CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

201292Orig1s000

PHARMACOLOGY REVIEW(S)

MEMORANDUM

Gilotrif (afatinib)

Date: April 30, 2013
To: File for NDA 201292
From: John K. Leighton, PhD, DABT
Acting Director, Division of Hematology Oncology Toxicology
Office of Hematology and Oncology Products

I have examined pharmacology/toxicology supporting review for Gilotrif conducted by Drs. Kufrin and Weis and secondary memorandum and labeling provided by Dr. Helms. I concur with Dr. Helms' conclusion that Gilotrif may be approved and that no additional nonclinical studies are needed for the proposed indication.

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/s/

JOHN K LEIGHTON
04/30/2013

MEMORANDUM

Date: April 29, 2013
From: Whitney S. Helms, Ph.D.
Supervisory Pharmacologist
Division of Hematology Oncology Toxicology for Division of Oncology Products 2
To: File for NDA #201292
Afatinib (GILOTRIF)
Re: Approvability of Pharmacology and Toxicology

The non-clinical pharmacology and toxicology data provided to support NDA 201292 for the use of Gilotrif in the treatment of patients with metastatic non-small cell lung cancer (NSCLC) whose tumors have epidermal growth factor receptor (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations as detected by an FDA approved test were reviewed in detail by Dubravka Kufrin, Ph.D., and Shawna L. Weis, Ph.D. The submission included studies of orally administered afatinib in mice, rats, minipigs, and rabbits that investigated the drug's pharmacology, pharmacokinetics, safety pharmacology, general toxicology, genetic toxicity (*in vivo* and *in vitro*), and reproductive toxicity.

The pharmacology studies submitted to this NDA demonstrate that afatinib covalently binds to the kinase domain regions of the EGFR family including EGFR, HER2, and HER4. Consistent with covalent binding, incubation with afatinib resulted in prolonged inhibition of EGF-induced EGFR phosophorylation compared to known reversible inhibitors of EGFR signaling. The majority of studies submitted to the NDA performed to investigate the mechanism of afatinib activity explored the inhibition of wild type EGFR or HER2. In kinase assays, afatinib was able to inhibit wild type EGFR and HER2 with IC_{50}s of 0.5 and 14 nM, respectively and was able to inhibit *in vivo* growth of multiple wild type EGFR of HER2-overexpressing cell lines. Upon inquiry additional data was submitted to demonstrate the activity of afatinib against EGFR exon 19 deletions and exon 21 (L8585R) substitution mutations. *In vitro*, afatinib inhibited the L858R mutant EGFR with an IC_{50} of approximately 0.43 nM and also showed activity against a L858R/T790M EGFR double mutant (10 nM). In cellular *in vitro* assays, afatinib was able to inhibit phosphorylation and/or proliferation of multiple cell lines representing models of overexpression of wild type EGFR, constitutively active HER2, and EGFR exon 19 deletion mutations, including an exon 19/T790M double mutant. Concentrations of afatinib required for activity in these models ranged from approximately 10 to 100 nM; effects on exon 19/T790M double mutants typically occurred at the high end of this range.

The major route of elimination for afatinib was in the feces. Afatinib was not highly metabolized in any species tested and the major metabolic products of the drug consist of protein adducts. Protein adducts include hemoglobin and afatinib was highly associated with red blood cells in all species examined including humans. The ability to form covalent adducts with endogenous proteins raises a theoretical concern for the occurrence of idiosyncratic drug reactions following treatment with afatinib; these reactions would be difficult to predict during a clinical trial but may be observed as a wider population of patients gain access to the drug.

Afatinib was positive in both the presence and absence of metabolic activation in one bacterial strain used to test for genotoxic potential in the bacterial reverse mutation assay (Ames) using the plate incorporation method. The drug was negative in all other *in vitro* and *in vivo* assays for genotoxicity. The positive finding in the Ames assay was discussed with the Associate Director of Pharmacology/Toxicology, Dr. David Jacobson-Kram. Based on these discussions, the team agreed that the weight of evidence suggests that afatinib is not genotoxic. A similar approach was used for a single impurity that was identified in *in silico* assays as potentially genotoxic. While this impurity was weakly positive in the absence of metabolic activation in a single bacterial strain in the Ames assay, it was negative in other assays. Weight of evidence again suggests that this impurity, (b) (4) was not genotoxic and it was treated as a non-genotoxic impurity for qualification purposes.

Han Wistar rats and Göettingen minipigs were the primary models used to investigate the toxicology of afatinib. The major target organ for toxicity in both rats and minipigs was the gastrointestinal tract. In rats the skin and the kidney were additional major target organs. The skin and renal toxicities were consistent with toxicities observed with other approved EGFR inhibitors. In general, epithelial cells were targets of afatinib, with epithelial atrophy noted in organs including the prostate, uterus, and vagina in rats, and the prostate, the seminal vesicles, cornea, upper respiratory tract, and respiratory mucous glands in the minipig. Gastrointestinal and cutaneous toxicities have been commonly reported clinically. The findings of epithelial atrophy in the minipig upper respiratory tract along with a histopathological finding of an increase in cholesterol clefts within foam cells in the lung in the long term study in rats are suggestive of lung toxicity noted clinically. Findings of corneal atrophy in minipigs are consistent with clinical reports of keratitis and conjunctivitis included in the label for Gilotrif. The male reproductive tract was also target for afatinib toxicity. In addition to the epithelial atrophy noted above, a dose-dependent increase in the incidence of low or no sperm count was observed in a dedicated fertility study. This finding has been included in the label for Gilotrif.

Based on the results of *in vitro* hERG testing (IC_{50} value of2.4 μM) and *in vivo* ECG monitoring in minipigs and rats, there does not appear to be a strong potential for afatinib-mediated QT prolongation at clinically relevant exposures; however, other types of cardiac toxicity do appear to be associated with afatinib exposure. In a single continuous intravenous administration study in domestic pigs, a decrease in left ventricular function was noted at a dose of 30 mg/kg. Cardiomyopathy characterized as cardiac failure, left ventricular dysfunction, or decreased ejection fraction has been described clinically in studies of afatinib and is included in the label for the drug.

Reproductive toxicity studies conducted for afatinib included embryofetal development studies conducted in Han Wistar rats and Himalayan rabbits. More significant signals for embyrofetal risk were observed in rabbits. In rabbit embryofetal studies at doses ≥ 5 mg/kg (approximately 0.24 times the exposure by AUC in humans at the recommended dose of 40 mg) there were increases in abortions at timepoints after the end of the dosing period. At this same dose, in the absence of clear maternal toxicity, increases in resorptions and the occurrence of visceral and skeletal variations, predominantly delays in ossification, along with lower fetal weights were observed. Additional studies were conducted in Han Wistar rats to assess potential effects of afatinib on fertility and pre- and postnatal development. In the fertility study female rats

displayed mild decreases in the number of corpora lutea and mild increases in pre-implantation loss and early resorptions at the high dose of 8 mg/kg (approximately 0.6 times the exposure by AUC in patients at the recommended dose). These observations were supported by decreases in ovarian weights at all dose levels in a 4 week general toxicology study in rats. In the pre- and postnatal development study, there were no effects noted on postnatal survival of offspring of rats treated at any dose of afatinib; while there was a treatment related effect on fetal weight that persisted throughout the study at doses ≥ 6 mg/kg, no effects on the attainment of developmental landmarks, sexual maturation, or behavioural performances were noted. Based on these studies and its mechanism of action, Pregnancy Category D is recommended for Gilotrif. Afatinib was also present at high concentrations in the milk of lactating rats. Women should not breastfeed while taking Gilotrif.

Recommendations: I concur with the conclusion of Drs. Kufrin and Weis that the pharmacology and toxicology data support the approval of NDA 201292 for the use of Gilotrif in the treatment of patients with metastaic non-small cell lung cancer whose tumors have EGFR exon 19 deletions of exon 21 (L8585R) substitution mutations. There are no outstanding nonclinical issues that would prevent the approval of afatinib for the proposed indication.

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/s/

WHITNEY S HELMS
04/29/2013

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:	201292
Supporting document/s:	2
Applicant's letter date:	11/14/2012
CDER stamp date:	11/15/2012
Product:	GILOTRIF (BIBW 2992; Afatinib)
Indication:	Locally advanced or metastatic non-small cell lung cancer with epidermal growth factor receptor mutation(s)
Applicant:	Boehringer Ingelheim Pharmaceuticals Inc.
Review Division:	Division of Hematology Oncology Toxicology in support of Division of Oncology Products 2 (DOP2)
Reviewer:	Dubravka Kufrin PhD Shawna L. Weis PhD
Supervisor/Team Leader:	Whitney S. Helms PhD
Division Director:	• John K. Leighton, PhD, DABT for DHOT, OHOP • Patricia Keegan, MD for DOP2, OHOP
Project Manager:	Deanne Varney

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1 Executive Summary

1.1 Introduction

Boehringer Ingelheim has submitted a New Drug Application (NDA) with data to support the use of afatinib (GILOTRIF™) for the treatment of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) whose tumors have epidermal growth factor receptor (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations as detected by an FDA-approved test. A once daily oral dose of 40 mg is proposed.
The epidermal growth factor receptor (EGFR) family is the cell-surface receptor family that consists of the EGFR (ErbB-1; HER1 in humans), and three other closely related receptors: HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB4). After binding to various cognate ligands, EGFR family members form homo or heterodimers, leading to activation of the kinase activity of the receptor. Activation of the receptor kinase domains leads to autophosphorylation and activation of signaling pathways with downstream effects including inhibition of apoptosis. These transmembrane glycoproteins are present on normal cells and play important roles in many normal cellular activities, but overexpression or aberrant function of these receptors has been implicated in the development and pathogenesis of many tumor types.

1.2 Brief Discussion of Nonclinical Findings

Boehringer Ingelheim submitted data showing that afatinib (BIBW 2992) binds to the kinase domains of EGFR, HER2, and HER4. X-ray structure analysis and mass spectrometry studies of EGFR in complex with BIBW 2992 show that the α,ß-unsaturated ketone group (the Michael acceptor group) of BIBW 2992 covalently binds EGFR at Cysteine 797. BIBW 2992 was also specifically shown to covalently bind the EGFR L858R substitution mutant. Inhibition of downstream signaling following incubation with aftatinib was demonstrated both biochemically and in cellular assays. In kinase assays, afatinib was able to inhibit wild type EGFR and HER2 with IC_{50}s of 0.5 and 14 nM, respectively. Afatinib showed similar potency against the L858R mutant EGFR (0.43 nM) as well as strong inhibition of the erlotinib resistant L858R/T790M EGFR double mutant (10 nM). In cellular *in vitro* assays, afatinib activity was demonstrated by inhibition of phosphorylation and/or proliferation of multiple cell lines, with representative models for overexpression of wild type EGFR, constitutively active HER2, and EGFR exon 19 deletion mutations, including an exon 19/T790M double mutant. Concentrations of afatinib required for activity in these models ranged from approximately 10 to 100 nM; effects on exon 19/T790M double mutants typically occurred at the high end of this range. Prolonged inhibition of EGF-induced EGFR phosphorylation, consistent with covalent binding of afatinib to the receptor, was also demonstrated. Finally afatinib was able to inhibit *in vivo* growth of multiple wild type EGFR- or Her2- overexpressing tumor cell lines in nude mice.

Afatinib does not undergo extensive metabolic biotransformation in humans or animals. In radiolabeled-ADME studies, the majority of drug-associated radioactivity was observed in feces and/or bile, primarily as unchanged parent. Excretion was primarily

through the feces with little renal involvement. Conjugates to glutathione and endogenous proteins were the most abundant metabolite species observed.

Afatinib is highly reactive and forms covalent adducts via Michael addition to cysteine-bound SH groups. As a result, it can form widespread adducts to endogenous proteins, including red blood cells (RBCs), which contained a significant proportion of drug-associated radioactivity in exposed animals. Afatinib association with RBCs also occurred in humans to a similar extent as all other species tested. Some accumulation was observed in the liver and kidney as well as other organs such as the adrenal, pituitary, thyroid and spleen; however, levels in these organs were considerably lower than the levels exhibited by RBCs, and in the case of the spleen, may reflect sequestered RBCs undergoing degradation. Distribution to the brain was low in all assays.

The presence of covalent adducts is of concern for the overall safety of afatinib, since adduction of reactive small molecules to foreign proteins has been repeatedly associated with adverse idiosyncratic drug reactions (IDRs), including fatalities, in patients. IDRs have been repeatedly associated with drug-induced hepatic failure, anaphylaxis, autoimmune disease (e.g. SLE), and thrombocytopenia, anemia and agranulocytosis, among others [1]. Because of the diverse array of potential adverse outcomes and the low frequency at which each type occurs, it is not possible to prospectively design monitoring or exclusion criteria for IDRs. Physicians should be apprised of the potential for IDRs in patients treated with afatinib

Safety pharmacology studies included *in vitro* and *in vivo* evaluation of the effects of afatinib on vital physiological functions. Assessments following single dose administration of afatinib in rats included those for telemetry/plethysomography, gastric emptying and secretion, GI transit, and renal function. Behavior was assessed by modified IRWIN test together with nocturnal motility observations in mice. There were no effects noted on general behavior or motility following administration of afatinib. At oral doses of 300 mg/kg afatinib prolonged gastric empting, and increased serum and liver enzymes in rats.

Afatinib inhibited hERG-mediated potassium current inHEK293 cells with an IC_{50} of 2.4 μM, suggesting low potential for QTc prolongation at clinically relevant concentrations. In telemetered rats, afatinib administration resulted in in increased arterial blood pressure and heart rate at 100 mg/kg, however, no effects were noted on respiration rate and tidal volume. In the 1-year minipig study, the effects of afatinib administration on blood pressure, heart rate, or ECG intervals and waveform rhythms were minimal. Additionally, no significant cardiac histopathological findings were noted in any of the toxicology studies conducted for afatinib. When domestic pigs were given an intravenous infusion of afatinib at doses of 10 and 30 mg/kg, however, decreased

[1] Knowles, Sandra R., Uetrecht, J., Shear, Neil H. 2000. Idiosyncratic drug reactions: the reactive metabolite syndromes. *The Lancet*. 356:1587-1591

LVdP/dt-max contractility was noted. Left ventricular dysfunction has been reported clinically in patients treated with afatinib.
Nonclinical toxicology evaluation of afatinib included dose-range studies in the mouse and rat, and repeat-dose toxicity studies in the Wistar Han rat and the Göettingen minipig. The gastrointestinal tract was identified as a major target organ in both rats and minipigs. Soft stool and diarrhea were common observations in both species, while single cases of occult fecal blood in high-dosed minipigs were also noted. Microscopically, atrophy of GI epithelium and focal ulcerations in the stomach of rats and minipigs were observed. Gastrointestinal toxicity was commonly reported clinically.

Studies in rats also identified the kidneys and skin as important target organs. Evidence of kidney toxicity included elevations in serum blood urea nitrogen (BUN) and in urinary markers of renal toxicity (b-NAG, creatinine and urinary protein). Evidence of renal papillary necrosis was also noted in premature descendents. Skin toxicity in the 4-week rat study manifested as scabs, thickening or swelling of muzzle and alopecia. Wavy, rough or dull fur and scaly tail skin were observed in long-term studies. Epidermal atrophy, inflammatory infiltrations, purulent/granulomatous folliculitis, purulent dermatitis, elevated WBCs- primarily elevated neutrophils, and increased axillary lymph node weight were also observed in rats. Serious cutaneous toxicity has been commonly reported in clinical trials with afatinib. Epithelial atrophy was noted in other organs as well, including the prostate, uterus, and vagina in rats, and the prostate, the seminal vesicles, cornea, upper respiratory tract, and respiratory mucous glands in the minipig. The findings of epithelial atrophy in the minipig upper respiratory tract along with findings at high dose levels in rats are suggestive of lung toxicity noted clinically. Findings of corneal atrophy are consistent with clinical reports of keratitis and conjunctivitis included in the label for afatinib. The majority of changes resolved or were resolving during the recovery period in each species, with the exceptions of kidney papillary necrosis and inflammatory skin lesions. The skin and renal toxicities were consistent with toxicities observed with other approved EGFR inhibitors.

The genotoxic potential of afatinib was tested in both *in vitro* and *in vivo* assays. While afatinib demonstrated mutagenic potential in a single strain (TA 98) in the *Salmonella-Escherichia coli* reverse mutation assay (Ames assay) by the plate incorporation method only both with and without mammalian microsomal activation, it was negative in all other tests. The weight of evidence therefore suggests that afatinib is not mutagenic at clinically relevant concentrations.

In a dedicated fertility study, male and female rats received afatinib daily by oral administration at doses of 4, 6, or 8 mg/kg. In males at doses of ≥6 mg/kg (approximately equal to exposure by AUC in patients at the recommended dose) afatinib increased the incidence of low or no sperm count; decreases in sperm count were supported by findings of atrophy in the testes, seminal vesicles, and prostate in general toxicology studies. In females, at the high dose of 8 mg/kg (approximately 0.6 times the exposure by AUC in patients at the recommended dose) there was a mild decrease in the number of corpora lutea and mild increases in pre-implantation loss and early resorptions were observed. In a 4-week general toxicology study, female rats had

decreased ovarian weights at all dose levels, which had not fully recovered by the end of a 2-week recovery period.

Han Wistar rats were used in embryo-fetal development and pre- and postnatal development studies of afatinib. In rats administration of afatinib at doses up to 8 mg/kg (approximately equal to the human exposure at the recommended dose) did not result in significant effects on embryofetal or postnatal development, though prolonged decreased fetal weight was observed. In an additional embryo-fetal development study at a high dose level of 16 mg/kg, increased total numbers of fetal abnormalities including both visceral and skeletal changes were observed. Embryofetal development studies were also conducted in rabbits. In rabbits at the 10 mg/kg dose level (approximately 0.74 times the exposure based on AUC in patients at the recommended dose) there was evidence of maternal toxicity and 3 females had complete abortions at late timepoints in the study. There was an additional late abortion in a single female at the 5 mg/kg dose level (approximately 0.24 times the human exposure by AUC at the recommended dose), a dose without clear increases in maternal toxicity. No differences were recorded in the mean values of corpora lutea, number of implantations, and the numbers of intrauterine deaths among control and treatment groups of the rabbit embryofetal study, though does with total litter loss were excluded from this analysis. Treatment of rabbits with afatinib doses of ≥5 mg/kg resulted in increased rates of late abortions, resorptions, lower fetal weights, and increased occurrence of visceral and skeletal variations, predominantly delays in ossification. Pregnancy Category D is recommended.

