
| 6 | ORG 25969 | 500 | 14/SC | 16 | 16 |
| 7 | Control | 0 | 21/IV | 16 | 16 |
| 8 | ORG 25969 | 120 | 21/IV | 16 | 16 |
| 9 | ORG 25969 | 500 | 21/IV | 16 | 16 |

# Expressed in terms of the active entity.
$ 4 litters (8 males and 8 females) per group killed at 24 hours after dosing, and 4 litters (8 males and 8 females) per group killed 14 days after dosing.
SC Subcutaneous
IV Intravenous

Parameters and endpoints evaluated:
Clinical observations, mortality, and body weight were recorded and physical examination was conducted weekly in dams and their offspring throughout the study. Little size and sex ratio of offspring were also recorded. At termination (SD1 and SD14), blood samples were collected from groups 4 to 9 for hematology and blood chemistry analysis. The parameters of hematology and blood chemistry examined were shown below. Additional blood samples were collected from animals with body weight ≥ 45 g for prothrombin time (PT) and activated partial thromboplastin time (APTT).

Hematology Blood chemistry

Haematocrit (Hct)
Haemoglobin (Hb)
Erythrocyte count (RBC)
Reticulocyte count (Retic)
Mean cell haemoglobin (MCH)
Mean cell haemoglobin concentration (MCHC)
Mean cell volume (MCV)
Total white cell count (WBC)
Differential WBC count
- Neutrophils (N)
- Lymphocytes (L)
- Eosinophils (E)
- Basophils (B)
- Monocytes (M)
- Large unstained cells (LUC)

Platelet count (Plt)

Alkaline phosphatase (ALP)
Alanine aminotransferase (ALT)
Aspartate aminotransferase (AST)
Creatine phosphokinase (CK)
Total bilirubin (Bili)
Urea
Creatinine (Creat)
Glucose (Gluc)
Total cholesterol (Chol)
Triglycerides (Trig)
Sodium (Na)
Potassium (K)
Chloride (Cl)
Calcium (Ca)
Inorganic phosphorus (Phos)
Total protein (Total Prot)
Albumin by chemical assay (Alb)
Globulin (GLOB)

Urinalysis: Urinalysis was only conducted in animals at age of 21 days after recovery period (age of 34 days). The appearance, volume, pH, and specific gravity (SG) of urine were recorded. Glucose, ketones, bile pigments, haem pigments were measured, and microscopic examination were conducted for detection of urine sediment including Epithelial cells, Leucocytes, Erythrocytes, Crystals, Spermatozoa and precursors, Casts, and Other abnormal components. Presence of protein, sodium, potassium, chloride and N-acetyl-β-D-glucosaminidase (UNAG) were also examined.

Necropsy: animals sacrificed at 24 hours after treatment (on Days 8, 15 or 22 of age) and 14 days after treatment (on Days 21, 28 or 35 of age) were subject to a detailed necropsy. For animals not selected for blood sampling all external features and orifices were examined visually. The cranium was removed and the brain examined in situ before removal. After ventral mid-line incision, the neck and associated tissues and the thoracic, abdominal and pelvic cavities and their viscera were examined in situ. For animals selected for blood sampling, the examination and removal of the brain was performed after ventral mid-line incision (to allow exposure of the abdominal aorta). Examination of the neck and associated tissue and the thoracic, abdominal and pelvic cavities were then performed. Abnormal position and morphology or interaction was recorded. External and cut surfaces of the organs and tissues were examined. Abnormality in the appearance or size of any organ and tissue was recorded and the required tissue samples preserved in appropriate fixative. Decedents were similarly examined. The following organs were dissected and fixed; selected organs were weighed, as shown in the following table (extracted from the sponsor's report).

| Tissue and regions to be examined | Weigh |
|---|---|
| Abnormalities | |
| Brain | * |
| Epididymides (total and cauda weights) | |
| Femur (longitudinal section through joint) @ | |
| Heart (including auricular and ventricular regions) | * |
| Kidneys | * |
| Liver (section from all main lobes) | * |
| Lungs (section from two major lobes including bronchi) – fixed by inflation | * |
| Ovaries | |
| Pituitary | |
| Prostate | |
| Seminal vesicles and coagulating gland | |
| Spinal cord (transverse and longitudinal sections at the cervical, thoracic and lumbar levels)@ | |
| Spleen | * |
| Teeth - incisors and molars (Groups 7 to 9 only) | |
| Testes | |
| Thymus | |
| Urinary bladder | |
| Uterus with oviducts and cervix | |

Histological examination were conducted in selected tissues (femur, kidneys, lungs, urinary bladder and any abnormalities for all groups, and the incisors and molars from animals in Groups 7 to 9)

**Results:**

Clinical signs and mortality: There were no treatment-related change observed

Body weight: There were no remarkable treatment-related changes observed in Org 25969 treated rats as compared to control.

Clinical path: There were no remarkable treatment-related changes observed as compared to control in parameters of hematology, blood chemistry, and urinalysis.

Necropsy: There was no remarkable treatment-related macroscopic necropsy findings observed.

Organ weight: Significant increase in liver weight was observed in male animals of 7 days old (15%) and female rats of 14 days old (7%) following 500 mg/kg Org 25969 treatment on SD1; this change was reversible since similar change was not observed in animals after 14-day recovery. However, these changes may be not treatment-related since there was no similar change observed in animals of the other gender at either time-point. In addition, there was no similar change observed in animals of 21 days old after treatment.

Histopathology examination:

Kidney: Minimal to slight vacuolation of tubules were observed at all age groups.
In rats of 7 days old, vacuolation of tubules were observed in 7/16 (3 large tubular vacuolation and 4 fine vacuolation) animals at 500 mg/kg one day after dosing. Similar change was not seen in animals of recovery group, indicating these changes are reversible. In rats of 14 days old, animals were observed with tubule vacuolation at 120 mg/kg (1/16 large tubular vacuoles) and 500 mg/kg (7/16 large tubular vacuoles and 1/16 fine tubular vacuoles). These changes were reversible since similar changes were only observed in one rat at 500 mg/kg after 2-week recovery. Tubular vacuolation were not

seen in animals of 21 days old on day 1 or 14 after dosing except one rat at 120 mg/kg on day 1 after dosing.

Teeth: Minimal disruption of the enamel epithelium with presence of amorphous material was observed in the incisors of one female after 2 week recovery period following 500 mg/kg Org 25969 treatment at age of day 21.

**2. Study title**: An extended single dose toxicity study in juvenile rats to assess the effects of Org 25969 on teeth

Study summary: the objective of the study was to investigate effect of a single dose treatment with Org 25969 (batch AE) on teeth discoloration in male and female juvenile rats. Juvenile Wistar rats were dosed once on day 7 of age with either 0 (0.9% NaCl), 30, 120 or 500 mg/kg of Org 25969. Three animals/sex/group/time-point were sacrificed on SD 1, 3, 7, 14, 21, 28, 35, or 42, and incisor and bone samples were collected. There were no treatment-related clinical signs and mortality and body weight of animals were not affected at all doses of animals as compared to control. Mild whitish discoloration of incisors (mottled appearance) was observed at all groups including control with 2 (mild), 4 (minimal: 1; mild: 2; more severe: 1), 2 (mild), 7 (minimal: 1; mild: 5; more severe: 1) at 0, 30, 120, and 500 mg/kg. Since white discoloration of incisors was also observed in control animals, the relevance to Org 25969 is hard to determine. Treatment at 500 mg/kg may slightly increase incisor white discoloration after single-dose treatment since increased incidence at this group was observed in this group as compared to the control based on results in this study. Histopathological assays in teeth and bone were not conducted since the sponsor did not consider this teeth effect to be treatment-related. Bone-specific analysis including bone turnover parameters and μCT assay were not conducted based on the findings in the 4-week juvenile toxicity study in rats (see below in study 4).

**3. Study title**: A two week pilot juvenile toxicity and toxicokinetic study with Org 25969 in male and female Wistar rats

Summary: This is a preliminary study which was conducted in compliance with GLP standard but without QA report included. The main purpose of the study was to preliminarily assess the toxicity of Org 25969 (batch AE) in juvenile rats at doses up to 500 mg/kg after 2-week administration. In addition, the concentration of Org 25969 in plasma of juvenile rats was also determined and toxicokinetic parameters were calculated. Animals were treated with control item (0.9% NaCl) or Org 25969 at doses of 30, 120, and 500 mg/kg from age of day 7 for 2 weeks and experimental parameters include mortality and morbidity, clinical signs, body weight, gross anatomy findings, organ weights and histopathology examinations, as well as TK analysis. There were no treatment-related clinical signs and body weight change were observed in groups treated with Org 25969 as compared to control animals. In addition, there were no treatment related gross necropsy findings and organ weight changes in contrast to control.

Histopathological examination found minimal to moderate changes in lung, kidney, and urinary bladder which were comparable to what were found in adult rats after dosing as shown in the following (extracted from the sponsor's report). Reversibility of these changes was not examined in this study.

***Lungs***

- Minimal increased foamy macrophages (alveolar) in the lungs in 1/8 males of the mid-dose group and 2/8 males of the high-dose group.

***Kidneys***

- Minimal tubular cell vacuolation (cortical) in 2/8 males and 1/8 females of the mid-dose group and in 4/8 males and 1/8 females of the high-dose group.
- Slight tubular cell vacuolation (cortical) in 2/8 males and 5/8 females of the high-dose group.
- Moderate tubular cell vacuolation (cortical) in 2/8 males and 2/8 females of the high-dose group.
- Minimal increased basophilia, tubular cell in 2/8 males and 2/8 females of the placebo group, in 2/8 males and 1/8 females of the low-dose group, in 3/8 males and 1/8 females of the mid-dose group and in 1/8 males and 2/8 females of the high-dose group. The basophilic tubular cells are coarsely vacuolated (as specified in the free text) in the reported 3 males and 1 female of the mid-dose group and in the 1 male and 2 females of the high-dose group.

***Urinary bladder***

- Minimal vacuolation of the umbrella cells in 1/8 males and 1/8 females of the mid-dose group and in 2/8 males and 2/8 females of the high dose group.
- Slight vacuolation of the umbrella cells in 3/8 males and 2/8 females of the high-dose group.

Blood samples were collected for TK analysis at 0 (pre-dose sample), 2.5, 5, 10, 20, 40 min, 1, 3, 7, and 24 hours. Plasma levels of Org 25969 were detected up to 1 hour (30 mg/kg), 3 hour (120 mg/kg), and 7 hours after single-dose administration at age of 7 days. After 2-week administration, Org 25969 was still detectable at 24 hours after termination of dosing at age of 21 days. There were no substantial difference between males and females. Systemic exposure was generally dose-proportional. The AUC levels on day 21 after multiple dosing were lower than those on day 7 after single dose.

| kinetic parameters | sex | 30 $mg \cdot kg^{-1}$ | | 120 $mg \cdot kg^{-1}$ | | 500 $mg \cdot kg^{-1}$ | |
|---|---|---|---|---|---|---|---|
| | | SD | MD | SD | MD | SD | MD |
| $AUC_{(0-3)}$ | M | 53.5 | 22.3[1)] | 170 | 155 | 1190 | 703 |
| ($\mu g \cdot h \cdot mL^{-1}$) | F | 46.0 | 20.5[1)] | 221 | 139 | 946 | 737 |
| $NAUC_{(0-3)}$ | M | 1.78 | 0.745[1)] | 1.41 | 1.29 | 2.38 | 1.41 |
| ($\mu g \cdot h \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | F | 1.53 | 0.682[1)] | 1.84 | 1.16 | 1.89 | 1.47 |
| $AUC_{(0-7)}$ | M | - | - | - | 162 | 1533 | 764[2)] |
| ($\mu g \cdot h \cdot mL^{-1}$) | F | - | - | - | 146 | 1085 | 796[2)] |
| $NAUC_{(0-7)}$ | M | - | - | - | 1.35 | 3.07 | 1.53[2)] |
| ($\mu g \cdot h \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | F | - | - | - | 1.21 | 2.17 | 1.59[2)] |
| $C_{max}$ | M | 29.6 | 28.5 | 122 | 119 | 595 | 514 |
| ($\mu g \cdot mL^{-1}$) | F | 39.2 | 27.8 | 133 | 127 | 526 | 641 |
| $NC_{max}$ | M | 0.987 | 0.950 | 1.01 | 0.992 | 1.19 | 1.03 |
| ($\mu g \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | F | 1.31 | 0.927 | 1.10 | 1.05 | 1.05 | 1.28 |
| $t_{max}$ | M | 1.0 | 0.33 | 0.67 | 0.17 | 1.0 | 0.17 |
| (h) | F | 0.67 | 0.33 | 0.67 | 0.33 | 0.67 | 0.33 |
| $t_{1/2}$ | M | - | 0.7 | - | - | - | - |
| (h) | F | - | - | - | - | 0.9 | - |

SD = single dose, MD = multiple dose

M = male, F = female

- : could not be determined due to a lack of data or to the irregular shape of the terminal portion of the curve (elimination phase)

1) : $AUC_{(0-1)}$ and $NAUC_{(0-1)}$ instead of $AUC_{(0-3)}$ and $NAUC_{(0-3)}$

2) : $AUC_{(0-24)}$ and $NAUC_{(0-24)}$ instead of $AUC_{(0-7)}$ and $NAUC_{(0-7)}$

**4. Study title**: Org 25969: toxicity study in the neonatal/juvenile rat by subcutaneous and intravenous administration

**Key study findings**:

- 4-week continuous administration of Org 25969 in juvenile rats from Day 7 of age elicited minor but statistically significant changes in bone parameters of femur as assessed by μCT including transiently increased trabecular BMD, bone volume/tissue volume trabecular metaphysis, and trabecular number and trabecular thickness at 500 mg/kg, along with decreased cortical thickness at both 120 and 500 mg/kg. The exact biological significance of these changes was not known.
- Moreover, 4-week continuous administration of Org 25969 caused minor decreased ulna length, femur length and weight with more severe effect on Day 91 (end of recovery period) at 120 and 500 mg/kg. These changes were not coincident with the slight body decrease occurred during the early stage of recovery period (Day 35-70). In addition, decrease in polar moment of inertia, a parameter indicative of the ability of bone to resist torsion, was observed on Day 91 in all groups of males and 500 mg/kg group of female. All these may suggest a prolonged effect of bone growth.
- The NOEL of bone effect can not be identified since decrease in polar moment of inertia occurred at all dose levels of Org 25969 in male rats. However, 30 mg/kg should be considered to be NOAEL since no other bone parameter were altered at this dose level.
- In teeth, abnormal color (white marbled/mottled banding), malocclusion and/or overgrowth of the incisors in the majority of animals receiving 500 mg/kg (21 of 22 animals per sex), which were generally became apparent following at least 2 weeks of treatment and persisted for up to five weeks into the recovery period. These changes were also seen in some animals of 120 mg/kg (2/22 per sex).
- In addition, minimally to markedly deposition of amorphous material in the molar enamel epithelial layer were found in 5/10 males and 5/10 females of the 500 mg/kg/day group. Recovery was complete in 8 weeks for the changes in the molars but not fully complete for the incisors (1/24 at 120 mg/kg and 2/24 at 500 mg/kg).
- 4-week administration of Org 25969 in juvenile rats caused minimal slight histopatholigcal changes in the lung, kidney, and urinary bladder, similar to those observed in the 4-week study in young adult rats. The NOAEL for these changes is 30 mg/kg.

**Document ID.**: INT00038389
**Study No.:** 063592
**Volume #, and page #**: eCTD
**Conducting laboratory and location**: (b) (4)
**Date of study initiation**: 04/19/2006
**GLP compliance**: Yes
**QA reports**: yes (X) no ( )

**Drug, lot #, and % purity**: Org 25969, Batch AE (TZ021), 90.0%

**Methods**

Doses: 0, 30, 120, 500 mg/kg active entity including Org 25969, Org 48302, and associated impurities

Species/strain: Wistar rats (Hsd/Cpb: WU (spf-bred) with age of 7 days in the beginning of dosing

Number/sex/group: 22/sex/group as shown in the following table as extracted from the sponsor's report.

| Group | Treatment | Dose (mg/kg/day) # | Number of juvenile rats $ | | Allocation to groups |
|---|---|---|---|---|---|
| | | | Males | Females | |
| 1 | Control | 0 | 22 | 22 | 1 male + 1 female per group within each litter |
| 2 | ORG 25969 | 30 | 22 | 22 | |
| 3 | ORG 25969 | 120 | 22 | 22 | |
| 4 | ORG 25969 | 500 | 22 | 22 | |

# Expressed in terms of the active entity.
$ 10 males and 10 females per group were killed at completion of 4 weeks treatment. 12 males and 12 females were killed after an eight-week recovery period, with blood samples collected for toxicokinetic assessment on the last day of treatment.

Twenty-two litters containing at least 4 male and 4 female neonates were selected with one pup of each sex in each litter assigned to each of the study groups. The juvenile rats were treated once daily from Day 7 to Day 34 of age, at doses of 0 (Control), 30, 120 or 500 mg/kg/day. The juvenile rats were treated once on Day 7 of age at doses of 0 (Control), 30, 120 or 500 mg/kg/day.

Route, formulation, volume, and infusion rate*: SC from day 7 – day 21, IV from day 22 - 35; 100mg/ml diluted with 0.9% NaCl to reach the appropriate concentration, 5ml/kg
**formulation analysis was conducted according to the principle of GMP, but not GLP*

Satellite groups used for toxicokinetics: For the single dose Toxicokinetic phase, animals treated with Org 25969 were allocated to the study for blood samples for assessment of toxicokinetic parameters after a single dose on day 7 of age. Eighteen litters containing 4 male and 4 female neonates were selected with 6 litters/group, resulting in 24/sex/group.

Toxicokinetic phase: Single dose on Day 7 of age

| Group | Treatment | Dose (mg/kg)# | Number of juvenile rats | | Allocation to groups |
|---|---|---|---|---|---|
| | | | Males | Females | |
| 2 | ORG 25969 | 30 | 24 | 24 | 6 litters per group, 4 pups per sex per litter treated at same dosage, killed at different time points |
| 3 | ORG 25969 | 120 | 24 | 24 | |
| 4 | ORG 25969 | 500 | 24 | 24 | |

# Expressed in terms of the active entity.

Study design: Mated female rats were allowed to deliver and selected litters were used for this study. Pups were treated for 4 weeks starting on age of day 7. From Day 7-20 of age the dose was administered subcutaneously and thereafter, from Day 21 to 34 of age, doses were administered intravenously. Ten males and ten females per group were sacrificed following the completion of four weeks of treatment. The remaining twelve males and twelve females were sacrificed after an eight week recovery period.

Serum samples were taken from 10 males and 10 females per group at the end of treatment and from 9 males and 9 females after eight weeks of recovery and sent to the Sponsor for measurements of osteocalcine. Urine samples were collected from all animals at the end of treatment and after eight weeks of recovery and sent to the Sponsor for analysis of urinary DPD (free and total deoxypyridinolines). Bone samples (femur/tibia and vertebrae) were collected from all animals at scheduled termination and sent to the Sponsor for further investigations. However, based on the results obtained from investigations relating to the femur samples, the other analyses scheduled to be performed by the Sponsor were not undertaken.

Parameters and endpoints evaluated:

Toxicokinetics: Blood samples were collected following completion of treatment from the recovery phase animals for assessment of toxicokinetic parameters at 0 (prior to dosing), 5, 10, 30, minutes, 1, 2, 3, and 5 hours after dosing (3 animals/sex/group/point). An additional 24 males and 24 females per treated group were allocated to the study for blood sampling for assessment of toxicokinetic parameters after a single dose on day 7 of age at 0 (pre-dosing), 0.5, 1, 2, 3, 4, 5, and 7 hours after dosing (3 animals/sex/group/time-point).

Observations: Clinical observations, mortality, and body weight were recorded in both dam and offspring animals throughout the study. Litter size and sex ratio of offspring were also recorded. Sex maturation of the offspring was also examined during the study. Parental females were killed on Day 21 of lactation as their offspring weaned, or on the day when their pups were sacrificed for study, with the numbers of implantation sites in each uterine horn was counted; there were no other examinations were conducted in parental females.

Clinical Pathology: Blood samples were collected in animals of the main phase study on the day after the termination of treatment and 8-week recovery for analysis of parameters of hematology and blood chemistry. These parameters were shown below as extracted from the sponsor's report. Urinalysis was also conducted in these animals

Hematology

Packed cell volume (Hct)
Haemoglobin (Hb)
Erythrocyte count (RBC)
Reticulocyte count (Retic)
Mean cell haemoglobin (MCH)
Mean cell haemoglobin concentration (MCHC)
Mean cell volume (MCV)
Total white cell count (WBC)
Differential WBC count
- Neutrophils (N)
- Lymphocytes (L)
- Eosinophils (E)
- Basophils (B)
- Monocytes (M)
- Large unstained cells (LUC)

Platelet count (Plt)
Prothrombin time (PT)
Activated partial thromboplastin time (APTT)

Blood chemistry

Alkaline phosphatase (ALP)
Alanine aminotransferase (ALT)
Aspartate aminotransferase (AST)
Creatine phosphokinase (CK)
Total bilirubin (Bili)
Urea
Creatinine (Creat)
Glucose (Gluc)
Total cholesterol (Chol)
Triglycerides (Trig)
Sodium (Na)
Potassium (K)
Chloride (Cl)
Calcium (Ca)
Inorganic phosphorus (Phos)
Total protein (Total Prot)
Albumin by chemical assay (Alb)
Globulin (GLOB)

Neurobehavioral examinations: The assays were performed on recovery phase animals.

- Motor activity on day 55/56 of age
- Sensory function (quantitative): Evaluation of sensory functions was assessed by auditory startle habituation on Day 57/58 of age. To further investigate equivocal results obtained in the startle habituation tests all animals were tested for assess the pre-pulse inhibition of startle on Day 78/79 of age.
- Sensory reactivity: During the treatment, "some animals, particularly females, appeared to be more reactive to handling and/or stimuli than would normally be expected", according to the sponsor. Sensory reactivity assessment including ease of removal from home cage, reactivity to handling, approach response, auditory startle reflex, tail pinch response, touch response were conducted to further investigate these findings on Day 77 of age.
- Learning and memory (Morris water maze) form Day 59 to 62.

Necropsy: Main phase animals were sacrificed after 4 weeks of treatment on Day 35 of age. Recovery phase animals were killed after 8 weeks of recovery on Day 91 of age. Toxicokinetic phase offspring on the first day of dosing were killed on Day 7 of age.

Macroscopic pathology examinations:

All animals sacrificed at Day 35 and Day 91 of age were subjected to a detailed necropsy. All external features and orifices were examined visually. The cranium was removed and the brain examined in situ before removal. After ventral mid-line incision, the neck and associated tissues and the thoracic, abdominal and pelvic cavities and their viscera were exposed and examined in situ. Abnormal position, morphology or interaction was recorded. External and cut surfaces of the organs and tissues were examined as appropriate. Any abnormality in the appearance or size of any organ and tissue was recorded and the required tissue samples preserved in appropriate fixative. The examination also included an assessment for the presence of milk in the stomach.

The length and weight of the left femur were recorded for all animals on Day 35 (termination of Org 259696 administration) and Day 91 of age (end of recovery period).

Abnormality in the appearance or size of any organ and tissue was recorded and selected tissue shown below (extracted from the sponsor's report) were weighted and preserved in fixative for histopathological examination. The femur, kidney, lungs, and urinary bladder were examined from animals of all groups, and all other selected tissues were only examined from animals of control and 500 mg/kg. It should be noted that both sides of femur were collected. The right one was sectioned for histopathological examination, while the left one for µCT assessment. The spinal cord was collected, but the assessment was not conducted.

| | Necropsy | | Histology | Pathology |
|---|---|---|---|---|
| **Tissue and regions to be examined** | **Weigh** | **Fix** | **Groups** | **Light microscopy** |
| Abnormalities | | * | All | All |
| Adrenals | * | * | 1/4 | 1/4 |
| Brain | * | * | 1/4 | 1/4 |
| Epididymides (total and cauda weights) | * | * | 1/4 | 1/4 |
| Femur (longitudinal section through joint) @ | * | b) | All | All |
| Heart (including auricular and ventricular regions) | * | * | 1/4 | 1/4 |
| Kidneys | * | * | All | All |
| Liver (section from all main lobes) | * | * | 1/4 | 1/4 |
| Lungs (section from two major lobes including bronchi) – fixed by inflation | * | * | All | All |
| Ovaries | * | * | 1/4 | 1/4 |
| Pituitary | * | * | 1/4 | 1/4 |
| Prostate | * | * | 1/4 | 1/4 |
| Seminal vesicles and coagulating gland | * | * | 1/4 | 1/4 |
| Spinal cord (transverse and longitudinal sections at the cervical, thoracic and lumbar levels)@ | | @ | @ | @ |
| Spleen | * | * | 1/4 | 1/4 |
| Teeth - lower incisors and molars | | * | # | # |
| Testes | * | * | 1/4 | 1/4 |
| Thymus | * | * | 1/4 | 1/4 |
| Thyroid with parathyroids | a) | * | 1/4 | 1/4 |
| Urinary bladder | | * | All | All |
| Uterus with oviducts and cervix | * | * | 1/4 | 1/4 |
| Vagina | | * | 1/4 | 1/4 |

a) Weighed after partial fixation.
b) Both hindlimbs retained, right sectioned where appropriate.
* Organs weighed, samples fixed or sections examined microscopically.
@ For special examination by Sponsor.
\# See Pathology section

Special assessment:

a. Limb measurements:

The length of the left ulna was recorded once every two weeks from Day 14 of age to termination for Recovery phase animals. The distance from the thumb (digit 1) to the olecranon of the ulna was measured using digital callipers.

b. Tooth color

A colorimetric designed tooth shade system, Candulor TCR Resin shade palette, which provides systematic coverage of tooth color shades in humans, was used for tooth color examination. Colors were divided into 7 main categories (J, M, S, A, B, C and D) that contained sub-categories, defined by shade numbers (suffix number) within categories.

Animals were scored by means of comparison of the color of the mid-section of their lower incisors with the colors provided by the teeth color palette. Assessments were performed once every two weeks from Day 21 of age when was considered to be the earliest feasible time point for assessment in juvenile rats to termination for Recovery phase animals.

c. Bone measurement: µCT analysis for bone mineral density and architecture assessment
Bone mineral density (BMD) measurement and architecture assessment were conducted in the femur by micro computed tomography (µCT) using Skyscan 1076 microCT-40 system (Skyscan, Belgium). This assay was not conducted in compliance to GLP standard. The following cortical and trabecular bone parameters were determined.

- trabecular BMD
- cortical BMD
- cortical thickness
- bone volume over tissue volume from trabecular bone in metaphysis
- trabecular number
- trabecular thickness
- trabecular separation
- degree of anisotrophy (measure of trabecular orientation)
- structure model index (plate/rod characterisation of trabecular bone)
- polar moment of inertia

Statistical analysis: Statistical analysis was applied where there was indication of possible meaningful inter-group differences. All statistical analyses were carried out using the individual animal (or litter) as the basic experimental unit. For categorical data, the proportion of animals was analyzed using Fisher's Exact test for each treated group versus the control. For continuous data, Bartlett's test was first applied to test the homogeneity of variance between the groups. If 75% of the data (across all groups) were the same value, treatment groups were compared using a Mantel test for a trend in proportions and also pairwise Fisher's Exact tests for each dose group against the Control. If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test was performed instead. If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. As the H1 test for monotonicity of dose-response was not significant at the 1% level, Shirley's test for a monotonic trend was applied.

Deviations from the protocol that may impact the integrity of the study*: None
* According to the protocol, analysis of bone turnover parameters including osteocalcine, CTx and urinary DPD would be conducted in serum or urine samples. However, the sponsor decided not to conduct these assays based on their evaluation on the results of µCT assay.