1.3 Recommendations

1.3.1 Approvability:

The pharmacology and toxicology data submitted to NDA 201292 is sufficient to support the approval of afatinib for the treatment patients with metastatic NSCLC whose tumors have epidermal growth factor receptor (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations as detected by an FDA-approved test.

1.3.2 Additional Non Clinical Recommendations: None.

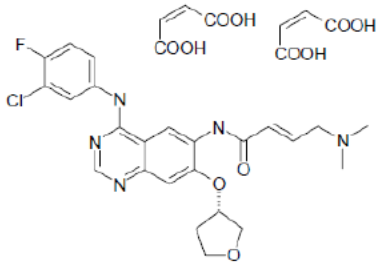
1.3.3 Labeling: A separate labeling review will be conducted.

2 Drug Information

2.1 Drug

CAS Registry Number:	850 140-73-7
Generic Name:	afatinib
Code Name:	BIBW 2992

Chemical Name	2-butenamide, *N*-[4-[3(-chloro-4-fluorophenyl)amino]-7-[[(3*S*)-tetrahydro-3-furanyl]oxy]-6-quinazolinyl]-4-(dimethylamino),(2*E*)-, (2*Z*)-2-butenedioate (1:2)
Molecular Formula/Molecular Weight:	718.1 g/mol (salt form) 485.9 g/mol (free base)
Structure:	



Pharmacologic class:	Kinase inhibitor
Relevant INDs/NDAs/DMFs	DMF [redacted] (b) (4) IND 67969

2.3 Drug Formulation

Afatinib (BIBW 2992) has two moieties, the salt form is known as afatinib dimaleate, while the active compound is a base, named "afatinib". Film-coated afatinib tablets are available in four dosage strengths: 20, 30, 40 [redacted] (b) (4) mg.

(excerpted from Applicant's submission)

Table 1: Drug Composition

Dosage strength		[20 mg]**	20 mg	30 mg	40 mg	[redacted] (b) (4)		
Part of tablet	Ingredient		[mg/coated tablet]				Function	Reference to Standards
Core	BIBW 2992 MA2 (BIBW 2992 free base)	29.5600 (20.0000)	29.5600 (20.0000)	44.3400 (30.0000)	59.1200 (40.0000)		Active	Company Standard
	Lactose monohydrate	[redacted] (b) (4)	[redacted] (b) (4)				[redacted] (b) (4)	NF
	Microcrystalline cellulose							NF
	Colloidal silicon dioxide							NF
	Crospovidone							NF
	Magnesium stearate							NF
Film-coat	Hypromellose [redacted] (b) (4)							USP
	Polyethylene glycol [redacted] (b) (4)							NF
	Titanium dioxide							USP
	Talc							USP
	FD&C Blue [redacted] (b) (4) (b) (4)							21 CFR 74.1102
	Polysorbate 80							NF
	[redacted] (b) (4)							USP
	Total mass	185.00	185.00	277.00	368.00			

BIBW 2992 MA2 contains a chiral center within the furanoyl moiety in the 3S configuration. The 2-buteneamide sub-structure allows for E/Z isomerism; BIBW 2992 MA2 is the E-isomer. It is white to brownish yellow powder by the physical appearance, with a melting point of 173 ±7° C, and logarithmic dissociation constants of pKa1 8.2 and 5 ±0.1. BIBW 2992 is hygroscopic and it takes in water above 70% relative humidity. BIBW 2992 MA2 is highly soluble in water and in aqueous buffer media up to pH 6 (>50 mg/ml). The solubility of the drug substance in these media significantly decreases at pH values of between 6 and 7; however the solubility still exceeds 1 mg/ml.

2.4 Comments on Novel Excipients

None.

2.5 Comments on Impurities/Degradants of Concern

An overview of by-products and degradation product organic impurities is presented in Table 2 (excerpted from Applicant's submission).

Table 2: By-products and Degradation Products

(b) (4)

[redacted]

Impurity	Classification	Origin of impurity
[redacted] (b) (4)		

To provide qualification of the specific drug substance and drug product impurities with proposed specification limits at or above the ICH limits, specifically [redacted] (b) (4) [redacted] (b) (4) the Applicant spiked the BIBW2992 MA2 drug substance batch (1040244SPK) used in a 13-week repeat, oral dose toxicity study in rats with these impurities. The Applicant specified, based on the outcome of the mentioned toxicology study, a safety margin of [redacted] (b) (4) for each impurity.

With regard to the specification limit of [redacted] (b) (4), a literature search was performed. In conclusion, a limit of no more than [redacted] (b) (4) in the drug product is considered toxicologically acceptable.

The Applicant evaluated the potential genotoxicity of all drug substance impurities by using DEREK (version 13) and MC4PC (version 2.3/2.4) systems. Impurities [redacted] (b) (4) [redacted] (b) (4) were identified by The Applicant as potentially genotoxic and subsequentially assessed in *in vitro* Ames tests. [redacted] (b) (4) was negative in the Ames test [redacted] (b) (4) was positive in one strain in the Ames test; however, a weight of evidence approach based on several additional genotoxicity tests conducted for this impurity suggests that it's mutagenic potential is similar to that of afatinib itself and that it is unlikely to be genotoxic at clinically relevant concentrations, thus, this impurity can be treated as a non-genotoxic impurity.

Specific impurities [redacted] (b) (4) were above ICH Q3B qualification levels. At the proposed specifications of NMT [redacted] (b) (4) respectively, patients would be exposed to these impurities at levels of [redacted] (b) (4) daily at the recommended dose. The Applicant conducted a 13-week study to qualify these impurities. Based on

the qualification study, in which animals received up to (b) (4) (b) (4) without significant increases in toxicity compared to afatinib alone. These impurities are therefore qualified from the safety perspective at the proposed specifications.

2.6 Proposed Clinical Population and Dosing Regimen

Patients with metastatic non-small cell lung carcinoma (NSCLC) whose tumors have EGFR exon 19 deletions or exon 21 (L858R) substitution mutations and who were not previously treated with EGFR TKI drugs are proposed for treatment with afatinib at the daily oral dose of 40 mg.

3 Studies Submitted

Pharmacology				
			Reviewed	Not reviewed
	16-02 U02-1351	Analysis of potency and selectivity of the epidermal growth factor receptor (EGFR) inhibitor BIBW 2992 BS in in vitro kinase assays	X	
	22-02 U02-1391	Effects of BIBW 2992 on endogenous EGFR and HER2 phosphorylation states and on Proliferation in various cell lines	X	
	17-02 U02-1534	Anti-tumor activity of the epidermal growth factor receptor (EGFR) inhibitor BIBW 2992 BS in nude mouse xenografts derived from the EGFR-overexpressing human epidermoid carcinoma cell line A431	X	
	25-02 U02-1614	Anti-tumor activity of the combined EGFR and HER2 inhibitor BIBW 2992 BS in nude mouse xenografts derived from the human gastric carcinoma cell line NCI-N87	X	
	26-02 U02-1660	Anti-tumor activity of the combined EGFR and HER2 inhibitor BIBW2992BS in nude mouse xenografts derived from the human ovarian carcinoma cell line SKOV-3	X	
	32-02 U02-1702	Minimum effective dose of the epidermal growth factor receptor (EGFR) inhibitor BIBW 2992 BS in nude mouse xenografts derived from the EGFR-overexpressing human epidermoid carcinoma cell line A431	X	
	31-02 U02-1703	Anti-tumor activity of the combined EGFR and HER2 inhibitor BIBW 2992 BS in nude mouse xenografts derived from the human breast carcinoma cell line MDA-MB-453	X	
	28-02 U013-1086	Inactivation of the EGF signaling pathway by BIBW 2992: A time course analysis	X	
	06-03 U03-1940	Anti-tumor activity of the EGFR/HER2 inhibitor BIBW 2992 BS in combination with the VEGFR inhibitor BIBF 1120 CL2 in nude mouse xenografts derived from the human ovarian carcinoma cell line SKOV-3	X	
	22-04 U04-2147	Evaluation of the combination of EGFR/HER2-tyrosine kinase inhibition by BIBW 2992 or BIBW 2669 with radiation therapy on the human squamous cell-carcinoma cell line FaDu	X	
	02-04 U05-1301-01	Anti-tumor activity of the EGFR/HER2 inhibitor BIBW 2992 BS in nude mouse xenografts derived from the human breast carcinoma cell line MCF-7		X

	114.360.449, 03-05 U05-1342	Treatment of SKOV-3 xenografts subcutaneously transplanted in female NMRI nude mice with compound BIBW 2992		X
	114.360.601 U05-2392	Treatment of SKOV-3 Xenografts Subcutaneously transplanted in female NMRI nude mice with compound BIA 92 and BIA 20 in Concomitant and weekly alternating schedules: Study 2		X
	U05-0266	Investigational Medicinal Product Dossier Supplement: Summary of combination studies with BIBW 2992 and BIBF 1120 - Non-clinical Pharmacology and Toxicology Data		X
	15-05 U05-2532	Anti-tumor activity of the EGFR/HER2 inhibitors BIBW 2992 and BI 7325 in comparison to Herceptin on xenografts derived from the human gastric cancer cell line NCI-N87 in nude mice	X	
	19-05 U05-2665	Anti-tumor activity of BIBW 2992 in combination with BIBF 1120 on xenografts derived from the human head and neck cancer cell line FaDu in nude mice		X
	114.360.515 U06-1036	Treatment of skow-3 human ovarian carcinoma xenografts subcutaneously transplanted into female nmri nude mice with compounds BIA92 and BIA20 in comcominant and weekly alternating schedules		X
	07-06 U07-1338-01	BIBW 2992, an irreversible dual EGFR/HER2 kinase inhibitor, shows activity on L858R - and L858R/T790M-EGFR mutants Report	X	
	Bircv15-09 U09-1454-01	Anti-tumor activity of the EGFR/HER2 inhibitor BIBW 2992 in a mouse model of human triple-negative breast cancer (cell line SUM-149)		X
	Bircv16-09 U09-1455-01	Anti-tumor activity of the EGFR/HER2 inhibitor BIBW 2992 in a mouse model of human HER2-positive breast cancer (cell line SUM-190)		X
	Bircv38-09 U10-1264-01	X-ray structure analysis of EGFR in complex with BIBW 2992	X	
	Bircv23-04 U10-1265-01	Potency of BIBW 2992 in various protein kinase assays	X	
	Bircv18-11 U11-2645-01	Potency of BIBW 2992 in HER4 kinase assays in vitro		X
	Secondary Pharmacodynamics			
	03-02 U02-1083	Study of BIBW 2992 BS, BIBW 3022 BS, and BIBW 3049 BS in various receptor binding assays	X	
	GP2002/047/PH5 U02-1467	General Pharmacology: BIBW 2992 BS Effects of BIBW 2992 BS on vital physiological functions in conscious rats using a telemetry/plethysmography system	X	
	GP2001/264/PH4 U02-1487	Effects of BIBW 2992 BS (30, 100, 300 mg/kg BW p.o.) on gastric emptying in rats	X	
	GP2001/262/PH4 U02-1488	Effects of BIBW 2992 BS (30, 100, 300 mg/kg BW p.o.) on gastrointestinal transit in rats	X	
	GP2001/263/PH4 U02-1489	Effects of BIBW 2992 BS (30, 100, 300 mg/kg BW p.o.) on gastric secretion in rats		X
	GP2001/265/PH4 U02-1490	Effect of BIBW 2992 BS (30, 100, 300 mg/kg p.o.) on renal function in conscious rats	X	

	GP2001/298/PH1 U02-1619	General Pharmacology: BIBW 2992 BS. Effects of BIBS 2992 BS on behaviour assessed by observation in a modified IRWIN-test and on nocturnal motility in mice after oral administration of 30, 100 and 300 mg/kg	X	
	GP2002/246/PH2 U03-1311	Effect of BIBW 2992 MA2 (0.3 - 30 mg/kg IV) on hemodynamic and electrocardiographic parameters in anesthetized domestic pigs	X	
	Safety pharmacology			
	GP2001/070/470/PH2 U02-1580	Influence of BIBW 2992 BS on HERG-mediated potassium current in HEK293 cells and on action potential configuration in isolated guinea pig papillary muscle	X	
	BOI270/032645 U03-1858	BIBW 2992 MA2: Modified IRWIN study in male and female rats including body temperature and locomotor assessment (single oral administration)	X	
	02B193-dup1 U03-1774	BIBW 2992 MA2: 4-week oral (gavage) toxicity study in the Goettingen minipig with a 2-week recovery period	X	
	BOI271/032845 U03-1859	BIBW 2992 MA2: Evaluation of respiratory parameters in the conscious male and female rat using whole body bias flow plethysmography (single dose administration)	X	
	Pharmacokinetics			
	0183-02rm	BIBW 2992 MA2: A method for the quantification of BIBW 2992 BS in minipig plasma using solid phase-extraction and HPLC-MS/MS: validation and stability data	X	
	M182_02RM	BIBW 2992 MA2: A Method for the Quantification of BIBW 2992 BS in Rat Plasma Using Solid Phase Extraction and HPLC-MS/MS: Validation and Stability Data	X	
		Synthesis of [quinazolinyl-2-14C] BIBW2992 MA2	X	
	B205-03RM	BIBW 2992 MA2: Partial Validation of a Modified Method for the Quantification of BIBW 2992 BS in Minipig Plasma with a Lower Limit of Quantification of 1 nmol/L	X	
	R259-04RM	BIBW 2992 MA2: A Modified Method for the Quantification of BIBW 2992 BS in Rat Plasma with a Lower Limit of Quantification of 1.00 nmol/L	X	
	M262-05RM	BIBW 2992 MA2: A Modified Method for the Quantification of BIBW 2992 BS in Minipig Plasma with a Lower Limit of Quantification of 0.200 nmol/L	X	
	V293-05RM	BIBW 2992 MA2: Revalidation of the HPLC-MS/MS Method for the Quantification of BIBW 2992 BS in Rat Plasma	X	
	B2820	BIBW 2992 MA2: Stability of BIBW 2992 BS in rat EDTA Plasma and Whole Blood	X	
	M374-08RM	BIBW 2992 MA2: A Method for the Quantification of BIBW 2992 BS in Rabbit Plasma using Solid Phase Extraction and HPLC-MS/MS: Validation and Stability Data	X	
	r456-10rm	Revalidation of the HPLC-MS/MS Method for the Quantification of BIBW 2992 in Rat Plasma	X	
	v435-10rm)	A Method for the Quantification of BIBW 2992 in Mouse Plasma (Concentration Range 1.00 - 1000 nmol/L) Using Solid Phase Extraction and HPLC-MS/MS: Validation and Stability Data	X	
	ADME			
	A102-02RB	Absorption, Distribution and Excretion of [14C]BIBW 2992 MA2 in the Rat	X	
	A239-05TE A129-	Absorption, Distribution and Metabolism of BIBW 2992 MA2 in Minipigs	X	

	05RB			
	A142-05RB	Pharmacokinetics of BIBW 2992 MA2 After Oral or Intravenous Administration in the Minipig	X	
		Excretion, Distribution in Blood and Pharmacokinetics of Radioactivity and Parent Compound After Oral or Intraduodenal Administration of [^{14}C]BIBW 2992 MA2 to Female Rabbits	X	
	a227-10rb	Absorption, Distribution and Excretion of [^{14}C]BIBW2992BS After Oral Administration to Mice	X	
	A058_03UB	BIBW 2992 MA2: Whole Body Autoradiography After Intravenous Administration of [^{14}C]BIBW2992 MA2 in Male Albino and Male Pigmented Rats and Oral Administration in Male Albino Rats	X	
	A224-02TE	Metabolism of BIBW 2992 MA2 in Rats and Covalent Binding of BIBW 2992 MA2 to Blood Constituents	X	
		[14C]BIBW 2992 MA2: Species Comparison of in Vitro Plasma Protein Binding and in Vitro Distribution into Blood Cells	X	
	A240-11RB	Transfer of BIBW 2992 to Milk After Oral (gavage) Administration of [^{14}C]BIBW 2992 to Lactating Rats	X	
	A277-08TE	Metabolism of BIBW 2992 MA2 in Female Rabbits	X	
	A035-08JS	Excretion, Distribution in Blood and Pharmacokinetics of Radioactivity and Parent Compound After Oral or Intraduodenal Administration of [^{14}C]BIBW 2992 MA2 to Female Rabbits	X	
		Metabolism of BIBW 2992 in Mice	X	
	A198-09RB	Tissue Distribution and Excretion After Multiple Oral Dosing of [^{14}C]BIBW 2992 MA2 in the Male Rat	X	
		The Effect of BIBW2992 MA2 and Known Model CYP Inducers on Hepatic Levels of Cytochrome P450 and Related Parameters in Male Wistar Rats After Administration for 4 Days	X	
	A240-11RB	Transfer of BIBW 2992 to Milk After Oral (gavage) Administration of [^{14}C]BIBW 2992 to Lactating Rats	X	
	Toxicology			
	Single dose			
	02B182 U03-1089	BIBW 2992 MA2: single dose toxicity study in mice by oral (gavage) administration	X	
	02B181 U03-1088	BIBW 2992 MA2: single dose toxicity study in mice by oral (gavage) administration	X	
	01B087 U04-1027	BIBW 2992 MA2: 26-week oral (gavage) toxicity study in rats with an 8-week recovery period	X	
	Repeat-dose toxicity-rats			
	01B087 U04-1027	BIBW 2992BS: Exploratory 2-week oral toxicity study in male rats-non-GLP	X	
	01B087 U04-1027	BIBW 2992 MA2: 2-week oral (gavage) dose range-finding toxicity study in Wistar rats	X	
	02B194 U03-1775	BIBW 2992 MA2: 4-week oral (gavage) toxicity study in rats with a 2-week recovery period	X	
	03B087 U04-1766	BIBW 2992 MA2: 13-week oral (gavage) toxicity study in Wistar rats followed by a 6-week recovery period	X	
	01B087 U04-1027	BIBW 2992 MA2: 26-week oral (gavage) toxicity study in rats with an 8-week recovery period	X	
	Repeat-dose toxicity-minipig			

	02B127 U04-1174	BIBW 2992 MA2: Escalating dose oral (gavage) toxicity study in the Gottingen minipig. Non-GLP	X	
	02B143 U04-1175	BIBW 2992 MA2: Fixed oral (gavage) pilot study in the Gottingen minipig. Non-GLP	X	
	02B144 U03-1780-02	BIBW 2992 MA2: 2-week oral (gavage) toxicity study in Gottingen minipig with a 2-week recovery period	X	
	02B193 U03-1774	BIBW 2992 MA2: 4-week oral (gavage) toxicity study in Gottingen minipig with a 2-week recovery period	X	
	02B090 U04-1795	BIBW 2992 MA2: 13-week oral (gavage) toxicity study in minipigs followed by a 6-week recovery period	X	
	05B018 U06-2251	BIBW 2992 MA2: 52-week oral (gavage) toxicity study in Gottingen minipigs	X	
	Genotoxicity			
	In vitro			
	03B095	Mutagenicity study using the S. typhimurium/mammalian-microsome assay (Ames test)	X	
	03B096	Mutagenicity study for chromosomal aberrations in human lymphocytes in vitro	X	
	In vivo			
	03B026	Oral (gavage) mutagenicity study using micronucleus analysis in rat bone marrow (Part of the 4-week oral toxicity study)	X	
	03B198	BIBW 2992 MA2: In vivo Comet assay in liver, kidney and jejunum of rats after oral dosing (gavage)	X	
	8240164	BIBW 2992 MA2:Induction of lacZ-mutations in tissues of orally treated Muta Mice	X	
	Carcinogenicity			
	ddb0083	BIBW 2992 MA2: Toxicity study by oral gavage administration to CD-1 mice for 4 weeks		X
	11-2264	BIBW 2992 MA2: An 8-week oral gavage toxicity study in TgrasH2 wild-type mouse		X
	ddb0084	BIBW 2992 MA2: Preliminary toxicity study by oral gavage administration to CD-1 mice for 13 weeks		X
	Reproductive and developmental toxicity			
	ddb0102	BIBW 2992 MA2: Fertility and early embryonic development toxicity study in the Han Wistar rat by oral (gavage) administration	X	
	boi0360	BIBW 2992 MA2: Preliminary study for effects on embryo-fetal development in Han Wistar rat by oral (gavage) administration	X	
	08B025	BIBW 2992 MA2: Dose-range finiding study for effects on embryo-fetal development in rabbits by oral (gavage) administration	X	
	08B026	BIBW 2992 MA2: Study for effects on embryo-fetal development in rabbits by oral (gavage) administration	X	
	ddb0103	Prenatal and postnatal development study in the Han Wistar rat by oral (gavage) administration	X	
	Local tolerance			
	02b183	BIBW 2992 MA2: Local dermal tolerance after single administration to rabbits	X	
	07B008	BIBW 2992 MA2: Acute eye irritation/corrosion study in rabbits	X	
	Other toxicity studies			
	impurities			
	02B191	Mutagenicity study with [redacted] (b) (4) using the S typhimuriom/mammalian microsome assay (Ames II)		X
	02B139	Mutagenicity study with [redacted] (b) (4) using the S		X

		typhimuriom/mammalian microsome assay (Ames II)		
	08B036	(b) (4) (impurity of BIBW 2992): Mutagenicity study using the S. thyphimarium/mammalian microsome assay test (Ames test)		X
	08B072	(b) (4) (impurity of BIBW 2992): Mutagenicity S. thyphimarium/mammalian microsome assay test (Ames test)		X
	08B146	(b) (4) (impurity of BIBW 2992): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test		X
	09B075	(b) (4) (chemical intermediate and possible impurity of BIBW 2992): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test		X
	09B075	(b) (4) impurity of BIBW 2992): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test		X
	09B053	(b) (4) (impurity of BIBW 2992): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test		X
	09B076	(b) (4) (chemical intermediate and possible impurity): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test		X
	09B074	(b) (4): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)		X
	09B130	(b) (4) (impurity of BIBW 2992): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)	X	
	ddB0048	(b) (4) (impurity of BIBW 2992): Bacterial reverse mutation test		X
	ddB0047	(b) (4) (impurity of BIBW 2992): Bacterial reverse mutation assay		X
	09B186	(b) (4) (impurity of BIBW 2992): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)		X
	09B131	(b) (4) (impurity of BIBW 2992): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)	X	
	09B096	BIBW 2992 MA2 and impurities 13 week oral (gavage) toxicity study in rats	X	
	10B032	(b) (4): Exploratory 3 day oral (gavage) toxicity study in male rats	X	
	09B188	(b) (4) (impurity) Mutagenicity study using the chromosomal aberration test in human lymphocytes in vitro		X
	10B067	(b) (4) (possible impurity): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)		X
	10B133	(b) (4) (impurity) rat bone marrow micronucleus test and detection of DNA damage in rats using the Comet assay	X	
	95178	In vitro mammalian chromosome aberration test in human lymphocytes with (b) (4)	X	
	112658	Reverse mutation assay using becteria (Salmonela thypimurium) with (b) (4)		X
	112657	Reverse mutation assay using becteria (Salmonela thypimurium) with (b) (4)		X
	12B141	(b) (4) (possible impurity): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)		X
	12B142	(b) (4) (possible impurity): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)		X
	12B143	(b) (4) (possible impurity): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)		X
	12B162	(b) (4) (possible impurity): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)		X
	12B069	(b) (4) (possible impurity): Mutagenicity study using the S. thyphimurium/mammalian microsome assay test (Ames test)		X
	Other			
	111201	In vitro 3T3 phototoxicity test with (b) (4)	X	
	05B190	(b) (4): 2 week combination dose range finding study		X

		with concomitant oral (gavage) administration in rats		
	05B252	(b) (4): 4 week combination dose range finding study with concomitant oral (gavage) administration in rats		X
	05B021	(b) (4): combination dose range finding study with alternating oral (gavage, 4-cycle) dose regimen in rats		X
	05B188	(b) (4): Combination toxicity study with alternating oral (gavage, 4-cycle) dose regimen in rats		X
	02B222	(b) (4) 4-week oral (gavage) toxicity study in the Gottingen minipig		X

3.3 Previous Reviews Referenced

Several previous reviews were used for this review. Specifically, reviews of studies previously submitted for afatinib under the IND conducted by Drs. Wei Chen, Sachia Khasar and Andrew McDougal were referenced throughout this review.

4 Pharmacology

4.1 Primary Pharmacology

U02-1351 Analysis of potency and selectivity of the epidermal growth factor receptor (Egfr) inhibitor BIBW2992BS in *in vitro* kinase assays (Study report No 16-02; BIBW2992BS, CI-1033 (Exbu1026) and ZD-1839 (Exbx0080))

The inhibitory potential of BIBW2992BS on the EGFR and HER2 tyrosine kinases was assessed in *in vitro* kinase assays. SF9 cells were transduced with constructs coding for the catalytic domains of EGFR and HER2 or four other tyrosine kinase domains representing specificity controls; kinase domains were recovered from the cells following expression. In this assay BIBW2992BS inhibited EGFR kinase with an IC_{50} of 0.5 nM andHER2 with an IC_{50} of 14 nM. BIBW2992BS did not inhibit the tyrosine kinase activities of HGFR, SRK, KDR or BIRK (IC_{50}s > 13000 nM).

U02-1391 Effects of BIBW2992 on endogenous EGFR and HER2 phosphorylation states and on proliferation in various cell lines (Report study No: 22-02; BIB2992, CI-1033 and ZD-1839))

The inhibitory activity of BIBW 2992 on the phosphorylation status of the EGF receptor was investigated after EGF stimulation of serum-starved A431 cells in the presence or absence of increasing concentrations of the study compound. The phosphorylation status of the either EGFR after incubation with EGF or constitutively active HER2 was measured by sandwich ELISA. Streptavidin coated plates were incubation with biotin-conjugated antibodies against either EGFR or HER2. After the addition of lysates from cell culture samples, receptor phosphorylation status was determined using an anti-phospotyrosine antibody as the detection antibody.The IC_{50} for BIBW2992 on EGFR phosphorylation in this assay was 13 nM, while competitor molecules with reported inhibitory activity against EGFR signaling, ZD-1839 (gefitinib) and CI-1033 (canertenib) were 35 nM and 22 nM, respectively.

A BIBW2992-mediated dephosphorylation of constitutively active HER2 was detected in NIH-3T3-HER2 cells with an IC_{50} of 71 nM, while ZD-1839 and CI-1033 cell lines

obtained 2330 nM and 85 nM values, respectively. Additional IC_{50} values obtained in the study were presented in the table below:

Table 3: Afatinib-Mediated Changes in EGFR/HER2 Phosphorylation

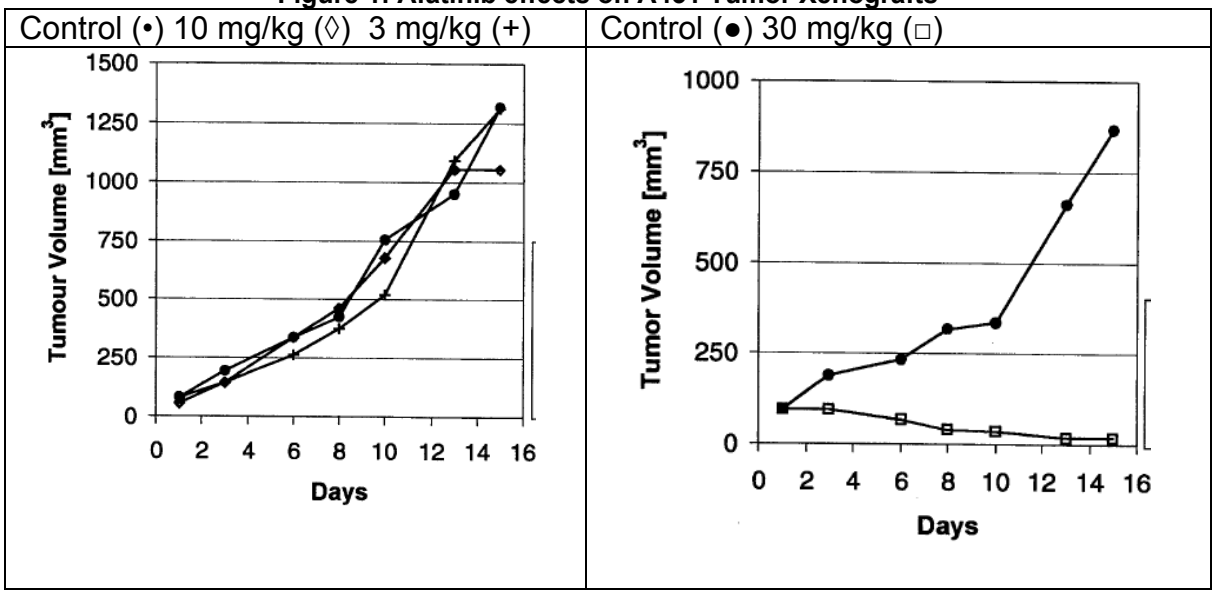
IC_{50} (nM)	EGFR Phosphorylation	HER2 dephosphorylation		HER2 anchorage-dependent proliferation	
	A431 cells	3T3 cells	N87 cells	BT-474	N87
BIBW 2992	13	71	48	12	4
ZD-1839	35	2330	541	1070	690
CI-1033	22	85	288	66	23

U02-1534 Anti-tumor activity of the epidermal growth factor receptor (EGFR) inhibitor BIBW 2992 BS in nude mouse human xenografts derived from the EGFR-overexpressed human epidermoid carcinoma cell line A431

Nude mice were subcutaneously injected with approximately $1x10^6$ A431 cells. After establishment of tumors (~100 mm^3 in volume), absolute volumes of individual A431 subcutaneous tumors (each of ~100 mm3 in volume) were measured on five to ten mice treated orally with BIBW 2992 at dose levels of 3, 10, and 30 mg/kg/day. Results were also obtained for additional mice given ZD-1839 at doses levels of 30, 100 or 300 mg/kg/day and vehicle controls.

Treatment of mice with BIBW2992BS at dose levels of 3 and 10 mg/kg/day resulted in tumor growth similar to that seen in the control-treated animals, however, inhibition of tumor growth was observed at the BIBW2992BS dose of 30 mg/kg/day of BIBW 2992 (Figure 1).

Figure 1: Afatinib effects on A431 Tumor Xenografts

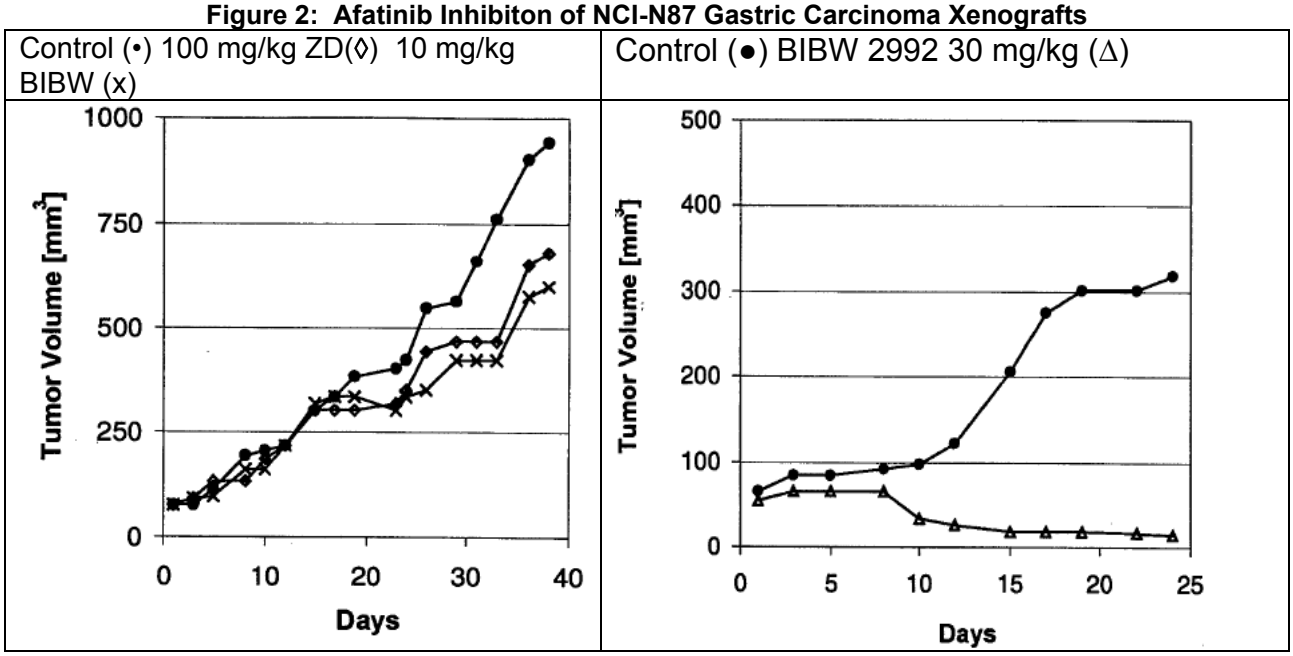


(excerpted from the Applicant's submission)

U02-1614 Anti-tumor activity of combined EGFR and HER2 inhibitor BIBW 2992 BS in nude mouse xenografts derived from the human gastric carcinoma cell line NCI-N87

NCI-N87 human gastric carcinoma cells were implanted subcutaneously in nude mice. The tumor growth was measured in mice treated orally with BIBW 2992 BS at doses of 10 or 20 mg/kg/day and compared with same number of mice treated with ZD-1839 at 100 mg/kg/day (Figure 2).

Figure 2: Afatinib Inhibiton of NCI-N87 Gastric Carcinoma Xenografts



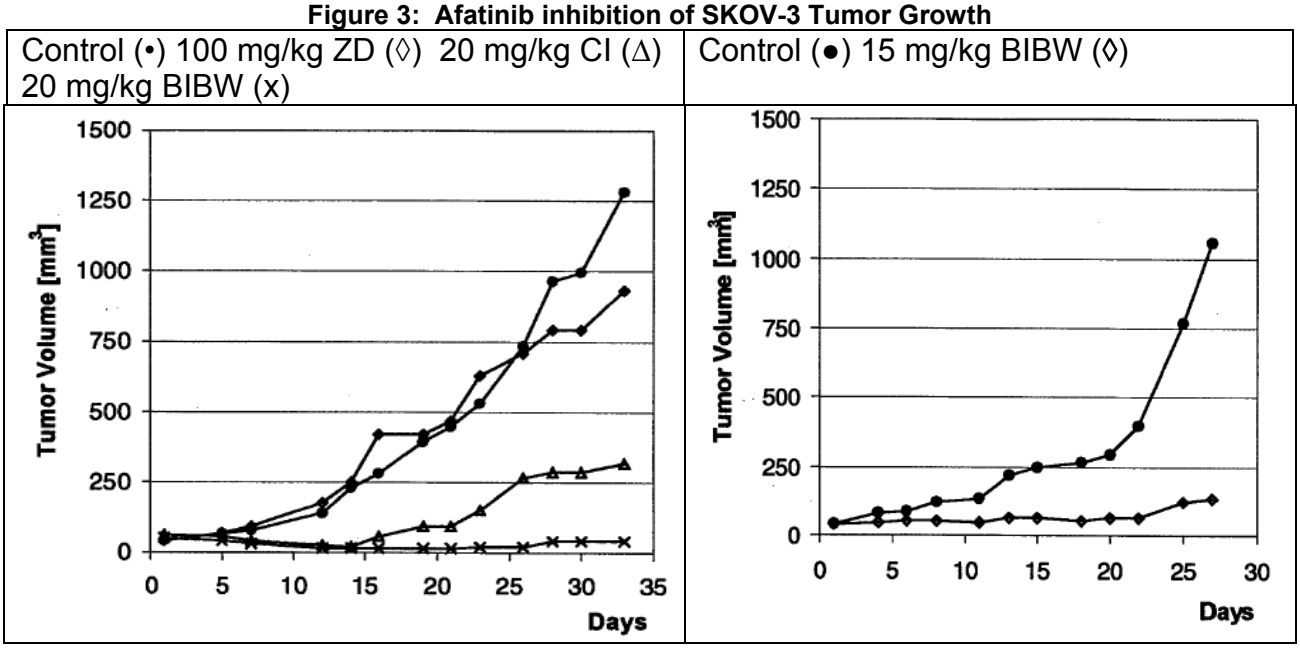
(excerpted from the Applicant's submission)

At 30 mg/kg of BIBW 2992 was able to significantly inhibit xenograft tumor growth. At the lower dose of 10 mg/kg BIBW 2992 had minor effects on tumor growth comparable to ZD-1839 at a dose of 100 mg/kg.