**Results**:

Toxicokinetics: *Note: it appeared that AUC values at age of day 7 were closer to adults.*

Plasma levels of ORG 25969 were comparable for males and females with a roughly proportional increase with increasing dose. Upon repeated dosing, plasma levels were

lower at 34 days of age after 28-day administration when compared with those following a single dose on Day 7 of age. The reason was not known. The sponsor suggested this was probably due to the increased glomerular filtration rate (GFR) at 34 days of age. (*Review's comment:* This explanation appeared to be not reasonable. This reviewer agreed that the difference in GFR may play important role in the systemic exposure of Org 25969. However, a comparison with the adult TK data indicated that systemic exposure levels of Org 25969 appeared to be closer to those in animals at age of 7 days. It is believed that the glomerular filtration function in rats is more mature and similar to that in adult at age of 34 days than age of 7 days. Therefore, GFR difference is not likely to be the reason for the difference in the systemic exposure in animals of 7 days old and 34 days old). Resorption from the subcutaneous dosing site was fast ($C_{max}$ reached at approximately 0.5 to 1 hour) and the elimination half-life shortened from 0.8 h at the age of 7 days to 0.3 h at the age of 34 days.

**Mean Org 25969 kinetic parameters of Org 25969 after single dose**

| | | Dose ($mg \cdot kg^{-1}$) | | |
|---|---|---|---|---|
| Org 25969 kinetic parameters | Sex | 30 | 120 | 500 |
| $AUC_{[0-3]}$ ($\mu g \cdot h \cdot mL^{-1}$) | M | 39.8 | 237 | 1087 |
| | F | 41.5 | 217 | 1119 |
| $NAUC_{[0-3]}$ ($\mu g \cdot h \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | M | 1.33 | 1.97 | 2.17 |
| | F | 1.38 | 1.81 | 2.24 |
| $AUC_{inf}$* ($\mu g \cdot h \cdot mL^{-1}$) | M | 42.7 | 266 | 1243 |
| | F | 42.8 | 242 | 1306 |
| $NAUC_{inf}$* ($\mu g \cdot h \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | M | 1.42 | 2.21 | 2.49 |
| | F | 1.43 | 2.02 | 2.61 |
| $C_{max}$ ($\mu g \cdot mL^{-1}$) | M | 33.3 | 145 | 570 |
| | F | 33.5 | 134 | 592 |
| $NC_{max}$ ($\mu g \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | M | 1.11 | 1.21 | 1.14 |
| | F | 1.12 | 1.12 | 1.18 |
| $t_{max}$ (h) | M | 0.5[1)] | 1.0 | 1.0 |
| | F | 0.5[1)] | 0.5[1)] | 0.5[1)] |
| $t_{1/2}$ (h) | M | 0.7 | 0.9 | 0.8 |
| | F | 0.5 | 0.9 | 0.8 |

M = male, F = female
NAUC=AUC/dose (in $mg \cdot kg^{-1}$) ; $NC_{max}=C_{max}$/dose (in $mg \cdot kg^{-1}$)
* : AUC under the dosing interval ($AUC_{[0-24]}$) was estimated by AUCinf
[1)] : first sampling point after dosing, hence AUC and $C_{max}$ values might be underestimted

**Mean toxicokinetic parameters of Org 25969 after multiple dose**

| | | Dose ($mg \cdot kg^{-1}$) | | |
|---|---|---|---|---|
| Org 25969 kinetic parameters | Sex | 30 | 120 | 500 |
| $AUC_{[0-1]}$ ($\mu g \cdot h \cdot mL^{-1}$) | M | 21.0 | 94.7 | 376 |
| | F | 21.7 | 85.0 | 413 |
| $NAUC_{[0-1]}$ ($\mu g \cdot h \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | M | 0.700 | 0.789 | 0.752 |
| | F | 0.723 | 0.708 | 0.827 |
| $AUC_{inf}$* ($\mu g \cdot h \cdot mL^{-1}$) | M | 22.3 | 104 | 416 |
| | F | - | 91.0 | 448 |
| $NAUC_{inf}$* ($\mu g \cdot h \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | M | 0.742 | 0.867 | 0.831 |
| | F | - | 0.759 | 0.896 |
| $C_0$ ($\mu g \cdot mL^{-1}$) | M | 69.3 | 456 | 1639 |
| | F | 100 | 339 | 1992 |
| $NC_0$ ($\mu g \cdot mL^{-1}$)/($mg \cdot kg^{-1}$) | M | 2.31 | 3.80 | 3.28 |
| | F | 3.35 | 2.82 | 3.98 |
| $t_{1/2}$ (h) | M | 0.3 | 0.3 | 0.4 |
| | F | - | 0.3 | 0.4 |
| CL ($L \cdot kg^{-1} \cdot h^{-1}$) | M | 1.35 | 1.15 | 1.20 |
| | F | - | 1.32 | 1.12 |
| V ($L \cdot kg^{-1}$) | M | 0.482 | 0.548 | 0.634 |
| | F | - | 0.560 | 0.573 |

M = male, F = female
NAUC=AUC/dose (in $mg \cdot kg^{-1}$) ; $NC_0=C_0$/dose (in $mg \cdot kg^{-1}$)
- : could not be determined due to the irregular shape of the terminal portion of the curve (elimination phase)
* : AUC under the dosing interval ($AUC_{[0-24]}$) was estimated by AUCinf

Clinical signs and mortality: no treatment-related mortality observed. Abnormal color (white marbled/mottled banding), malocclusion and/or overgrowth of the incisors in the majority of animals receiving 500 mg/kg, which were generally became apparent in Week 4 of age (following at least 2 weeks of treatment) and persisted for up to five weeks into the recovery period. These changes were also seen in some animals of 120 mg/kg as shown below as extracted from the sponsor's report. Other clinical signs observed during the course of the study were generally unremarkable and not likely to be treatment-related. There was no difference from control in sexual development.

Clinical signs - group distribution of observations

| Group | : | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Compound | : | Control | ORG 25969 | ORG 25969 | ORG 25969 |
| Dose (mg/kg/day) | : | 0 | 30 | 120 | 500 |

| Weeks 3 - 13 of age | | | Number of animals affected | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Group/sex: | 1M | 2M | 3M | 4M | 1F | 2F | 3F | 4F |
| Category | Observation | Number in group: | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| Muscle Reaction | Tremor | | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | Tremor, Slight | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Posture | Hunched | | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Skin | Encrustation, Dorsal Body Surface | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| | Encrustation, Lower Jaw (Right) | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | Encrustation, Upper Dorsal Thorax (Right) | | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | Exfoliation, Upper Dorsal Thorax (Left) | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Skin Colour | Pallor | | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Staining | Abnormal Colour, Brown, Head | | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| | Abnormal Colour, Brown, Nose | | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | Abnormal Colour, Brown, Upper Dorsal Thorax | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Teeth | Abnormal Colour | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | Abnormal Colour, White | | 0 | 0 | 1 | 21 | 0 | 0 | 1 | 21 |
| | Abnormal Colour, White, Lower Jaw (Left) | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | Abnormal Colour, Yellow | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | Absent | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | Broken | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | Malocluded | | 0 | 0 | 1 | 6 | 0 | 1 | 0 | 2 |
| | Malocluded, White | | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | Overgrown | | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 6 |

Body weight: Body weight gain of male and females juvenile rats from Day 7 to Day 35 of age was unaffected by treatment at doses up to 500 mg/kg/day. Minor decrease in mid and high dose (5-8%) in males was observed during recovery period; this change was statistically significant during Day 35-70. Changes in females were smaller than males (≤5%).

Bodyweight change - group mean values (g) for males

- Recovery phase

| Group | : | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Compound | : | Control | ORG 25969 | ORG 25969 | ORG 25969 |
| Dose (mg/kg/day) | : | 0 | 30 | 120 | 500 |

| Group /Sex | | Days 35-42 | Days 35-49 | Days 35-56 | Days 35-63 | Days 35-70 | Days 35-77 | Days 35-84 | Days 35-91 |
|---|---|---|---|---|---|---|---|---|---|
| 1M | Mean | 45 | 87 | 128 | 160 | 187 | 208 | 227 | 222 |
| | SD | 7.1 | 11.9 | 15.3 | 17.8 | 20.7 | 23.9 | 26.5 | 23.6 |
| | N | 12 | 11 | 11 | 11 | 11 | 11 | 11 | 11 |
| 2M | Mean | 46 | 89 | 131 | 162 | 191 | 212 | 232 | 225 |
| | SD | 4.3 | 8.5 | 12.4 | 11.3 | 16.5 | 15.8 | 16.3 | 17.8 |
| | N | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| 3M | Mean | 43 | 82 | 122 | 151 | 174 | 195 | 212 | 207 |
| | SD | 3.6 | 6.5 | 9.0 | 9.8 | 12.0 | 14.9 | 16.7 | 17.0 |
| | N | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| 4M | Mean | 43 | 81 | 119 | 147 a | 171 a | 193 | 214 | 209 |
| | SD | 4.6 | 7.1 | 10.7 | 15.3 | 17.6 | 21.6 | 23.3 | 21.8 |
| | N | 11 | 11 | 11 | 11 | 11 | 11 | 11 | 11 |

Significant when compared with Group 1: a - $p<0.05$

Clinical pathology
Hematology: not remarkable
Serum chemistry: not remarkable

Urinalysis: There was no clear evidence of an adverse effect of treatment with ORG 25969 on the composition, characteristics or volume of the urine of animals sampled

Behavioral examination: no remarkable treatment-related effects in motor activity, auditory startles response habituation, Morris water maze, sensory reactivity, and auditory startle pre-pulse inhibition

Macro-pathology
Macroscopic findings for Main phase animals on Day 35 of age and for Recovery phase animals on Day 91 of age were generally unremarkable. Five of 10 males and four of 10 females allocated to the Main phase of the study which received 500 mg/kg/day were found to have pale incisors, confirming in-life findings. This change appeared to be reversible in rats since there was only one male was observed with similar finding in the recovery group at 500 mg/kg.

Organ weight: Reversible changes were observed in liver, lung, and male seminal vesicles.

| | Male | | | Female | | |
|---|---|---|---|---|---|---|
| Organ weight | 30mg/kg | 120 mg/kg | 500 mg/kg | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| Liver | ↑0.5% | ↑10%* | ↑14%* | ↑ 0.5% | ↓ 1.0% | ↑5.1% |
| Lung + Bronchi | ↑14% | ↑21% | ↑32%* | ↑ 7.0% | ↑ 2.9% | ↑18%* |
| Seminal vesicle | ↓18% | ↓18% | ↓20%* | | | |

There was a minor but statistically significant decrease (<3%) in femur length of males on SD35 after treatment in a dose-independent manner as shown below. There was no statistically significant decrease of femur length in females treated with Org 25969, but a trend of decrease in femur length observed as shown below. There was no statistically significant change in femur weight. These results may be of little toxicity significance due to the magnitude and dose-independence.

SD35

| femur | C | 30 mg/kg | 120 mg/kg | 500 mg/kg | C | 30 mg/kg | 120 mg/kg | 500 mg/kg |
|---|---|---|---|---|---|---|---|---|
| Sex | M | M | M | M | F | F | F | F |
| Length (mm) | 23.4 | 22.8* | 23.0* | 22.9* | 22.7 | 22.8 | 22.6 | 22.2 |
| Weight (mg) | 0.424 | 0.408 | 0.426 | 0.420 | 0.386 | 0.373 | 0.395 | 0.371 |
| Weight/BW (%) | 0.414 | 0.418 | 0.409 | 0.412 | 0.425 | 0.417 | 0.443 | 0.419 |

On SD91 after 8-week recovery, minor change in femur length and weight were observed in 120 and 500 mg/kg groups (<4%).

SD91 - recovery

| femur | C | 30 mg/kg | 120 mg/kg | 500 mg/kg | C | 30 mg/kg | 120 mg/kg | 500 mg/kg |
|---|---|---|---|---|---|---|---|---|
| Sex | M | M | M | M | F | F | F | F |
| Length (mm) | 34.1 | 33.9 | 33.4 | 32.9* | 31.1 | 30.8 | 30.3* | 30.2* |
| Weight (g) | 1.006 | 1.038 | 1.000 | 0.956 | 0.780 | 0.754 | 0.727* | 0.729* |
| Weight/BW (%) | 0.302 | 0.303 | 0.315 | 0.299 | 0.377 | 0.372 | 0.363 | 0.363 |

* Statistically significant, $p < 0.05$ or $p < 0.01$

Overall, Org 25969 administration caused a minor but irreversible decrease in femur length and weight.

Histopathology

Lung: Reversible slight to minimal foamy macrophages (alveolar) in 10/10 M and 10/10 F at 500 mg/kg,

Kidney: Minimal cortical tubular cell vacuolation were observed in 4/9 M and 4/9 F of the 120 mg/kg group. Slight to moderate cortical tubular cell vacuolation in 10/10 M and 10/10 F of the 500 mg/kg were observed. All these changes were reversible after 8-week recovery.

Urinary bladder: Findings including Minimal vacuolation of the umbrella cells in 4/9 M and 4/9 F of the 120 mg/kg and minimal to moderate vacuolation of the umbrella cells in 10/10 M and 10/10 F of the 500 mg/kg. These changes did not appear to be reversible since similar changes were seen in recovery groups: minimal in 5/12 M and 4/12 of the 120 mg/kg; minimal to slight in 11/12 M and 12/12 of F of the 500 mg/kg

Teeth:
A. Disruption of the enamel epithelial layer in the incisor

| | C | 30 mg/kg | 120 mg/kg | 500 mg/kg |
|---|---|---|---|---|
| minimal | 1/10 F | | 3/9 M, 2/8 F | 1/10 M, 2/10 F |
| slight | | | 1/9 M, | 1/10 M, 4/10 F |
| moderate | | | | 4/10 M, 2/10 F |
| marked | | | | 1/10/M |
| **Total** | 1/20 | | 6/17 | 15/20 |

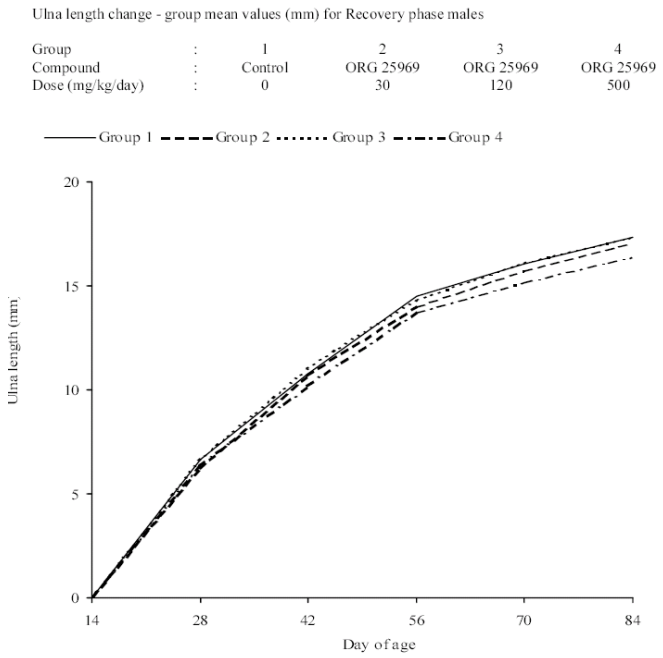
B: deposition of amorphous material in the incisor enamel epithelial layer

| | C | 30 mg/kg | 120 mg/kg | 500 mg/kg |
|---|---|---|---|---|
| minimal | | | 2/9 M, 2/8 F | 2/10 M, 4/10 F |
| slight | | | | 1/10 M, 3/10 F |
| moderate | | | | 3/10 M, 2/10 F |
| marked | | | | 2/10/M |
| **Total** | | | 4/17 | 17/20 |

In addition, minimally to markedly deposition of amorphous material in the molar enamel epithelial layer were found in 5/10 males and 5/10 females of the 500 mg/kg/day group.

Recovery was complete in 8 weeks for the changes in the molars but not fully complete for the incisors (1/24 at 120 mg/kg and 2/24 at 500 mg/kg).

Special assessment:
Ulna length: A minor dose-dependent decrease was observed in 120 and 500 mg/kg groups (< 5%) after Org25969 administration as shown below (extracted from the sponsor's report). Similar change was also observed in recovery groups at 120 and 500 mg/kg.




Tooth color

At the first recording occasion on Day 21 of age, all animals showed "A" grade tooth color, in the white range of the color shade palette. There were approximately half of the animals receiving 500 mg/kg/day with the color of the incisors recorded as being more white than the whitest shade of the color palette (-A1) from Day 35 of age to Day 49 of age. This change was not found at the end of recovery except in one male rat at 500 mg/kg. This extreme whiteness of the incisors represented a partial absence of enamel from the mid-section of the teeth.

Normal progression of tooth color through the color shade palette from the white range towards the orange/brown range (M shades) was observed as the animals aged, with the majority of animals, including those which received 500 mg/kg/day, displaying a tooth colour of M4+ (more orange than available on the colour palette) by the end of the recovery period. However, the process at 500 mg/kg appeared to be slow as compared to control.

Bone parameters: µCT assessment showed statistically significant increase in trabecular bone mineral density (BMD), trabecular bone volume/tissue ratio in metaphysis, trabecular number and trabecular thickness in animals of 500 mg/kg as shown in the table below. Cortical BMD was significantly decreased at 120 mg/kg and 500 mg/kg in a dose-independent manner. All these changes returned to normal after 8-week recovery (no significant difference from control). In addition, >10% decrease in structure model index was observed at 120 mg/kg and 500 mg/kg in both male and female rats with statistical significance in female of 500 mg/kg; the change appeared to be reversible. Femur length was statistically decreased on Day 91 (end of 8-week recovery) at 120 mg/kg and 500 mg/kg in a dose-dependent manner. µCT assessment also showed a likelihood of slight decrease in femur medial-lateral and anterior-posterior diameters.

Polar moment of inertia, a quantity used to predict the ability to resist torsion, was also decreased significantly at all doses of males and 500 mg/kg of females on Day 91.

| Sex | Day | Trabecular bone mineral density (mg/ml) | | | |
|---|---|---|---|---|---|
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 325 | 302 | 318 | 353 (↑9%)* |
| F | 91 | 358 | 368 | 347 | 332 (↓7%) |
| M | 35 | 285 | 289 | 334 (↑17%) | 336 (↑18%)* |
| M | 91 | 259 | 255 | 248 | 248 |
| Sex | Day | Cortical bone mineral density (mg/ml) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 1460 | 1477 | 1475 | 1434 |
| F | 91 | 1165 | 1189 | 1191 | 1180 |
| M | 35 | 1443 | 1433 | 1415 | 1393 |
| M | 91 | 1125 | 1132 | 1130 | 1145 |
| Sex | Day | Cortical thickness (mm) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 0.319 | 0.300 | 0.288 (↓10%)* | 0.292* |
| F | 91 | 0.546 | 0.560 | 0.554 | 0.556 |
| M | 35 | 0.302 | 0.294 | 0.274 (↓9%)* | 0.280* |
| M | 91 | 0.576 | 0.578 | 0.572 | 0.588 |
| Sex | Day | Bone Vol Tissue Vol ratio trabecular metaphysis (%) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 27.3 | 26.9 | 28.9 (↑6%) | 32.5 (↑19%)* |
| F | 91 | 35.8 | 36.3 | 34.6 | 32.8 (↓8%) |
| M | 35 | 25 | 25.5 | 30.1 (↑20%) | 30.9 (↑24%)* |
| M | 91 | 25.1 | 25.3 | 24.8 | 24.6 |
| Sex | Day | Bone Vol Tissue Vol ratio mid diaphysis (%) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 26.2 | 25.6 | 23.8 (↓9%) | 24.2 |
| F | 91 | 39.4 | 40.2 | 39 | 39.7 |
| M | 35 | 24.1 | 24.4 | 23.2 | 23.5 |
| M | 91 | 39.5 | 41.7 | 40.1 | 42.1 (↓6%) |
| Sex | Day | Trabecular number (1/mm) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 3.30 | 3.25 | 3.34 | 3.73 (↑13%)* |
| F | 91 | 3.53 | 3.47 | 3.42 | 3.22 (↓10%) |
| M | 35 | 3.16 | 3.15 | 3.64 | 3.63 (↑15%)* |
| M | 91 | 2.65 | 2.68 | 2.55 | 2.57 |
| Sex | Day | Trabecular thickness (mm) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 0.0821 | 0.0825 | 0.0866 | 0.0869 (↑6%)* |
| F | 91 | 0.101 | 0.104 | 0.100 | 0.101 |
| M | 35 | 0.0791 | 0.0806 | 0.0826 | 0.0851 (↑8%)* |

| | | | | | |
|---|---|---|---|---|---|
| M | 91 | 0.0942 | 0.0941 | 0.0965 | 0.0957 |
| Sex | Day | Trabecular Separation (mm) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 0.182 | 0.191 | 0.198 | 0.174 |
| F | 91 | 0.174 | 0.180 | 0.181 | 0.194 (↑11%) |
| M | 35 | 0.192 | 0.194 | 0.169 | 0.178 (↓7%) |
| M | 91 | 0.256 | 0.252 | 0.271 | 0.259 |
| Sex | Day | Anisothrophy | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 3.05 | 3.09 | 3.06 | 3.02 |
| F | 91 | 2.53 | 2.55 | 2.45 | 2.45 |
| M | 35 | 3.30 | 3.25 | 3.11 | 3.15 |
| M | 91 | 2.67 | 2.70 | 2.57 | 2.54 |
| Sex | Day | Structure model index | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 1.41 | 1.43 | 1.24 (↓12%) | 1.11(↓21%) |
| F | 91 | 0.853 | 0.795 | 0.924 (8%) | 1.03 (↑21%) |
| M | 35 | 1.50 | 1.47 | 1.25 (↓17%) | 1.18 (↓21%)* |
| M | 91 | 1.34 | 1.37 | 1.36 | 1.40 |
| Sex | Day | Polar moment of inertia (mm4) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 3.64 | 3.73 | 4.03 (↑11%) | 3.85 (↑6%) |
| F | 91 | 9.60 | 8.52 (↓11%)* | 8.17 (↓15%)* | 8.37 (↓12.8%)* |
| M | 35 | 4.25 | 4.09 | 4.10 | 4.26 |
| M | 91 | 13.8 | 14.3 | 13.6 | 12.5 (↓9%)* |
| Sex | Day | Femur length (mm) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 2.34 | 2.32 | 2.32 | 2.27 |
| F | 91 | 3.18 | 3.16 | 3.11* | 3.09* |
| M | 35 | 2.39 | 2.33 | 2.38 | 2.34 |
| M | 91 | 3.48 | 3.49 | 3.43* | 3.39* |
| Sex | Day | Anterior-posterior diameter femur (mm) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 2.38 | 2.35 | 2.43 | 2.42 |
| F | 91 | 2.85 | 2.73* | 2.72* | 2.69 (↓6%)* |
| M | 35 | 2.46 | 2.41 | 2.46 | 2.45 |
| M | 91 | 2.99 | 3.10 | 3.07 | 2.92 |
| Sex | Day | Medial-lateral Diameter Femur (mm) | | | |
| | | control | 30 mg/kg | 120 mg/kg | 500 mg/kg |
| F | 35 | 2.89 | 2.98 | 3.07(↑6%)* | 3.04 (↑5%)* |
| F | 91 | 3.57 | 3.47 | 3.41 (↓5%)* | 3.46 |
| M | 35 | 3.04 | 3.05 | 3.07 | 3.14 |
| M | 91 | 4.03 | 4.07 | 3.96 | 3.89 (↓3.5%)* |

### 2.6.6.7 Local tolerance

A local tolerance study with Org 25969 in female New Zealand white rabbits. The objectives of the study were to assess the local toxicity of Org 25969 in rabbits with single intravenous, intramuscular, subcutaneous, intra-arterial, and paravenous routes route of administration. The concentration of the Org 25969 (batch V) solution was 100 mg/ml and the vehicle control was NaCl 0.9%. Six animals (7 month of age, approximately 3.5 - 4.0 kg) were treated with single-dose of Org 25969 (left side of the animals) and NaCl (right side of the animal) via different routes of administration following a treatment regimen below (extracted from the sponsor's report).

| Route of administation | Org 25969 (concentration 100 mg/mL) | | Vehicle | |
|---|---|---|---|---|
| | Volume (mL) | Site of treatment | Volume (mL) | Site of treatment |
| Intravenous | 0.5 | Marginal ear vein - Right | 0.5 | Marginal ear vein - Left |
| Intramuscular | 0.5 | Biceps femoris - Right | 0.5 | Biceps femoris - Left |
| Subcutaneous | 0.5 | Dorsally* - Right | 0.5 | Dorsally* - Left |
| Intraarterial | 0.5 | Ear artery - Right | 0.5 | Ear artery - Left |
| Paravenous | 0.2 | Adjacent to marginal ear vein - Right | 0.2 | Adjacent to marginal vein - Left |

* : The sites used for injection were situated rather low and were nearly at the flanks.

Mortality and morbidity, clinical signs, body weight were recorded after treatment, 3 animals were sacrificed 24 hours after dosing while the rest 3 were sacrificed 96 hours after dosing for gross necropsy and histopathology examination. The results did not show Org 25969 treatment-related effects during 96 hours after tratment, indicating Org 25969 is tolerable to different routes of single-dose injection (intravenous, intramuscular, subcutaneous, intra-arterial, and para-venous) up to 50 mg (approximately 12 mg/kg) in rabbits.

To examine the blood compatibility of Org 25969, an in vitro study was conducted in the presence and absence of Org 9426 in male human blood. The objectives of this study were to determine the effect of Org 25969 (batch U) on hemolysis and osmotic red cell fragility in human blood. Human blood samples from 5 volunteers were incubated with Org 25969 at concentrations of 0 (NaCl, 0.9%), 0.011, 0.22, 0.54, 3.26 mM in the absence or presence of approximately same concentration of Org 9426 for 60 min at 37 °C. The results of this study indicated that Org 25969 at concentration up to 3 mM cause no hemolysis and does not affect RBC osmotic fragility, in the presence or absence of Org 9426 (rocuronium), suggesting compatibility with blood. Similarly, additional study showed that Org 48302 is also compatible with blood in human without effect on hemolysis and red cell fragility.

### 2.6.6.8 Special toxicology studies

Effects of bone and teeth

**1. Study Title:** An investigational single dose toxicity study to assess the effects of Org 25969 on bone quality, structure and turnover in male and female Wistar rats.

**Key study findings**:

- Single-dose 2000 mg/kg administration caused statistically significant change in bone parameters via μCT assay on day 21 after dosing as compared to the control, including decrease in trabecular bone volume/tissue volume (18% in female; 8% in male), trabecular number (↓10%), along with increased trabecular separation (31% in female; 11% in male). Trabecular bone mineral density also appeared to be decreased (female ↓18%, male ↓8%), although statistical significance was not reached. Bone strength assessment indicated decreased femur stiffness (femur indentation test: ↓19% in female, ↓47% in male; 3-point bending test: ↓10%) or stress (femur indentation test: ↓27% in female, ↓50% in male) on day 21 after dosing. Femur indentation test also showed decreased energy, maximal load, stiffness, and stress, indicating compromised bone length of the distal femur on day 21. BTM assay showed increased serum CTx and urine DPD, markers for bone resorption, primarily in females at this time-point. All these suggest a minor bone resorptive effect.
- Decreased trabecular number and increased trabecular separation test lasted up to day 42 after dosing, suggesting a prolonged bone effect. In addition, there were decreased stiffness in the femoral mid shaft by femur 3-point bending test and increased stiffness of the trabecular bone in the marrow cavity of the distal femur by indentation test on day 43 after dosing, further indicative of bone effect by Org 25969. In contrast, changes on day 42 appeared to be less severe than those on day 21, indicative of reversibility of the bone effect. Stiffness and stress in the marrow cavity of distal femur was significantly increased as compared to control on day 42 after dosing, which may suggest a compensatory response.
- μCT assay and bone strength assessment were not conducted on day 0 and day 7 after dosing. Examinations in BTM did not suggest a remarkable change in bone during this period.
- There was no effect on incisor after single-dose of 25969 at 2000 mg/kg.

Document ID: INT00015425
**Study no.**: 050396
**Volume #, and page #**: eCTD
**Conducting laboratory and location**: The major part of the study was conducted in N.V. Organon, Department of TDD, Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands, bone specific parameter analysis was conducted in the department of PHO of N.V. Organon.

**Date of study initiation**: 12/23/2005
**GLP compliance**: Yes
**QA reports**: yes (X)* no ( )

* The in-life part of the study, and the determination of plasma ALP, calcium and inorganic phosphate and the urinalysis with the exception of the determination of DPD were conducted in compliance with GLP standard. All other analyses including statistics were not in compliance with GLP.