U02-1660 Anti-tumor activity of the combined EGFR and HER2 inhibitor BIBW 2992 BS in nude mouse xenografts derived from the human ovarian carcinoma cell line SKOV-3

To assess whether BIBW 2992 can inhibit growth of the SKOV-3 human ovarian tumor line, ten mice carrying established SKOV-3 tumors were treated orally with BIBW2992 or an alternative test substance at dose levels of 15 or 20 mg/kg/day. Starting tumor volume was ~50-100 mm^3, and tumor volume was measured 3 times weekly following initiation of test-article administration.

Figure 3: Afatinib inhibition of SKOV-3 Tumor Growth



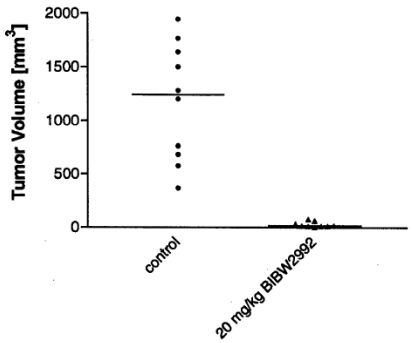
(excerpted from the Applicant's submission)

At doses of 15 and 20 mg/kg/day, BIBW 2992 suppressed the growth of the human ovarian cell line tumor, similarly to CI-1033.

U02-1702 Minimum effective dose of the epidermal growth factor receptor (EGFR) inhibitor BIBW 2992 BS in nude mouse xenografts derived from the EGFR-overexpressing human epidermoid carcinoma cell line A431

Mice implanted with subcutaneously established A431[2] tumors were treated for 25 days with 20 mg/kg/day BIBW 2992 or a vehicle control. Blood samples were taken for measurement of afatinib plasma levels before and after the last treatment.

Figure 4: Afatinib inhibition of A431 Tumor Growth



(excerpted from the Applicant's submission)

[2] The A431 cell line was derived from vulval squamous cell carcinoma tissues

A431 tumor-bearing mice treated with BIBW2992 at a dose of 20 mg/kg/day had smaller total tumor volumes, indicating that afatinib was able to inhibit A431-derived tumor cell growth *in vivo*.

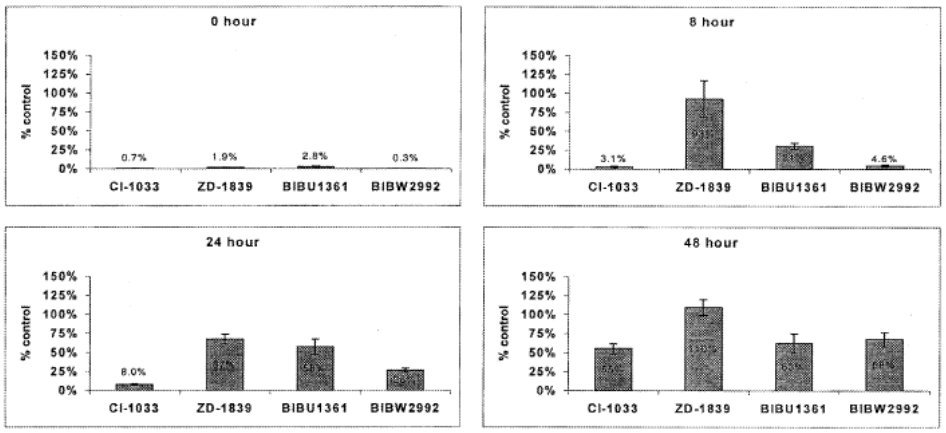
31-02 Activity of BIBW 2992 BS in nude mouse xenografts derived from the human breast carcinoma cell line MDA-MB-453

This study was done similarly to those described above using CI-1033 and ZD-1839 as comparators. All three compounds were able to suppress tumor growth; however, BIBW 2992 showed the same potency at doses less than or equal to those of CI-1033 and ZD-1839.

U03-1086 Inactivation of the EGF signaling pathway by BIBW 2992: A time course analysis

The purpose of this study was to assess the duration of the inhibitory activity of BIBW 2992 in cellular assays by monitoring the ability of EGF to re-stimulate tyrosine phosphorylation of the EGF receptor. A431 cells were incubated with BIBW2992 or comparator EGFR inhibitors for 1 hour at concentrations of 80 or 400 nM followed by washout. Additional washouts occurred at 2,4, 8 and 24 hours. At the selected timepoints of 0, 8, 24, and 48 hours post-washout, EGF was added to cultures for 15 minutes and EGF induced phosphorylation of EGFR was measured. The results, obtained by ELISA, show that inhibition of EGFR phosphorylation occurred following incubation with all EGFR inhbitors immediately following washout. By 8 hours post-washout, the reversible inhibitors were no longer able to prevent EGFR phosphorylation; however BIBW2992 and CI-1033, another reported irreversible binder of EGFR, were both retained the ability to inhibit phosphorylation. By 24 hours cells can begin to display new receptors. At this timepoint, BIBW2992-mediated phosphorylation began to recover and by 48 hours, incubation with EGF resulted in phosphorylation at least 50% that seen with control cells for all cells, regardless of the inhibitor (Figure 5). This study suggests that afatinib exposure can result in prolonged inhibition of EGFR phosphorylation.

Figure 5: Time-dependent Inhibition of EGFR Phosphorylation

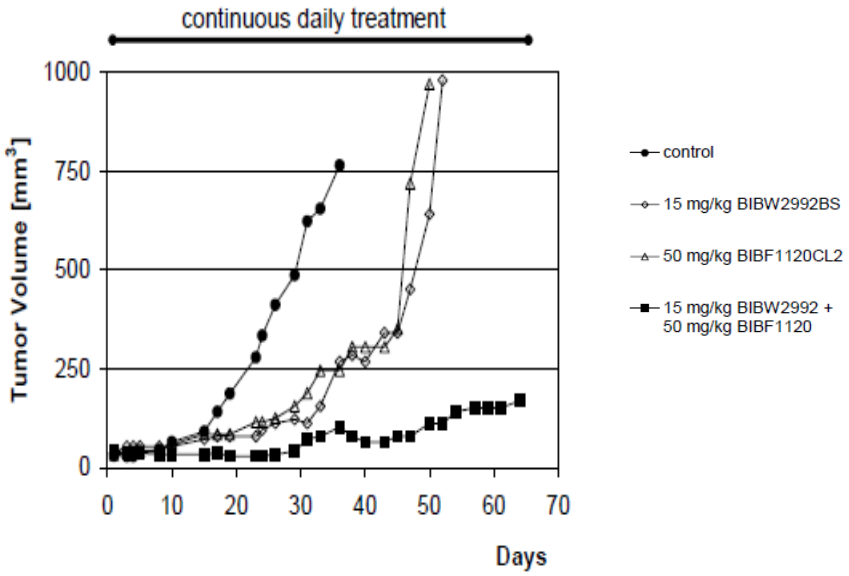


(excerpted from the Applicant's submission)

U03-1940 Anti-tumor activity of the EGFR/HER2 inhibitor BIBW 2992 BS in combination with the VEGFR inhibitor BIBW 1120 Cl2 in nude mouse xenografts derived from the human ovarian carcinoma cell line SKOV-3

In experiments conducted using nude mice with xenografts derived from the human ovarian cancer cell line SKOV-3, BIBW2992 alone was able to inhibit tumor growth. BIBW2992 in combination with another inhibitor, BIBW 1120 CL2, a reported inhibitor of VEGFR signaling, had an enhanced effect on delay of tumor growth compared to the effects of either inhibitor alone (Figure 6).

Figure 6: Afatinib +VEGFR inhibition of SKOV-3 Xenografts

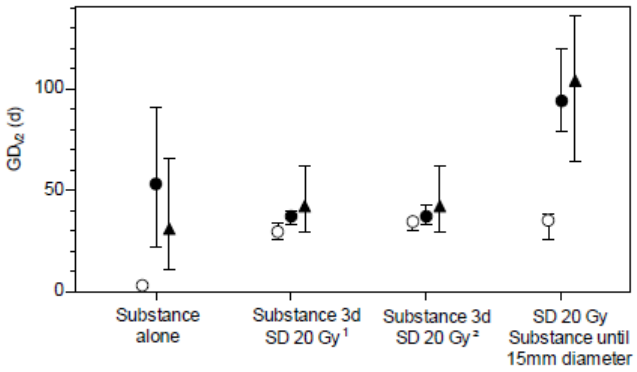


(excerpted from the Applicant's submission)

U04-2147 Evaluation of the combination of EGFR/HER2-tyrosine kinase-inhibition by BIBW 2992 or BIBW 2669 with radiation therapy on the human squamous cell-carcinoma cell line FaDu

The Applicant conducted a study to investigate the effects of EGFR inhibition on radiosensitization of cell lines. Nude mice were implanted with a squamous cell-carcinoma cell line, FaDu, known to express EGFR. After the establishment of tumors mice were treated with either BIBW 2992 or BIBW2669, EGFR/HER2-tyrosine kinase inhibitors for 3 days followed by a single dose of radiation, or with radiation alone. An additional cohort was treated only with test article in the absence of radiation as a control In this assay treatement with EGFR inhibitors showed no clear radiosenstizing effect; however, treatment with an EGFR inhbitor following single dose irradiation did delay tumor growth compared to radiation alone (Figure 7).

Figure 7: EGFR inhibition has no effect on radiosensitization



BIA 2669 (●), BIA 2992 (▲), or carrier (o).
y axis: day at which tumor volume double from start of treatment
(excerpted from the Applicant's submission)

07-06 BIBW 2992, an irreversible dual EGFR/HER2 kinase inhibitor, shows activity on L858R and L858R/T790M-EGFR mutants

The ability of BIBW2992 to inhibit EGFR, the EGFR L858R activating mutant, and the erlotinib-resistant EGFR L858R/T790M double mutant was tested in a series of biochemical kinase and cellular assays. In two independent experiments BIBW 2992 interfered with the tyrosine kinase activity of all EGF receptors tested, including wild-type EGFR, the activated mutant L858R and the erlotinib-resistant L858R/T790M double mutant with the IC_{50} values of ~1, ~0.43 and 10 nm (Table 4).

Table 4: Inhibition of EGFR WT, L858R, and L858R/T790M Kinase Domains

IC 50 nM	BIBW 2992	Gefitinib	Erlotinib	Lapatinib
WT	0.99	1.7	No data	No data
L 358 R EGFR	0.43	0.8	1.2	2
L 858 R/T790M	10	1013	1520	>4000

NSCLC cell lines carrying different EGFR mutated isoforms[3] were tested to assess the inhibitory effect of BIBW 2992. BIBW 2992 inhibited EGF-induced EGFR phosphorylation in all NSCLC cells tested independent of their mutational status. The IC_{50} vlaues were 7 nM (H1666; wild type EGFR), 6 nM (H3255; L858R mutant) and 93 nM (NCI-H1975; L858R/T790M double mutant) (Table 5). A duplicate experiment resulted in IC_{50}s of 12, 5 and 61 nM, for the listed murtations, respectively.

[3] H1666 is a wildtype EGFR; H3255 is L858R EGFR mutant and NCI-H1975 is L858R/T790M EGFR double mutant

Table 5: Aftatinib Inhibition of EGF Induced Autophosphorylation of EGFR in WT and EGFR mutant cells

Cell line	IC_{50}[nM]
H1666	6.9
H1666	12
H3255	5.9
H3255	5.7
NCI-H1975	93
NCI-H1975	61

(excerpted from the Applicant's submission)

BIBW 2992 was able to inhibit proliferation of NCI-H1975 cells carring the L858R/T790M double mutant with similar activity that the drug inhibited the proliferation of EGFR wild type cells (EC_{50} values of 99-116 nM vs. 37-60 nM, Table 6).

Table 6: Aftatinib Inhibition Proliferation of EGFR in WT and EGFR mutant cells

Cell line[a]	EC_{50}[nM]
H1666	60
H1666	37
H3255	0.7
H3255	0.08
NCI-H1975	99
NCI-H1975	116

(excerpted from the Applicant's submission)

While concentrations of afatinib resulting inhibition of EGFR autophosphorylation in cells were approximately 10 times higher in L858R/T790M double mutants vs. wild type under the conditions studied, inhibition of anchorage-independent proliferation occurred at similar concentrations and direct inhibition of L858R/T790M kinase activity occurred at much lower concentrations of afatinib than other EGFR inhibitors used for comparison, suggesting that further exploration of afatinib in the T790M patient population could be explored.

U11-2645-01 bircv18-11 Potency of BIBW 2992 in HER4 kinase assays *in vitro*

An assessment of the inhibitory activity of afatinib was performed at 50 μM ATP concentrations (Km=1.86 μM; saturating levels) on the catalytic domain of HER4 that was subcloned into a baculovirus expression system and affinity purified. Based on the

results of four independent experiments, afatinib was potent inhibitor of the kinase activity of HER4 with IC_{50} values ranging from 0.6 to 1.7 nM, and a mean IC_{50} of 1.1 nM.

U10-1264-01 X-ray structure analysis of EGFR in complex with BIBW 2992

The Applicant employed standardized techniques to determine the crystal structure of BIBW 2992 in complex with the EGFR receptor. In Figure 8 and Figure 9, ,EGFR is shown as a ribbon, while BIBW 2992 is shown as a stick model.

(excerpted from Applicant's submission)

Figure 8: Structure of EGFR and BIBW 2992

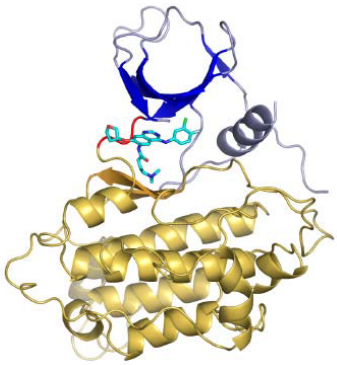
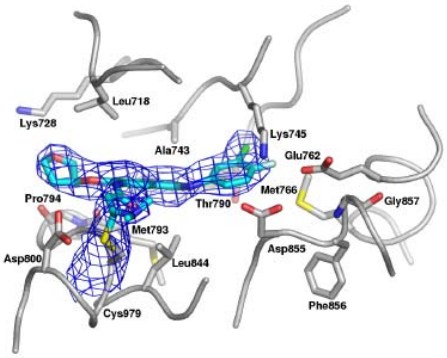
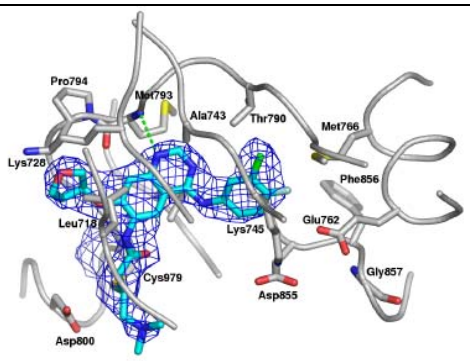


Figure 9: EGFR binding pocket with respect to BIBW 2992 positions in starting orientations and 90° rotation

Binding pocket of EGFR containing the ligand BIBW 2992 in starting orientation	90° rotation
	

BIBW 2992 was shown to covalently bind EGFR by this crystal structure analysis. Specifically, in addition to noncovalent bonds, the electron density indicates a covalent bond between Cysteine 797 at the edge of the active site and the Michael acceptor group of the inhibitor.

U10-1265-01 Potency of BIBW 2992 in various protein kinase assays

Two independent screens were conducted to assess the potency of BIBW 2992 against non-EGFR targets. The activity of protein kinases in the presence of 10 µM BIBW 2992 MA2 was expressed as percentage of activity in the presence of a DMSO control (Table 7).

Table 7: The activity of different protein kinases treated with BIBW 2992 from two different screens.

Kinase	April 2001 screen		June 2004 screen	
	% activity remaining	S.D.	% activity remaining	S.D.
MKK1	**93**	1	**78**	0
MAPK2/ERK2	**92**	5	**83**	3
JNK1/SAPK1c	**97**	8	**76**	7
SAPK2a/p38α	**17**	1	**12**	1
SAPK2b/p38β2	**77**	4	**35**	0
SAPK3/p38γ	**97**	6	**84**	17
SAPK4/p38δ	**100**	0	**93**	5
RSK1	-	-	**85**	4
RSK2	**75**	1	-	-
MAPKAP-K2	**80**	6	**96**	4
MSK1	**99**	2	**83**	5
PRAK	**84**	3	**82**	3
PKA	**68**	6	**96**	1
PKCα	**88**	10	**79**	14
PDK1	**114**	1	**91**	3
PKBα	**52**	6	**88**	6
SGK	**54**	3	**89**	9
S6K1	**100**	4	**90**	9
GSK3β	**101**	6	**83**	7
ROCK-II	**96**	5	**86**	13
AMPK	**84**	6	**65**	8
CHK1	**88**	4	**74**	9
CK2	**99**	3	**100**	8
PHK	**11**	3	**20**	4
LCK	**36**	3	**3**	1
CSK	**51**	1	**41**	1
CDK2/Cyclin A	**95**	2	**92**	4
PI3-K	**109**	22	-	-
DYRK1a	**64**	1	**40**	7
CK1	-	-	**50**	1
NEK6	-	-	**95**	4
NEK2a	-	-	**94**	1

(excerpted from the Applicant's submission)

The results show that BIBW 2992 was able to inhibit enzymatic activity of SAPK2a/p38a, LCK, and phosphorylase kinases by more than 60% at a 10 µM concentration. IC_{50} values were then calculated for BIBW 2992 against targets with significant inhibition at the 10 µM concentration and are presented in Table 8.

Table 8: Potential Non-EGFR Targets of Afatinib

Kinase	IC_{50} (Molar)	R^2
LYN	1.33E-06	0.9795
LYN	1.83-06	0.9886
LYN	1.42E-06	0.9916
SRC	2.67E-06	0.9702
SRC	1.59-06	0.9829
LCK	1.72-06	0.9678
LCK	1.99E-06	0.9253
SAPK2α/p38α	2 E-06	
PHK	2.62E-07	
PHK	2.8E-07	
PHK	1.8E-06	
LCK	1E-07	
LCK	6.5E-07	

(excerpted from the Applicant's submission)

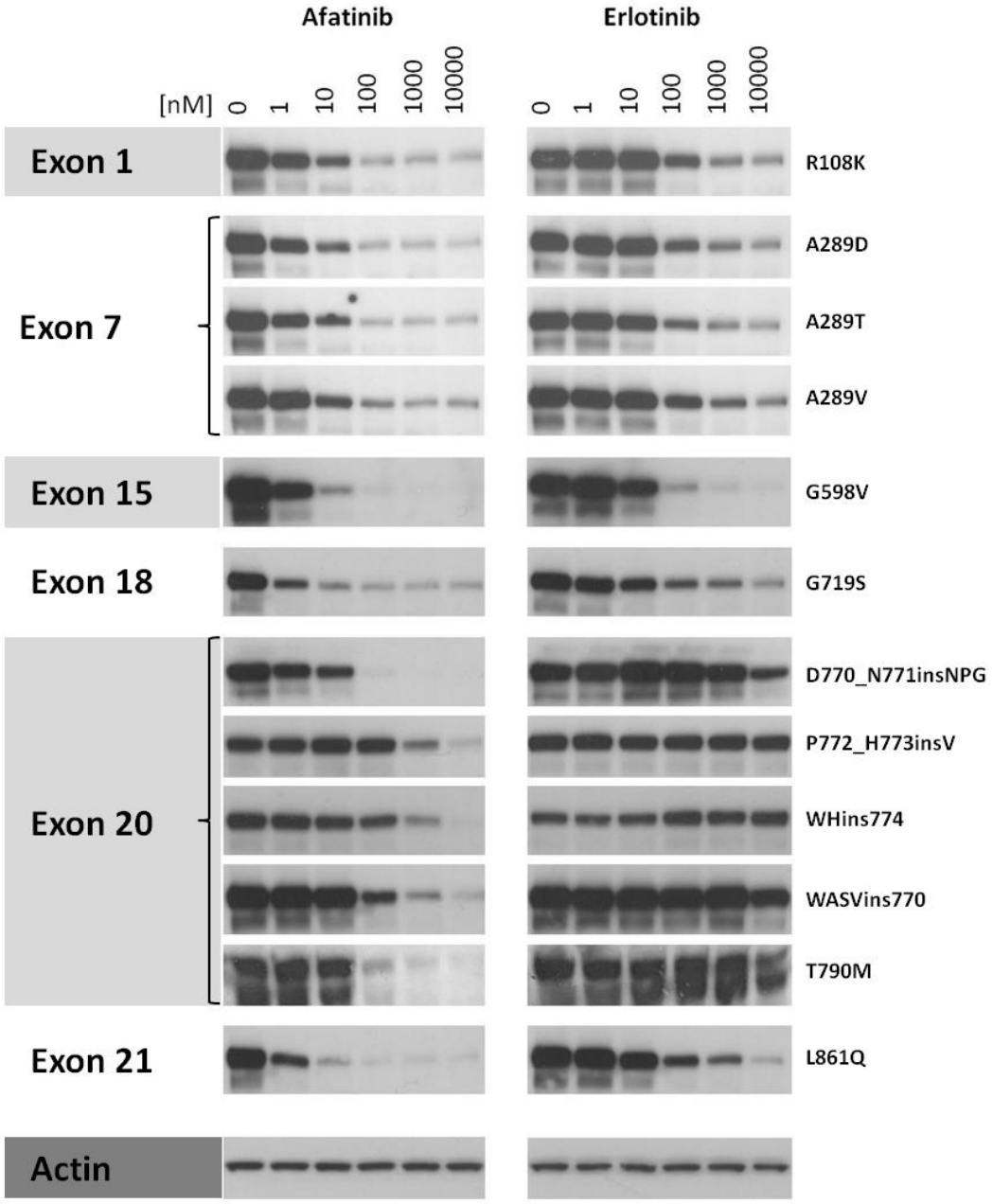
This data showed that BIBW 2992 inhibits LYN, SRC, LCK and SAPKa/p38a kinases at low micro-molar concentrations; however most inhibition occurred at concentrations of 1000 nM or more. In one experiment, the IC_{50} for BIBW 2992 against LCK was determined as approximately 100 nM, which represents a concentration that could be achieved clinically.
Additional nonclinical studies (p08-14760, p12-09544, and U13-1320) were submitted following a nonclinical information request for analysis of the inhibitory potential of afatinib on EGFR mutants. In addition the Applicant directed FDA to supplementary data included in a literature reference submitted at the time of original NDA submission (p08-06904).

U13-1320 bircv02-13 Inhibition of EGFR mutant protein autophosphorilation by afatinib and erlotinib in cellular assays

The inhibition of afatinib on autophosphorylation of uncommon activating EGFR mutants expressed in transfected mouse NIH/3T3 fibroblasts is presented in Figure 10. Specifically, point mutations in exons 18, 20, and 21 encoding the kinase domain of EGFR (G719S, T790M, L861Q), exon 20 insertions (WASVins770, D770_N771insNPG, P772_H773insV, WHins774) and activating missense mutations in exons 1, 7, and 15

encoding the extracellular domain of EGFR (R108K, A289D, A289T, A289V, G598V) were tested.

Figure 10. Effect of afatinib treatment on EGFR-Y1068 phosphorylation in NIH-3T3 cells ectopically expressing EGFR mutants.



(excerpted from Applicant's submission)

Afatinib inhibited the autophosphorylation of most tested mutants, including T790M, at concentrations of 100 nM or less. Incubation with erlotinib at concentrations of up to 100 nM had no effect on phosphorylation of T790M.

P12-09544 Target binding properties and cellular activity of afatinib (BIBW 2992), an irreversible ErbB family blocker by Flavio Solca et al, 2012, *Journal of Pharmacology and Experimental Therapeutics* 343:342

In this paper, the authors show the covalent binding of afatinib to EGFR, EGFR L858R/T790M, HER2, and HER4 by X-ray christalography and mass spectometry studies. Furthermore, studies shows that the acrilamide group is responsible for covalent binding of BIBW 2992 to Cys 797 at the edge of the active site. The applicant also synthesized a compound called "BI 37781", with close structural characteristics to afatinib, but without the reactive double bond and thus incapable of forming a covalent bond to cysteine residues in the catalystic site. Side by side comparisons of BI 37781 and BIBW2992 showed that BI37781 did not inhibit proliferation of the NCI-H1975 cell line which carries the EGFR L858R/T790M mutation (EC 50 >3000 vs 92 for afatinib) or the BT474,HER 2 gene amplification, cell line (EC 50 > 4000, vs 54 for afitinib). Additional experiments demonstrated afatinib mediated inhibition of the kinase activity of EGFR family proteins and mutants as well as afatinib-mediated inhibition of phosphorylation of the same proteins (Table 9).

(*excerpted from Applicant's submission, Solca et. al.)*

P08-06904 BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chirieac LR, Padera RF, Shapiro GI, Baum A, Himmelsbach F, Rettig WJ, Meyerson M, Solca F, Greulich H, Wong KK. *Oncogene*. 2008 Aug 7;27(34):4702-11.

In this paper the authors once again showed afatinib-mediated inhibition of EGFR L858R and EGFR L858R/T790M mutant carrying cell lines in both phosphorylation and proliferation assays. For the L858R mutant inhibition was also observed following incubation with erlotinib; however aftatinib inhibited the cells at lower concentrations. In a supplemental figure from this paper the authors showed inhibition of Ba/F3 cells that were transfected with a series of EGFR exon 19 deletion mutants or exon 19 deletion/T790M double mutants. Ba/F3 cells normally require supplementation with IL-3 for survival. Ectopic expression of active EGFR mutants can overcome the IL-3 dependence of these cells. Incubation with afatinib inhibited IL-3 independent survival of EGFR exon 19 deletion mutant expressing Ba/F3 cells at low nanomolar concentrations regardless of their T790M status.

Table 10: Afatinib inhibits survival of Ba/F3 cells ectopically expressing various EGFR mutants

	Afatinib EC_{50} (nM)
L858R	4
L858R + T790M	119
E746_A750del5	0.9
E746_A750del5 + T790M	64
S752_I759del8	0.2
S752_I759del8 + T790M	103
L747_A750del4insP	1
L747_A750del4insP + T790M	60
L747_P753del7insS	2
L747_P753del7insS + T790M	49
E746_S752del7insV	0.2
E746_S752del7insV + T790M	102
vIII (variant III deletion)	0.9

(excerpted from Applicant's submission)

4.2 Secondary Pharmacology

03-02-8810241 Study of BIBW 2992 BS, BIBW 3022 BS, and BIBW 3049 BS in various receptor binding assays * summary

Binding affinities of BIBW 2992 BS, and similarly derived inhibitors screened in a radio-ligand binding assay against a panel of receptors. The compounds were tested at 5 µM. The submitted results showed that at the 5 µM concentration BIBW 2992 inhibited the H2 receptor by 68%, and M1 receptors by 78%. BIBW 3049 BS inhibited CCKa receptors by 64%, H2 receptors by 72%, and M1 receptors by 81%. BIBW 3022 BS had no binding affinity for any of the measured receptors.

4.3 Safety Pharmacology

U02-1467 Gp2002-047-ph5 Effects of BIBW 2992 BS on vital physiological functions in conscious rats using a telemetry/plethysmography system

An assessment of the effects of orally administered (PO) BIBW2992BS on systolic arterial pressure (SAP), heart rate (HR), temperature, motility and respiration rate and volume was conducted in unrestrained, telemetered, conscious male rats. A plethysmograph system was also used in this study. A baseline period of 60 min was monitored before dosing. The doses of 10, 30 or 100 mg/kg of BIBW 2992 were used in 8 males per dose-group. Measurements were recorded continuously for 7 hours. There was a dose-dependent increase in arterial blood pressure, with the highest dose resulting in a significant increase over placebo over the 7 hr measurement period (Figure 11). There was also a transient increase in heart rate in the high-dose received males (Figure 12).

Figure 11: Effects of increasing doses of BIBW2992 on systolic arterial blood pressure in rats

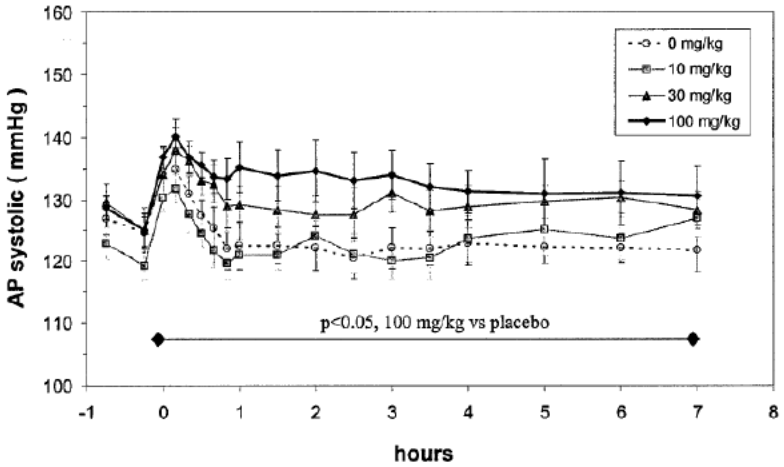
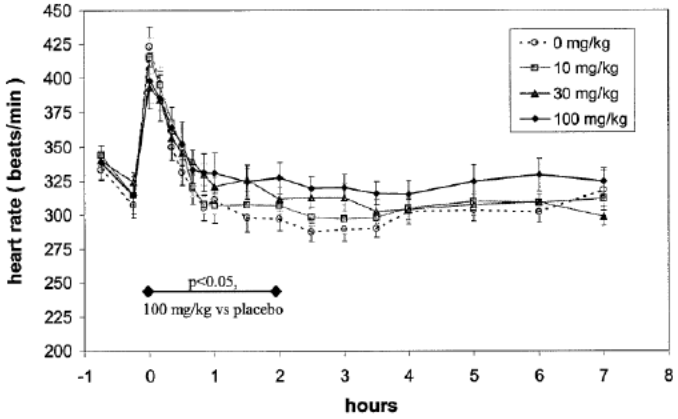


Figure 12: Effects of increasing dose of BIBW2992 on HR in rats



(excerpted from the Applicant's submission)

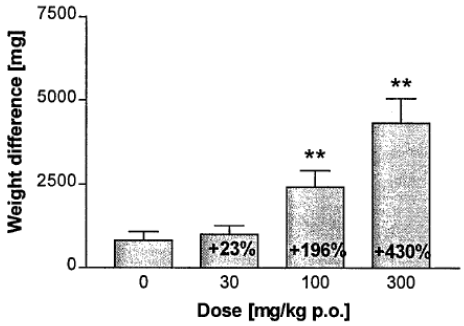
In conclusion, an oral dose of 100 mg/kg of BIBW 2992 BS significantly increased systolic blood pressure over a 7 hour period in conscious male rats. Heart rate was

also slightly and transiently increased at the same dose. There were no effects on body temperature, respiration rate, tidal volume and motility at any of the doses tested.

U02-1487 gp2001-264-ph4 Effects of BIBW 2992 BS (30, 100, 300 mg/kg BW po) on gastric emptying in rats

The study to assess the effects on gastric emptying was performed at afatinib doses of 30, 100 and 300 mg/kg in conscious rats. Gastric emptying was estimated by calculating the difference in weight between stomachs of control versus treated mice 1 hour after the administration of 10g of rat feed by gavage. Figure 13 shows a dose-dependent decrease in the rate of gastric emptying, where the animals given 100 mg/kg had 196% more of the gastric matter present when compared to the control animals and animals given 300 mg/kg had an increase of ~ 430%.

Figure 13: Effect of Afatinib on gastric empyting



(excerpted from Applicant's submission)

U02-1488 gp2001-262-ph4 Effects of BIBW 2992 (30, 100 and 300 mg/kg BW po) on gastrointestinal transit in rats *summary

The experiment was performed on five CRL: WI (GIx/BRL/HAN) IGS BR rats after fasting for 24 hr. A dose-dependent inhibition of gastrointestinal transit (~66% decrease in the highest-dosed animals) was noted. Although the animals at the lower doses also experienced inhibition (~3% and ~22% less in low and mid-dosed animals, respectively, compared to controls), a significant result was only noted for the animals at the 300 mg/kg dose level BIBW2992.

U02 1489 gp2001-263-ph4 Effects of BIBW 2992 BS (30, 100 and 300 mg/kg BW id) on gastric secretion in rats * summary

Gastric juice volume and acid output were recorded in 4 groups of 6-8 male Wistar rats given afatinib at dose levels of 30, 100, or 300 mg/kg per group. Gastric secretions was evaluated 4 hours after the BIBW 2992 application. . BIBW2992 reduced gastric

secretion at the highest dose level of 300 mg/kg in male rats when compared to control and lower-dose group rats.
Afatinib exposure in rats at the 300 mg/kg dose level is expected to exceed the clinical exposure by at least 10-fold.

U02 1490 gp2001-256-266 Effects of BIBW 2992 BS (30, 100 and 300 mg/kg po) on renal function in conscious rats

Four groups of 20 animals (10/sex/group) were used to investigate the effects of BIBW 2992 BS on renal and liver function. Urine was collected 4, 8, and 24 hours after the administration of BIBW 2992 BS at dose levels of 30, 100 and 300 mg/kg. Volume, pH, osmolality, protein concentration, creatinine, glucose, electrolytes (sodium, potassium, chloride, calcium, and magnesium), and enzymes (N-acetyl-b-D-glucosaminidase, aspartate aminotransferase, alanine aminotransferase, g-glutamyl transferase, lactate dehydrogenase and alkaline phosphatase) were measured at each time-point. Osmolarity and the concentrations of Na+, K+, Cl-, Ca++, Mg++, protein, creatinine, glucose, blood urea nitrogen (BUN), total bilirubin (TBIL), conjugated bilirubin (FBIL), triglycerides, free fatty acids, and cholesterin, and the enzymes AST, ALP, GGT, LDH glutamate dehydrogenase (GLDH), ALP and creatine kinase (CK) were also determined in serum isolated at the same intervals.

Results from urine are presented in the table below:

Table 11: Renal Function Parameters

Findings (dose related times)	0-4h	4-8h	8-24 h	0-24h
↑Glucose excretion		30, 100 300	100, 300	100, 300
↓ urine volume and pH		300		
↑ osmolarity		300		
↑ AST		300		
↓ Mg++ excretion			300	300
↑ LDH			300	300

In addition, a trend of an increase was seen for AST (8-24h and 0-24h), ALT (4-8h, 8-24h and 0-24h), LDH (4-8h), ALP (4-8h) and b-NAG (4-8h) at the dose of 300 mg/kg.

Serum-obtained results:

- A dose of 30 mg/kg of BIBW 2992 did not change any of the measured parameters
- A dose of 100 mg/kg increased Mg++ concentration and protein amount at 4 h. At 24h, Ca++ and glucose levels increased, when compared to the control animals.
- A dose of 300 mg/kg had following effects: Mg++ was increased at 4h, and decreased at 24 h; Ca++ was decreased at 8 and 24 h, while osmolarity was reduced at 24 h; glucose was increased at 4, 8 and 24 h, protein was increased at 4h, and free fatty acids were decreased at 24 h; AST was increased at 4 h, while ALT was increased at 4 and 8 h.

In conclusion, oral doses of 30, 100 and 300 mg/kg increased urinary glucose excretion when measured up to 24 h after the ingestion. The dose of 300 mg/kg of BIBW 2992 led to sustained but mild enhancement of serum glucose levels compared to controls and animals at lower dose levels, and increases in serum and urine enzymes, suggesting that BIBW 2992 is able to cause liver and renal toxicity in rats.