**Drug, lot #, and % purity**: Batch AE

**Methods**

Doses: 0 (0.9% NaCl), 2000 mg/kg/day drug substances for single dose administration

Species/strain: adult Wistar rats (Dsd/Cpb:WU(spf-bred)) with body weight of 261-313 g for males and 147-188 g for females

Number/sex/group: 16/sex/group rats were used as shown in the following table as extracted from the sponsor's report.

| Group No | Group size and Sex | | Dose level $mg \cdot kg^{-1}$ | Treatment |
|---|---|---|---|---|
| | Male | Female | | |
| PL1 | 16 | 16 | 0 | Control item |
| DS2 | 16 | 16 | 2000 | Org 25969 |
| | | | | |

Route, formulation, volume, and infusion rate:

The test or control item was administered intravenously. A total dosing volume of 20 mL/kg was administered in all animals given as 2 subsequent injections (10 ml/kg) with approximately 1 h interval.

Satellite groups used for toxicokinetics: none

Parameters and endpoints evaluated: Clinical observations and body weight of animals were recorded during the study. Samples were collected for examination according to the following scheme:

| Dose ($mg \cdot kg^{-1}$) / Group | Number of animals | T = 24 h | T = 7 days | T = 20/21 days | T= 41/42 days |
|---|---|---|---|---|---|
| 0/PL1 | 16M + 16F | BTM 16M+16F | BTM 16M+16F | BTM 16M+16F<br>µCT 8M+8F<br>BS 8M+8F | BTM 8M+8F<br>µCT 8M+8F<br>BS 8M+8F |
| 2000/DS2 | 16M + 16F | BTM 16M+16F | BTM 16M+16F | BTM 16M+16F<br>µCT 8M+8F<br>BS 8M+8F | BTM 8M+8F<br>µCT 8M+8F<br>BS 8M+8F |

M = males; F = females; BTM = biochemical bone turnover markers; µCT = micro computed tomography; BS = bone strength

Biochemical bone turnover markers examined included plasma alkaline phosphatase (ALP), calcium (CA), inorganic phosphate (PHOS), and Osteocalcin; serum CTx (C-terminal cross-linked telopeptides of type-1 collagen); urine creatinine (CREA), CA, PHOS, DPD (free and total deoxypyridinolines). The determination of DPD was performed by a not-validated assay. Osteocalcin, a product of bone resorption, was determined by (b) (4) in a scientific research lab ( (b) (4),

(b) (4)) using radioimmunoassay. The details of the procedure were not provided. CTx, a parameter considered to be specific for bone resorption, was determined using the RatLaps ELISA assay.

Animals were sacrificed 21 and 42 days after dosing (8 males and 8 females of each group). Immediately after animal sacrifice, femur, vertebral bodies (L4 and L5), and tibia were dissected. However, the assessments of bone parameters were only conducted in femurs in this study. The length and the dimensions of the cross section of the left femur were measured by a caliper.

Bone mineral density (BMD) measurement and architecture assessment were conducted in the femur by micro computed tomography (µCT) using Skyscan 1076 microCT-40 system (Skyscan, Belgium). BMD of trabecular bone was measured in a total area of 30 slices (1mm), starting 65 slices (2.3 mm) proximal to the end of the epiphyseal disc. Cortical bone was measured in a total area of 30 slices (1mm), starting 265 slices (9.3 mm) proximal to the end of the epiphyseal disc. The following cortical and trabecular bone parameters were determined:

- trabecular BMD
- cortical BMD
- cortical thickness
- bone volume over tissue volume
- trabecular number
- trabecular thickness
- trabecular separation
- degree of anisotrophy (measure of trabecular orientation)
- structure model index (plate/rod characterisation of trabecular bone).
- Polar moment of inertia

Bone strength assessments were in femur including trabecular indentation test, 3-point bending test and femur neck cantilever test.

*Indentation test of the distal femur*
An indentation test determines the mechanical properties of the trabecular bone in the marrow cavity of the distal femur. A 4 mm section of the distal femoral metaphysic is cut directly proximal to the femoral condyle (+/- 5 mm from the distal end) under constant saline irrigation. A 1.6-mm diameter cylindrical indenter (with flat testing face) attached to the load cell is applied to the centre of the marrow cavity on the distal face of the section at a constant displacement velocity of 6 mm/min. The indenter is allowed to penetrate the cavity to a depth of 2 mm before load reversal. From load-displacement curves, maximum force (N), displacement (mm), energy (area under the curve, J), stress ($N/mm^2$) and stiffness (N/mm) were determined. The maximum force and maximum displacement was determined by locating the initial peak, in which the height of the initial peak represents the maximum force and the consequent displacement is the maximum displacement. The structural rigidity of the tested bone, also known as the stiffness, is determined by calculating the slope of the linear portion of the initial peak. Stress is defined as force per unit area and was calculated by dividing the maximum force by the cross sectional area of the cylindrical indentor.

*Three-point bending test of femoral shaft*
The three-point bending test determines the mechanical properties of the femoral mid shaft. The femur is placed freely on 4 mm-diameter supporting rollers with the anterior aspect down. The rollers are fixed on the crosshead and were separated by a span of 15 mm. Vertical load is applied in the middle of the femur by moving the loading roller downwards at a speed of 1 mm/min until fracture occurs. From load-displacement curves, maximum force (N), displacement (mm), energy (area under the curve, J) and stiffness (N/mm) were determined. The maximum force and maximum displacement was determined by locating the initial peak, in which the height of the initial peak represents the maximum force and the consequent displacement is the maximum displacement. The stiffness, is determined by calculating the slope of the linear portion of the initial peak.

*Cantilever bending test of femoral neck*
The cantilever bending test, also known as femoral neck bending test, is used to determine the mechanical properties of the femoral neck. The whole femur is fixed in a specially constructed fixation device and the fixation unit is then placed in a mechanical testing machine. A vertical load is conducted on top of the femoral head with a hollow-surfaced brass cylinder. The loading unit moves downwards at a constant rate of 2 mm/min until fracture of the femoral neck. From load-displacement curves, maximum force (N), displacement (mm), energy (area under the curve, J) and stiffness (N/mm) were determined. The maximum force and maximum displacement was determined by locating the initial peak, in which the height of the initial peak represents the maximum force and the consequent displacement is the maximum displacement. The structural rigidity of the tested bone, also known as the stiffness, is determined by calculating the slope of the linear portion of the initial peak.

No μCT or strength assessments were done on the Lumbar vertebral bodies

Color determination of teeth
Possible teeth discoloration due to Org 25969 were evaluated by determination of the color of the (upper part) of the lower incisors of the rats. This was done prior to dosing and approximately every ten days thereafter up to sacrifice of the animals. Teeth color was scored visually with comparison to a tooth color palette (Candulor TCR Resin ®, Swiss). The Candulor TCR Resin shade guide has 2 shade systems that are chromatographically arranged. The first system is the Candulor system which has 9 shades - J1, J2, J3, M1, M2, M3, M4, S1 and S2 (these are more yellow/orange shades). The second system is the Vita system with 11 shades - A1, A2, A3, A3.5, A4, B2, B3, C2, C3, D2 and D3 (these are more white/grey/brown shades). Teeth were scored according to these 2 systems. In addition, a color code was supplemented with + or ++ in case the color was darker than provided within the shade system but not represented within the given chromatographic range (eg M4+, more yellow than M4 but not within the S-shades). Individual scores throughout the experiment are reported in a table.

Statistical analysis of the result was performed with SAS software. All variables were summarized by means of descriptive statistics (mean, standard deviation, minimum, median and maximum) per time, gender and treatment group, and analyzed with a three way ANOVA with factors treatment, gender and time and (at least) their first order interactions. The significance level was set at 0.05. The interaction between time and treatment, and the interaction between gender and treatment was investigated if statistically significance of main effect was detected .

**Results:**

In-life observation

Single dose intravenous administration of Org 25969 (2000 mg/kg) induced no drug-related clinical signs and body weight change.

Analysis of biochemical bone turnover markers

There was no significant difference in arterial and venous plasma alkaline phosphate, calcium, and osteocalcin observed in animals of 2000 mg/kg groups as compared to control. For inorganic phosphorous, a minor but significant increase was only observed in arterial plasma of females (≤6%), and no significant difference was observed in arterial of males and venous of both male and females.

| Sex | Day after dosing | *Plasma Alkaline phosphatase* | | | |
|---|---|---|---|---|---|
| | | control | | 2000 mg/kg | |
| | | Arterial | Venous | Arterial | Venous |
| F | 1 | | 170.00 | | 187.50 |
| | 7 | | 156.75 | | 176.13 |
| | 21 | 123.5 | 136.13 | 118.75 | 133.50 |
| | 42 | 85.88 | 91.00 | 92.5 | 110.25 |
| M | 1 | | 257.63 | | 256.57 |
| | 7 | | 217.88 | | 217.50 |
| | 21 | 158.38 | 187.13 | 154.88 | 170.88 |
| | 42 | 122.75 | 141.00 | 121.88 | 135.50 |
| Sex | Day after dosing | *Plasma Calcium* | | | |
| | | control | | 2000 mg/kg | |
| | | Arterial | Venous | Arterial | Venous |
| F | 1 | | 2.84 | | 2.84 |
| | 7 | | 2.77 | | 2.77 |
| | 21 | 2.58 | 2.64 | 2.57 | 2.60 |
| | 42 | 2.66 | 2.61 | 2.65 | 2.56 |
| M | 1 | | 2.89 | | 2.94 |
| | 7 | | 2.87 | | 2.87 |
| | 21 | 2.57 | 2.71 | 2.60 | 2.68 |

| | | | | | |
|---|---|---|---|---|---|
| | 42 | 2.66 | 2.67 | 2.66 | 2.62 |
| Sex | Day after dosing | *Plasma Osteocalcin (ng/ml)* | | | |
| | | control | | 2000 mg/kg | |
| | | Arterial | Venous | Arterial | Venous |
| F | 1 | | 239.96 | | 220.31 |
| | 7 | | 201.91 | | 203.10 |
| | 21 | 115.08 | 157.74 | 109.55 | 151.85 |
| | 42 | 89.64 | 123.23 | 86.20 | 108.18 |
| M | 1 | | 265.13 | | 237.59 |
| | 7 | | 249.48 | | 238.70 |
| | 21 | 146.88 | 195.49 | 148.88 | 181.68 |
| | 42 | 117.36 | 166.10 | 119.98 | 177.50 |
| Sex | Day after dosing | *Plasma phosphate (mM)* | | | |
| | | control | | 2000 mg/kg | |
| | | Arterial | Venous | Arterial | Venous |
| F | 1 | | 2.35 | | 2.50 |
| | 7 | | 2.05 | | 2.16 |
| | 21 | 1.70 | 2.05 | 1.80 (↑6%)* | 2.10 |
| | 42 | 1.81 | 1.90 | 1.98 (↑5%)* | 1.93 |
| M | 1 | | 2.65 | | 2.87 |
| | 7 | | 2.48 | | 2.56 |
| | 21 | 1.95 | 2.54 | 1.93 | 2.50 |
| | 42 | 1.87 | 2.10 | 1.83 | 2.17 |

* $p < 0.05$

Statistical analysis showed a significant increase on day 21 after dosing in arterial serum CTx with no gender difference. There was no statistical significance detected on day 42 after dosing, indicating this change was reversible. However, since the CTx level was not examined at day 0 and 7 after dosing, it is not know whether significant change also occurred in these time-points. For venous plasma, significant increase was only observed at day 1 after dosing, indicating an acute and transient change. Overall, Org 25969 may cause reversible increase of CTx in the blood at 2000 mg/kg, indicative of bone resorption.

| Sex | Day after dosing | *Serum CTx* | | | |
|---|---|---|---|---|---|
| | | control | | 2000 mg/kg | |
| | | Arterial | Venous | Arterial | Venous |
| F | 1 | | 41.01 | | 42.51 |
| | 7 | | 32.89 | | 34.78 |
| | 21 | 17.38 | 30.17 | 21.77 (↑25%)* | 33.60 |
| | 42 | 15.58 | 12.27 | 14.95 | 12.38 |
| M | 1 | | 56.01 | | 69.19 (↑24%)* |
| | 7 | | 43.01 | | 49.15 |
| | 21 | 33.71 | 55.39 | 34.24 (↑2%)* | 53.39 |
| | 42 | 26.85 | 18.76 | 26.50 | 18.2 |

* $p < 0.05$

Urine analysis indicated a significant decrease in phosphate at day 0 of dosing with no significant change in later time-points, indicating a reversible effect. DPD was significantly decreased on day 1 after dosing in females (5%) and males (14%) while increased on day 21 after dosing in both females (23%) and on day 42 after dosing in males (11%).

| Sex | Day after dosing | *Urine calcium (mM/mM)* | |
|---|---|---|---|
| | | control | 2000 mg/kg |
| F | 1 | 1.23 | 1.25 |
| | 7 | 1.17 | 1.17 |
| | 21 | 1.02 | 1.24 |
| | 42 | 1.03 | 1.07 |
| M | 1 | 1.03 | 1.01 |
| | 7 | 0.76 | 0.80 |
| | 21 | 0.53 | 0.51 |
| | 42 | 0.38 | 0.41 |
| Sex | Day after dosing | *Urine phosphate (mM)* | |
| | | control | 2000 mg/kg |
| F | 1 | 2.46 | 1.71 |
| | 7 | 2.16 | 2.09 |
| | 21 | 3.04 | 3.42 |
| | 42 | 3.32 | 3.11 |
| M | 1 | 5.02 | 3.59 |
| | 7 | 4.27 | 5.26 |
| | 21 | 3.50 | 3.98 |
| | 42 | 4.16 | 3.82 |
| Sex | Day after dosing | *Urine Creatinine (μM)* | |
| | | control | 2000 mg/kg |
| F | 1 | 4916.25 | 4858.25 |
| | 7 | 588456 | 5550.81 |
| | 21 | 5308.56 | 4902.75 |
| | 42 | 6011.63 | 5265.50 |
| M | 1 | 5586.13 | 6091.94 |
| | 7 | 7030.13 | 7198.31 |
| | 21 | 6552.50 | 6838.31 |
| | 42 | 8873.38 | 9447.00 |
| Sex | Day after dosing | *Urine volume (ml)* | |
| | | control | 2000 mg/kg |
| F | 1 | 9.38 | 8.94 |
| | 7 | 8.81 | 7.69 |
| | 21 | 11.25 | 11.19 |

| | | | |
|---|---|---|---|
| | 42 | 10.88 | 11.38 |
| M | 0 | 11.44 | 10.25 |
| | 7 | 10.81 | 9.88 |
| | 21 | 14.88 | 13.19 |
| | 42 | 12.50 | 10.25 |
| Sex | Day after dosing | *Urine DPD (nM)* | |
| | | control | 2000 mg/kg |
| F | 1 | 234.39 | 222.68 (↓5%) * |
| | 7 | 233.82 | 211.89 |
| | 21 | 186.26 | 230.93 (↑24%)* |
| | 42 | 121.67 | 116.85 |
| M | 1 | 261.85 | 223.99 (↓14%) * |
| | 7 | 253.98 | 252.31 |
| | 21 | 214.92 | 215.94 |
| | 42 | 182.09 | 201.26 |

* $p < 0.05$

Bone parameters

There was no treatment-related change in femur length and diameters.
Significant decrease in trabecular bone volume/tissue volume ratio of trabecular bone in metaphysics of femur on day 21 of dosing. In addition, bone mineral density also appeared to be decreased at this time-point, though there was no statistically significant. There were no changes in trabecular mineral density and bone volume/tissue volume ratio observed on day 42 of dosing. Trabecular number was significantly decreased while trabecular separation increased in both male and female rats on day 21 and 42 after dosing. Additionally, anisotrophy was found to be minor but significant increased at both day 21 and 42 of dosing.

| Sex | Day | *Bone volume/tissue volume ratio of mid diaphysis(distal) of femur (%)* | |
|---|---|---|---|
| | | control | 2000 mg/kg |
| F | 21 | 26.43 | 24.59 (↓7.0%) |
| | 42 | 33.61 | 33.06 (↓1.6%) |
| M | 21 | 37.99 | 31.94(↓16%) |
| | 42 | 27.94 | 27.28 (↓2.4) |
| Sex | Day | *Bone volume/tissue volume of trabecular bone in metaphysics* (proximal) *of femur (%)* | |
| | | control | 2000 mg/kg |
| F | 21 | 43.51 | 35.59 (↓18%)* |
| | 42 | 53.02 | 49.17 (↓7.3%)* |
| M | 21 | 25.24 | 23.29 (↓7.7%)* |
| | 42 | 32.24 | 28.57 (↓11%)* |
| Sex | Day | *Cortical bone mineral density (mg/cm$^3$)* | |
| | | control | 2000 mg/kg |
| F | 21 | 1258.25 | 1234.5 |
| | 42 | 1287 | 1285.13 |

| | | | |
|---|---|---|---|
| M | 21 | 1203.5 | 1196.38 |
| | 42 | 1230.13 | 1241 |
| Sex | Day | *Cortical bone thickness (mm)* | |
| | | control | 2000 mg/kg |
| F | 21 | 0.49 | 0.48 |
| | 42 | 0.51 | 0.51 |
| M | 21 | 0.51 | 0.51 |
| | 42 | 0.60 | 0.59 |
| Sex | Day | *Anisotropy* | |
| | | control | 2000 mg/kg |
| F | 21 | 2.09 | 2.29 (↑9.6)* |
| | 42 | 1.8 | 1.8 * |
| M | 21 | 2.28 | 2.51 (↑10%)* |
| | 42 | 2.16 | 2.25 (↑4.2%)* |
| Sex | Day | Structure model index | |
| | | control | 2000 mg/kg |
| F | 21 | 0.76 | 1.12 (↑47%) |
| | 42 | 0.31 | 0.52 (↑67%) |
| M | 21 | 1.52 | 1.56 (↑2.6%) |
| | 42 | 1.12 | 1.28 (↑14%) |
| Sex | Day | Polar moment of inertia ($mm^4$) | |
| | | control | 2000 mg/kg |
| F | 21 | 8.7 | 9.24 (↑6.2%) |
| | 42 | 8.83 | 8.62 (↓2.4%) |
| M | 21 | 13.1 | 14.39 (↑9.8%) |
| | 42 | 19.77 | 18.35 (↓7.2%) |
| Sex | Day | Trabecular bone mineral density ($mg/cm^3$) | |
| | | control | 2000 mg/kg |
| F | 21 | 321.5 | 271.38 (↓18%) |
| | 42 | 378.75 | 389.25(↑2.8%) |
| M | 21 | 191 | 175.88 (↓7.9%) |
| | 42 | 242.13 | 215.25 (↓11.1%) |
| Sex | Day | Trabecular number (1/mm) | |
| | | control | 2000 mg/kg |
| F | 21 | 2.36 | 2.09 (↓11%)* |
| | 42 | 2.73 | 2.39 (↓12%)* |
| M | 21 | 1.58 | 1.43 (↓9.5%)* |
| | 42 | 1.85 | 1.71 (↓7.6%)* |
| Sex | Day | Trabecular separation (mm) | |
| | | control | 2000 mg/kg |
| F | 21 | 0.29 | 0.38 (↑31%)* |
| | 42 | 0.23 | 0.25 (↑8.7%)* |
| M | 21 | 0.57 | 0.63 (↑11%)* |
| | 42 | 0.54 | 0.57 (↑5.5%)* |
| Sex | Day | Trabecular thickness (mm) | |

| | | control | 2000 mg/kg |
|---|---|---|---|
| F | 21 | 0.18 | 0.17 |
| | 42 | 0.19 | 0.20 |
| M | 21 | 0.16 | 0.16 |
| | 42 | 0.17 | 0.17 |

* $p < 0.05$

Bone strength assessment

Femur indentation test for mechanical properties of in the marrow cavity of the distal femur indicated that maximum load, and stress decreased significantly on day 21 after dosing. Stiffness was decreased with no statistically significant and increased day 42 significantly. In addition, stress was also likely to be increased as compared to the control on day 42 after dosing.

| Sex | Day | Femur indentation test displacement (mm) | |
|---|---|---|---|
| | | control | 2000 mg/kg |
| F | 21 | 0.11 | 0.07 |
| | 42 | 0.17 | 0.16 |
| M | 21 | 0.05 | 0.04 |
| | 42 | 0.06 | 0.09 |
| Sex | Day | Femur indentation test energy (J) (log-transformed) | |
| | | control | 2000 mg/kg |
| F | 21 | -6.38 | -7.31* |
| | 42 | -5.99 | -5.68 |
| M | 21 | -8.21 | -8.71* |
| | 42 | -7.87 | -7.85 |
| Sex | Day | Femur indentation test maximal load (N) | |
| | | control | 2000 mg/kg |
| F | 21 | 29.01 | 21.21* |
| | 42 | 49.06 | 30.86 |
| M | 21 | 6.98 | 3.51* |
| | 42 | 5.89 | 6.74 |
| Sex | Day | Femur indentation test Stiffness (N/mm) | |
| | | control | 2000 mg/kg |
| F | 21 | 302.66 | 244.35 (↓19%) |
| | 42 | 232.98 | 418.46 (↑80%)* |
| M | 21 | 77.72 | 41.55 (↓47%) |
| | 42 | 46.78 | 56.53 (↑20%)* |
| Sex | Day | Femur indentation test Stress ($N/mm^2$) | |
| | | control | 2000 mg/kg |
| F | 21 | 14.43 | 10.50 (↓27%)* |
| | 42 | 15.35 | 24.40 (↑59%) |
| M | 21 | 3.47 | 1.75 (↓50%)* |
| | 42 | 2.93 | 3.35 (↑14%) |

Femur 3-point bending test showed significant decrease in stiffness on day 21 and 42 after dosing was observed. In addition, there was significant decrease in maximal bending load at day 21 but not day 42.

| Sex | Day | Femur 3-point bending displacement (mm) | |
|---|---|---|---|
| | | control | 2000 mg/kg |
| F | 21 | 0.51 | 0.52 |
| | 42 | 0.43 | 0.49 |
| M | 21 | 0.52 | 0.60 |
| | 42 | 0.55 | 0.57 |
| Sex | Day | Femur 3-point bend energy (J) (log-transformed) | |
| | | control | 2000 mg/kg |
| F | 21 | -3.58 | -3.77 |
| | 42 | -3.63 | -3.43 |
| M | 21 | -3.27 | -3.2 |
| | 42 | -2.83 | -2.99 |
| Sex | Day | Femur 3-point bending maximal load (N) | |
| | | control | 2000 mg/kg |
| F | 21 | 92.95 | 82.89 |
| | 42 | 115.75 | 108.40 |
| M | 21 | 122.78 | 117.91 |
| | 42 | 177.60 | 170.20 |
| Sex | Day | Femur 3-point bending Stiffness (N/mm) | |
| | | control | 2000 mg/kg |
| F | 21 | 251.29 | 223.71 (↓11%)* |
| | 42 | 377.88 | 342.40 (↓9%)* |
| M | 21 | 334.44 | 301.69 (↓10%)* |
| | 42 | 496.26 | 449.54 (9%)* |

Femur cantilever test parameter: No remarkable change.

| Sex | Day | Femur Cantilever displacement (mm) | |
|---|---|---|---|
| | | control | 2000 mg/kg |
| F | 21 | 0.86 | 0.98 |
| | 42 | 0.87 | 0.92 |
| M | 21 | 1.02 | 0.87 |
| | 42 | 0.96 | 1.06 |
| Sex | Day | Femur cantilever energy (J) (log-transformed) | |
| | | control | 2000 mg/kg |
| F | 21 | -3.25 | -3.10 |
| | 42 | -3.19 | -3.07 |
| M | 21 | -3.26 | -3.32 |
| | 42 | -3.11 | -2.96 |
| Sex | Day | Femur cantilever maximal load (N) | |
| | | control | 2000 mg/kg |
| F | 21 | 86.18 | 85.68 |

| | | | |
|---|---|---|---|
| | 42 | 91.58 | 93.13 |
| M | 21 | 76.44 | 78.27 |
| | 42 | 87.91 | 91.35 |
| Sex | Day | Femur cantilever Stiffness (N/mm) | |
| | | control | 2000 mg/kg |
| F | 21 | 107.35 | 102.25 |
| | 42 | 115.88 | 106.98 |
| M | 21 | 86.74 | 100.16 |
| | 42 | 98.37 | 89.76 |

Color determination of teeth: no treatment related change.

**2. Study Title:** Second investigational single dose toxicity study to assess the effects of Org 25969 on bone quality, structure and turnover in male and female Wistar rats.

**Key study findings**: There were no significant changes in bone parameters after single-dose administration of Org 25969 at dose level up to 500 mg/kg as assessed by µCT asssay

Document ID: INT00033963
**Study no.**: 060338
**Volume #, and page #**: eCTD
**Conducting laboratory and location**: N.V. Organon, Department of TDD, Nistelrooisebaan 3, NL-5374 RE Schaijk, The Netherlands

**Date of study initiation**: 10/24/2006
**GLP compliance**: Yes*
The bone mineral density and architecture assessment and all statistics performed at the test site are excluded from GLP.

**QA reports**: yes (X) no ( )
**Drug, lot #, and % purity**: Batch AE

**Methods**
Doses: 0 (0.9% NaCl), 30, 120, 500 mg/kg drug substances for single dose administration

Species/strain: adult Wistar rats (Dsd/Cpb:WU(spf-bred)) with body weight of 250-289 g for males and 163-206 g for females at start of dosing

Number/sex/group: 24/sex/treated group rats were used as shown in the following table as extracted from the sponsor's report. In addition, there was an untreated control group with 16 animals (8/sex) in this study.

| Group No. | Group size and sex | | Dose level | Treatment |
|---|---|---|---|---|
| | Male | Female | ($mg \cdot kg^{-1}$) | |
| PL1 | 24 | 24 | 0 | Control item |
| PL2# | 8 | 8 | untreated | untreated |
| DS3 | 24 | 24 | 30 | Org 25969 |
| DS4 | 24 | 24 | 120 | Org 25969 |
| DS5 | 24 | 24 | 500 | Org 25969 |

# The animals allocated to PL2 were used as an untreated control group (these animals were not treated at all) and were randomly selected at arrival and put into experiment 060338S(2). The other dosing groups were put into experiment 060338S(1). Animals of PL2 were weighed at the start of the pre-dose and sacrificed on the day of start dosing of experiment 060338S(1).

Route, formulation, volume, and infusion rate:

The test or control item was administered intravenously once via the tail vein. A total dosing volume of 5 ml/kg was administered in all animals.

Satellite groups used for toxicokinetics: none

Parameters and endpoints evaluated: Clinical observations and body weight of animals were recorded during the study. 3/sex/group animals were sacrificed on the following time-points (SD1, 3, 7, 14, 21, 28, 35, 42) and bone samples were collected for µCT analysis. Blood and urine samples were also collected, but bone turnover parameters were not measured.

The following sampling scheme was applicable for experiment 060338S(1):

| Dose ($mg \cdot kg^{-1}$) | Animals in total | Sacrifice at day x of dosing | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 7 | 14 | 21 | 28 | 35 | 42 |
| 0 | 24M+24F | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 |
| 30 | 24M+24F | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 |
| 120 | 24M+24F | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 |
| 500 | 24M+24F | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 | 3+3 |

M = males; F = females.
The animals in experiment 060338S(2) (8 M + 8 F) were sacrificed on the day of start dosing (t=0) of experiment 060338S(1).

**Bone mineral density and architecture assessment by µCT assay**
The following cortical and trabecular bone parameters were determined:

- trabecular BMD
- cortical BMD
- cortical thickness
- bone volume over tissue volume mid diaphysis femur
- bone volume over tissue volume trabecular bone metaphysis femur
- trabecular number
- trabecular thickness
- trabecular separation
- degree of anisotrophy (measure of trabecular orientation)
- structure model index (plate/rod characterisation of trabecular bone).
- polar moment of inertia

Statistical analysis: Analysis were performed with SAS software

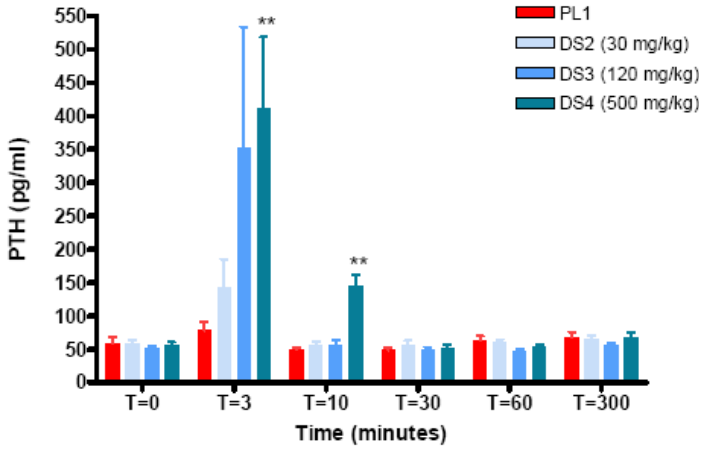
All variables were summarized by means of descriptive statistics (mean, standard deviation, minimum, median and maximum) per time, gender and treatment group. In addition, all variables were analyzed with a three way ANOVA with factors treatment, gender and time and (at least) their first order interactions. The significance level was set at 0.05. The interaction between time and treatment, and the interaction between gender and treatment was investigated.

**Results:**

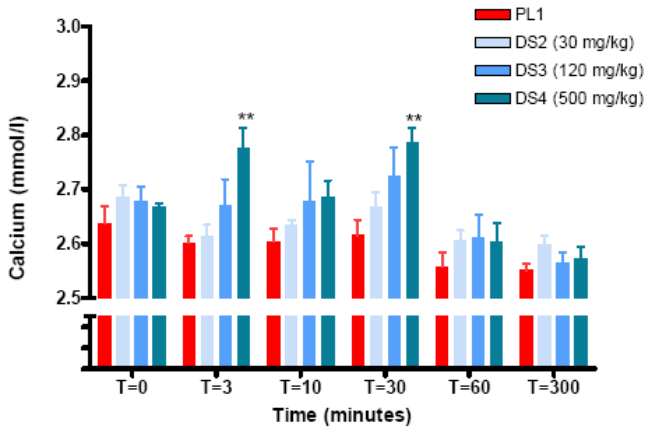
No drug-related clinical signs
Bone parameters by µCT: There were no treatment-related changes observed.

**3. Study Title:** the effect of a single intravenous dose Org 25969 on PTH release and urinary Ca2+ excretion in female Wistar rats

The objectives of this study were to study the effect of a single bolus injection of Org 25969 (batch AE) on parathyroid hormone (PTH) levels in serum and urinary Ca2+ excretion in rats.
A single dose of Org 25969 was administered intravenously to female Wistar rats via the tail vein at dosages of 0 (0.9% NaCl), 30, 120, and 500 mg/kg (6 rats/group). Parameters evaluated included mortality, clinical signs, body weight and clinical pathology. Blood on behalf of serum for clinical pathology (calcium, phosphate and PTH) was collected after a single dose at the following time points: 0 (pre-dose sample), 3, 10, 30, 60, and 300 min after dosing. In addition, urine for clinical pathology (volume, calcium, phosphate and creatinine) was collected between 1 and 5 h after dosing and between 5 and 24 h after dosing. This study was conducted in accordance with the OECD principles of GLP except the determination of PTH and the statistical analysis of the clinical pathology parameters.
The results of the study demonstrated statistically significant increase in PTH level at 500 mg/kg at 3 (>5 fold) and 10 minutes (approximately 3 fold) after Org 25969 administration as shown in the figure below (extracted from the sponsor's report). This change was transient since similar change was not observed after 30 minutes of administration. At 3 minutes after administration, trend of increase was also observed in animals of 30 and 120 mg/kg group.




Mild but significant increase in serum Ca2+ concentration was also observed at 3 minutes (6.9%) and 30 minutes (6.5%) after dosing of 500 mg/kg as shown below (extracted from the sponsor's report). There was no significant change in serum inorganic phosphate concentration.




In the urinalysis, increased phosphate excretion was observed between 5 and 24 hours urine collection after dosing at 500 mg/kg Org 25969. There were no treatment-related changes observed. Overall, Org 25969 induced a transient PTH surge which was probably associated with a slight increase in total serum Ca2+ and urine phosphate.

In addition, effect on plasma PTH was also examined in dogs in the 3-week toxicokinetic study (see Section 26.4.3 Absorption), a single IV bolus injection of Org 25969 to beagle dogs at dose levels of 25, 80 or 250 mg/kg resulted in a transient increase in plasma PTH in all dogs at 250 mg/kg and 2 out of 6 dogs at 80 mg/kg. The increase appeared to be dose dependent in height and duration and did not last for more than approximately 15 minutes. However, dispersion in results and limited animal numbers restrained solid statistic analysis in this study.

Immunogenicity

1. **Study Title:** Determination of the potential immunomodulating effects of Org 25969 (batch U) by means of the plaque forming cell assay in 2-week intravenous dosing study in male and female Wistar rats

The direct plaque forming cell (PFC) assay was initially developed in 1961, primarily accessing humoral IgM antibody responses to antigen. In this study, rats of 7 weeks old were treated with Org 25969 or vehicle control via intravenous injection for 2 weeks in group 2-4 following the design below as extracted from the sponsor's report. The positive control was only treated once during the treatment on SD8 during the treatment

| Group/ Treatment | Dose level (mg/kg/day) | Dose volume (mL/kg/day) | Dose concentration (mg/mL) | Number of animals | |
|---|---|---|---|---|---|
| | | | | Males | Females |
| 1. Control | 0 | 5 | 0 | 8 | 8 |
| 2. Low dose | 30 | 5 | 6 | 8 | 8 |
| 3. Intermediate dose | 120 | 5 | 24 | 8 | 8 |
| 4. High dose | 500 | 5 | 100 | 8 | 8 |
| 5. Positive control | 50 | 5 | 10 | 8 | 8 |

The vehicle control was 0.9% NaCl, and positive control was cyclophosphamide. On SD10 (males) or 11 (females), all animals were immunized with $4 \times 10^9$ sheep red blood cells (SRBC)/kg. All surviving animals were sacrificed 4 days after SRBC immunization, and a direct plaque forming cell (PFC) assay was performed on spleenocytes. The results of this study showed that there were no changes in spleen weight or decrease in plaque forming in animals treated with Org 25969 at all dose levels tested as compared to vehicle control. In contrast, cyclophosphamide, the positive control, elicited statistically significant decrease in plaque formation, indicative of immunosuppression. These suggested that Org 25969 is not likely to be immunosuppressive. Instead, these data showed slight increase in PFC formation in animals treated with Org 25969 as compared to the vehicle control as shown below (extracted from the sponsor's review). Although this change was not dose-dependent, and was not statistically significant at 120 and 500 mg/kg, it is likely to suggest a potential of immuno-stimulation of Org 25969.

PLAQUE FORMING CELL ASSAY
Group Mean Values

| | Day 14 Log PFC/1E5c | Day 14 PFC/1E5c | Day 14 PFC/g/spleen 1E3 | Day 14 SpleenWeight g | Day 14 Cells/spleen 1E6 | Day 14 PFC/spleen 1E3 |
|---|---|---|---|---|---|---|
| Male, 0 mg/kg/day | | | | | | |
| Mean | 2.04 | 140 | 946 | 0.7626 | 515 | 747 |
| SD | 0.30 | 118 | 793 | 0.1062 | 118 | 645 |
| N | 8 | 8 | 8 | 8 | 8 | 8 |
| Male, 30 mg/kg/day | | | | | | |
| Mean | 2.34 | 253 | 1973** | 0.7293 | 547 | 1412* |
| SD | 0.26 | 134 | 1168 | 0.0589 | 73 | 822 |
| N | 7 | 7 | 7 | 7 | 7 | 7 |
| Male, 120 mg/kg/day | | | | | | |
| Mean | 2.22 | 187 | 1342 | 0.7864 | 558 | 1031 |
| SD | 0.24 | 110 | 816 | 0.1286 | 88 | 581 |
| N | 8 | 8 | 8 | 8 | 8 | 8 |
| Male, 500 mg/kg/day | | | | | | |
| Mean | 2.25 | 215 | 1509 | 0.8149 | 567 | 1185 |
| SD | 0.28 | 162 | 1207 | 0.1695 | 152 | 910 |
| N | 8 | 8 | 8 | 8 | 8 | 8 |
| Male, Positive control | | | | | | |
| Mean | 1.29*** | 28** | 117** | 0.5393** | 209*** | 67** |
| SD | 0.37 | 32 | 143 | 0.0914 | 57 | 89 |
| N | 8 | 8 | 8 | 8 | 8 | 8 |

Key: Experimental Unit = Animal

PFC: Plaque Forming Cell

c: cell.

PFC/1E5c: number of plaque forming cells per $10^5$ cells.

PFC/g/spleen: number of plaque forming cells per gram of spleen.

PFC/spleen: number of plaque forming cells per spleen.

2. **Study Title:** A popliteal lymph node assay (PLNA) with Org 25969 in female mice

The objective of this study was to demonstrate whether Org 25969 has sensitizing properties when applied in a Popliteal Lymph Node Assay (PLNA) in mice after subcutaneous (SC) footpad injection at three different dose levels (0.3, 1.2 and 5 mg/animal). Based on the data of the mean body weight of each group (around 18 grams), the Org 25969 dosing levels were 17, 67, and 278 mg/kg. A positive control group receiving $HgCl_2$ was included in the study for study validation. The following table (extracted from the sponsor's report) shows the schematic study design of this study.

| Group, colour | Study substances | Number of animals per group, sex | Dose (in 50 µl) day 0 (3 February 2004) | Necropsy Day 7 (10 February 2004) |
|---|---|---|---|---|
| A White | Vehicle | 8♀ | - | 8♀ |
| B Blue | Org 25969 low | 8♀ | 0.3 mg/animal | 8♀ |
| C Green | Org 25969 mid | 8♀ | 1.2 mg/animal | 8♀ |
| D Red | Org 25969 high | 8♀ | 5 mg/animal | 8♀ |
| E yellow | $HgCl_2$ | 8♀ | 50 µg/animal | 8♀ |

After SC injection of a constant volume of 50 µl vehicle control (NaCl 0.9%), Org 25969, and the positive control ($HgCl_2$) on SD0 into the left hind footpad (heel to toe direction), all animals received an IV injection of [$^3$H]-thymidine on SD7. Five hours later, the weight and the [$^3$H]-thymidine incorporation in the ipsilateral PLN was determined and

compared with untreated contra lateral PLN. Statistical evaluation was performed by analysis of (co)-variance followed by Dunnett's multiple comparison tests.

$HgCl_2$, a known sensitizer, resulted in statistically significant increase in [$^3$H]-thymidine incorporation in the ipsilateral PLN (26-fold ↑) with significant increased PLN weight (approximately ↑80%) as compared with vehicle control. In contrast, there was no significant difference in the contralateral PLN weight and [$^3$H]-thymidine incorporation between the group of positive control and vehicle control. There was no significant change in ipsi- and contra-lateral PLN weight in all groups of Org 25969-treated as compared to that of the vehicle control group. However, in the animals treated with the high dose Org 25969 (5 mg/animal), a statistically significant increase (2.8 fold ↑) in $^3$H-thymidine incorporation was observed in the ipsilateral PLN compared to the vehicle control as shown below (extracted from the sponsor's report). This change was not seen in low and mid-dose groups. In addition, increased [$^3$H]-incorporation was not observed in contralateral PLN in all Org 25969-trated groups. These results indicated that Org 25969 is likely to be compound with weak immuno-stimulatory effect.

A popliteal lymph node assay (PLNA) with Org 25969 in mice
TNO Nutrition and Food Research
Study : 5593

Table 3 Mean 3H thymidine incorporation

| F E M A L E S | | PopLN L dpm | PopLN R dpm | PLN index |
|---|---|---|---|---|
| vehicle | Mean | 177 | 165 | 1.1 |
| | sd | 133 | 147 | 0.3 |
| | n | 8 | 7 | 7 |
| Org25969low | Mean | 186 | 132 | 2.3 |
| | sd | 131 | 82 | 2.7 |
| | n | 7 | 7 | 7 |
| Org25969mid | Mean | 133 | 139 | 1.3 |
| | sd | 26 | 63 | 1.1 |
| | n | 8 | 8 | 8 |
| Org25969high | Mean | 495* | 115 | 4.9 |
| | sd | 427 | 50 | 4.0 |
| | n | 8# | 8 | 8 |
| HgCl2 | Mean | 4545*** | 316 | 23.5 |
| | Sd | 2496 | 270 | 20.9 |
| | n | 8 | 8 | 8 |

3. **Study Title:** Assessment of contact hypersensitivity to Org 25969 in the mouse (local lymph node assay)

The purpose of the study was to evaluate whether the test substance induce contact hypersensitivity in female mice after 3 consecutive topical applications (1 application/day) of 3 different concentrations (10%, 25%, and 50%) of 25 µl Org 25969 (batch V). The vehicle for Org 25969 solution preparation was dimethyl sulfoxide.

For LLNA assay, a preliminary irritation assay needs to be conducted in order to select the highest test substance concentration to be used in the main study. In principle, this concentration should be well tolerated systemically and produce ≤ moderate irritation (grade 3). In this study, the sponsor tested 50% concentration of Org 25969 and found no

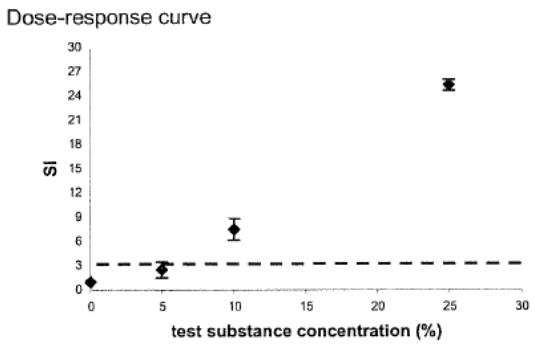
sign of irritation based on erythema and edema grading. The concentration of 50% was thus used as the highest concentration in the LLNA assay.

In the main study, 25 µl 50% Org 25969 solution and vehicle control (DMSO) was initially applied on the dorsal surface of both ears of animals of the designated groups for 3 consecutive days during the induction phase. Three days after the termination of treatment (SD6), each animal was injected with 0.25 ml of PBS containing 20 µCi [$^3$H]-methyl thymidine. After 5 hours, animals were sacrificed by intra peritoneal injection with an overdose of pentobarbital. The draining (auricular) lymph node of each ear was excised. The relative size of the nodes (as compared to normal) was estimated by visual examination. Afterwards, the nodes were pooled for each animal in 3 ml PBS and the radioactivity (DPM) of each sample was determined. Lower concentrations (25% and 10%) of Org 25969 were examined in additional animal groups with concurrent DMSO control (5/group). A stimulation index (SI: DPM/treated group vs. DPM/control group) was calculated for each concentration. Based on the guideline and recommendations from ICCVAM, a test substance may be regarded as a skin sensitizer if the SI is ≥ 3.

The results of this study showed that the SI at either Org 25969 concentration was not ≥ 3 as shown below (extracted from the sponsor's report), and therefore, concluded by the sponsor that Org 25969 was not a skin sensitizer.

| group | treatment | induction | mean DPM ± SD | SI ± SD |
|---|---|---|---|---|
| 3** | experimental | 10% test substance | 213 ± 70 | 1.0 ± 0.5 |
| 4** | experimental | 25% test substance | 151 ± 32 | 0.7 ± 0.4 |
| 2* | experimental | 50% test substance | 292 ± 249 | 0.7 ± 0.9 |
| 1* | vehicle control | Dimethyl sulphoxide | 407 ± 90 | 1.0 |
| 3** | vehicle control | Dimethyl sulphoxide | 221 ± 75 | 1.0 |

There was no concurrent positive control in this study, however, the reliability check is carried out at regular intervals to test the sensitivity of the test system and the reliability of the experimental techniques used by the lab in which the study was conducted (b) (4) ). This LLNA study was conducted on August 7, 2003. A reliability check study was conducted in August, 2003 (date of the study was not indicated) in animals of 7-8 weeks old using α-hexylcinnamic aldehyde in acetone: olive oil (4:1). The results indicated that this test system was valid and reliable as shown below (extracted from the sponsor's report).




*Reviewer comment:* It was not clear why the sponsor chose 50% but not the undiluted Org 25969in DMSO as the highest concentration. The preliminary irritation test study only examined one concentration and the results indicated there was no skin irritation at this concentration, and the skin irritation effect of undiluted Org 25969 was not examined. In addition, the topical absorption rate of 50% and undiluted Org 25969 were not examined; it is not know whether Org 25969 were absorbed after topical application. Lack of skin sensitization following topical application may be due to low or none absorption of the test article with large molecule size. Therefore, this topical application study does not appear to be appropriate for evaluating the immunotoxic effects of Org 25969 for human use via IV administration.

**2.6.6.9 Discussion and Conclusions**

During the development of Org 25969 drug substance, non-clinical studies including genotoxicity, general toxicity, reproductive toxicity, local toxicity, and special toxicity studies including studies on bone and teeth effects and immunogenicity studies have been conducted.

*Genotoxicity:*

*In vitro* and *in vivo* genotoxicity studies were conducted to characterize the genotoxic potential of the drug substances. To investigate the genotoxicity potential of Org 25969, in vitro bacterial reverse mutation tests and chromosomal aberration tests in human peripheral blood lymphocytes have been conducted with batches L, T, V, and AE which containing different levels of impurities. All these studies showed negative results at concentration up to 5000 µg/plate or 5000 µg/ml. In vivo micronucleus assay in mice after single-dose administration at 1772 mg/kg Org 25969 drug substance and rats after 28-day administration at 500 mg/kg Org 25969 drug substance did not find increased micronucleus formation. In mice, the high dose level was intended to be 2000 mg/kg active entity, however due to improper corrections for [redacted] (b) (4) and [redacted] (b) (4) [redacted]/impurity content the actual Org 25969 dose was slightly lower than that is advised for a single-dose limit test from OECD guideline protocol for mammalian bone marrow chromosome aberration test. Since there were no toxic effects observed at this dose level (1774 mg/kg) and genotoxicity of γ-cyclodextrin is not expected due to the relatively large size of molecule, a full study using 3 dosing levels were not conducted by the sponsor. This is considered to be acceptable. The rat micronucleus assay using samples from the secondary 4-week repeat-dose toxicity study using batch AE was primarily used

to characterize the *in vivo* mutagenicity of Org 48302; however, since Org 25969 was about 90% in this drug substance batch, the study thus provide useful information to characterize the genotoxicity of the primary active ingredient. The results of all these genotoxicity studies indicated that Org 25969 is mutagenic or clastogenic.

*General toxicity:*
Although a number of general toxicity studies were submitted for this application, studies in rats and dogs with complete histopathological examination and appropriate recovery period for evaluation of the reversibility of the adverse findings were considered to be pivotal to support the proposed dosing levels in human. These studies included a single-dose study in rats treated with batch AE at 0, 200, 600, and 2000 mg/kg; two 4-week repeat-dose studies in rats treated with batch U and batch AE respectively at 0, 30, 120, and 500 mg/kg; a 28-day repeat-dose study in dogs treated with batch U at 0, 25, 80, and 250 mg/kg. In the repeat-dose rat study, the target organs identified were lungs (minimal to marked foamy macrophages at 500 mg/kg), kidneys (minimal to moderate vacuolated cortical tubule cells at ≥ 120 mg/kg), and urinary bladder (minimal to moderate foamy cytoplasm in umbrella cells at ≥ 120 mg/kg). Changes in the lung or kidney showed complete or partial recovery respectively, but the change in the urinary bladder was not reversible at 500 mg/kg after 8-week recovery period. Based on these results, the NOEL of Org 25969 is considered to be at 30 mg/kg and reversible NOAEL is at 120 mg/kg in the 4-wk rat toxicity studies. It should be noted that the severity of the changes in urinary bladder did not increase and these changes did not cause subsequent cellular damage and associated with changes in clinical pathology parameters at dosing termination or after 8-week treatment-free period, indicating limited toxicity significance. In the single-dose rat study, similar but less severe changes were observed in the lungs and kidneys; there were no change observed in urinary bladder. These changes appeared to be reversible and no other remarkable adverse effects were observed. Therefore, the NOEL for single-dose administration is considered to be 200 mg/kg, and reversible NOAEL may be considered to be at 2000 mg/kg. In dogs, there were no remarkable treatment-related change observed after treatment and NOAEL is considered to be at 250 mg/kg (MTD). The following table showed the margin of exposure/safety of these studies based on AUC levels.

| Species | Study | Dose (mg/kg/day) | $AUC_{0-\infty}$ (µg·hr/ml) | Proposed Maximal Human Dose 16 mg/kg $AUC_{0-\infty}$ (170 µg.hr/ml) |
|---|---|---|---|---|
| Rat | Single-dose tox | 200 (NOEL) | 380 | 2.2 |
| | | 600 | 1015 | 6 |
| | | 2000 ( NOAEL) | 4140 | 24 |
| Rat | Repeat-dose tox | 30 (NOEL) | 47 | 0.3 |
| | | 120 (NOAEL) | 203 | 1.3 |
| | | 500 | 952 | 5.6 |
| Dog | Repeat-dose tox | 250 (NOAEL) | 903 | 5.3 |

It should be noted that the AUC levels of rats and dogs as shown above were not derived from the same toxicity studies but based on estimation from other studies of the same species. In rats, the results of the TK analysis in the second 4-wk study (batch AE) were discarded because the bioanalysis part was outside of GLP scope. TK analysis was not conducted in this single-dose toxicity study for safety margin calculation. However, TK analysis was conducted in the first 4-week toxicity study. In addition, the sponsor also conducted a single-dose PK study in rats with batch AE; dosing levels in these studies were 120, 500, and 2000 mg/kg. The results of these two studies indicated that $C_{max}$ and AUC levels of Org 25969 were increased in proportion to the escalation of the dosing levels and there was no apparent accumulation after 4-wk administration. The AUC values shown in the above table are an estimation based on the results of these two studies. This approach is considered to be adequate by this reviewer based on the PK/TK profile identified from these studies. Toxicokinetics were conducted in the 4-week dog study but were found to be conducted outside of GLP and the resulting data in error; therefore, no direct TK can be obtained from this study but the Sponsor considers the data from a 3-week PK study in the dog with identical dose levels to be informative. This is accepted since PK data from the 3-week dog study indicated that there were no apparent accumulation after repeat-dose administration and plasma concentration of Org 25969 generally increased in a dose-proportional manner. The safety margins shown in the above table indicated that general toxicity studies in rats and dogs provided sufficient safety margins to support the safety of the proposed maximal dosage for human administration (16 mg/kg).

General toxicity of Org 25969 was also evaluated in juvenile animals to support clinical trials in the pediatric population. Results from both single- and 28-day repeat-dose studies in juvenile rats were comparable with findings in adults.

*Reproductive and developmental toxicity*
Reproductive and developmental studies have been conducted to investigate the fertility and early embryonic developmental toxicity in rats (segment I), the embryo-fetal developmental toxicity in rats and rabbits (segment II), and prenatal and postnatal developmental toxicity in rats (segment III).

The fertility and early embryonic development study in Sprague-Dawley rats revealed that the NOAEL of Org 25969 for effects on fertility and early embryonic development is at 500 mg/kg/day. In addition, histopathological examination of the male and female reproductive organs in the 4-week rat and dog toxicity studies did not reveal any adverse effects on fertility. In the embryo-fetal development studies in rat and rabbit, the NOAEL for embryofetal development was identified as 500 and 200 mg/kg/kg, respectively. In both of these species, teratogenic effects were not noted. Maternal toxicity as indicated by slightly lower body weight gain and food consumption was recorded at 500 mg/kg in rats and 200 mg/kg in rabbits. The lower mean fetal weight recording in rabbits at 200 mg/kg was likely due to maternal body weight loss and an increased mean litter size in this group.

In the pre- and postnatal development studies in rat (Segment III), slight neonatal mortality (postnatal) was noted during the first 4 days after delivery at 120 and 500 mg/kg. Cannibalism was likely to be the explanation of the decreased neonatal survival. There is no evidence of abnormal fetal or pup development which might have been a cause of decreased pup survival or trigger maternal cannibalistic behavior. To investigate the potential mechanism of the increased cannibalization as observed in the regular peri- and postnatal development study, the sponsor conducted two follow-up studies. In the first study, increased postnatal loss was not observed after 500 mg/kg treatment from gestational day 6 to postnatal day 4 which mimicked the treatment scheme of the high dose group in the regular segment III study, indicating the finding of neonatal loss in the regular pre- and postnatal development studies may be incidental. In addition, fetuses obtained by cesarian section one day prior to delivery from animals treated with 500 mg/kg Org 25969 from gestational day 6 showed good viability and a normal distribution of live and dead fetuses, which may indicate no intrinsic abnormalities within the fetuses that caused the dams to start cannibalizing their young. The purpose of the second follow-up study was to investigate whether ex-utero or in-utero exposure of Org 25969 is the possible cause of the increased incidence of cannibalism in dams with Org 25969 administration. To investigate effect of the ex-utero exposure of Org 25969, pups from two groups of pregnant females (22-24/group) without any treatment during pregnancy were swabbed with saline or 0.5 mg/ml Org 25969 after birth and observation of maternal nesting and nursing behavior, neonatal mortality or cannibalism were recorded. Results showed neonatal survival was comparable in the litters in which the pups were swabbed with 0.5 mg/kg Org 25969 as compared to the litters in which the pups were swabbed with saline. To investigate in-utero exposure of Org 25969, pups from two groups of pregnant rats treated with 500 mg/kg Org 25969 and 0.9% saline from GD6-21 were delivered by C-section on GD21 and cross fostered to untreated dams to observe for pup death and cannibalization from PND0-4. The results of this study showed a slight increase in total fetal death in the in-utero Org 25969 exposed litters as compared to the in-utero placebo exposed litters (neonatal survival rate: 79.4% vs. 85.1%). These results likely suggest odors of neonates due to in-uterus Org 25969 exposure may play a role in increased cannibalization. If that is the case, this effect may have little clinical significance, and therefore, the NOAEL for clinically relevant effects is considered to be at 500 mg/kg. The NOAEL (500 mg/kg) identified from the 3 rat reproductive studies provided a 6-fold safety margin, and the NOAEL in rabbits for embryofetal toxicity provided an 8-fold safety margin for the maximal human dosing level (16 mg/kg) based on the AUC comparison of the non-clinical model to the human.

Overall, reproductive toxicity studies did not show impaired fertility, teratogenicity, or clinical relevant effect during peri- and postnatal development. Therefore, a category B for pregnancy may be considered.

*Effect of Org 25969 drug substance on bone*

Distribution studies indicated that Org 25969 is rapidly distributed to the bone of rats and is retained in these tissues for extended periods of time with mean $t_{1/2}$ of 172 days (range from 67 – 210 days). Retention of Org 25969 was also seen in teeth as examined in rat incisors with $t_{1/2}$ of 8 days. Binding to bone and teeth was not easily saturated and bone

concentration (mg/g) increased in less than dose-proportional manner at doses up to 400 mg/kg, and the absorbed amount of Org 25969/gram bone and teeth after each individual administration was generally decreased in a multiple-dose study. At a dose of 30 mg/kg retention in pooled bones was 13% (juvenile), 3% (young adult), and 0.5% (aged rats), at 24h post dose. The increased bone retention in juvenile rats is probably due to rapid growth of bone containing multiple sites of bone resorption, formation and mineralization. To evaluate the effect of Org 25969 in the bone and teeth, dedicated bone study were conducted in adult and juvenile rat model.