U02-1619 gp2001-298ph1-329ph1 Behavior assessed by observation in a modified IRWIN-test and on nocturnal motility in mice after oral administration *summary

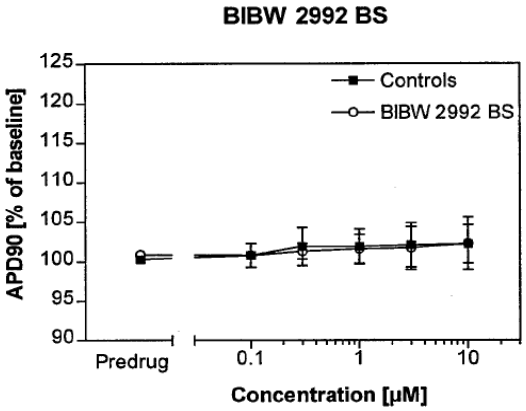
Mice were given doses of 30, 100, or 300 mg/kg PO; general behavior was measured through series of observations and compared to control animals at 15, 30, and 60 min, and at 24 h after administration of afatinib. There were no serious adverse effects on behavior at any dose tested following single oral administration of BIBW 2992. The single clinical observation recorded was that of light colored feces in animals given doses of 100 or 300 mg/kg one hour after administration. By the 24 hour timepoint, this finding had resolved.

U02-1580 gp2001-070-470 Influence on HERG-mediated potassium current and on action potential configuration in isolated guinea pig papillary muscle

An additional electrophysiologic evaluation of BIBW 2992 BS was done using the human ether-a-go-go (hERG)-mediated potassium current assay due to the previously noted potential of BIBW2992 to prolong myocardial effects. This experiment was performed using HEK293 cells that stably express the hERG-potassium channel. The patch-clamp technique was used on the whole-cell extracts in the presence of cumulative afatinib concentrations of 0.1, 0.3, 1, 3 and 10 µM (n=5). Effects on the HERG-mediated current amplitude were measured for each test concentration over 5 min. An additional control group of cell extracts were incubated with equivalent concentrations of the DMSO vehicle control. Action potential measurements were done at a stimulations frequency of 0.33 Hz (20 cycles/min) and included action potential duration to 10%, 30%, and 90% re-polarization (APD10, APD30, APD90), resting membrane potential (RPM), maximal velocity of phase 0 upstroke (V-max), AP overshoot (OS), AP amplitude (APA), and the force of concentration (FOC).
The IC_{50} of BIBW 2992 BS in the hERG assay was 2.4 µM.
There were no changes caused by BIBW 2992 BS on the action potential configuration at concentrations of up to 10 µM in the guinea pig papillary muscle (Figure 14).

(excerpted from Applicant's submission)

Figure 14: Afatinib effects on in vitro APD



U03 1858 boi-270-032645 Modified Irwin study in male and female rats including body temperature and locomotor assessment-single oral administration (Irwin test) *summary

Single oral doses of 4, 8.5, or 18 mg/kg BIBW 2992 were given to both SPF CD Crl:CD(SD)IGSBR and SPF Wistar Han Crl: WI(GIx/BRL/Han)IGSBR rats (4/sex/group) in order to assess effects on general behavior, body temperature and spontaneous locomotor activity at 60, 120, 240, and 360 min and at 24 hr following afatinib administration.
There were no changes in any parameters observed in any of the tested animals during the duration of the study. In addition, there were no other signs of toxicity observed in the animals up to day 7 after administration of aftatinib at any dose level.

U03 1859 boi-271-032845 Evaluation of respiratory parameters in the conscious male and female rat using whole body bias flow plethysmography after single dose administration

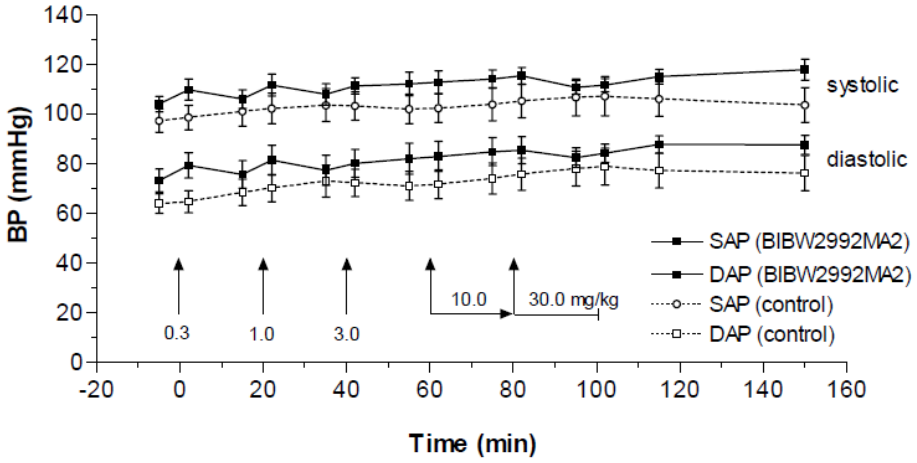
Respiratory parameters were measured in Wistar rats after the administration of 4, 8.5, or 18 mg/kg of BIBW 2992 BS or morphine sulphate (200 mg/kg) as a positive control. There were no effects seen on respiration rate, tidal volume, or minute volume in rats given BIBW 2992. Morphine sulphate treatment resulted in statistically significant decreases in respiration rate and minute volume in both male and female rats at 60 minutes post-dose. Tidal volume was also increased after the morphine application.
In conclusion, BIBW 2992 administration at single doses of up to 18 mg/kg does not change respiratory parameters in Wistar rats.

Effect of BIBW 2992 MA2 (0.3-30 mg/kg IV) on hemodynamic and electrocardiographic parameters in anesthetized domestic pigs U03-1311

Summary
To determine the influence of BIBW 2992 MA2 on cardiovascular and electrocardiographic parameters *in vivo*, eight Phenobarbital-anesthetized domestic male pigs were randomized into two groups of four. One group received graded doses of BIBW 2992 MA2 (0.3, 1, 3, 10 and 30 mg/kg); the first 3 doses were delivered as bolus injections while doses of ≥10 mg/kg were delivered as continuous infusions over 20 minutes. The other group received equivalent volumes of the vehicle.

Slight increases in systolic and diastolic pressure occurred immediately after the administration of .3, 1 and 3 mg/kg BIBW 2992, or during infusion of 10 and 30 mg/kg BIBW 2992. The measurements suggests that any increases are of uncertain significance.

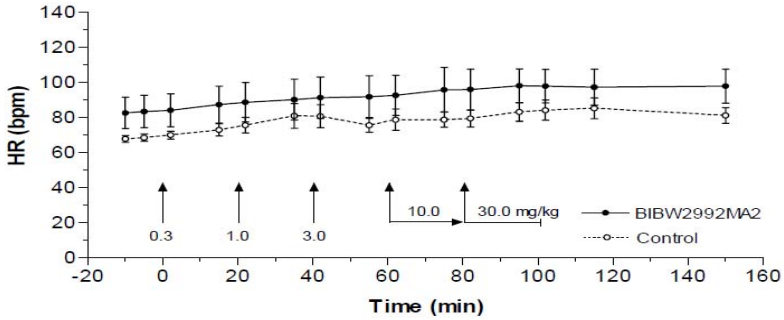
Figure 15: Blood Pressure in Domestic Pigs



(excerpted from Applicant's submission)

There were no changes observed in the heart rate of the animals on treatment, as visible in the following Figure:

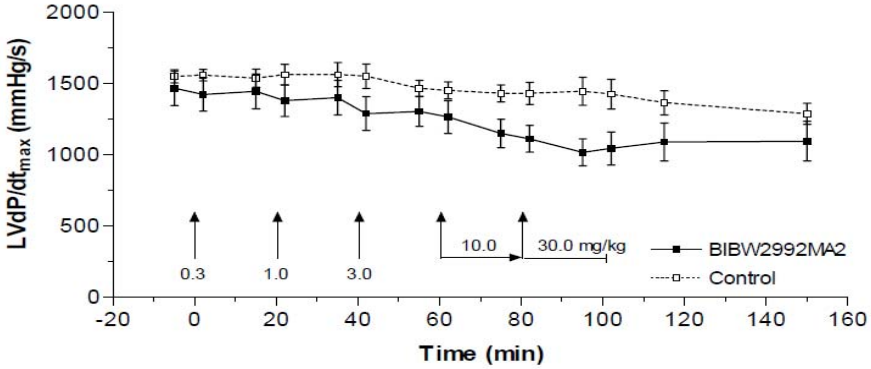
Figure 16: Heart Rate in Domestic Pigs



(excerpted from Applicant's submission)

A moderate and statistically significant decrease in the LVdP/dt-max levels was observed after infusion of the 30 mg/kg dose of BIBW 2992 MA2, visible in the following Figure:

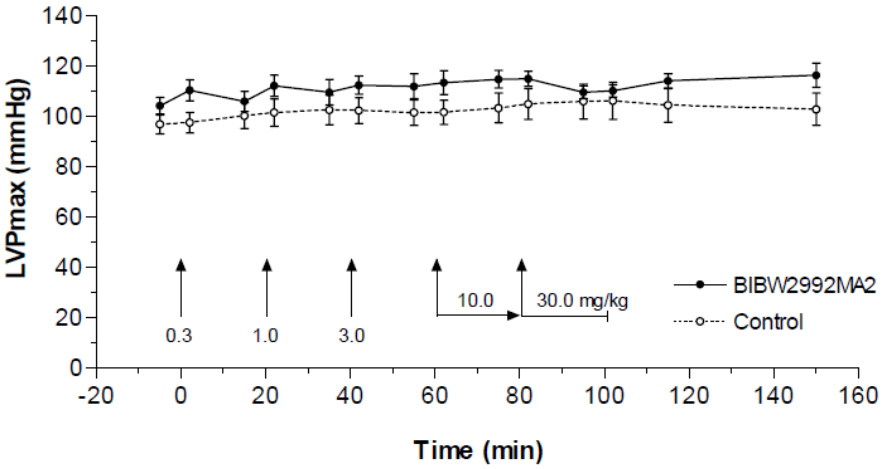
Figure 17: Cardiac Function in Domestic Pigs



(excerpted from Applicant's submission)

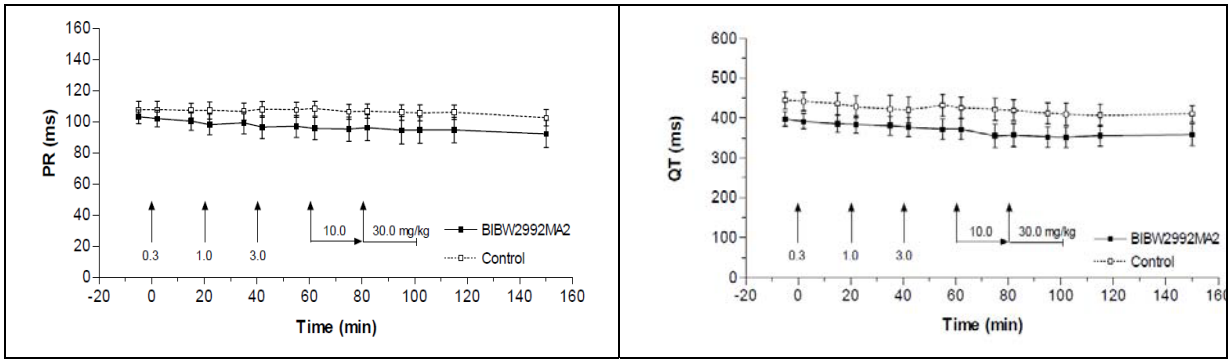
No siginificant changes were observed in maximal left ventricular pressure analysis.

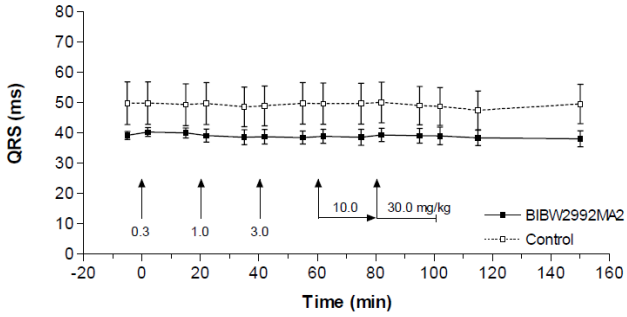
Figure 18: LVP max in Pigs



(excerpted from Applicant's submission)

ECG interval (QT, QRS, and PR) measurements did not show any significant changes in the animals on the study.





(excerpted from Applicant's submission)

In conclusion, administration of BIBW 2992 MA2 to phenobarbital anesthetized pigs resulted in minimal changes in blood pressure, heart rate, ECG measurements, or maximum ventricular pressure that quickly disappeared at all dose levels; however a negative inotropic effect was noted at the doses of 10 and 30 mg/kg.

5 Pharmacokinetics/ADME/Toxicokinetics

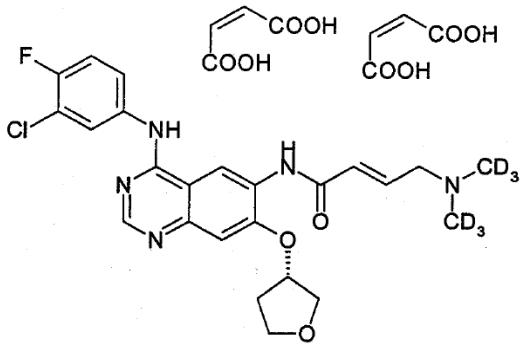
5.1 PK/ADME

Method Validation Reports

BIBW 2992 MA2: A method for the quantification of BIBW 2992 BS in minipig plasma using solid phase-extraction and HPLC-MS/MS: validation and stability data. (0183-02rm)

The purpose of this study was to develop and validate an HPLC-MS/MS method for the quantification of BIBW2992 base (BS) in EDTA-plasma from the Göttingen minipig, a toxicology species for BIBW2992BS preclinical development. This study also included an evaluation of incurred sample reanalysis (ISR) from bioanalytical samples collected during BIBW2992 minipig toxicology studies.
Drug-spiked blank plasma samples underwent solid-phase extraction followed by chromatographic separation on a C18 reverse-phase column. Quantification was performed using tandem MS detection in positive electrospray ionization mode. The internal standard was a deuterated (D6) isoform of BIBW2992 BS (Applicant-**Figure 19**).

Figure 19: Structure of BIBW2992 IS and Position of Deuterium Labels



D = H-2 label

Mean recovery was approximately 56-65% over the range from 8.0-1300 nM, the level of quantitation (LOQ) of this assay was 2 nM and calibration curves were linear over the range of 2-1000 nM BIBW2992BS. There was minimal interference from endogenous compounds.
Accuracy and precision, measured as percent deviation of replicate analyses from theoretical values in QC samples were acceptable (both intra- and inter-batch).

BIBW2992BS was stable after 3 freeze-thaw cycles in minipig plasma, and storage stability of BIBW2992BS was demonstrated in spiked blank EDTA-anticoagulated minipig plasma for a period of 52 weeks when maintained at -20° C.
Finally, ISR from Study BIBW2992 MA2 (an oral minipig study) was performed on samples stored under frozen conditions (-20° C) for 24 weeks. Relative % difference at the end of the storage interval was between 75.3-112.5% of the initial result, which is considered acceptable. The concentrations evaluated for ISR ranged from 2 – 6 nM.

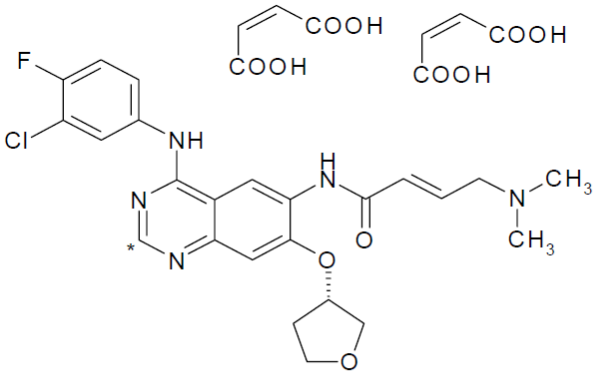
BIBW 2992 MA2: A Method for the Quantification of BIBW 2992 BS in Rat Plasma Using Solid Phase Extraction and HPLC-MS/MS: Validation and Stability Data (M182_02RM)

The purpose of this study was to develop and validate an HPLC-MS/MS method for the quantification of BIBW2992 BS in EDTA-plasma from the Han Wistar rat, a species used during the preclinical development of BIBW2992BS.
Drug-spiked blank plasma samples underwent solid-phase extraction followed by chromatographic separation on a C18 reverse-phase column. Quantification was performed using tandem MS detection in positive electrospray ionization mode. The internal standard was a deuterated (D6) isoform of BIBW2992 BS (Applicant-**Figure 19**).
The LOQ was 2 nM. Mean recovery was approximately 47-73% over the range from 8.0-1300 nM, and the calibration curves were linear over the range of 2-1000 nM BIBW2992BS. There was no interference from endogenous compounds, and carryover was minimal. Accuracy and precision, measured as percent deviation of replicate analyses from theoretical values in QC samples were acceptable (both intra- and inter-batch).
BIBW2992BS was stable in rat plasma through 3 freeze-thaw cycles. Although extended storage under ambient light at 15 ^{o}C was associated with slow degradation (as observed by decreased analyte concentration over the storage interval), acceptable ambient storage was demonstrated for 16 hours sufficient to permit handling and analysis of samples. Long-term freezer stability of BIBW2992BS was not assessed in this study.

Synthesis of [quinazolinyl-2-14C] BIBW2992 MA2

This report details the synthesis of [quinazolinyl-2-^{14}C]BIBW2992 MA2 (the isotope used in mass balance studies), by the introduction of a ^{14}C radiolabel at position 2 of the quinazoline part of the molecule (Applicant-**Figure 20**).
The specific activity was 732 MBq/mmol (~ 19.8 mCi/mmol). This corresponds to a radioactive dose of 1.020 MBq/mg (or ~ 27.6 µCi/mg).

Figure 20: Structure of BIBW2992MA2 and the Position of the ^{14}C Radiolabel (*)



* = C-14 label

BIBW 2992 MA2: Partial Validation of a Modified Method for the Quantification of BIBW 2992 BS in Minipig Plasma with a Lower Limit of Quantification of 1 nmol/L (B205/03RM)

The purpose of this study was increase the sensitivity of the HPLC-MS/MS bioanalytical method for BIBW2992BS by lowering the LOQ from 2 nM to 1 nM. The assay remained largely the same as that used to validate the 2 nM LOQ – the samples were extracted by solid-phase extraction and chromatographed on a C18 reverse-phase column, and the quantification was performed by tandem MS in positive electrospray ionization mode. Accuracy and precision, measured as perecent deviation of replicate analyses from theoretical values in QC samples were acceptable (both intra- and inter-batch). The same internal standard was used.