The first single-dose study in adult rat to assess the bone effect of Org 25969 indicated slightly increased resorption after single-dose 2000 mg/kg Org 25969 administration as demonstrated by decrease in μCT bone parameters such as trabecular bone density, bone volume/tissue volume, trabecular number and separation and increased bone resorption marker CTx and DPD. This effect was further confirmed by bone strength assessment with slightly decreased max load, energy, stiffness, and stress primarily at distal femur. Femur length was not affected after 2000 mg/kg Org 25969 single-dose administration. A subsequent single-dose bone study in rats with dosing levels at 0, 30, 120, 500 mg/kg did not find any change in μCT bone parameters, indicating a NOAEL for bone effect at 500 mg/kg. This provided an approximate 6-fold margin of safety for the maximal human dose (16 mg/kg) based on AUC. In the 28-day repeat-dose general toxicity rat study, regular histopathological examination did not find any change in femur of animals at dosing level up to 500 mg/kg, suggesting minimal bone effect in extent. The following table compares the AUC and bone concentration in rats following single dose 500 mg/kg (NOAEL for bone and teeth effect) administration and human following the maximal proposed dose (16 mg/kg).

| Safety margin based on | Rat | Human | Safety margin |
|---|---|---|---|
| Dose (mg/kg) | 500 | 16 | 31 |
| AUC (μg·hr/ml) | 1050 | 160 | 6 |
| Bone/teeth concentration (μg/g) | 313 | 18 | 17 |

It should be noted that the calculation of Org 25969 bone concentration was based on some assumptions. For rat, bone distribution study showed ≤ dose-proportional bone absorption at single dose administration up to 400 mg/kg (maximal dosing level examined) with concentration of 250 μg/g. It is assumed that bone absorption will be comparable at 500 mg/kg, and thus bone concentration at 500 mg/kg is equal to 500/400 × 250 μg/g = 313 μg/g. For human, mass balance trial and other single dose clinical trials indicated that the recovery of Org 25969 in urine is at minimum 85% (worst-case estimate from reliable mass balance recovery figures), it can thus be calculated that ≤15% of the recommended human doses of 2, 4 and 16 mg/kg (assuming 75 kg subject), i.e. 22.5 mg, 45 mg and 180 mg, respectively, are retained in bone and teeth in a worst-case scenario. Assuming an approximately even distribution in the bone and teeth (10 kg total weight of bones and teeth) the concentration in human bone or teeth is estimated as 2.25, 4.5 or 18 μg/g bone or teeth. These calculations based on assumptions appeared to be

acceptable. Therefore, dedicated bone and teeth study supported the safety in bone and teeth of org 25969 with the proposed maximal single-dose administration (16 mg/kg) in human. However, the dedicated bone studies does not appear to have properly addressed the concern of the potential effect of the drug product on fractured bone in adult patients during healing process. Increased Org 25969 absorption is expected during the healing process of fractured bone, and it is not known how Org 25969 may affect the process of healing. It is recommended that the sponsor conduct a study in an appropriate non-clinical model with bone fracture to address this issue. These studies also indicated Org 25969 does not cause incisor discoloration in adult rats.

Studies in juvenile were conducted to support clinical trials in pediatric patients. Since high amount of bone and teeth absorption to Org 25969 was absorbed in juvenile (15%) as compared to adults (13%) in rats. Assessment of bone and teeth effects of Org 25969 were performed in juvenile animals following single and repeat-dose administration. A 28-day repeat-dose rat study was conducted with dosing initiated on day 7 of age to investigate the bone and teeth effect of Org 25969 in juvenile. Study assessments included bone growth (length), structure (µCT), and histopathology. Bone resorption or formation markers and bone strength were not measured. Results of the study showed minor decrease in bone growth as represented by small but significant decrease in femur and ulna length at the end of dosing and after 8-week treatment-free period (< 5%). In addition, significant decrease of femur weight was observed in female rats of 120 and 500 mg/kg after 8-week recovery period (< 10%). The sponsor has indicated that this is due to the minor decrease in body weight of animals (5-9% in males; < 5% in females) observed during the recovery period. This possibility can not be excluded. However, it is also possible that the Org 25969 caused a direct effect on bone which plays a role in body weight decrease or not associated with body weight decrease because 1) significant decrease in femur length was observed at termination of dosing when no body weight change was observed; 2) greater decrease in femur weight was observed in females than males while smaller body weight decrease in females than males. Although small in extent, the consequence may be severe if similar changes also occur in human if juveniles who expose to Org 25969 drug product multiple-times may be at risk of about 5% smaller in size in adulthood than those without exposure of the drug. Therefore, further studies are recommended to examine the reversibility and mechanism of these changes. µCT assay showed slightly increased trabecular BMD, bone vol/tissue vol, number in femur at 500 mg/kg, but a decrease in cortical thickness at 120 and 500 mg/kg. The biological significance of these changes is unknown. Bone strength assessment was not conducted since there were only minimal and biologically unknown changes observed with µCT assay according to the sponsor. In teeth, abnormal color (white marbled/mottled banding), malocclusion and/or overgrowth of the incisors in the majority of animals receiving 500 mg/kg, which were generally became apparent following at least 2 weeks of treatment and persisted for up to five weeks into the recovery period. These changes were also seen in some animals of 120 mg/kg. Histopathological examination showed dose-dependently increased disruption of enamel epithelial layer in the incisor at 120 and 500 mg/kg. Deposition of amorphous material in the incisor enamel epithelial layer was also observed at these dosing levels. In addition, minimal to marked deposition of amorphous material in the molar enamel epithelial was found at

500 mg/kg. Recovery was complete after 8-week treatment-free period in the molar but not for incisors at 500 mg/kg. Based on this study, the NOAEL for bone and teeth effect of Org 25969 with repeat-dose administration is 30 mg/kg; at this dosing level, AUC level was approximately 21 μg·hr/ml, and at least ≥ 150 μg/g bone was reached based on a distribution study treated with 4-week repeat-dose 30 mg/kg [$^{14}$C]-Org 25969 in young adult rats since bone absorption is much higher in juvenile than in adult.

Single-dose juvenile toxicity study was also conducted with attempts to examine the bone and teeth effect of Org 25969. Slight teeth effect including increased incidence of incisor discoloration and minimal disruption of the enamel epithelium with presence of amorphous material. Regular histopathological examination did not find change in the femur. Although samples have been collected for assays of μCT and bone turnover marks, tests were not conducted due to the minimal bone effect in the 4-week juvenile study according to the sponsor. Therefore, the NOAEL for teeth effect of Org 25969 with single-dose administration was 120 mg/kg. For bone, NOAEL can not be identified since the proper assessments were not conducted. Body weight was not changed with single-dose administration at a dosing level up to 500 mg/kg and bone length effect which was observed in repeat-dose juvenile study was not measured. However, additional single-dose study to assess bone effect may not be helpful. Changes observed in the 28-day repeat-dose juvenile rat may not be able to be detected with a single-dose administration due to the small extent. To initiate a repeat-dose clinical trial or seek approval of sugammadex for pediatric use, the mechanism and reversibility of the effects on bone length and body weight should examined with additional study in juvenile rats. In addition, this reviewer noted that the parameter values had a large variation in μCT parameter and bone strength assessment, which may produce error or false positive/negative results. It is recommended that the μCT assay is conducted in the future required juvenile rat study with the method validated with an appropriate positive control.

The discoloration of the incisors and disruption of the enamel forming epithelium of juvenile rats are most likely driven by the local concentration of Org 25969 in teeth. To evaluate the appropriateness of the animal models and clinical significance of the teeth effects observed in juvenile animals. Our pharmacology and toxicology review team has consulted the dental team in Division of Dermatology and Dental Product (DDDP). According the review of Dr. Fred Hyman, "in the rat, the incisors are a good model for developing human teeth; the rat molars, which have only an extremely limited ability to continue growth, are a good model for permanent human teeth, as in individuals over the age of 7." In juveniles, the 28-day repeat-dose study in juvenile rats revealed incisor discoloration and disruption of the enamel forming epithelium in incisors. In addition, deposition of amorphous material in the incisor and molar enamel epithelial layer were observed. The NOAEL for incisor and molar changes is at 30 mg/kg and 120 mg/kg, respectively, with 28-day repeat-dose administration. The NOAEL for incisor change is at 120 mg/kg with minimal disruption of the enamel epithelium with amorphous material in the incisors of one animal at 500 mg/kg after single-dose administration. With single-dose administration, the NOAEL for molars is ≥ 500 mg/kg in juvenile rats. Since adult human teeth have been fully mineralized and therefore do not have the ability to produce

more enamel, these effects in juvenile may not reflect potential change in adult human. However, these effects in juvenile animals may have impact on the consideration for children trial initiation. The incisors in rats are a good model for developing human teeth; the rat molars, which have only an extremely limited ability to continue growth, are a good model for permanent human teeth, as in individuals over the age of 7. Therefore, clinical study for pediatric patients is suggested to be initiated from patients greater than 7 years old based on safety concern on teeth effect of Org 25969 in pediatrics. Clinical trial under 7 years old may be initiated if the safety of the maximal dosing level is supported by NOAEL for incisor effect derived from these juvenile studies.

In vitro and in vivo studies are needed to investigate the binding site of Org 25969 and distribution within bone and teeth. The sponsor hypothesized that Org 25969 may bind to hydroxyl apatite in bone and teeth, but there were no data provided to support this hypothesis. A micorautoradiography study was submitted with this NDA to evaluate the distribution of [$^{14}$C]-Org 25969 within femur and scapula in rats; however, the study was found to have deficiencies. Firstly, the method used appeared to have limitation since variation was found between sections of the same tissue form the same animal and no differences in intensity could be detected between animals in which different amount of Org 25969 was supposed to be absorbed. Secondly, the sponsor stated that the results of the study indicated Org 25969 was not found at the growth plates, but within the limited sample size (3 animals), only one animal was found to have growth plates identified in both femur and scapula, one was found with growth plate identifiable in scapula but not in femur, and no growth plate was identifiable in either femur or scapula in the last animal. Therefore, the reliability of the results from this study was questionable. Therefore, a study with appropriate methodology is recommended to further investigate distribution of Org 25969 within the bone.

*Other toxicity studies to evaluate the safety of Org 25969 drug substance*
Immunotoxicity: three immunotoxicity studies have been conducted including plague forming cell (PFC) assay in rats, PLNA assay in mice, and contact hypersensitivity assay in mice. The first 2 studies indicated that Org 25969 is likely to be immunostimulatory, but the result in the 3$^{rd}$ study was negative. However, Org 25969 was administered via topical application. The topical absorption of Org 25969 was not examined; it is not known whether Org 25969 is absorbed after topical application. Actually, with the large molecular size, the skin absorption will be expected to be very limited. Lack of skin sensitization following topical application may be due to low or no absorption of the test article. Therefore, this topical application study does not appear to be appropriate for evaluating the immunotoxic effects of Org 25969 for human use via IV administration. Based on the results of the three immuno-toxicity studies submitted by the sponsor, it is concluded that Org 25969 is mild immuno-stimulatory. However, these non-clinical studies provided little information to predict the immunotoxic effect of Org 25969 in human. It was not known whether anti-Org 25969 antibody was produced *in vivo* based on the results of these studies. Indeed, anti-Org 25969 antibody production in human will be still difficult to predict even if anti-Org 25969 antibodies had been observed because of species difference.

Local toxicity: local tolerance and blood compatibility studies did not show local toxic effect of Org 25969 and Org 48302.

Toxicity profile and safety evaluation of Org 48302

During the drug development, a (b) (4) Org 48302, has always been prominently present in the drug substance batches manufactured with a concentration range of (b) (4)%. In a teleconference with the Division on August 4th, 2004, the sponsor proposed to "define the content of the active entity as the sum of Org 25969 and Org 48302" for the drug substance. The Division generally agreed with this approach but indicated that adequate toxicology studies needed to be conducted to characterize the safety of the drug substance containing concomitant components and demonstrate no additional safety concerns relative to Org 25969. In this NDA submission, the acceptance criterion of Org 48302 is less than (b) (4)%. With 16 mg/kg administration in human, the maximal dosing level of Org 48302 is (b) (4) mg/kg

Affinity binding assays demonstrated that Org 48302 has about half of Org 25969 binding affinity for rocuronium in isothermal microcalorimetry. *In vitro* assays in isolated mouse phrenic nerve-hemi-diaphragm preparation showed that Org 48302 had similar potency on reversal of rocuronium-induced NMBs. Additional studies in mouse diaphragm preparation indicated that mixture of Org 25969 and Org 48302 containing up to 20% Org 48302 had similar potency to Org 25969 with 100% purity. Studies conducted in anaesthetized guinea pigs showed that a mixture of 80% Org 25969 and 20% Org 48302 caused a similar speed of reversal as compared to 100% Org 25969.

To evaluate the potential effects in cardiovascular system, the effect of Org 48302 on cardiac action potential was assessed in isolated Purkinje fibers of dogs. The results indicated that Org 48302 induced increase in APD50, APD70, and APD90 similar to Org 25969 with a little less extent, indicating that Org 48302 is not likely to add additional risk with the presence of Org 25969. In addition, PK/TK analysis indicated that Org 48302 had a similar PK profile to Org 25969.

To assess the toxicity profile and evaluate the safety of Org 48302, additional toxicity studies were conducted including *in vitro* and *in vivo* genotoxicity studies using Org 48302 batch and single- and repeat-dose general toxicity studies in rats using Org 25969 drug substance batch AE containing about 5.4% Org 48302. The results of i*n vitro* Ames and chromosomal aberration assays indicated that Org 48302, like Org 25969, does not appear to impose risk for mutagenesis or clastogenesis. This was further confirmed by an in vivo micronucleus assay on bone marrow samples obtained from animals of control and 500 mg/kg Org 25969 batch AE (27 mg/kg Org 48302) treated groups in a 4-week general toxicity study in rats treated. In this 4-week rat toxicity study treated with Org 25969 batch AE, animals were treated with the drug substance at 0, 30, 120, and 500 mg/kg corresponding to 0, 1.6, 6.5, and 27 mg/kg Org 48302 for 4-week with a recovery period of 8-weeks. The results of this study showed comparable findings at the same dosing levels in contrast to the first 4-week rat toxicity study using Org 25969 batch U

containing 1.2% Org 48302 and the NOAEL for the both studies was 30 mg/kg, indicating that Org 48302 is not likely to produce additional and/or unique toxic effects in this animal species. In the additional single-dose toxicity study in rats treated with Org 25969 drug substance batch AE, adult animals were dosed with the drug substance at 0, 200, 600, and 2000 mg/kg corresponding to 0, 10.8, 32.4, and 108 mg/kg. The findings were similar to those observed in the repeat-dose studies including vacuolation in kidney and increased foamy macrophage in the lungs with less severity. These studies, in combination with other studies conducted to assess the general and reproductive/developmental toxicity of Org 25969 following administration of different Org 25969 drug substance batches, are considered to be sufficient to support the proposed maximal dosing level of Org 48302 (16 mg/kg × 7% = 1.12 mg/kg). The following table shows the qualified levels of Org 48302 (NOAEL) for general and reproductive toxicity and safety margins for the proposed maximal human dosing level of Org 48302 at 16 mg/kg administration based on AUC comparison. It should be noted that the AUC levels of Org 48302 in the non-clinical models and human were estimated values based on the % of Org 48302 in each drug substance batch. This reviewer thinks it is appropriate based on the results of the PK/TK analysis in these animal models, i.e. the PK profile of Org 48302 is similar to that of Org 25969. The table below showed that the general toxicity and reproductive toxicity studies general support the safety of maximal Org 48302 dosing level in human with 16 mg/kg Org 25969 drug product administration.

| Species | Study (Batch No.: % Org 48302) | NOAEL of Org 25969 (mg/kg) | NOAEL of Org 48302 (mg/kg) | Estimated Org 48302 $AUC_{0-\infty}$ (µg·hr/ml) | Proposed Maximal Human Dose<br>16 mg/kg containing ≤ 7% Org 48302<br>Estimated Org 48302 $AUC_{0-\infty}$ (12 µg.hr/ml) |
|---|---|---|---|---|---|
| Rat | Single-dose general tox (AE: 5.4%) | 200 | 10.8 | 20 | (b) (4) |
| Rat | Repeat-dose general tox (AE: 5.4%) | 120 | 6.5 | 11 | |
| Dog | Repeat-dose general tox (U:1.2%) | 250 | 3 | 11 | |
| Rat | Fertility and early embryonic development (U:1.2%) | 500 | 6 | 12 | |
| Rat | Embryofetal development (U:1.2%) | 500 | 6 | 12 | |
| Rabbit | Embryofetal development (U:1.2%) | 200 | 2.4 | 16.8 | |
| Rat | Prenatal and postnatal development (W: 4.2%) | 500 | 21 | 40 | |

Impurity qualification

During the synthesis of Org 25969 in the (b) (4)

(b) (4) The acceptance criterion of these impurities and degradants in the drug substance and drug product was listed below.

| Acceptance criteria for impurities and degradation products | | | | |
|---|---|---|---|---|
| Impurities/Degradation product | Drug substance acceptance criterion | Shelf life acceptance criterion | Release acceptance criterion | Potential dosing levels (mg/kg) in human with highest dosing level of the product (16 mg/kg) |
| *Specified identified:* | | | | |
| (b) (4) | (b) (4) | | | |
| *Specified unidentified:* | | | | |
| (b) (4) | | | | |
| *Unspecified (others):* | | | | |
| Each individual (b) (4) Each individual (other) | (b) (4) | | | |

*Assumption: average body weight in human is 60 kg.

The total dosing amount of Org 25969 drug product is about (b) (4) mg, which is less than (b) (4). According to the ICH guidance for industry Q3A Impurities in new drug substances, the identification and qualification threshold for impurities in drug substance is (b) (4)%, respectively, if the maximal daily dose is (b) (4) For drug product degradants, Q3B guidance recommended that both the identification and qualification threshold is (b) (4)% (or (b) (4)mg) if the maximal daily dose is (b) (4) Since the acceptance criteria of the unspecified impurities and degradants (Unspecified: Each

individual (other) in the above table) are lower than the identification respective threshold as shown in the above table, identification is not needed.

During the development of the drug product, the sponsor proposed qualification for (b) (4)% for NDA submission. In a teleconference between the Division and the sponsor on Aug 5th, 2004, the Division agreed that "the threshold for qualification of (b) (4) impurities is acceptable if adequate toxicology studies support the proposed maximal exposure to the impurity"; however, the Division indicated qualification threshold may be reduced to below (b) (4)% for any impurity containing a structural alert for mutagenicity. Based on these, all impurities with an acceptance level ≥ (b) (4)% are needed to be qualified. According to the Q3A and Q3B guidance, genotoxicity (point mutation and chromosomal aberration) and general toxicity studies in one species (usually 14-90 days) are generally needed for impurity and degradants qualification. For this specific product, a single dose toxicity study with histopathological analysis and at least 14-day recovery observation is also considered to be adequate for qualify the impurities and degradants in consideration of the single-dose treatment for this drug product. (b) (4) is a (b) (4) with structural alert for mutagenesis, therefore, the qualification level for this CD should be at (b) (4)% for mutagenesis. In this NDA submission, the submitted toxicity studies including genotoxicity and general toxicity studies are considered to be sufficient for qualification for the specified (b) (4).

The following table extracted from the sponsor's report is an overview of Org 25969 batches and (b) (4) levels tested in *in vitro* and *in vivo* genotoxicity studies. Org 25969 drug substance batch L, T, V, AE, and AM were used in *in vitro* Ames test in bacteria and chromosomal aberration test in cultured human peripheral blood lymphocytes with maximal concentration of 5000 µg/plate or 5000 µg/ml, respectively. Since the (b) (4) were presented in the drug product with different levels in the batches tested, the genotoxic potential of these compounds were considered to be addressed in some extent. In addition, specially batches of batch AJ with enriched impurities the degraded batch with combination of batch U, W, and Z were also tested for potential genotoxic effects using Ames and chromosomal aberration test at concentrations up to 5000 µg/plate or 5000 µg/ml, respectively. In comparison to the acceptance criterion for the identified organic impurities, all identified impurities were tested at an overall higher percentage. Considering the large number of the impurities/degradants in this drug substance/product, the approach used for qualification of the mutagenicity of impurities/degradants appears to be adequate. Additionally, the mutagenicity of (b) (4) was specifically tested using in vitro Ames and chromosomal aberration test at concentrations up to 5000 µg/plate or 5000/ml. All these in vitro genotoxicity studies showed negative results, indicating lack of mutagenicity of these impurities/degradants.

| Batch code | L | S | T | U | V | AE | AM | AJ [a)] | U, W, Z [b)] |
|---|---|---|---|---|---|---|---|---|---|
| Genotoxicity tests performed | Ames CAT | MNT (SD) [c)] | Ames CAT | MNT (MD) [d)] | Ames CAT | Ames CAT | Ames | Ames CAT | Ames CAT |
| Organic impurities: Specified identified (% m/m): (b) (4) | (b) (4) | | | | | | | | |
| Specified unidentified (% a/a): (b) (4) | | | | | | | | | |
| Total unidentified impurities ≥ (b) (4) %:[e)] | | | | | | | | | |

Abbreviations used: Ames = Bacterial Reverse Mutation Test; CAT = Chromosomal Aberration Test; MNT = micronucleus test (in vivo); n.q. = not quantifiable, see footnote a); RRT = Relative retention time; MD = multiple dose; SD = single dose

(b) (4)

Two in vivo micronucleus assays were conducted in mice or rats using batch S at 1774 mg/kg for single-dose administration and batch AE at 500 mg/kg for 28-day treatment, respectively. As shown in the following table, the highest dosing levels of each individual impurity in the *in vivo* MN studies were higher than the corresponding acceptance levels. The results from these two in vivo MN studies also negative in micronucleus formation.

| Impurities | Highest dosing level tested in the *in vivo* MN tests (mg/kg) | Potential maximal level in human with maximal proposed dose (16 mg/kg) administration (mg/kg) |
|---|---|---|
| (b) (4) | | |
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*Assumption: average body weight in human is 60 kg.

Overall, the genotoxicity potential of the impurities of the drug substance and degradant have been adequately characterized and the results of these genotoxicity studies implied no risk of genotoxicity.

General toxicity studies conducted by the sponsor are considered to be adequate for impurity and degradants qualification. The following table is an overview of maximum human doses, toxicological qualified levels (NOEL)and safety margins of Org 25969 (b) (4) in drug substance and drug product extracted from the sponsor's report. This reviewer generally agreed with NOEL level the sponsor identified in these studies The calculation of safety margin in he following table was based on correction factors for interspecies difference based on the ratio of AUC/dosing (NAUC) of Or 25969 in the pre-clinical species vs. human. The approximate NAUCs were approximately 10, 4, and 2 µg·h/ml per mg/kg dosing in human, dogs, and rats, and the corresponding correction factor in rats and dogs were 5 and 2.5 respectively. The interspecies difference in NAUC is considered to be mainly due to the difference in GFR in these species. This approach has been accepted by the Division in previous discussions with the applicant. Additionally, these values were close to the correction factor calculated based on the body surface area (6.3 in rats and 1.8 in dogs).

| (b) (4) identified and unidentified | Proposed accept. Criterion @ | Max. human dose (b) (4) (mg) | Tox. Qualified based on NOEL (mg) | Calculation of toxicologically qualified level of (b) (4) in mg (NOEL (in mg/kg) x % in tox study x body weight (in kg)/ correction factor for exposure) | Safety margin to 16 mg/kg in man |
|---|---|---|---|---|---|
| (b) (4) | | | | | |

| (b) (4) identified and unidentified | Proposed accept. Criterion @ | Max. human dose (b) (4) (mg) | Tox. Qualified based on NOEL (mg) | Calculation of toxicologically qualified level of (b) (4) in mg (NOEL (in mg/kg) x % in tox study x body weight (in kg)/ correction factor for exposure) | Safety margin to 16 mg/kg in man |
|---|---|---|---|---|---|
| | | | | | (b) (4) |

As shown above, the safety of the maximum dosing level of each impurity or degradant in human was supported by toxicology studies in at least one species based on the NOEL except (b) (4) a degradant of drug product which is not found in the drug substance with acceptance criterion of ≤ (b) (4)% for release and (b) (4)% for shelf life. The maximum potential dosing level is (b) (4) mg in human following administration of 16 mg/kg, assuming the average body weight in human is 60 kg. The maximal safety margin for this level was (b) (4) from non-clinical studies based on the NOEL (200 mg/kg) in a single-dose rat toxicity study using degraded Org 25969 batch containing (b) (4) (b) (4). However, this evaluation is rather conservative since it is based on the NOEL. The NOAEL of this study was considered to be 600 mg/kg because all changes found at this dosing level were minimal and reversible, which provide approximately 2-fold safety margin for (b) (4) level in human with 16 mg/kg drug product administration. In this single dose study which utilized higher dose levels of degraded Org 25969 (0, 200, 600, and 2000 mg/kg) vs. non-degraded Org 25969 (2000 mg/kg), increased number of spermatids (3/6 animals) and hypospermia (1/6 animals) were observed at 2000 mg/kg with degraded but not non-degraded Org 25969, indicating effect of these impurities or degradants contained in this batch may have the potential for effects on reproductive organs. These changes were reversible after 2-week recovery period, suggesting limited toxicology significance at this dosing level with single-dose administration. Therefore, single-dose toxicity study with degraded Org 25969 batch have adequately qualified impurities and/or degradants contained in this batch. A two-week repeat-dose toxicity study in rats treated with degraded Org 25969 also found changes in reproductive organs including decreased weight of seminal vesicle at all doses in a dose-independent manner (-26%, -25%, and -20% for 50, 100, and 200 mg/kg vs. control), and ruptured epididymal duct (1/8 animals) and sperm granulomas (3/8 animals) were found at 200 mg/kg. Since there were no recovery groups in the repeat-dose study, it was not known whether these changes were reversible. These changes were not seen in the 4-week repeat-dose study with non-degraded batches, indicating these changes were associated with impurities or degradants contained in the degraded but not in the non-degraded such as (b) (4). The degraded batch used in this study was especially prepared containing relatively high level of impurity/degradant including (b) (4) The findings in this 2-week study with degraded Org 25969 batch raised concern of multiple-dose administration in human. This reviewer agreed that a truly "multiple-dose" administration like the 2-week daily dosing may not occur in human, instead, the treatment of this drug may be more akin to intermittent treatment which might be considered single-dose with washout. However, warning should be included in the label to prevent any potential consecutive daily use before additional non-clinical studies are conducted to completely characterize the effect of the degraded batch or the individual impurity/degradant contained in this batch on reproductive system and provide sufficient safety margin for the maximal human dosing.

For the unspecified individual (b) (4), the sponsor increased the identification and qualification levels to (b) (4)% with the agreement from the Division based on the following justification, which is considered to be accepted

The toxicological justification is based on, among other considerations, the low toxicity of (b) (4) in general and experimental results on studies with various Org 25969 batches. In short, no evidence of genotoxic potential was observed in the many bacterial reverse mutation tests, chromosomal aberration tests and in vivo micronucleus tests performed with Org 25969 and Org 48302 containing many different (b) (4) in the various batches tested. In addition (b) (4) containing structural alerts for genotoxicity the monobromide (b) (4) and an Org 25969 batch in which the (b) (4) concentrations were artificially increased by a factor (b) (4) to increase genotoxicity assay sensitivity, did not show mutagenic or clastogenic potential when tested in vitro. Furthermore, the very low potential for metabolic activation of the (b) (4)) the absence of intrinsic secondary pharmacological activity and the intended single dose use of Org 25969 provide additional reasons to accept an identification and qualification threshold above those indicated by ICH Q3A and Q3B. In view of the low risk of toxicity including genotoxicity of (b) (4), a limited qualification is proposed for (b) (4) impurities at a level of >(b) (4)%.

## OVERALL CONCLUSIONS AND RECOMMENDATIONS

**Overall Summary:**

Org 25969 is a compound structurally belonging to the molecular family of γ-cyclodextrins (CDs). It is developed as a selective relaxant binding agent that binds to the hydrophobic molecular part of steroidal NMB drug rocuronium and vecuronium with high affinity resulting in reversal of neuromuscular blockade. The Org 25969 drug substance batches contains numerous (b) (4) including Org 48302 with a typical range (b) (4) (b) (4) which was added as the secondary active ingredient and others with lower levels which were considered as impurities. Non-clinical studies were conducted to identify the toxic effects and evaluate the safety of Org 25969 and Org 48302. In addition, studies were also conducted to qualify the impurities and degradants. The pharmacodynamics of Org 25969 was examined using physicochemical techniques (isothermal titration microcalorimetry), in-vitro techniques (tissue bath studies), in-vivo methods (muscle contraction in anaesthetized animals) and side effect profile studies. The pharmacodynamics of Org 25969 (b) (4) formed during Org 25969 synthesis were also examined and compared with that of Org 25969. In addition, the sponsor also compared the pharmacodynamic effect of various synthetic batches of the drug product which containing different composition of Org 25969, Org 48302, and (b) (4) .

These studies have shown that Org 25969 was a potent and effective agent for the reversal of neuromuscular blockade induced by the steroidal neuromuscular blocking agents (NMBA) rocuronium, vecuronium and pipecuronium, but had very limited effect against non-steroidal neuromuscular blocking agents such as succinylcholine, atracurium, cis-atracurium and mivacurium. Org 25969 does not act as an acetylcholinesterase inhibitor. The Org 25969 (b) (4), which are formed during the synthesis process, also show a similar affinity for the steroidal neuromuscular blocking agents. Administration of Org 25969 or Org 259569 (b) (4) causes reversal of neuromuscular blockade in the *in-vitro* mouse diaphragm model and *in-vivo* models in rat, Guinea pig, cat, and rhesus monkeys. The studies of primary pharmacodynamics of manufactured batches of Org 25969 showed a similar NMB reversal effect in batch V, AE, AM, AN, AP, AQ, L00027721, L00027960, L00027961, and L00027962, indicating

that small variation in composition of factory batches of Org 25969 are not likely to affect the ability of Org 25969 to encapsulate steroidal neuromuscular blocking agents like rocuronium and vecuronium.

Safety pharmacology

For evaluation of the effects of Org 25969 in the cardiovascular system, a hERG inhibition assay in HEK-293 cells demonstrated a 22% inhibition of hERG tail current at 1.5 mM of Org 25969. Higher concentrations of Org 25969 could not be evaluated due to an undesirable increase of the osmolarity of the buffer system. An action potential duration (APD) assay in Purkinje fibers of dogs demonstrated that Org 25969 produced increase in action potential duration ($APD_{50, 60}$, and $_{90}$) for approximately 5% and 20% at 150 and 1500 μM under normal (1 Hz) and low stimulations (0.3 Hz); similar results were observed for Org 48302. In contrast, hydroypropyl-β-cyclodextrin and γ-cyclodextrin only slightly increased APD90 of Purkinje fibers of dog at 1.5 mM concentration (< 5.0%) with no change in APD50 and APD70. An in vivo hemodynamic and electorcardiographic study with Org 25969 and rocuronium bromide in anesthetized beagle dogs indicated that a dose of 1.8 mg/kg rocuronium followed after 2 min by administration of 250 mg/kg Org 25969 resulted in approximately 7% increase in corrected QT duration (both Bazett's and Fridericia's correction) at the end of a 5 min perfusion. These studies suggested a potential of Org 25969 for QT prolongation. The concentration/dose producing effects in these *in vivo and in vitro* studies roughly provided 10-fold exposure margin for the proposed maximal human dosing level (16 mg/kg) based on plasma concentration. However, based on the small extent of the findings in these studies, the risk for QT prolongation may be negligible in human with the 16 mg/kg administration due to the small extent of the effects detected and large exposure margins as described above. The effect of Org 48302 on cardiovascular system is likely to be comparable to Org 25969. No CNS or respiratory effects were observed in rats following single-dose injection of Org 25969 at 500 mg/kg and 92 mg/kg, respectively.

ADME/PK

Org 25969 is indicated for IV injection therefore bioavailability is 100%; the systemic exposure levels appeared to be generally dose-proportional in rats, dogs, and rabbits. There was no apparent accumulation after multiple-dose administration and no remarkable difference found between male and female of any species. The elimination $t_{1/2}$ was short in these animal species (< 1 hour) and human (approximately 2 hours) by HPLC-MS assay. In addition, the distribution volume was relatively low in rats (~ 0.4 L/kg), dogs (~ 0.25 L/kg), rabbits (0.19 L/kg in F), and human (0.18 L/kg). The PK profile of Org 48302 appeared to be comparable to that of Org 25969. In contrast, a kinetics study using [$^{14}$C]-Org 25969 radioactivity assay demonstrated a very long $t_{1/2}$ in rats (~ 15 hours) and dogs (~ 8 hours). This is likely to be due to a difference in limit of quantitation (LOQ) between the liquid-chromatography-Mass Spectrometry (LC-MS) and radioactivity method, and the "tailing" of a long terminal elimination phase noted in the rat and dog is likely to represent slow elimination from a deeper compartment such as bone.

Distribution studies in the rat indicated that Org 25969 is rapidly distributed to the bone and is retained in these tissues for extended periods of time with mean $t_{1/2}$ of 172 days (range from 67 – 210 days). Retention of Org 25969 was also seen in teeth to a reduced degree as compared to bone with a $t_{1/2}$ of 8 days demonstrated in incisor. Injection of rocuronium immediately after sugammadex injection at a half molar dose diminished the extent of binding in bone and teeth by about 40%. The binding site and distribution of Org 25969 within these tissues have not been completely characterized. A microautoradiography study in young male adult rats showed labeled Org 25969 in periosteal and endosteal aspects of cortical bone and endosteal aspects of trabecular bone in femur and scapula, but not to growth plates. Bone formed subsequent to labeled dose administration did not contain radiolabeled Org 25969. This study did not provide sufficient detail to characterize the binding site of Org 25969 as cells or extracellular matrix. Binding to bone was not easily saturated and bone concentration (mg/g) increased in less than dose-proportional manner at doses up to 400 mg/kg, and the absorbed amount of Org 25969 in the bone and teeth after each individual administration (30 mg/kg) was generally decreased but absorption was still observed (approximately 50% of initial absorption ratio) on the last day of treatment in a 28-day multiple-dose study. At a dose of 30 mg/kg retention in pooled bones was 13% (juvenile), 3% (young adult), and 0.5% (aged rats), at 24h post dose. The increased bone retention in juvenile rats is probably due to rapid growth of bone containing multiple sites of bone resorption, formation and mineralization.

Distribution studies also demonstrated that accumulation was also observed in prostate and vagina, kidneys, and urinary bladder, but the amount was substantially reduced and appeared to be not different from other organs after 168 hours.

Org 25969 was found to cross the placenta and distributed into various fetal tissues with highest concentration in the femur (< 0.5% dose/gram) followed by urinary bladder and GI tract with no remarkable accumulation found in the vital organs such as the heart, brain, and lung in rats. The amount of Org 25969 in fetal blood was about 2-9% that of maternal blood at one hour after maternal administration, but the elimination was slower in the fetus than the dam. In addition, Org 25969 was found to be excreted into milk; maximal concentration was observed at 30 minutes after single-dose IV administration with the concentration similar to the plasma concentration at this time-point.

In vitro studies demonstrated that there was only a limited amount of Org 25969 uptake into hepatocytes, indicating hepatic metabolism may be minimal. Minimal metabolism likely occurs after Org 25969 administration in rats, dogs, and human. The primary metabolites were Org 198958-2 and Org 199425-1 in these species, which are also found in Org 25969 drug substance. There were no remarkable unique metabolites in human as compared to rats and dogs. In both rats and dogs, Org 25969 excreted primarily though urine (78% in rats; 85% in dogs) with minor fecal excretion (< 7% in rats; <1% in dogs).

General toxicology:
Single-dose studies in mice and rats indicated that maximum non-lethal dose of Org 25969 in these species exceeds 2000 mg/kg with minor and transient clinical signs such

as reduced activity, ptosis, and cramps after intravenous injection of this dose. A single-dose intravenous toxicity study in male and female rats was conducted to assess the acute toxicity profile of Org 25969 at 0, 200, 600, or 2000 mg/kg (containing 10.7, 32.1, and 107 mg/kg Org 48302). The target organs identified in this study were the kidney (tubular vacuolation at ≥ 600 mg/kg) and the lung (foamy alveolar macrophages at 2000 mg/kg). All changes were minimal and appeared to be reversible, indicating limited toxicologic significance. Therefore, the reversible NOAEL is considered to be at 2000 mg/kg, which provides an approximately 25-fold safety margin for the maximal proposed human dosing level of Org 25969 (16 mg/kg) and Org 48302 (1.12 mg/kg) based on AUC comparison.

Two 4-week toxicity studies were conducted in rats with the same dosing scheme (0, 30, 120, and 500 mg/kg Org 25969). The study design of these two studies were similar except that (1) the drug substance was batch U containing around 1.2% Org 48302 in the first study vs. batch AE containing around 5.4% Org 48302 in the second study; (2) the recovery period was 3 weeks in the first study vs. 8 weeks for the second study. The second 4-week toxicity study was proposed to provide sufficient safety margin for the maximal dosing levels of Org 48302 as well as Org 25969 in human and examine the reversibility of the microscopic findings with a longer recovery period (8 weeks vs. 3 weeks). The findings in these two studies were comparable. Target organs identified after 4-week administration were lungs (minimal to marked foamy macrophages at 500 mg/kg), kidneys (minimal to moderate vacuolated cortical tubule cells at ≥ 120 mg/kg), and urinary bladder (minimal to moderate foamy cytoplasm in umbrella cells at ≥ 120 mg/kg). Changes in the lung and kidney showed complete or partial recovery respectively, but the change in the urinary bladder was not reversible at 500 mg/kg after 8-week recovery period. Based on these results, the NOEL of Org 25969 is considered to be at 30 mg/kg and reversible NOAEL is at 120 mg/kg in the 4-wk rat toxicity studies. The AUC levels of Org 25969 and Org 48302 in rats at 120 mg/kg Org 25969 (6.5 mg/kg Org 48302) were approximately equal to those in human with 16 mg/kg Org 25969 administration. It should be noted that although the change in urinary bladder at 500 mg/kg in the second 4-week toxicity study did not show evidence of recovery after an 8-week treatment-free period, the severity of this change did not increase with time and the finding was not associated with subsequent cellular damage or associated with changes in clinical pathology parameters, indicating limited toxicologic significance. This dosing level provides an approximately 5-fold exposure margin for the proposed maximal human dosing level of Org 25969 as well as Org 48302.

Single-dose and 2-week toxicity studies in rats administered a degraded Org 25959 drug substance batch were conducted to assess the acute toxicity profile of the degraded Org 25969 drug product in comparison with non-degraded drug product. The batch was specially prepared containing relatively high level of certain degradants. The results of these studies indicated that the toxic effects produced by degraded and non-degraded Org 25969 were generally comparable except for some changes associated with reproductive systems in male rats. In the single dose study which utilized degraded Org 25969 (0, 200, 600, and 2000 mg/kg) and non-degraded Org 25969 (2000 mg/kg), an increased number of spermatids (3/6 animals) and hypospermia (1/6 animals) in epididymides were

observed at 2000 mg/kg with degraded but not non-degraded Org 25969. These changes were not seen in animals after a 2-wk recovery period. In the 2-wk repeat-dose study, decreased weight of seminal vesicle was observed at all doses in a dose-independent manner (-26%, -25%, and -20% for 50, 100, and 200 mg/kg vs. control), and ruptured epididymal duct (1/8 animals) and sperm granulomas (3/8 animals) in epididymides were found at 200 mg/kg. These changes were not seen in the 4-week repeat-dose study with non-degraded batches. Since there were no recovery groups in the repeat-dose study, it was not known whether these changes were reversible. Collectively, these changes suggested that degraded batch of Org 25969 drug substance which contains relatively high level of certain impurities or degradants including (b) (4) may cause effects on reproductive organs which were not seen treated with non-degraded batches. A NOAEL of 600 mg/kg based on the single-dose study provides sufficient safety margin for single-dose use of sugammadex, but further studies may be needed to further investigate these effects of degraded Org 25969 batch to support multiple dosing administration in human.

A 4-week IV toxicity study was also conducted in dogs to evaluate the toxicity of Org 25969. In this study, beagle dogs (4/sex/group) were treated with 0, 25, 80, and 250 mg/kg Org 25969 consecutively for 4-weeks. The results of this study indicated that intravenous administration of Org 25969 to beagle dogs for 4 weeks at the maximum feasible dose (MFD) did not result in remarkable systemic toxicity. Therefore, the NOAEL is identified as 250 mg/kg Org 25969 (3 mg/kg Org 48302), which provides around a 6-fold safety margin for the proposed maximal human Org 25969 dosing level of 16 mg/kg, and about 1-fold safety margin for proposed maximal human Org 48302 exposure based on AUC estimation with a 16 mg/kg clinical dose.

Overall, the general toxicity of Org 25969 and Org 48302 has been adequately characterized in rats and dogs, and the results of these studies appeared to provide sufficient evidence to support the safety of the proposed maximal dosing level of Org 25969 and Org 48302. In addition, levels of impurities/degradants in general toxicity studies adequately covered the maximal exposure levels in human with 16 mg/kg drug product for single-dose administration. Additional study may be needed to further investigate the potential effect of degraded Org 25969 drug substance on the reproductive system.

Reproductive toxicology:
In the segment I fertility study, both male and female rats (22/sex/group) were treated with Org 25969 at 0, (0.9% NaCl), 20, 100, and 500 mg/kg via intravenous bolus injection in the tail vein. The results showed slight decrease in body weight of female animals (~10%) indicating minimal maternal toxicity at 100 and 500 mg/kg. No Org 25969 treatment-related macroscopic or organ weight changes were observed in reproductive organs of male and female rats. There were no treatment-related changes in mating and fertility parameters including mating index, fertility index, pre-coital interval, live concepti number, and gestational index in Org 25969-treated groups as compared to control. The NOAEL for fertility and early embryonic development is considered to be

at 500 mg/kg for rat based on the results of this study, corresponding to a 6-fold human AUC safety margin at the maximal human dose of 16 mg/kg.

Embryofetal development (segment II) studies were conducted in both rats and rabbits. In the rat study, pregnant animals were treated with Org 25969 at 0, 20, 100, and 500 mg/kg during fetal organogenesis. Minimal maternal toxicity was observed at 100 and 500 mg/kg represented by minimal decrease in body weight gain (~7%) likely due to slight decrease in food consumption (~7%) during the treatment period. Hysterectomy did not show any treatment-related change in pre- and post-implantation loss or live fetus numbers. There were no treatment-related changes in fetal soft tissue and skeleton except increased incidence of dilated renal pelvis in the offspring of dams treated with 500 mg/kg Org 25969 (6.5%) as compared to control (0.7%). However, this change is considered to be a variation but not malformation and the recorded value (6.5%) was still within the laboratory historical range (mean = 4.9%; range 0 – 8.5%) which suggests this is not related to drug. Therefore, the NOAEL for embryofetal developmental toxicity in rats is considered to be at 500 mg/kg Org 25969 which corresponds to a 6-fold human AUC safety margin at the maximal human dose of 16 mg/kg.

In the rabbit embryofetal development study, pregnant New Zealand white rabbits were treated with Org 25969 at 0, 20, 65, 200 mg/kg during fetal organogenesis, and the animals were sacrificed at the end of gestation for embryofetal developmental toxicity evaluation. The results of the study showed a reversible dose-dependent decrease in body weight gain in dams during the treatment period at 20 (↓5.4%), 65 (↓16%) and 200 mg/kg (↓31%) as compared to the control, likely due to decreased food consumption. Macroscopic examination in dams indicated changes in gonads (conjunctive tissue, periovarian region: serous cysts) in 1/20 animals of all drug-treated groups, but not in control. This change was not dose-dependent, and has been reported to occur in animals of this strain at this age. Decrease in offspring body weight was also observed in all groups of animals with approximately 5%, 10%, and 14% at 20, 65, and 200 mg/kg, respectively, likely due to maternal body weight decrease or increased litter size in the treated groups as compared to the control. This reduced body weight is not considered to be adverse or directly related to drug on the fetus. Skeletal examination showed increased skeletal variation including increased percentage of incomplete ossification of $1^{st}$ to $4^{th}$ sternebra and $6^{th}$ sternebra, and unossified $1^{st}$ metacarpal at 200 mg/kg, likely to be due to the lower fetal weight in this group. These changes were within historical control value range except the incomplete ossification of $1^{st}$ and $4^{th}$ sternebra (12.6% vs. 1.4 – 11% in historical control). However, incomplete ossification of sternebra is considered variation but not malformation, which does not indicate any toxicity. Malformations such as thoracic hemivertebra and misshapen thoracic vertebra, misaligned sternebra, and fused rib were found in one fetus in the high-dose group, which was not seen in the control. These were considered to be incidental because of the low incidence and similar malformations were also reported in animals of historical control. Overall, the primary change in maternal and offspring rabbits with Org 25969 administration during fetal organogenesis was a minor decrease in body weight, which is monitorable in the mother and of minimal toxicologic significance in the offspring; therefore, the NOAEL for embryofetal toxicity in rabbits is considered to be at 20 mg/kg,

but the NOAEL for teratogenicity is considered to be 200 mg/kg, corresponding to approximately 8-fold safety margin for Org 25969 in human with the maximal 16 mg/kg administration based on AUC comparison.

In the segment III perinatal and postnatal development study, pregnant rats (22/group) were treated with vehicle or Org 25969 at 30, 120, 500 mg/kg from gestational day (GD) 6 to postnatal day (PND) 21 (corresponding to the beginning of organogenesis through parturition and subsequent pup weaning). No abnormalities were observed in dams ($F_0$ animals). There were no Org 25969-related effects on the mean number of implantation sites, the gestation or parturition duration, and the pregnancy rate was comparable between Org 25969 treated and control groups. Neonatal mortality appeared to be increased during PND0-4 in the high-dose (500 mg/kg) and mid-dose group (120 mg/kg). Initial examination indicated increased postnatal loss from the first litter check on day 0 to 4 post partum was found to be increased in the high dose group compared to control (19.2% vs. 10.4%) with observed total litter loss of 2, 0, 2, and 3 dams in groups of control, 30, 120, and 500 mg/kg, respectively, during this period. However, in group 3, two dams had extremely small litter size (2 or 4 pups) as compared to others at the first litter check; while the implantation sites of these two animals were 13 and 18, respectively, indicating cannibalization occurred in these two dams immediately after birth and before the first litter check. In addition, there was one complete litter loss each in group 2 and group 3 before the first litter check after delivery, which is also most likely due to cannibalization since the body weight of these two animals were comparable with others. Collectively, there appeared to be increased postnatal loss in group 3 (120 mg/kg) and group 4 (500 mg/kg) from the birth on postnatal day 1 to postnatal day 4, most likely due to cannibalization. Histopathological examination of heart, lungs, liver, and kidneys of the dead or partially cannibalized $F_1$ pups did not identify any change that might cause pup death or trigger cannibalization. There were no remarkable abnormalities observed in the surviving $F_1$ pups during lactation period. In addition, there were no remarkable treatment-related changes in $F_1$ surviving animals in physical development, behavior evaluation, necropsy, and reproduction evaluation. Overall, the primary change was postnatal $F_1$ pup loss during postnatal day 0-4. The NOAEL for postnatal development was considered at 30 mg/kg.

To investigate the potential mechanism of increased postnatal loss observed in Org 25969 treated groups at 120 and 500 mg/kg, two follow-up studies were conducted. Results of the first study following similar dosing regimen in 22 pregnant rats/group showed no difference in neonatal death between control and 500 mg/kg groups, indicating that the increased postnatal loss of offspring in the regular prenatal and postnatal development study may be incidental. In addition, fetuses obtained by cesarian section from animals treated with 500 mg/kg Org 25969 showed good viability and a normal distribution of live and dead fetuses, which may indicate no intrinsic abnormalities within the fetuses to cause the dams to cannibalize their young. The purpose of the second follow-up study was to investigate whether ex-utero or in-utero exposure of Org 25969 was the possible cause of the increased incidence of cannibalism in animals with Org 25969 administration. To investigate effect of the ex-utero exposure of Org 25969, pups from two groups of pregnant females without any treatment during pregnancy were swabbed

with saline or 0.5 mg/ml Org 25969 after birth and observation of maternal nesting and nursing behavior, neonatal mortality or maternal cannibalism were recorded. Results showed neonatal survival was comparable in the litters of Org 25969 swabbing as compared to the litters swabbed with saline (95.8% and 92.1%, respectively). To investigate in-utero exposure of Org 25969, pups from two groups of pregnant rats treated with 500 mg/kg Org 25969 and 0.9% NaCl during gestational day 6 to 21 were delivered by C-section on gestational day 21 and cross-fostered to untreated dams to observe for pup death and cannibalization from postnatal day 0-4. The results showed a slight increase in total pup death in the in-utero Org 25969 exposed litters as compared to the in-utero saline exposed litters (neonatal survival ratio: 79.4% vs. 85.1%). Collectively, results of these studies likely suggest that in-utero exposure of Org 25969 may play a role in triggering cannibalization due to change in odor or taste but not malformation or abnormality of pups. This effect in rats is not likely to be clinically relevant; therefore, 500 mg/kg is considered to be NOAEL for clinically relevant effects in peri- and postnatal development.

Juvenile Toxicity Evaluation

In support of pediatric trials, developmental toxicity studies in juvenile rats with single- and repeat-dose administration were conducted to further investigate the potential toxic effects of Org 25969 during the juvenile developmental period. In addition, bone and teeth effect of Org 25969 were specifically examined in juvenile rats because of the high amount of bone and teeth absorption in juvenile animals as observed in the distribution studies. In a single-dose treatment study, animals at ages of 7 days, 14 days, or 21 days were injected subcutaneously (for animals of 7 days old only) or intravenously with Org 25969 at 0 (0.9% NaCl), 30, 120, and 500 mg/kg. Reversible minimal to slight tubular vacuolation in kidney were observed upon14 days after dosing primarily at 120 and 500 mg/kg in animals administered drug at age 7 days. Minimal disruption of the enamel epithelium with presence of amorphous material was observed in the incisors of one female rat treated at age 21 days with 500 mg/kg on day 14 after dosing. The NOAEL is considered to be at 500 mg/kg Org 259659 for general toxicity and 120 mg/kg Org 25969 for the tooth effect. Another single-dose toxicity study showed slightly higher incidence of whitish discoloration at 500 mg/kg Org 259659 as compared to control.

The adverse effects of Org 25969 after multiple dose administration was investigated in juvenile rats following 4-week administration (n=10/sex/group) with an 8-week treatment-free period observation (n=12/sex/group). The dosing levels utilized in this study were 0, 30, 120, and 500 mg/kg Org 25969. Animals were administered drug or vehicle starting at day 7 after birth via SC injection for two weeks, and then switched to IV injection for 2 weeks. TK analysis showed that plasma levels of Org 25969 were comparable for males and females with a roughly proportional increase with dose escalation. Upon repeated dosing, plasma levels were lower at 34 days of age after the 28-day administration when compared with those following a single dose on Day 7 of age. The reason for this difference was not known. General toxicity effects in juvenile rats after 28-day administration were comparable to those observed in adult rats primarily including minimal to slight histopathological changes in the lungs, kidneys, and urinary bladder. Neurobehavioral examinations including motor activity, sensory function and

reactivity, and learning and memory (Morris water maze) after treatment did not show any sign of abnormality. There was no body weight change during the treatment period, but minor decrease in body weight gain of both males (5-8%) and females (<5%) at 120 and 500 mg/kg Org 25969 were observed as compared to the control during the 8-week treatment free period. At the end of 8-week recovery period, the body weight of animals treated with Org 25969 at 120 and 500 mg/kg were still slightly lower than that of control in both males (4%) and females (3%). Additional observations in this study included an analysis of the effects of Org 25969 in teeth and bones. In bones, 4-week continuous administration of Org 25969 caused minor decreased ulna length (≤5%), femur length (≤3.5%) and weight (≤6.5%) with more severe effects at the end of 8-week recovery period at 120 and 500 mg/kg Org 25969 dose levels. It can not be determined whether the decrease of bone length was due to the slight body weight decrease. However, these results indicated that Org 25969 may have a potential to cause slightly smaller size and height lasting until the adulthood if administered during pediatric period. Minor but statistically significant changes in bone parameters of femur as assessed by µCT including increased trabecular bone mineral density (9% in M, 18% in F versus control), bone volume/tissue volume of trabecular metaphysis (19% in M, 24% in F versus control) , and trabecular number (13-15%) and trabecular thickness (6-8%) at 500 mg/kg, along with dose-independently decreased cortical thickness at both 120 (9-10%) and 500 mg/kg (7-8%) after Org 25969 administration, which were reversible after 8-week treatment-free period. The biological significance of these changes is not clear. Bone turnover markers or bone strength were not evaluated. Histopathological examination found a very low incidence of chondromucinous degeneration in the Org 25969 120 and 500 mg/kg (one animal at each group) and fibrosis at 500 mg/kg (one animal) group in femur. Based on these results, the NOAEL for bone effect in juvenile is considered to be 30 mg/kg. In teeth, abnormal color (white marbled/mottled banding), malocclusion and/or overgrowth of the incisors in the majority of animals receiving 500 mg/kg and some animals of 120 mg/kg, which were generally became apparent following at least 2 weeks of treatment and persisted for up to five weeks into the recovery period. Histopathological examination showed dose-dependently increased disruption of enamel epithelial layer in the incisor of 1/20 (minimal severity), 0/20, 6/17 (5 minimal, 1 slight), and 15/20 rats (3 minimal, 5 slight, 6 moderate, 1 marked) at 0, 30, 120, and 500 mg/kg Org 25969, respectively. Deposition of amorphous material in the incisor enamel epithelial layer were observed at 120 (4/17, minimal) and 500 mg/kg (17/20, minimal to marked). Minimally to marked deposition of amorphous material in the molar enamel epithelial layer were also found in 5/10 males and 5/10 females of the 500 mg/kg group. Recovery was complete in 8 weeks for the changes in the molars but not fully complete for the incisors. The NOAEL was at 30 mg/kg for incisors but 120 mg/kg for molars.

Special toxicology:

*3. Special studies regarding potential effects on bone and teeth*

In a single-dose study, adult male and female rats (16/sex/group) were injected intravenously with 2000 mg/kg Org 25969 or 0.9% NaCl. Bone turnover markers (BTM) were measured on day 1, 7, 21, and 42 days after doing, and evaluation of µCT bone parameter and bone strength were conducted on day 21 and 42. Single-dose 2000 mg/kg

administration caused statistically significant change in bone parameters via μCT assay on day 21 after dosing as compared to the control, including decrease in trabecular bone volume/tissue volume (18% in female; 8% in male), trabecular number (↓10%), along with increased trabecular separation (31% in female; 11% in male). Trabecular bone mineral density also appeared to be decreased (female ↓18%, male ↓8%), although statistical significance was not reached. In addition, bone indentation test showed decreased energy, maximal load, stiffness, and stress, indicating compromised bone integrity of the distal femur on day 21. BTM assay showed increased serum CTx and urine DPD, markers for bone resorption, primarily in females at this time-point. All these findings suggest a minor bone resorptive effect with greater severity in females than males. Decreased trabecular number and increased trabecular separation test were also seen on day 42 after dosing, suggesting a prolonged bone effect. μCT assay and bone strength assessment were not conducted on day 0 and day 7 after dosing. Examinations of BTM did not suggest a remarkable change in bone during this period. Possible teeth discoloration due to Org 25969 was evaluated by determination of the color of the (upper part) of the lower incisors of the rats. There was no effect of discoloration on incisor after a single-dose of 25969 at 2000 mg/kg. Overall, this study indicated that Org 25969 at 2000 mg/kg produced a resorptive effect in femur after 21 days of dosing but no effect on teeth discoloration after single-dose administration. The bone effect of Org 25969 was likely to be reversible since the changes in μCT parameters and BTM appeared to be less severe on day 42 as compared to those on day 21. In the second study of bone effect, rats were treated with Org 25969 at 0, 30, 120, and 500 mg/kg for single-dose administration. μCT assay did not indicate any remarkable adverse effects in the bone, and therefore, NOAEL is considered to be at 500 mg/kg for bone effect in this study, corresponding to a 6-fold or 17-fold safety margin for human administration at 16 mg/kg based on AUC or bone concentration estimation. There was no effect on teeth discoloration observed in either of the studies.