BIBW 2992 MA2: A Modified Method for the Quantification of BIBW 2992 BS in Rat Plasma with a Lower Limit of Quantification of 1.00 nmol/L (R259_04RM)

The purpose of this study was to validate an HPLC-MS/MS method for detection of BIBW2992BS at a level of 1 nM in rat plasma. The linear range of the new assay is 1.00 to 1000 nM BIBW2992BS in plasma. Accuracy and precision, measured as percent deviation of replicate analyses from theoretical values in QC samples were acceptable (both intra- and inter-batch).
This method used different equipment (HPLC and MS detectors) and conditions from those used in study M182_02RM. Parameters such as carryover, extraction recovery, and matrix effect were not re-validated, as the relevant equipment and processes did not change. The Applicant used the same internal standard as was used in other validations (Applicant-**Figure 19**).

BIBW 2992 MA2: A Modified Method for the Quantification of BIBW 2992 BS in Minipig Plasma with a Lower Limit of Quantification of 0.200 nmol/L (M262/05RM)

The purpose of this validation study was to produce a reliable HPLC-MS/MS bioanalytical method for the detection of BIBW2992BS in minipig plasma with a 5-fold increased sensitivity (i.e. from an LOQ of 1.0 nM to an LOQ of 0.2 nM).
This method used different equipment (HPLC and MS detectors) and conditions from those used in study B205/03RM. Parameters such as carryover, extraction recovery, and matrix effect were not re-validated, as the relevant equipment and processes did not change. Accuracy and precision, measured as percent deviation of replicate analyses from theoretical values in QC samples were acceptable (both intra- and inter-batch).
The Applicant used the same internal standard as was used in other validations (Applicant-**Figure 19**). The linear range of the assay was from 0.2 - 200 nM.

BIBW 2992 MA2: Revalidation of the HPLC-MS/MS Method for the Quantification of BIBW 2992 BS in Rat Plasma (V293_05RM)

The purpose of this method revalidation was to develop an HPLC-MS/MS bioanalytical method that used rat rather than human plasma as the diluent for the eluate from the solid phase extraction. Methods such as linearity, precision and accuracy were therefore re-validated; other parameters were considered to be unaffected. The linear range of this method was 1.00-1000 nM for BIBW2992BS. Accuracy and precision, measured as percent deviation of replicate analyses from theoretical values in QC samples were acceptable (both intra- and inter-batch).
The method employed the same internal standard that was used in other method validation studies (Applicant-**Figure 19**).

BIBW 2992 MA2: Stability of BIBW 2992 BS in rat EDTA Plasma and Whole Blood (B2820)

The purpose of this study was to evaluate the stability of BIBW2992BS in rat EDTA plasma for the purposes of supporting development of an HPLC-MS/MS bioanalytical method. ISR was also evaluated using rat plasma samples collected from toxicology studies, in this study.
Short- and long-term stability (0.5 and 24 hours, respectively) of BIBW2992BS in rat plasma incubated at room temperature and on ice and under ambient lighting, was evaluated. Samples were purified by SPE, chromatographed on a C18 reverse-phase column and concentrations measured with tandem MS/MS detection in the positive electrospray ionization mode.
Room Temperature conditions: BIBW2992BS was found to be stable for at least 0.5 hrs when stored at room temperature; however, it began to degrade (as indicated by a gradual decrease in concentration) after prolonged storage from 16-24 hours. Stability

was acceptable for 16 hours, which the Applicant argues permitted sufficient time for handling and analysis. This stability was demonstrated over the concentration range of 5-800 nM (Applicant-**Table 12** and Applicant-**Table 13**). BIBW2992BS was also found to be stable under stress conditions (i.e. following 3 cycles of freezing and thawing).

Table 12: BIBW2992BS Stability When Stored at Room Temperature in Rat Plasma (Low Concentration)

Date of analysis	16 Sep 2002	12 Sep 2002	23 Sep 2002	23 Oct 2002	23 Oct 2002
Stress condition	3 x frozen	24 h RT	24 h RT	4 h RT	16 h RT
Concentration [nM]	5.00	5.00	5.00	5.00	5.00
Determined conc. [nM]&	4.39	4.34	4.03	4.86	4.59
	5.09	4.23	4.30	4.88	4.63
	4.60	4.54	4.35	5.54	5.24
	5.22	4.26	4.27	5.69	5.10
	4.42	3.87	4.86	4.88	4.99
	4.76	3.93	4.33	5.14	4.95
Mean [nM]	4.75	4.20	4.36	5.17	4.92
CV (%)	7.3	6.0	6.3	7.1	5.3
Relative to nominal (%)	94.9	83.9	87.1	103.3	98.3

Table 13: BIBW2992BS Stability When Stored at Room Temperature in Rat Plasma (High Concentration)

Date of analysis	**16 Sep 2002**	**12 Sep 2002**	**23 Sep 2002**	**23 Oct 2002**	**23 Oct 2002**
Stress condition	**3 x frozen**	**24 h RT**	**24 h RT**	**4 h RT**	**16 h RT**
Concentration [nM]	800	800	800	800	800
Determined conc. [nM]&	773	746	738	770	761
	725	746	736	786	772
	769	714	718	799	752
	754	745	742	809	743
	789	757	702	827	737
	812	760	704	820	764
Mean [nM]	770.33	744.67	723.33	801.83	754.83
CV (%)	3.9	2.2	2.5	2.7	1.8
Relative to nominal (%)	96.3	93.1	90.4	100.2	94.4

& rounded to 3 significant digits in KinLims; basic statistics calculated using these values
method used: [U03-1191]
For completeness of stability data within one report this table is reproduced from report [U03-1191].

Freezer stability: BIBW2992BS was stable in rat EDTA plasma for at least 127 days in the freezer when stored at -20C. ISR was performed from an animal study in which samples were re-analyzed after a period of 155 days of freezer storage and found to be stable (Applicant-**Table 14** and Applicant-**Table 15**).

Table 14: Stability of BIBW2992BS Under Conditions of Freezer Storage (Low Concentration)

Preparation of calibration standards	17 Oct 2002	24 May 2005	27 Feb 2003
Preparation of stability samples	09 Sep 2002	17 Jan 2005	09 Sep 2002
Date of analysis	21 Oct 2002	24 May 2005	03 Mar 2003
Freezer storage*	38 days	127 days	171 days
Concentration [nM]	5.00	2.50	5.00
Determined conc. [nM]&	4.98	2.51	4.46
	4.48	2.65	3.65
	5.26	2.89	3.80
	4.53	2.85	4.31
	4.71	2.44	4.14
	4.85	2.73	4.45
Mean [nM]	4.80	2.68	4.14
CV (%)	6.2	6.8	8.2
Relative to nominal (%)	96.0	107.2	82.8

Preparation of calibration standards	25 Aug 2003	25 Aug 2003	17 Jan 2005
Preparation of stability samples	27 Feb 2003	09 Sep 2002	09 Sep 2002
Date of analysis	17 Sep 2003	17 Sep 2003	17 Jan 2005
Freezer storage*	179 days	350 days	861 days
Concentration [nM]		5.00	5.00
Determined conc. [nM]&		4.80	2.86
	Only high	4.55	2.97
	concentration	4.29	2.41
	(800 nmol/L)	4.38	2.63
	analysed	4.82	2.50
		4.74	2.64
Mean [nM]		4.60	2.67
CV (%)		4.9	8.0
Relative to nominal (%)		92.0	53.4

* difference between calibration standards and stability samples (in days)

& rounded to 3 significant digits in KinLims;
mean and CV (%) calculated in KinLims before rounding;
relative percentage calculated in Excel using mean values (rounded to 3 significant digits)

methods used: in 2003 [U03-1191] and in 2005 [U05-1932]

Table 15: Table 9: Stability of BIBW2992BS Under Conditions of Freezer Storage (High Concentration)

Preparation of calibration standards	17 Oct 2002	24 May 2005	27 Feb 2003
Preparation of stability samples	09 Sep 2002	17 Jan 2005	09 Sep 2002
Date of analysis	21 Oct 2002	24 May 2005	03 Mar 2003
Freezer storage*	38 days	127 days	171 days
Concentration [nM]	800	800	800
Determined conc. [nM]&	767	878	700
	799	877	705
	760	816	693
	780	868	696
	770	839	702
	760	830	735
Mean [nM]	773	851	705
CV (%)	1.9	3.1	2.1
Relative to nominal (%)	96.6	106.4	88.1

Preparation of calibration standards	25 Aug 2003	25 Aug 2003	17 Jan 2005
Preparation of stability samples	27 Feb 2003	09 Sep 2002	09 Sep 2002
Date of analysis	17 Sep 2003	17 Sep 2003	17 Jan 2005
Freezer storage*	179 days	350 days	861 days
Concentration [nM]	800	800	800
Determined conc. [nM]&	911	765	414
	828	735	NA
	917	669	435
	845	711	425
	811	712	437
	--	671	417
Mean [nM]	862	710	426
CV (%)	5.6	5.2	2.4
Relative to nominal (%)	107.8	88.8	53.3

* difference between calibration standards and stability samples (in days)
& rounded to 3 significant digits in KinLims;
mean and CV (%) calculated in KinLims before rounding;
relative percentage calculated in Excel using mean values (rounded to 3 significant digits)
methods used: in 2003 [U03-1191] and in 2005 [U05-1932]

Whole blood: In contrast, BIBW2992BS was found to be less stable in whole blood. Concentrations decreased by 3 hours in storage, to approximately 40% and 75% of initial concentrations when stored at RT and on ice, respectively (Applicant-**Table 16**).

Table 16: Stability of BIBW2992BS in Whole Blood When Maintained at Room Temperature or on Ice

	Spiked sample		*Ex vivo* samples			
Time / storage condition	[nmol/L]	Relative (%)	Rat male [nmol/L]	Relative (%)	Rat female [nmol/L]	Relative (%)
5 min / ice	194	100.0*	95.0	100.0*	55.4	100.0*
30 min / ice	209	107.7	107	112.6	64.2	115.9
60 min / ice	202	104.1	90.8	95.6	55.1	99.5
180 min / ice	147	75.8	79.0	83.2	40.0	72.2
5 min / RT	204	105.2	107	112.6	55.7	100.5
30 min / RT	180	92.8	84.4	88.4	48.4	87.4
60 min / RT	159	82.0	75.7	79.7	43.2	78.0
180 min / RT	74.4	38.4	44.3	46.6	24.8	44.8

* set to 100 % within a column
method used: [U05-2640]
date of analysis: 04 November 2005

Incurred Samples: Freezer Stability of toxicology study samples (02B126) were assessed for method stability (ISR) following a period of 155 days of freezer storage. The net difference between the initial analysis and the concentrations re-assessed at the end of the storage period was within ±20% and met the acceptance criteria for ISR (Applicant-**Table 17**).

Table 17: BIBW2992BS Freezer Stability of Incurred Samples from Rat Study 02B126

	First analysis 08 October 2002*	Re-analysis 12 March 2003	Difference in storage time: 155 days
Method used	U03-1191	U03-1191	
Raw data storage	study 02B126	M182/02RM	
Sample ID§	[nmol/L]	[nmol/L]	Difference (%)
U261A01/2	250	256	2.4
U261A01/4	103	116	12.6
U261A01/8	44.2	40.7	-7.9
U261A01/24	3.19&	3.01	-5.6
U261A14/2	143	159	11.2
U261A14/4	147	137	-6.8
U261A14/8	47.6	46.4	-2.5
U261A14/24	5.53	4.44	-19.7
U312B01/2	354	368	4.0
U312B01/4	187	193	3.2
U312B01/8	60.6	51.0	-15.8
U312B01/24	<2.00	2.89	NA
U312B14/2	297	289	-2.7
U312B14/4	427	385	-9.8
U312B14/8	106	105	-0.9
U312B14/24	7.58	7.60	0.3
		N	15
		Mean	-2.6
		SD (%)	8.9
		Min	-19.7
		Max	12.6

* reported within study no. 02B126 [U03-1783]

§ Sample coding: U(unknown) - animal number - treatment - study day / sampling time, e.g. U261A01/2 = animal 261, A = low dose (8 mg/kg/day), day 01, 2 hours post dose; U312B14/24 = animal 312, B = mid dose (16 mg/kg/day), day 14, 24 hours post dose

& analysed on 10 October 2002

BIBW 2992 MA2: A Method for the Quantification of BIBW 2992 BS in Rabbit Plasma using Solid Phase Extraction and HPLC-MS/MS: Validation and Stability Data (M374_08RM)

The purpose of this study was to validate an HPLC-MS/MS method for detection of BIBW2992BS at a level of 1 nM in rabbit plasma. The parameters validated included assay linearity, stability under stress (3 repeated freeze-thaw cycles), and short- and long-term storage under RT and frozen conditions, sufficient to cover the period of sample collection, handling and pre-analysis storage. The Applicant used the same internal standard used in other bioanalytical method validations (Applicant-**Figure 19**). Extraction recovery ranged from 54-68% over the concentration range evaluated (2.50-800 nM). Column carryover was 0.84% and 0.41% following the first and second blanks, respectively. Accuracy and precision, measured as percent deviation of replicate analyses from theoretical values in QC samples were acceptable (both intra- and inter-batch). The LOQ was 1 nM and calibration curves were linear over a 3-log range (up to 1000 nM).

The molecule was found to be stable to freeze/thaw for at least 3 cycles, and was stable for at least 77 days of storage at -20C; by Day 182, acceptance criteria were not met (Applicant-**Table 18**).

Table 18: Evaluation of BIBW2992BS Long-Term Freezer Stability in Rabbit Plasma

Experimental information:				
Batches: 374S1, 374S2A				
Analyte: BIBW 2992 BS				
Species/matrix: rabbit plasma				
Stability sample preparation: 17.04.2008				
Calibration sample preparation: 03.07.2008 / 16.10.2008				
Stress conditions	**77 days at -20°C** (analysed 03 Jul 2008)		**182 days at -20°C** (analysed 16 Oct 2008)	
Sample label	UQ02_F_1	UQ04_F_1	UQ02_F_2	UQ04_F_2
Nominal conc. [nmol/L]	2.50	800	2.50	800
	2.51	899	2.78	928
	2.65	814	2.78	963
	2.65	780	2.84	935
	2.41	783	2.60	923
	2.28	772	2.83	932
	2.33	786	2.62	940
Mean conc. [nmol/L]	**2.47**	**806**	**2.74**	**937**
Imprecision (CV)	**6.3 %**	**5.9 %**	**3.8 %**	**1.5 %**
Inaccuracy (dev.)	**-1.2 %**	**0.8 %**	**9.6 %**	**17.1 %**

Remarks:
Values for mean concentration and imprecision are calculated in KinLims.
Calculation of inaccuracy is done in Microsoft Excel.

When stored in whole blood, loss of analyte (≥15% loss) was observed within 60 minutes when maintained at room temperature; however, when stored on ice, the loss was < 15% after 90 minutes (Applicant-**Table 19**).

Table 19: Evaluation of BIBW2992BS Stability in Whole Rabbit Blood

Batch: 374MV1 Analyte: BIBW 2992 BS Species/matrix: rabbit whole blood						
Storage on ice						
Animal no.	151		152		153	
Storage [min]	**BIBW 2992 BS** [nmol/L]	**Relative** (%)	**BIBW 2992 BS** [nmol/L]	**Relative** (%)	**BIBW 2992 BS** [nmol/L]	**Relative** (%)
0	291	100.0	118	100.0	174	100.0
10	267	91.8	115	97.5	165	94.8
30	258	88.7	113	95.8	157	90.2
60	259	89.0	114	96.6	169	97.1
90	249	85.6	106	89.8	151	86.8
Storage at room temperature						
Animal no.	151		152		153	
Storage [min])	**BIBW 2992 BS** [nmol/L]	**Relative** (%)	**BIBW 2992 BS** [nmol/L]	**Relative** (%)	**BIBW 2992 BS** [nmol/L]	**Relative** (%)
0	330	100.0	148	100.0	202	100.0
10	326	98.8	143	96.6	205	101.5
30	296	89.7	140	94.6	199	98.5
60	283	85.8	126	85.1	187	92.6
90	268	81.2	120	81.1	193	95.5

To evaluate stability under the conditions of analysis, samples were held at 16°C (the temperature of the autosampler) found to be stable for at least 49 hours (Applicant-**Table 20**).

Table 20: Evaluation of BIBW2992BS Stability at 16°C

Experimental information: Batches: 374VC Species/matrix: rabbit plasma Analyte: BIBW 2992 BS						
Stress conditions	**24 h in the autosampler at 16°C**		**49 h in the autosampler at 16°C**		**138 h in the autosampler at 16°C**	
Sample label	UQ02AS1	UQ04AS1	UQ02AS2	UQ04AS2	UQ02AS3	UQ04AS3
Nominal conc. [nmol/L]	2.50	800	2.50	800	2.50	800
	2.46	890	3.12	918	2.41	960
	2.25	870	2.72	924	2.32	933
	2.25	865	2.60	910	2.29	922
	1.91	846	2.70	883	2.36	891
	2.18	865	2.76	894	2.47	920
	1.76	883	2.66	924	2.30	943
Mean conc. [nmol/L]	**2.14**	**870**	**2.76**	**909**	**2.36**	**928**
Imprecision (CV)	**12.0 %**	**1.8 %**	**6.7 %**	**1.9 %**	**3.0 %**	**2.5 %**
Inaccuracy (dev.)	**-14.4 %**	**8.8 %**	**10.4 %**	**13.6 %**	**-5.6 %**	**16.0 %**

Remarks:
Values for mean concentration and imprecision are calculated in KinLims.
Inaccuracy and duration of stress are calculated in Microsoft Excel.

There was little endogenous interference observed. No assessment of repeatability (ISR) was performed in this validation.

Revalidation of the HPLC-MS/MS Method for the Quantification of BIBW 2992 in Rat Plasma (r456-10rm)

The purpose of this study was to revalidate the bioanalytical method for detection of BIBW2992BS in Han Wistar rat plasma to incorporate the use of new equipment and solvents. The modified method was assessed for linearity, accuracy, precision and autosampler stability. Other parameters were considered unaffected.
The internal standard was used in prior methods (Applicant-**Figure 19**). The calibration curves were linear over the range from 1.00-1000 nM. Precision and accuracy met pre-specified acceptance criteria, and the samples were stable for up to 42 hours at autosampler temperature (15°C), sufficient time to permit completion of the analysis.