As a possible hormonal explanation for the reduced trabecular strength and volume, a PTH surge was observed in both rats and dogs upon Org 25969 administration. In the rat (30, 120, 500 mg/kg Org 25969) this increase was maximal at 3 min post dose (a statistically significant 4-fold increase at 500 mg/kg compared to controls), and returned to baseline by 30 min. Serum $Ca^{2+}$ was increased at 3 and 30 minutes after dosing and urine phosphate excretion was increased over 24 hours. In dogs, the PTH surge lasted about 15 min at ≥ 80 mg/kg. A transient effect is probably not relevant in the reduction of trabecular mass and strength since bone changes persisted through 3-6 weeks following single dose administration. However, it should be noted that the maximal dose examined for PTH release was 500 mg/kg, a dosing level without any observable changes in bone. The effect of Org 25969 on plasma PTH and $Ca^{2+}$/phosphate concentration after 2000 mg/kg, the dosing level that produced bone effect, was not examined.

*4. Immunogenicity:*

There were 3 studies conducted to examine the immunogenic property of Org 25969. First, a Plaque-Forming Cell (PFC) assay in which sheep red blood cells (SRBCs) were injected into the rat after 2-week IV injection of Org 25969 at 30, 120, and 500 mg/kg and splenic cells were subsequently harvested and incubated with SRBCs to evaluate the

integrity of the T-cell dependent antibody response and any potential immunosuppressive effect. However, Org 25969 treated groups showed slight increase in plaque formation in a dose-independent manner, suggesting immuno-stimulation. The second study was a popliteal lymph node assay (PLNA) assay conducted in mice to examine the potential for an immuno-stimulatory effect of Org 25969 after subcutaneous (SC) footpad injection at three different dose levels (0.3, 1.2 and 5 mg/animal). The results showed a small but statistically increased [$^3$H]-thymidine incorporation in PLN at 500 mg/kg (2-fold higher for Org 25969 compared with 26-fold higher for positive control) as compared to vehicle control, indicating weak immuno-stimulation. The 3$^{rd}$ study was a local lymph node assay (LLNA) assay to examine skin contact hypersensitivity to Org 25969 after topical application on the ears of animals. The results showed no increase in [$^3$H]-thymidine incorporation in auricular lymph node suggestive of lymphocyte stimulation. However, skin absorption of Org 25969 has not been examined. Based on the results of the three immuno-toxicity studies submitted by the sponsor, it is concluded that Org 25969 is mildly immuno-stimulatory. However, these non-clinical studies provided little information to predict the immunotoxic effect of Org 25969 in human. It was not known whether anti-Org 25969 antibody was produced *in vivo* based on the results of these studies. Indeed, anti-Org 25969 production in human will be still difficult to predict even anti-Org 25969 antibodies had been observed in nonclinical studies because of species difference.

**Recommendations:**

1) Approvability: Approval of (b) (4) (sugammadex) is recommended from the pharmacology and toxicology perspective.

2) Labeling revision: revision of labeling is recommended based on the results of non-clinical studies

| Label originally proposed by the Sponsor | Recommended revision | *Rationale* |
|---|---|---|
| **8.1 Pregnancy** | **8.1 Pregnancy** (b) (4) | *Toxic effect observed in the prenatal and postnatal developmental toxicity (segment III) study should be described in this section.* (b) (4) |

| | | |
|---|---|---|
| (b) (4) | | |
| (b) (4) | | 1) *The value of* (b) (4) *deleted because it may be various with different injection dosing level.*<br>2) (b) (4)<br>3)<br>4) |
| **13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility** | **13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility** | (b) (4) |
| (b) (4) | | |

(b) (4)

| 13.2 Animal Toxicology and/or Pharmacology | 13.2 Animal Toxicology and/or Pharmacology | *Unnecessary parts have been deleted.* |
|---|---|---|
| (b) (4) | Bone and teeth retention of sugammadex occurred in rats after IV injection, with the mean $t_{1/2}$ of 172 and 8 days, respectively. The binding site is not known, and the distribution within these tissues has not been well-characterized. Studies in adult rats have shown that single-dose administration of 2000 mg/kg sugammadex slightly increased bone resorption, but had no effect on teeth color and appearance. In juvenile rats, 28-day administration of sugammadex at 120 and 500 mg/kg caused slightly decreased body weight and bone length which did not recover within an 8-week treatment-free period. Whether an association between these effects exists is not known. μCT assay showed alterations in bone parameters including such as increased trabecular bone mineral density and trabecular thickness at 500 mg/kg, along with decreased cortical thickness at both 120 and 500 mg/kg after Org 25969 administration, but the biological significance is not clear. Bone strength was not assessed in juvenile rat studies. Whitish discoloration was also observed in the incisors and disturbance of enamel formation was observed upon repeated dosing in the incisors and the molars at these dosing levels. | |

3) Recommendation for non-clinical studies

*For adult use:*

1) The effect of sugammadex on bone healing after fracture has not been evaluated in non-clinical models. A study in rat or other appropriate non-clinical models with bone fracture to evaluate the effect of sugammadex on bone healing process is recommended.
2) A repeat-dose study in rats (≥ 14 days) with appropriate treatment-free recovery period is needed to examine the equivocal effects in reproductive organs in males of sugammadex and its reversibility observed in the single-dose and 2-week repeat-dose study in rats treated with degraded drug substance batch which contained high levels of certain impurities/degradants.

*For pediatric use:* Additional bone studies are needed before multiple-dose clinical trial in pediatric patients and/or for approval of the drug to be used in pediatrics.

1) A repeat-dose study (≥ 28-day) of sugammadex in the juvenile rat with multiple recovery periods of varying length ≥ 8 weeks is recommended to evaluate the mechanism and reversibility of the slight but lasting decreased bone length and body weight as observed in the 28-day repeat-dose study. Within this study, assays of μCT, bone turnover markers, and bone strength assessment are necessary with concurrent positive control in order to clearly clarify the bone effects of sugammadex in juvenile rats.
2) The binding site of sugammadex will need to be conclusively determined and distribution of sugammadex within the bone will need to be further investigated in order to provide mechanistic information for potential findings in the bone in animal studies. The sponsor hypothesized that sugammadex is likely to bind with hydroxyapatite, a compound of extracellular matrix in the bone, but there was no study conducted to directly investigate the binding site of sugammadex in the bone or tooth. The microautoradiography study conducted to examine the distribution of sugammadex in the bone does not appear to be adequate with deficiencies in method and, small sample size, and inappropriate data presentation.

Signatures (optional):

Reviewer Signature _____

Supervisor Signature_____ Concurrence Yes ___ No ___

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/s/
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Zengjun Xu
6/20/2008 01:22:32 PM
PHARMACOLOGIST

Adam Wasserman
6/20/2008 01:25:33 PM
PHARMACOLOGIST
I concur that this application may be approved.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

# Supervisory Pharmacologist Memorandum

| | |
|---|---|
| NDA NUMBER: | **22-225** |
| SERIAL NUMBER: | **000** |
| DATE RECEIVED BY CENTER: | **30-OCT-2007** |
| PRODUCT: | (b) (4) **™; Sugammadex sodium** |
| INTENDED CLINICAL POPULATION: | **routine reversal of shallow or profound neuromuscular blockade induced by rocuronium or vecuronium, and immediate reversal of neuromuscular blockade at 3 minutes after administration of rocuronium.** |
| SPONSOR: | **Organon USA, a Division of Schering-Plough** |
| DOCUMENTS REVIEWED: | **eCTD submission, Primary review of Zengjun Xu, Ph.D., DABT, Consults of Bone Evaluations from Gemma Kuijpers Ph.D., Division of Metabolic and Endocrine Products and Dental Evaluations from Fred Hyman, DDS, Division of Dermatologic and Dental Products** |
| REVIEW DIVISION: | **Division of Anesthesia, Analgesia and Rheumatology Products (HFD-170)** |
| PHARM/TOX REVIEWER: | **Zengjun Xu, Ph.D., DABT** |
| PHARM/TOX SUPERVISOR: | **Adam Wasserman, Ph.D.** |
| DIVISION DIRECTOR: | **Bob Rappaport, M.D.** |
| PROJECT MANAGER: | **Allison Meyer** |

Date of review submission to Division File System (DFS): 6/16/08

## I. BACKGROUND

(b) (4)™ (sugammadex sodium/Org 25969) is a modified gamma cyclodextrin (γ-CD) developed by Organon, USA which is designed to capture, and therefore functionally inactivate, aminosteroidal neuromuscular blocking agents (NMBAs) rocuronium (Zemuron®) and vecuronium (Norcuron®), also manufactured by Organon. Sugammadex sodium 2, 4, or 16 mg/kg is proposed for intravenous administration at the conclusion of a surgical case for which the NMBA has been used to cause paralysis. The cyclodextrin-NMBA forms a stable inclusion complex which reverses the normal concentration gradient, drawing the NMBA away from the neuromuscular junction and reversing the block of nicotinic acetylcholinergic receptors (nAChR). The current practice for reversal of neuromuscular block is to administer the acetylcholinesterase inhibitor neostigmine in order to increase levels of acetylcholine at the neuromuscular junction and outcompete the NMBA at the nAChR, restoring neurotransmission. The use of a cyclodextrin, modified to be specific for a particular target drug, to physically remove a ligand from its site of action represents a novel mechanism of action.

γ-CDs are defined as a ring of 8-glucose units which form a hydrophobic cavity/pore with while maintaining a hydrophilic outer surface, capable of excellent solubility in water. These properties have allowed γ-CDs to become highly useful in food with potential intake of grams/day by the oral route (WHO Food Additive Series 42, 1999). When consumed orally, γ-CDs unlike the 6- and 7-unit α-CD and β-CDs are digested by salivary and pancreatic amylases; radiolabeled $^{14}C$ indicates the majority of radiolabel is exhaled as $CO_2$ suggesting entry into the standard glucose metabolism pathway. Prior evaluation of γ-CD has not identified genotoxicity or reproductive toxicity (Verhagen et al., 1991; Waalkens-Berendsen et al., 1992). Subacute and subchronic toxicity studies of intravenously administered γ-CD conducted in rats have identified the kidneys, lungs, and urinary bladder as the principal target organs (Fuchs et al., 1992; Ehling e tal., 1992). Renal reabsorption vacuoles, pulmonary histiocytosis, and hyperplasia of the urinary bladder – all of which reportedly reverse – were described. Additionally, some decrease in red cell parameters, considered due to a direct toxicity of CDs on red blood cell membranes, was observed at higher dose levels with secondary increase in spleen weights in the rat. This profile is generally consistent with that generated by nonclinical studies of Org 25969, as described in the following sections.

The primary nonclinical review of NDA 22-225 was undertaken by Zengjun Xu, Ph.D., DABT. Consult requests were made of the Division of Metabolic and Endocrine Products (DMEP) to address bone findings as well as to the Division of Dermatologic and Dental Products (DDDP) to address the toxicologic findings and implications of Org 25969 distribution to teeth. The DMEP and DDDP consults obtained were from Dr. Gemma Kuijpers and Dr. Fred Hyman, respectively. Additionally, a meeting of the Anesthetics and Life Support Drugs Advisory Committee (ALSDAC) was held on March 11, 2008 for which clinical discussion of nonclinical issues was sought.

## II. MAJOR ISSUES RAISED BY PRIMARY REVIEW

### A. Target Organs

General toxicology studies conducted by the Sponsor to evaluate the toxicity of Org 25969 are considered adequate with acceptable safety margins demonstrated for human use and identification of several target organs in single-dose and 4-week repeat-dose toxicology studies in the rat. Target organs were not identified in the dog with intravenous administration of Org 25969 up to the maximum feasible dose (MFD). Though the purpose of these studies is to identify toxicity, demonstration of acceptable safety margins and use of a maximum feasible dose is considered sufficient to allow use as part of a safety assessment. Target organs identified in the rat with administration of Org 25969 were the kidney, lung, and urinary bladder. In single-dose toxicity studies in the rat, Org 25969 was associated with renal tubular vacuoles at ≥ 600 mg/kg, foamy alveolar macrophages in the lung at 2000 mg/kg, and in two 4-week repeat-dose toxicology studies an additional finding of foamy umbrella cells of the urinary bladder with Org 25969 dose levels ≥120 mg/kg and kidney and lung findings at ≥120 and 500 mg/kg, respectively. Alterations in the lung and kidney were reversible and minimal in severity with a 2-week treatment-free period, while in the 4-week repeat-dose studies these target organ findings largely reversed within 3 or 8 week recovery period associated with each study.

Renal tubule vacuolation, urinary bladder hypertrophy/hyperplasia, and accumulation of macrophages in the lungs are a well known toxicologic finding of cyclodextrins when administered parenterally (Irie and Uekama, 1997; Donnabauer et al., 1998). Renal reabsorption vacuoles are though to occur as these compounds when administered intravenously are renally excreted and are highly concentrated in the tubular fluid of the proximal tubule. With α-CDs and β-CDs nephrotoxicity is characterized by an increase in apical vacuoles and the development of giant lysosomes. Although reuptake vacuoles are considered an adaptive response to osmotic agents and are seen with high dose administration of glucose, mannitol and dextran, toxicity has been observed with unsubstituted α- or β-CDs due to the inability of the lysosome to degrade the cyclodextrin and subsequent precipitation of the compound which forms microcrystals toxic to renal cells. The development of substituted β-CDs, used as a vehicle in six approved and marketed products (as Captisol, a sulfobutyl ether-β-CD or hydroxypropyl-β-CD), was driven in part by the need for a CD with greater solubility which would avoid the precipitation that occurs upon the high concentrations in the lysosome. γ-CDs are known to have higher solubility than α-CDs or β-CDs and have a reduced ability to interact with membrane lipids. This likely explains the limited toxicity with even high dose Org 25969 observed in the rat and dog toxicology studies. In addition to the non-progressing nature of the renal findings and reversibility, also reassuring was the absence of clinical chemistry changes which would indicate renal dysfunction. Foamy macrophages observed in the lungs with single- and multiple-dose Org 25969 exposure in the rat likely represents uptake and slow degradation and is not indicative of an adverse effect. I agree with Dr. Xu and the Sponsor that this likely is a clearance mechanism and it is also reassuring that no evidence of damage to the lung tissues themselves was observed in any study in rat or dog up to 4-weeks duration. Alterations in umbrella cells of the urinary bladder (foamy hypertrophy), observed only in the 4-week rat toxicology study did not

reverse within the 8-week recovery period. The Sponsor argued that the findings did not progress towards significant signs of cellular damage and that turnover of these cells is on the order of many months to a year and is therefore not adverse. I am in agreement that this does not represent a significant toxicity. Also, the urinary bladder and renal findings were not observed in the dog, which as the Sponsor noted has a 3X higher GFR than the rat. This suggests that the lower renal clearance of Org 25969 in the rat may be the cause of the greater urinary tract findings as the concentration of drug in the tubular fluid and bladder would be expected to be significantly greater in this species. Consistent with this line of reasoning, the human, which has a 2X and 6X higher GFR than the dog and rat, respectively, may be expected to have a reduced risk relative to either species.

### B. Drug Distribution to Bone & Teeth

One characteristic of Org 25969 which has elicited more detailed evaluation is the observation that after intravenous administration (the intended route) the compound preferentially distributes to bone and teeth with deposition of a significant amount of a single dose (up to nearly 2% in adult animals) where it remains (at least in bone) for long periods with a terminal mean half-life of 170 days. Additional data indicates that binding is enhanced in regions/states where active bone growth is occurring; therefore, there are concerns with use in pediatric populations as well as in surgical populations in which bone trauma (fracture, osteotomy) occurs as part of the precipitating event or corrective action taken.

A formal consult was requested from both the Division of Metabolic and Endocrine Products (DMEP) and the Division of Dermatologic and Dental Products (DDDP) to address data from drug distribution studies for bone and teeth and the follow-up adult and juvenile toxicity studies requested by the Agency. The nonclinical DMEP consult for bone concerns was provided by Gemma Kuijpers, Ph.D. while the DDDP consult for teeth issues was performed by Fred Hyman, DDS. Relevant aspects of their respective consults will be discussed at appropriate points in the following sections.

**Org 25969 Distribution Data**

The Sponsor has conducted several studies of Org 25969 distribution, primarily in the young adult rat. Studies examining the effect of single and multiple administration (the latter up to 4 weeks), effects of prior administration of rocuronium on Org 25969 distribution, placental transfer, fetal distribution, and effects of age have been submitted. These studies have generally focused on distribution to bone and teeth.

***Adult and Juvenile Data:***

[$^{14}$C]-Org 25969 administered as a single intravenous dose of 8 mg/kg to the young adult rat resulted in 74% eliminated in urine over the first 24 hr but this rose to only 78% at 7-days after administration. Evaluation of radiolabel indicated a deposition of 1.1 – 1.9% of dose/gram bone tissue while in incisors deposition was approximately 0.45% of dose/gram teeth within 24 hr -- with a slight increase over the first 7 days to >0.5% which may reflect a redistribution. This represents a 30 – 60-fold higher radiolabel concentration in bone and 15-fold higher level in teeth than found in cartilage. By Study Day 84 (SD84) levels were around 0.36% dose/gram tissue for bones though radiolabel was largely absent from teeth. The disappearance of label from teeth appears to be due to

incisor wear and turnover in the rodent rather than redistribution or exit of radiolabel. Elimination of radiolabel appears to follow a 2 (or perhaps 3) compartment model, with $t_{1/2}\alpha$ being approximately 21 days while the mean $t_{1/2}\beta$ for bones was approximately 172 days (longest up to 210 days) while in teeth $t_{1/2}$ was approximately 8 days.

Notably, pre-treatment with non-radiolabeled Org 25959 followed by [$^{14}$C]-Org 25969 revealed saturation of binding in bones and teeth did not occur and was suggestive of a high binding capacity. A follow-up bone and teeth retention study in the rat was designed to assess the cumulative effect (up to 4-weeks) of higher levels of Org 25969 on binding as well as examine juvenile, young adult, and aged rats to determine the influence of age and bone growth rate on this parameter. This study also examined whether rocuronium, administered prior to Org 25969, affects the distribution of radiolabeled Org 25969 to bone and teeth. Results indicated absorption of Org 25969 in bones and teeth increases non-linearly with increasing dose from 30 – 400 mg/kg. Some saturation of bone appeared to occur over 28-days of dosing but this was not fully complete as [$^{14}$C]-Org 25969 administered on SD28 labeled bone at approximately 50% of the level of SD1 even with 27 prior days of non-labeled Org 25969 dosing. Saturation was not evident in teeth, likely due to the continual wearing down and regenerative growth in this species.

The effect of age on distribution and binding of Org 25969 was examined using a single 30 mg/kg dose of [$^{14}$C]-Org 25969 in juvenile, young adult, and aged rats followed by sacrifice. Results indicate the juvenile rat absorbed a significantly greater amount of [$^{14}$C]-Org 25969: in contrast to the young adult rat in which ~ 3% of a single-dose was retained in pooled bones, in juvenile rats approximately 13% of the entire single dose was retained.

Administration of the neuromuscular blocking agent rocuronium at a 50% molar dose compared with [$^{14}$C]-Org 25969 reduced radiolabel binding in bone by roughly 1/3 – 1/2. Therefore, in the surgical setting the presence of rocuronium will lead to complexation with Org 25969 and reduction of deposition in bones and teeth of the patient.

***Drug Distribution in Pregnancy:***

Placental transfer studies were conducted in rat and rabbit using [$^{14}$C]-Org 25969 administered over various gestation periods (GD20, GD6-20, GD16-20). Limited transfer of radiolabeled drug was observed to cross the placental barrier (estimated at approximately 0.1%). Although the transfer level was considered low, the majority of the radiolabel is found in the bones of the fetus as measured by both liquid scintillation counting and whole body microautoradiography.

***Binding site***:

Microautoradiography and whole body autoradiography studies of the rat indicate Org 25969 binds readily to bone and teeth. Org 25969 is an anionic compound, containing 8 thiopropinate groups, this polycarboxylic structure has been proposed by Sponsor to interact with the hydroxylapatite crystals forming the major inorganic mineral content of bone and teeth. Tetracycline and bisphosphonates are other examples of polycarboxylic compounds with significant affinity for hydroxyapatite of bone and teeth.

Microautoradiographic evaluation of [$^{14}$C]-Org 25969 deposition in the bones of a young adult rat indicated that radiolabel was confined to the periosteal and endosteal aspects of cortical bone as well as the endosteal aspect of trabecular bone in the diaphysis region. No binding to metaphysis/growth plate region or epiphyseal region was apparent.

**Toxicity data:**
The toxicologic significance of Org 25969 retention in bones and teeth were evaluated in four types of studies: general toxicity studies in the (young) adult rat and dog, toxicity studies in the juvenile rat, several dedicated studies to evaluate the effect of intravenous Org 25969 on bone quality, integrity and turnover in the young adult rat as well as reproductive toxicity studies in rat and rabbit

***Adult models:***
Four-week general toxicity studies in the young adult rat and dog have been conducted in which no evidence of bone abnormalities were noted at doses up to 500 mg/kg/day and 250 mg/kg/day, respectively, although bone-specific evaluation (such as µCT) were not conducted. Dedicated bone studies in young adult rats utilizing single-dose intravenous administration of Org 25969 at 0 or 2000 mg/kg and evaluation of bone-specific clinical chemistry and urinalysis parameters (throughout study) as well as µCT of long with sacrifice either on SD21 or SD42 revealed a possible slight resorptive effect on trabecular bone on SD21 (increased urinary DPD, increased plamsa CTx, slight decrease in bone volume/tissue volume in metaphasis of femur, increased trabecular numbers and separation without change in trabecular bone mineral density or thickness; increased femur indentation) without apparent effect on cortical bone with recovery of femur trabecular bone changes by SD42. With the exception of increased femur indentation which the Sponsor suggests does not reflect a meaningful weakening of bone, no evidence of compromised bone strength was noted in the majority of the 3-point bending test parameters (though stiffness was slightly reduced) and femur neck cantilever test. A subsequent study with Org 25969 administered as a single intravenous dose up to 500 mg/kg with sacrifice and evaluation of subgroups at SD1, 3, 7, 14, 21, 28, 35 and 42 after single doses did not reveal any of the previously described changes and was considered a single-dose NOAEL level for bone integrity and structure by the Sponsor for which Dr. Xu was in agreement.

***Juvenile Models:***
A 4-week toxicology study in the juvenile rat was conducted in which rats were dosed from PND7-34 with 0, 120 or 500 mg/kg/day (SC followed by IV as rat matured) with sacrifice on SD35 or 8-week recovery sacrifice on SD91. The NOEL for bone was identified at 30 mg/kg/day. At doses ≥ 120 mg/kg/day in-life evaluation indicated shortened ulna compared with controls, altered trabecular bone parameters (↑bone mineral density [BMD], bone volume/tissue volume ratio [BV/TV], trabecular #, trabecular thickness), and mild ↓cortical thickness (no effects on cortical BMD) at SD35 but not observed at SD91 whereas decreased femur length (evaluated ex vivo) was noted at the SD91 evaluation only. Body weight gain was reduced with higher dose levels and the Sponsor argues that the effects on the femur and ulna is due to a general "growth retardant" effect due to the non-specific toxicity of the compound at high doses and is not indicative of a direct effect on bone growth. No bone strength measurements were

conducted as the Sponsor considered the effects to be insufficient to merit further evaluation. Two additional single-dose studies in juvenile rat model are less informative due to reduced (or absent) bone endpoints.

*Note*: *The DMEP consult provided by Gemma Kuijpers, Ph.D. notes that femurs had to be "reoriented" in relation to control femurs for evaluation due to the slight difference in femur length and diameter and that this may lead to an artifactual alteration in trabecular measurements (if the equivalent portion of the diaphysis is not evaluated between groups). Dr. Kuijpers considered the lack of reversibility of longitudinal bone growth suggests a potential relationship to bone retention, and though no binding has been observed to the growth plate, the Sponsor did not conduct growth plate morphometry. Dr. Kuijpers was not convinced that the decreased bone length is due to a general toxicity and growth retardation effect as there was no clear temporal relationship. Dr. Kuijpers states that Org 25969 may possess reabsorption inhibiting properties similar to tetracycline or bisphosphonates but bone turnover biomarkers were not evaluated. Alendronate, a bisphosphonate, binds avidly to bone hydroxyapatite (HA) and inhibits resorption and in has been previously reported to reduce bone length during the growth phase of the rodent, rabbit and pigs though this has not been observed in all nonclinical models. Though the clinical relevance of the findings are unclear to Dr. Kuijper, and the safety margins at the NOAEL of 30 mg/kg/day are low (1X) based on AUC the margin is much higher when based on Sponsor's estimation of bone concentration (33X) which she believes has some merit but may not utilize the most appropriate methods of estimation.*

In regards to effects on teeth in the 4-week juvenile toxicity study, tooth discoloration was observed beginning in the 3rd week of the 4-week dosing period for rats receiving 500 mg/kg/day; also apparent in some of these animals were other dental abnormalities including overgrown incisors and malocclusion which can occur when incisors hardness is increased (such as what happens with fluoride) and interferes with normal incisor wear. Although the Sponsor suggests there is recovery over the 8-week period, it seems probable this represents incisor turnover. Disruption of the enamel epithelial layer of the incisor with deposition of "amorphous material" was noted at doses ≥ 120 mg/kg/day. This "amorphous material" has not been identified further by the Sponsor. Molars were less affected though similar findings were noted at 500 mg/kg/day and effects were not noted with 8-weeks of recovery. As molars do not grow continuously in the rat, this apparent recovery may indicate true reversibility. To examine the risk of single dose exposure to Org 25969, the Sponsor conducted an extended single-dose study in which juvenile rats were administered drug intravenously on Post-Natal Day (PND) 7, 14, or 21 at doses of 0, 120, or 500 mg/kg and were sacrificed either acutely (1-day post-dosing) or 2-weeks later. No findings were noted in juvenile rats exposed to drug on PND 7 or 14. PND 21 rats did not demonstrate drug-related effects on the teeth at 0 or 120 mg/kg but a single animal (1/16) in the 500 mg/kg group sacrificed 2-weeks after administration was observed to have minimal disruption of the enamel epithelium with the presence of amorphous material in the incisors. An additional study in which juvenile animals were

dosed once on PND 7 and followed for 6 weeks did not detect defects in amelogenesis with doses as high as 500 mg/kg.

The NOAEL for teeth disturbances determined from the 4-week study in juvenile rats was identified by both Sponsor and Dr. Xu as 30 mg/kg/day. The Sponsor suggests the NOAEL for single-dose amelogenesis disruption and discoloration in the juvenile rat is 500 mg/kg if the single finding represents background incidence though the Sponsor notes this may also represent the LOAEL.

Dr. Kuijpers overall impression of the data from bone evaluations is that NOAEL safety margins were large (>18X) and supports use in adult patients. The only low AUC-based safety margin was observed in the 28-day multiple dose juvenile rat study (1X). Though bone concentration estimates do not produce as conservative margins as with AUC-based margins, it is unclear which is the more appropriate value but the bone concentration margins can be used carefully to provide support for situations of slim AUC-based margins. Safety margins for children are uncertain as we do not have this (pediatric) information at this time. The age range of the juvenile rat used in the study (PND7 – PND91) approximates a 2-10 y/o child. Dr. Kuijpers does not believe the increased trabecular bone parameters to be adverse, nor the cortical bone changes, and notes the 28-day duration of the study mitigates the significance of the animal findings for single-dose clinical treatment. However, she does not dismiss the possibility for risk of impaired bone growth with multiple-dose administration in the pediatric population.

Further data which Dr. Kuijpers believes would be helpful in ascertaining the nature of the effect of Org 25969 include:

1. Provide data on growth plate morphology to help understand the longitudinal growth reduction observed in the 28-day juvenile rat study; samples may be obtained from this study if still available.
2. Evaluation of vertebrae would be helpful to interpret bone effects since they have a more homogenous trabecular structure
3. Reevaluate bone localization (microautoradiography) study would be helpful as presentation of data was poor and errors in descriptions were noted.
4. Additional studies to address mechanism of action include in vitro bone resorption assay ($^{45}$Ca release), assessment of bone turnover markers, hydroxyl-apatite crystal growth and dissolution assay, effect of in vitro bone decalcification on sugammadex retention.
5. Further studies if requested should add positive control arm of bisphosphonates to prove sensitivity of the assay and extend the period of recovery to 4-6 months (>2X $t_{1/2\beta}$ Org 25969).
6. Fracture repair study in a nonclinical model.

I am in general agreement with Dr. Kuijpers expert and experienced evaluation. I find I am more comfortable with the use of estimated bone concentrations to develop safety margins than she appears to be. I agree that a safety margin based on the much more conservative AUC value will satisfy the need for a safety margin for human use; however, when evaluating risk I believe use of the AUC value is too conservative. While it is true that the Sponsor has made assumptions which may underestimate the risk to

humans they also have made assumptions for deposition in human bone which appears to be as conservative as possible (i.e. maximal retention) which I believe largely counteracts this concern. Also, safety margins based on bone concentration are generally large enough that should true margins be several-fold lower there would still be an adequate safety margin provided.

Dr. Hyman evaluated toxicologic data from teeth obtained in general toxicology, juvenile toxicology and dedicated bone and teeth studies. He noted that the rat incisors, which continue to grow throughout the rat's life, are a good model for developing human teeth whereas the rat molars (which have only an extremely limited ability to continue growth) are a good model for permanent human teeth – equivalent to humans > 7 y/o. White spots on teeth appear similar in nature to that observed with dental fluorosis, which is considered a cosmetic alteration and not a pathologic finding according to the Surgeon Generals Report on Oral Health (2000). Data indicated Org 25969 could interfere with enamelization (amelogenesis) in the juvenile rat which may confer some theoretical risk to children with developing teeth but he states "the extremely high doses and chronicity of exposure required to produce detectable adverse effects on the developing enamel provides a margin of safety for its labeled (single) use in humans under the age of 8" and for all children over the age of 8 regardless of lifetime frequency. Dr. Hyman agrees with the review team that the margins drop if multiple-doses are planned for either clinical trials or in clinical usage upon approval; however, it was reassuring that disrupted amelogenesis and deposition of "amorphous material" was not observed until 3 weeks of daily administration at high dose levels (>30X highest planned human dose) had already taken place. No recommendation was provided by Dr. Hyman on the number of administrations that would be supported by the data without further study. Dr. Hyman noted the low transfer of Org 25969 through the placenta to the fetus and therefore was unsurprised by the lack of dental findings in the reproductive toxicology studies and noted that should effects on primary teeth occur through gestational exposure, these would likely be replaced by normal permanent teeth later in the child's development. Dr. Hyman states "there is no evidence of a risk for adverse effects occurring in teeth of children over the age of 7, the age at which the enamel of all permanent dentition has been mineralized". He notes that sugammadex approximates the tetracycline model more than the fluoride model due to the IV delivery of drug to the bones, and the lack of benefit to teeth to offset risk of impaired dental development. Furthermore, "Until further safety is established, dental effects are considered a possible adverse event."

Further data which Dr. Hyman believes may be helpful for determining the risk profile in the pediatric population include:

1. Evaluation of extracted/shed teeth from children exposed during development (i.e. primary teeth)
2. Evaluation of Org 25969 effect on the teeth of a more analogous model for human dentition such as the monkey.

While I agree this information would be informative, the safety margins are significant even with repeated dosing and deposition in teeth would most likely be a cosmetic issue. Although possible, post-approval monitoring for toxicity to teeth in the clinical setting

would be very difficult; should reports of dental abnormalities be reported, further evaluations can be requested of the Sponsor.

### C. Cardiac Signal

Nonclinical safety studies of Org 25969 have identified a potential cardiac signal: High concentrations of Org 25969 (1.5 mM) produced a mild inhibition (22%) of the hERG delayed inwardly-rectifying potassium channel cloned into human embryonic kidney cells; the concentration producing 50% inhibition, a standard benchmark for comparing relative propensity for block was not reached. Bath application of Org 25969 to canine purkinje fibers, which contains multiple types of ion channels involved in cardiac conduction, revealed a mild to moderate prolongation in action potential duration ($APD_{50}$, $APD_{70}$, and $APD_{90}$: 4 – 17%) at 150 μM and 1500 μM tested at 1.0 [normal] or 0.3Hz [bradycardic] stimulation rates compared to baseline control. In vivo evaluation of cardiovascular safety in the dog indicated that 25 mg/kg Org 25969, with or without the presence of rocuronium, did not affect QT interval though 250 mg/kg Org 25969 did produce an increase of 6.9% over control QT duration. As noted by Dr. Xu in his primary review, this dose level represents a 10-fold higher plasma concentration than is produced with clinical use of the drug; therefore, the toxicologic significance of this finding was considered negligible by Dr. Xu, an opinion which I share. Furthermore, dedicated thorough QT (tQT) studies have been conducted with clinical subjects exposed to supra-therapeutic doses of Org 25969 with or without rocuronium and in neither study was there evidence of QT prolongation.

### D. Immunotoxicity signal

As noted in the primary review, 3 studies were conducted to explore the effects of Org 25969 administration on the immune system. In vitro evaluation of immunosuppressive potential of a test compound is usually evaluated in the Plaque Forming Cell Assay, designed to test the integrity of the T-cell Dependent Antibody Response (TDAR). A mild increase in the TDAR was noted in rats administered Org 25969 up to 500 mg/kg intravenously for 2 weeks. A popliteal lymph node assay (PLNA) was subsequently conducted in mice to determine if exposure to Org 25969 would cause an increase in cellularity in the draining lymph node of the foot pad site (where Org 25969 was injected). Results of this study revealed a statistically increased level of radiolabel incorporation into cells of the PLN (~3-fold vs. control) at 278 mg/kg (NOAEL 67 mg/kg) though as a signal for sensitization/immuno-stimulation this appears to be relatively mild when considering the positive control, $HgCl_2$ (26-fold higher than control). The 3rd study, a local lymph node assay in mice in which dermal application of Org 25969 to the ear did not generate evidence of contact hypersensitivity through examination of the auricular lymph node. Though the primary reviewer appears to discount the findings as he appropriately notes no evidence of dermal penetration (systemic exposure) to Org 25969 and the large size of the molecule which would preclude dermal passage, the vehicle used in the study, 50% DMSO, is an excellent penetration enhancer and it has been previously reported that transdermal passage of CDs can occur with use of absorption-promoting vehicles (Irie and Uekama, 1997). Therefore, the negative results in this study are inconsistent with the findings of the previous study but overall nonclinical data appears to indicate that sensitization and immuno-stimulation, may be a risk in humans. Although these tests are not highly

predictive of human risk, I disagree with Dr. Xu that they provide little information to describe the risk. The PLNA in particular has been suggested to be useful in the determination of immune-mediated drug hypersensitivity reactions (Goebel et al., 1996; Bloksma et al., 1995) and has been reported to detect more than 70 low molecular weight compounds that are associated with autoimmune-like and allergic reactions in humans (Vial et al., 1997; Gutting et al., 2002). Generally, compounds below a certain mass threshold "low molecular weight compounds" (LMWC) are not thought to be of sufficient size to generate an antibody-mediated immune response unless they form a hapten with another larger molecule. Proteins and polypeptides with molecular masses > 5,000 to 10,000 Da are frequently immunogenic. Org 25969, is not a LMWC by definition as the molecular mass is approximately 2,000 Da, nor is it large enough for which an immune response may be predictable. This 1,000 – 5,000 Da mass range has been considered to be inconsistently immunogenic (De Weck, A.L. (1974). Long chains of similarly linked glucose residues such as the plasma expander Dextran 40 and 70 are known to be associated with anaphylaxis (Zinderman et al., 2004) but these are large carbohydrate chains, 40 and 70,000 Da, respectively.

Some evidence for potential immune hypersensitivity reactions have been found in the clinical safety database and therefore this concern will be well addressed should further clinical studies be requested. Further nonclinical studies are not necessary at this time.

### E. Reproductive toxicology findings

The Sponsor has conducted an adequate evaluation of fertility in the rat which showed no evidence of Org 25969-associated impairment at dose levels which afforded large safety margins. General toxicology studies have not found significant pathology of reproductive organs in the rat or dog with up to 4 weeks of continual dosing of standard batches of Org 25969; however, studies with degraded Org 25969 did detect apparent test-article-related changes in male reproductive tissues in both single-dose (↑spermatids or hypospermia in epididymis; not observed in recovery group animals) as well as repeat-dose (2-week) studies in the rat (sperm granuloma, epididymal rupture, decreased seminal vesicle weight) which were not observed with the standard drug product. However, single-dose studies with degraded Org 25969 identified a safety margin for single-dose use (described in detail below).

Embryofetal development studies were conducted in both the rat and rabbit. These studies were designed to determine toxicity, and in particular teratogenicity, with exposure during the major period of organogenesis in these species. Standard methods and evaluations were used. The Sponsor contends that no teratogenicity or embryofetal developmental toxicity was observed in either study. Dr. Xu has noted his agreement with this evaluation. Although an apparent increase in the incidence of renal pelvis dilation was observed in rats treated with the highest dose of Org 25969 when compared with controls (6.5% vs. 0.7%, respectively), and the difference appears suspicious given the knowledge that sugammadex is: 1) renally eliminated; 2) the kidney is identified as a target organ in adult and juvenile general toxicity studies, and 3) the kidney function is immature at this stage of development; the Sponsor provided data that this level of observation is within the laboratory's historical control data (mean 4.9%; range 0 – 8.5). I note from a search of the Mid-Atlantic Reproductive Toxicity Association

(MARTA)/Midwest Teratology Association (MTA) joint database -- which collects results of control data from a number of contract laboratories –that constraining the search to data from studies conducted over 2000-2008 in this strain of rat indicates that the mean incidence) of this abnormality is 1.56% with a maximum mean in a control group of 3.3% with 4,625 rat pups evaluated from 666 litters in 28 studies. Although not a current dataset, the incidence of embryofetal abnormalities in the control groups of reproductive toxicology studies conducted between 1992 – 1994 has been published by Charles River laboratories in which 64,789 rat pups from 4,935 total litters in 229 studies revealed that dilated renal pelvis was the 3rd most commonly observed visceral abnormality in the same strain of rat. Notably, however, the mean and standard deviation for incidence is 1.17 ± 2.67%. This is not inconsistent with the more recent data provided by the MARTA/MTA database above. The maximum observed in a single study in the Charles River dataset was reported as 19.66% indicating that it is possible for high levels of this abnormality to be expressed in controls. The historical control data from the laboratory conducting the study appears to indicate this is unlikely to a drug-related effect; regardless of this determination I would not be inclined to consider this an approvability issue for several reasons: 1) this type of abnormality is considered a variation and is though to represent a transient finding; 2) no evidence of a more serious hydronephrosis was described which would indicate potential blockage of outflow; 3) no evidence of impaired renal function was noted in $F_1$ pups in the pre- and postnatal (segment 3) study; 4) similar findings were not observed in the 2nd species (rabbit).

The effects of Org 25969 on offspring pre- and postnatal development was evaluated in the rat in a series of studies. Administration of Org 25969 to pregnant rats from GD6 through lactation day 21 (LD21 = PND21 = weaning) revealed a marginally increased rate of post-natal offspring loss during PND0-4 which became statistically significant at ≥ 120 mg/kg. As described by Dr. Xu in his primary review, the apparent reduction in viability appears to be secondary to or, more properly, associated with an increase in cannabilization of pups by dams. The Sponsor's presentation of data is confusing on this issue as it is difficult to determine the level of differences between treatment groups. The Sponsor reports the number of dams cannibalizing their whole litter prior to PND4 is 2, 0, 2, and 3 animals in the 0, 30, 120, and 500 mg/kg treatment-groups. Additionally, one dam in the LD and MD group were considered to have cannibalized their litter prior to the first litter check as pregnancy was verified and weight was equivalent to litter-generating dams but no pups were found on the first litter check on PND0. Also complicating this interpretation is that 1 in the MD and 1 in the HD had very few pups at the first litter check despite the fact that on necropsy they were observed to have had 18 and 13 implantation sites in the uterus, respectively. Therefore, the reduced litter number is ascribed to a significant degree of cannibalism of pups prior to the first litter check. Taken together, the number of dams in each treatment group with whole/near complete litter loss through cannibalization can be described as 2, 1, 4, and 4. As noted by Dr. Xu, no evidence of developmental toxicity could be found in surviving progeny, nor was an evaluation of stillborn/dead pups or partly cannibalized pups illuminating as to any defects which might explain maternal behavior.

The Sponsor conducted a series of studies to further clarify, evaluate and ultimately confirm or refute the observation of increased pup cannibalism in response to high dose sugammadex. In the first follow-up study, dams (~22/group) were intravenously administered sugammadex at a dose of 500 mg/kg over varying gestation/lactation (i.e. postpartum) periods to determine if the finding could be replicated. Dosing of dams from GD6 – PND4 (the dosing period consistent with that in the original study in which an effect was observed) did not reveal any apparent increase in cannibalistic behavior or increased neonatal mortality. No gross evidence of abnormality in the pups was noted compared with controls. This is inconsistent with the prior study finding. Other dosing periods were also examined within the study. Administration of sugammadex during the period of GD16-21, however, produced an increased rate of cannibalization of litters (5/21 litters in the HD group compared with 1/19 in the control). At this time there is no data to explain this discrepancy; this may simply indicate a large variability in the maternal responses of the rat. As Dr. Xu notes, the Sponsor's argument that the 500 mg/kg group dosed over GD16-21 was not appropriately acclimated to the dosing procedure does not appear to have face validity – neither did the controls or any other dose group. A second follow-up study was a pseudo-cross-fostering study in which the pups of sugammadex-treated dams (500 mg/kg/day) were delivered by C-section and transferred to a naive animal. Survival/cannibalism was compared against control pups delivered from a vehicle-treated dam and transferred to a separate, naive dam. Also, pups from a 3rd dam (treatment-naive) were delivered and Org 25969 was applied directly to the body to determine if a direct smell or taste could drive the treated-dam to cannibalize pups. The results were mixed: an increase in postnatal death in pups with in utero exposure to Org 25969 compared with control was noted (20.6 vs. 14.9%, respectively) with no effect of direct application of drug.

Maternal culling through cannibalization is not an infrequent phenomenon in rodent studies, this is one reason why in embryofetal development studies pups are delivered the day prior to expected delivery by C-section, in order that pups with terrata or are otherwise unhealthy will not be consumed by the mother. In general there are a number of explanations put forward for this behavior: 1) Nutritional deficiency in the mother, 2) stress, 3) hormone dysregulation, 4) pup physical or behavior abnormalities. The data provided by the Sponsor does not clearly indicate a risk; increased postnatal death was not consistently observed between studies and there are several explanations for why such a result, if due to cannibalization, might be seen – only one of which, pup abnormality, confers risk for human developmental exposure. Maternal body weight and food consumption was not reportedly affected in the study though this is difficult to judge due to the variability in number of offspring; nevertheless, no evidence of nutritional deficiency was apparent. Stress and hormone dysregulation was not monitored for alteration; however, as a cyclodextrin, little penetration of the CNS occurs (3% estimated by Sponsor) though potential binding of Org 25969 to sex steroids such as progesterone (12X lower than rocuronium) and estradiol (24X lower than rocuronium) could play a role with repeated dosing at very high dose levels. Finally, though there is a possibility that there could be an occult structural or behavioral alteration which could lead to this action by the mother, no evidence of abnormality were found in surviving pups of the Seg 3 study though it is noted this may be a truly "selected" population. Nevertheless, the

absence of teratogenicity in rats exposed to Org 25969 for embryofetal development studies and in the follow-up examination to the pre- and postnatal study suggest a structural abnormality is not likely the explanation. Dr. Xu states that the Sponsor's hypothesis – that in utero exposure to Org 25969 results in a change in odor or taste which alters the maternal response – is the most likely explanation for this finding and the NOAEL with clinically relevant toxicity is >500 mg/kg. I am unconvinced of this; it is possible that a direct effect on the fetus could occur and has not been ruled out – behavioral teratogenicity has not been examined in animals which might have otherwise been consumed if the dam was allowed to deliver. I believe the data should not be entirely ignored in labeling though further follow-up studies may address this concern. Regardless, a NOAEL was identified at 30 mg/kg which provides safety margins for clinical use.

### F. Impurities/Degradants/Additional API

The manufacture of Org 25969 is a (b) (4) result in impurities which the Sponsor has had some limited success in reducing to levels within ICH Q3A & Q3B thresholds. (b) (4) A number of impurities fall above Q3A and Q3B thresholds for identification and qualification and a series of discussions with the Agency have taken place in order to determine a path forward. Agreement was reached that due to the low toxicity potential of (b) (4) impurities would not require identification and qualification at levels (b) (4)% when such impurities were determined to be (b) (4) The Sponsor will perform a partial identification once an impurity rises above (b) (4)% to determine conformance to the above listed criteria. One impurity, (b) (4) is found at levels of (b) (4)% in the drug substance batches and for which the Sponsor has requested a specification of NMT (b) (4)%. (b) (4) As discussed in the primary review of Dr. Xu, specified impurities have been qualified through general or genetic toxicology studies with inclusion in non-degraded or, where necessary to achieve acceptable exposure coverage, degraded toxicology batches of drug substance. All specified impurities in the drug product were qualified in the rat though some qualification studies for impurities in the drug substance were conducted in the dog. Dr. Xu has appropriately identified an apparent differential toxicity profile with the degraded batch used in single-dose and 2-week repeated-dose toxicology studies when compared with non-degraded drug substance. Effects on male reproductive organs (epididymis, seminal vesicle) were noted with degraded drug substance that was not observed with up to 4-week repeat-dose studies with non-degraded Org 25969 with similar dosing levels. I agree with Dr. Xu that the intended usage of sugammadex in practice will be more similar to the single-dose with recovery (i.e. washout) study in which reversible and inconsistent effects on the epididymis (hypospermia, increased spermatids) were

observed with the 2000 mg/kg level. The NOAEL in the study, 600 mg/kg, provides a reasonable safety margin ([redacted] (b) (4)) for the maximal human exposure to the degradant impurities contained in the degraded batch which are also specified impurities in the drug product. It should be noted that additional impurities were generated in the degraded batch which are not observed in the manufacturing batches which could have contributed to the toxicity observed. Finally, the inconsistency and reversibility of the finding is reassuring. I do not believe further studies are necessary to characterize this differential toxicity.

## III. ADVISORY COMMITTEE

The Anesthetic and Life Support Drugs Advisory Committee (ALSDAC) met on March 11, 2008 to address issues raised by the present NDA. Nonclinical questions put forward to the committee included the following (paraphrased), with this reviewer's interpretation and comments of general committee conclusions from the AC Meeting Minutes added:

> *1) Based on the nonclinical data submitted, has the risk for adults including those with fractures or bone injury been adequately characterized?*
>
> The committee considered the risk to adult patients to be acceptable due to the limited bone effects observed and high safety margins though they noted issues of fracture repair and healing were not directly addressed by the Sponsor. Post-marketing surveillance was recommended as was wording to be included in the label to note that this has not been studied in fracture settings or nonclinical models of fracture and bone repair. The committee thought that such statements should not be worded to dissuade its usage in this population, however. A study in a nonclinical fracture model could address this concern.

I concur that the risk of impaired fracture healing in adults with single, or even multiple exposures to sugammadex is relatively limited given the low level of distribution to adult bone and that description of the absence of fracture healing in the label would be reasonable. Additionally, we will recommend a study of sugammadex in a nonclinical fracture model to evaluate potential negative effects on bone healing.

> *2) Based on the nonclinical data submitted, has the risk for pediatric patients been adequately characterized?*
>
> The committee felt that additional data was necessary to address concerns prior to approval of sugammadex for a pediatric population as the committee believes it may become the drug of choice (with the amino-steroid NMB companion) for use in the pediatric population due to concerns with succinylcholine and the latter's preferred use in this population. In particular, the recommendation was made that more complete functional study such as load-bearing and fracture healing be conducted to complement the histologic and distribution data provided for the juvenile model. Concern was raised by the Sponsor that bone strength assessments in the juvenile can be complicated by secondary reductions in bone dimensions due to non-specific general toxicity and reduced body weight. As the juvenile models examined are considered to have attained mature renal function status, further committee concerns related to the lack of adequate nonclinical assessment in a model of immature renal function as would be expected in a neonate or infant. The Sponsor stated that they did not see any issues in the embryofetal or pre- and postnatal developmental toxicity studies in regards to renal function and therefore they do not expect an issue with this population.

The Sponsor has raised an important point about the dimension effect on bone strength assessments, as their hypothesis is that reductions in bone length are due to reductions in body weight from general toxicity of the drug, and that decreased bone strength would

naturally result from this difference in size alone. This confounding variable may be minimized by intermittent administration of Org 25969 – with the long $t_{1/2}$ of drug in the bone, significant concentrations may be achieved with more infrequent dosing which would be less likely to impact food consumption and body weight allowing for a more direct evaluation of drug deposition on bone strength, dimension, and material properties.

I do not believe the embryofetal or pre- post-natal developmental toxicity studies support use in neonates with immature renal function. In neither of these studies were offspring directly dosed with Org 25969. Therefore, the argument that as no renal or systemic toxicity was observed in these fetuses/pups there is unlikely to be any risk to the neonate is not supported by data. At this time, unless safety data can be provided with dosing of a neonatal model with immature renal function I do not believe there is support for dosing below the age of renal maturity, ~ 2 years of age in the human.

> *3) Does the nonclinical data provided support the safety of sugammadex for clinical trials in the pediatric population?*
> The committee was in agreement that the data provided support single-dose clinical trials in pediatric patients (ages not specified). Further studies in the juvenile animal such as functional studies mentioned previously, examinations of effects on steroid hormones, and information on bone fracture and healing should be provided prior to multiple-dose studies.

Fracture studies will be required prior to multiple-dose clinical trials or approval as previously described. I do not believe that an evaluation of the potential toxicity resulting from Org 25969 capturing steroid hormones is necessary at this time: though it is apparent that Org 25969 binds to steroid hormones such as progesterone and estradiol (12X and 24X weaker affinity than rocuronium), respectively, the drug is to be administered acutely and is eliminated rapidly. Short term derangement of these hormones is not expected to present a significant risk to the pediatric or adult population

> *4) Are there additional studies or information you believe would be necessary to support use in the adult or pediatric population?*
> See Q3 answer.

## IV. RECOMMENDATIONS

### A. Recommendation on approvability

I concur with the primary review of Dr. Zengjun Xu that the application may be approved for the adult population based on the nonclinical pharmacology/toxicology perspective.

### B. Recommendation for nonclinical studies

The application may be approved for the adult indication proposed without further nonclinical evaluation.

Nonclinical studies are also not required to initiate single-dose clinical trials in pediatric patients. However, several nonclinical evaluations will be necessary prior to any

multiple-dose pediatric trials, approval of a pediatric indication, or inclusion of pediatric data in the label:

1. The Sponsor must provide an evaluation of sugammadex in a nonclinical model of bone fracture to examine potential effects of the drug on bone healing. The Sponsor is encouraged to submit protocols of such studies to the Agency for comment prior to their conduct.
2. The Sponsor must provide data on growth plate morphology to help understand the longitudinal growth reduction observed in the 28-day juvenile rat study; samples may be obtained from this study if still available.
3. The Sponsor must provide a repeat-dose study of sugammadex in the juvenile rat with extended period of recovery, such that bone length, material properties and integrity at full skeletal maturity may be evaluated in order to clarify the observation of slight but lasting decreased bone length and body weight observed in the 28-day juvenile toxicity study. Within this study, μCT, bone turnover markers, and bone strength assessment should be obtained and evaluated. Also, the study should incorporate positive control arms to verify assay sensitivity. Lastly, though not required, the inclusion of vertebrae evaluation would be helpful to interpret bone effects since they have a more homogenous trabecular structure. The Sponsor is encouraged to consider an alternative, intermittent dosing paradigm in order to minimize effects of Org 25969 on body weight while allowing for significant drug accumulation in the skeleton.
4. The Sponsor must provide a reevaluation of bone localization of Org 25969 as presentation of the data was not clear and errors in descriptions were noted.

Although not required for approval in the pediatric population, the following nonclinical studies are recommended:

1. Additional studies to address the mechanism of action in bone such as an in vitro bone resorption assay ($^{45}Ca$ release), assessment of bone turnover markers, hydroxyl-apatite crystal growth and dissolution assay, effect of in vitro bone decalcification on sugammadex retention.

## C. Recommendations on labeling

I am in general agreement with the recommendations made by Dr. Xu in his primary review with the exception that I believe a Pregnancy category of C is appropriate until the mechanism for the increased neonatal mortality observed in several of the pre- and post-natal studies can be determined with greater confidence. As the specific language remains to be finalized internally and with Sponsor, a subsequent memo will address the label specifically.

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/s/
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Adam Wasserman
6/16/2008 08:11:31 PM
PHARMACOLOGIST
Recommend Approval from Nonclinical Perspective

# Memorandum to File

NDA 22-225
Bridion Injection

Date: December 12, 2007
From: Alan Schroeder, DPAI, ONDQA
Through: Ali Al-Hakim, Chief, Branch II, DPAI, ONDQA
To: Adam Wasserman and Zengjun Xu, DAARP

Re: impurities evaluation (informal consult)

Please evaluate impurities in the drug substance and drug product for safety.

**The following comments pertain to the drug substance.**

Evaluate drug substance specifications for safety [See Quality section, 3.2.S.4.1 ("Specification"), for drug substance specifications]. This includes evaluation of proposed maximum limits for the following classes of impurities: Organic impurities, inorganic impurities (e.g., heavy metals (b) (4)), and residual solvents. (b) (4)

See Quality section, 3.2.S.4.5 ("Justification of Specification") for chemical structures of specified, identified impurities and other, at least partially identified impurities (pp. 19-26). See for example, the decision tree for reporting, identification and qualification of unspecified organic impurities (pg. 8). See for example, the toxicological justification of specifications (pp. 9-10; pg. 26-28).

See Quality Section 3.2.S.4.4 ("Batch Analyses of Development Batches") for observed impurity levels described in batch analyses of various batches used for pre-clinical, clinical and stability studies.

The following additional concerns are listed:

Evaluate the safety of impurity (b) (4) which is limited in the drug substance to a maximum of (b) (4)%. (b) (4) contains a (b) (4)

Impurities resulting from reaction of (b) (4) have not been found in the drug substance at levels above (b) (4)% according to the applicant. Therefore, (b) (4) are controlled in the (b) (4) to a maximum of (b) (4)% and a maximum of (b) (4)%, respectively. Evaluate safety of (b) (4) of the drug substance as potential impurities in the drug substance at levels of (b) (4)% and (b) (4)%, respectively, as a worst case scenario.

(b) (4) are not controlled in the drug substance batches (and have not been detected there). Both of these impurities have been detected in (b) (4) so far to not have been detected in the drug substance. Evaluate the safety of these impurities in the worst case situation where they might appear in the drug substance at the maximum levels observed or controlled (b) (4)

Note that batches of drug substance prior to Batch L are early laboratory batches and HPLC impurity profiles were not determined for these batches. Yet, certain of these batches (Batches A, B, E, H) are listed as having been used in pre-clinical studies [see page 34 of the section on Impurities (first document under Section

S.3.2)]. Consider if this presents a problem for evaluation of the preclinical studies which were performed using these early laboratory batches.

This is for your information. See page 31 of the section on Impurities (first document under Section S.3.2) for the applicant's list of qualifed levels of specified organic impurities in the drug substances, cross referenced to the relevant pre-clinical studies. The applicant considers drug substance batches AE and later batches to be representative of the commercial manufacturing process.

**The following comments pertain to the drug product:**

Please evaluate the safety of the assay specification for impurity (b) (4), the impurity specifications, and the specification for particulate matter in the Quality section P5.1.

(b) (4) is known to be formed as an impurity (b) (4). The applicant has indicated that its level is below the reporting threshold (0.1%, according to ICH), and it is therefore not specifically limited in the drug product. Evaluate the safety of this level o (b) (4) since it has (b) (4)

For your information:

> Quality section P.5.6 (Justification of Specifications) provides a toxicological justification of the safety of the color specification (since discoloration has been demonstrated to be due to the formation of degradation products). Please evaluate this toxicological justification for safety (pp. 14-17 of this section).
>
> Chemical structures of the degradation products are provided on page 24 of Quality section P.5.6.
>
> Shelf life acceptance criteria are said to be qualified by toxicological studies (see Table 10, pg. 29 of Quality section P.5.6).

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/s/

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Alan Schroeder
1/2/2008 03:07:31 PM
CHEMIST
memo to file; informal pharm/tox consult

Ali Al-Hakim
1/3/2008 09:44:48 AM
CHEMIST