A Method for the Quantification of BIBW 2992 in Mouse Plasma (Concentration Range 1.00 - 1000 nmol/L) Using Solid Phase Extraction and HPLC-MS/MS: Validation and Stability Data (v435-10rm)

The purpose of this study was to validate a method for the quantification of BIBW2992BS in CD-1 mouse EDTA plasma over the concentration range of 1.0-1000 nM. The internal standard was the same as that used in all other methods (Applicant-**Figure 19**). This method was developed to support interpretation of experiments performed in Muta™Mice and Tg.rasH2 mice. Parameters evaluated included specificity, linearity, accuracy and precision, limits of quantitation, stability, carry-over and extraction recovery. The method developed in CD-1 mouse plasma was then evaluated for its applicability to muta™Mice and Tg.rasH2 mice. Parameters evaluated included specificity, sensitivity (LOQ), intra=batch accuracy and stability at RT.
The method met acceptance criteria for precision and accuracy (± 20%). The LOQ was 1 nM, and the calibration curves were linear over the range from 1.00-1000 nM. There was no endogenous interference observed.
Extraction recovery ranged from 62-72% over the concentration range tested (2.50-800 nM). Carry-over was 0.99% and 0.34% after the first and second blanks, respectively.
BIBW2992BS was stable in EDTA-mouse plasma for at least 6 hours at RT, and were stable following 3 freeze-thaw cycles:

Table 21: Room Temperature Stability of BIBW2992BS in EDTA-Mouse Plasma

Experimental information: Batches: 435S1, 453S3* Species/matrix: mouse plasma Analyte: BIBW 2992 BS				
Stress conditions	**6 h at room temperature**		**6 h at room temperature**	
Sample label	**UQ02_RT**	**UQ04_RT**	**UQ02_RT6H**	**UQ04_RT6H**
Nominal conc. [nmol/L]	2.50	800	2.50	800
	2.47	730	2.60	833
	2.36	790	2.77	861
	2.50	814	2.47	854
	2.30	849	2.65	836
	2.23	837	2.73	858
	2.38	843	2.70	869
Mean conc. [nmol/L]	**2.37**	**810**	**2.65**	**852**
Imprecision (CV)	**4.3%**	**5.5%**	**4.0%**	**1.7%**
Inaccuracy (dev.)	**-5.2%**	**1.3%**	**6.0%**	**6.5%**

Remarks:
Values for mean concentration and imprecision are calculated in KinLims.
Inaccuracy and duration of stress are calculated in Microsoft Excel.
* initial (435S1) and modified (435S3) IS conditions

(excerpted from Applicant's submission)

Table 22: Stability of BIBW2992BS After 3-Cycles of Freezing and Thawing

Experimental information: Batches: 435S1 Species/matrix: mouse plasma Analyte: BIBW 2992 BS		
Stress conditions	**3 Additional freeze/thaw cycles**	
Sample label	**UQ02_FT**	**UQ04_FT**
Nominal conc. [nmol/L]	2.50	800
	2.18	828
	2.18	817
	2.27	808
	2.28	807
	2.25	835
	2.32	842
Mean conc. [nmol/L]	**2.25**	**823**
Imprecision (CV)	**2.6%**	**1.8%**
Inaccuracy (dev.)	**-10.0%**	**2.9%**

Remarks:
Values for mean concentration and imprecision are calculated in KinLims.
Inaccuracy and duration of stress are calculated in Microsoft Excel.

(excerpted from Applicant's submission)

BIBW2990BS was stable for up to 202 days when stored at -20C. When stored in whole blood (on ice), BIBW2992BS was stable for up to 60 minutes. BIBW2992BS was also stable when stored in EDTA-mouse plasma at 16°C for 43 hours.

Table 23: Stability of BIBW2992BS After 43 Hours at 15°C

Experimental Information: Batches: 435S2 Species/Matrix: mouse plasma Analyte: BIBW 2992		
Stress conditions	**43 h in the autosampler at 15°C**	
Sample label	**UQ02_AS42**	**UQ04_AS42**
Nominal conc. [nmol/L]	2.50	800
	2.30	752
	2.39	757
	2.56	710
	2.55	757
	2.38	734
	2.39	779
Mean conc. [nmol/L]	**2.43**	**748**
Imprecision (CV)	**4.3%**	**3.2%**
Inaccuracy (dev.)	**-2.8%**	**-6.5%**

Remarks:
Values for mean concentration and imprecision are calculated in KinLims.
Inaccuracy and duration of stress are calculated in Microsoft Excel.

(excerpted from Applicant's submission)

Table 24: Stability of BIBW2992BS After Long-Term Storage at -20°C in EDTA-Mouse Plasma

Experimental information: Batch: 435S2 Analyte: BIBW 2992 Species/matrix: mouse plasma Stability sample preparation: 21.06.2010 / 26.05.2010 Calibration sample preparation: 14.12.2010				
Stress conditions	**176 days at -20°C**		**202 days at -20°C**	
Sample label	**UQ02_210610**	**UQ04_210610**	**UQ02_260510**	**UQ04_260510**
Nominal conc. [nmol/L]	2.50	800	2.50	800
	2.59	849	2.52	808
	2.71	808	2.40	791
	2.16	836	2.55	769
	2.54	873	2.25	837
	2.46	850	2.44	803
	2.67	808	2.25	846
Mean conc. [nmol/L]	**2.52**	**837**	**2.40**	**809**
Imprecision (CV)	**7.9%**	**3.1%**	**5.3%**	**3.6%**
Inaccuracy (dev.)	**0.8%**	**4.6%**	**-4.0%**	**1.1%**

Remarks:
Values for mean concentration and imprecision are calculated in KinLims.
Calculation of inaccuracy is done in Microsoft Excel.

(excerpted from Applicant's submission)

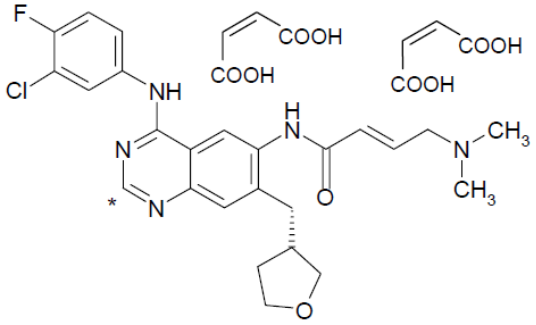
ISR was performed from samples obtained on Study DDB0084. Overall, 22 of the 24 percent met acceptance criteria following reanalysis; thus reproducibility was successfully demonstrated.
The method was also evaluated for applicability to Tg.RasH2 and Muta™Mice. There was no interference from plasma obtained from these two strains of mice. Accuracy and precision met acceptance criteria over the range evaluated (2.50-800 nM) and the stability was comparable under all conditions tested.

ADME

Absorption, Distribution and Excretion of [14C]BIBW 2992 MA2 in the Rat (A102_02RB)

The purpose of this study was to evaluate the ADME profile of BIBW2992BS in male Wistar rats following a single oral dose of 8 mg/kg or a single IV dose of 4 mg/kg. The potential for BIBW2992BS to partition to red blood cells was evaluated in whole blood from male and female Wistar rats.

Figure 21: Structure of [^{14}C]BIBW2992, Indicating the Position of the ^{14}C Radiolabel (*)



(excerpted from Applicant's submission)

Following a single oral or IV dose, plasma measurable concentrations were achieved for up to 216 hours (9 days) in whole blood; however, clearance from plasma occurred within 24 hours (Applicant-**Figure 22** and Applicant-**Figure 23**).
The Applicant then evaluated partitioning to RBCs and found that uptake by RBCs was rapid (detectable by 30 mins) and the ratio of RBC to plasma concentration increased over time (Applicant-**Table 26)**, indicating selective partitioning to RBCs. Importantly, AUCs of non-drug radioactivity were several orders of magnitudes higher than drug-associated AUCs in whole blood (Applicant-**Table 25**), indicating that in addition to some accumulation of the drug in the RBC fraction, metabolite-associated radioactivity exhibited strong association with the cellular compartment of the blood.

Figure 22: Mean Concentration-Time Profile in Plasma and Blood of [14C]BIBW2992BS After a Single IV Dose of 4 mg/kg to Male Wistar Rats

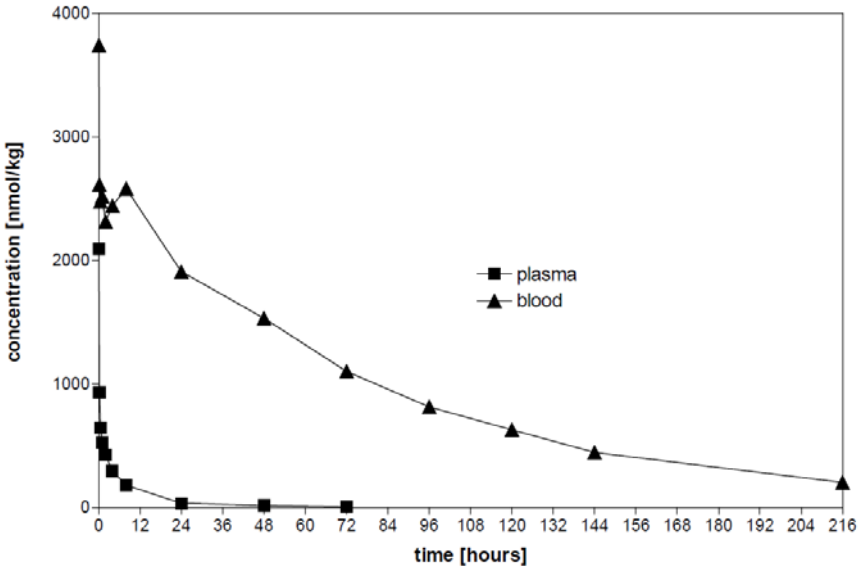


Figure 23: Mean Concentration-Time Profile in Plasma and Blood of [14C]BIBW2992BS After a Single Oral Dose of 8 Mg/Kg to Male Wistar Rats

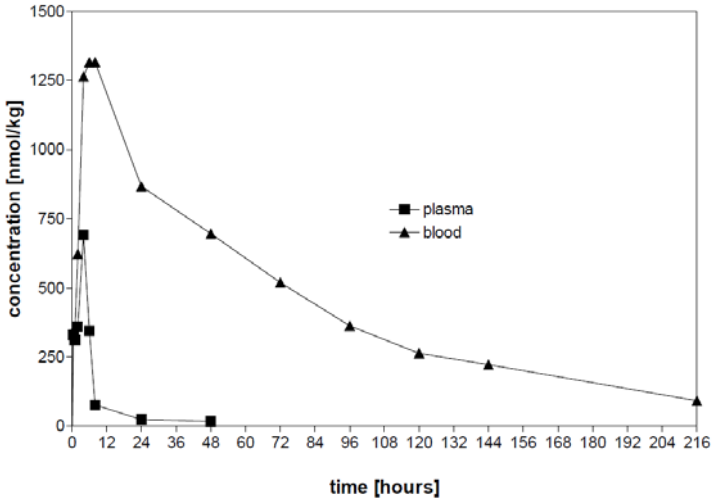


Table 25: Summary of Mean Pharmacokinetic Parameters of Drug and Radioactivity in Blood and Plasma after IV or PO Administration of BIBW2992BS in Male Wistar Rats

Parameter	Unit	Radioactivity				BIBW 2992	
		plasma		blood		plasma	
		i. v.	p. o.	i. v.	p. o.	i. v.	p. o.
Dose	[mg/kg]	4	8	4	8	4	8
C(max)	[nmol/L]	1220	468	3750	1360	1620	397
t(max)	[h]	-	4*	-	7*	-	4*
t(1/2)	[h]	24.4	22.1	60.2	62.6	5.22	4.54
AUC(0-∞)	[(nmol·h)/L]	4160	5630	222000	101000	2920	2600
AUC(0-24h)	[(nmol·h)/L]	3190	4320	55900	24100	2500	2540
CL(/F)	[mL/(min*kg)]	33.2	64.7	0.624	2.78	55.3	108
MRT	[h]	17.7	19.6	82.6	85.4	4.95	6.65
V(ss)(/F)	[L/kg]	35.3	78.3	3.09	14.2	16.2	43.6
V(z)(/F)	[L/kg]	70.1	133	3.24	15.2	25.0	42.8

* median

Table 26: Ratio of the Concentration in RBCs and Plasma (CC/CP) After Single IV or Oral Administration of [^{14}C]BIIB2992BS to Male and Female Rats.

time	i.v.		p.o.	
[h]	male	female	male	female
0.5	8.6	8.8	4.9	8.2
4	28.4	23.2	15.8	20.7
24	129.9	192.2	188.1	262.9

The calculated steady-state volume of distribution (3.3 and 6.2 for IV and oral administration, respectively) suggests extensive tissue penetration. Whether this is accounted for primarily by the extensive erythrocyte partitioning, is unclear.
Elimination was evaluated in both intact and bile duct-cannulated (BDC) rats. The majority of the radioactivity was eliminated in feces, as illustrated in Applicant-**Figure 24** and Applicant-**Figure 25**. Only about 3% of the total dose was eliminated in urine over the 96 hour collection period.
In BDC Rats, biliary excretion was measured for 6 hours following IV and intraduodenal (ID) eliminated. Elimination via bile accounted for 28.3% and 12.9%, respectively, of eliminated dose (Applicant-**Figure 26** and Applicant-**Figure 27**); however elimination was not complete at the end of the collection period.

Figure 24: Cumulative Excretion of Radioactivity After IV Administration of 4 mg/kg [^{14}C]BIBW2992BS to Rats

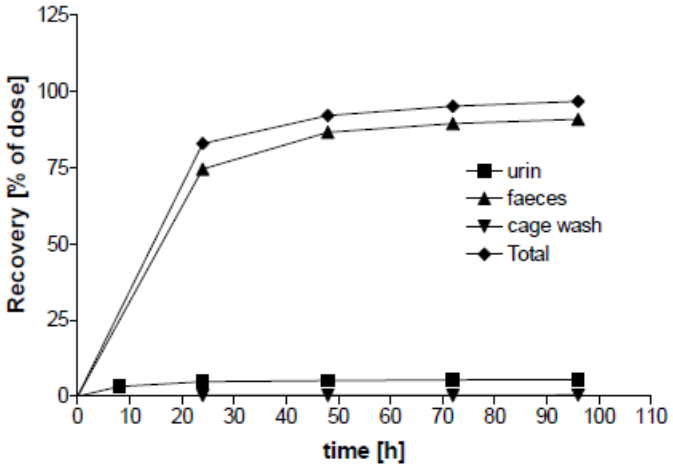


Figure 25: Cumulative Excretion of Radioactivity After Oral Administration of 8 mg/kg [^{14}C]BIBW2992BS to Rats

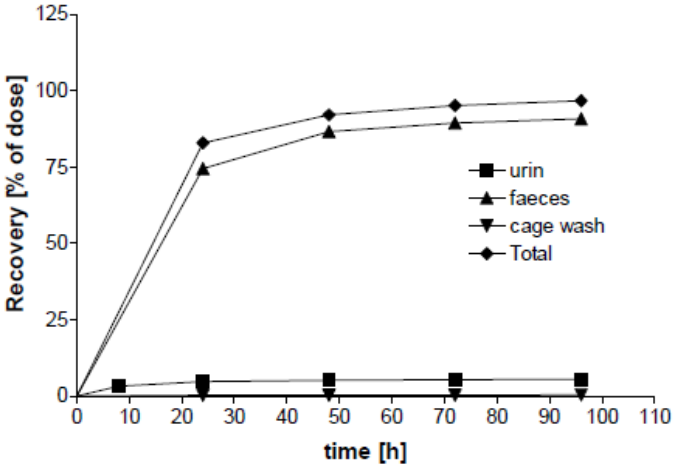


Figure 26: Individual and Average Cumulative Biliary Excretion of Radioactivity After IV Administration of 4 mg/kg [^{14}C]BIBW2992BS to rats.

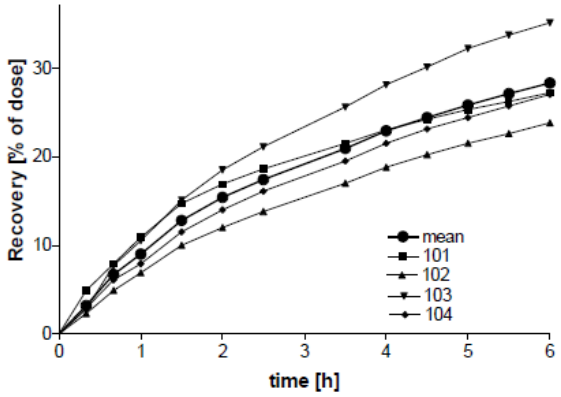
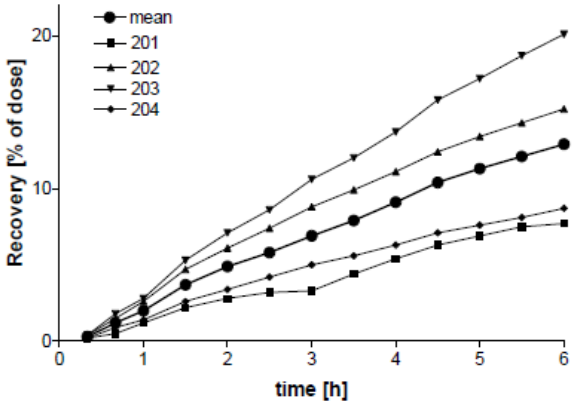


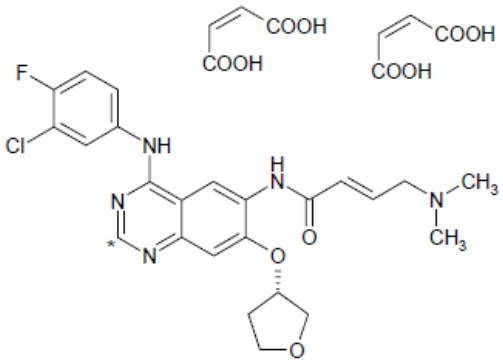
Figure 27: Individual and Average Cumulative Biliary Excretion of Radioactivity After ID Administration of 8 mg/kg [^{14}C]BIBW2992BS to rats.



Absorption, Distribution and Metabolism of BIBW 2992 MA2 in Minipigs (A239_ 05TE A129_05RB)

The minipig was used as the nonrodent species to characterize the nonclinical safety profile of BIBW2992BS; accordingly, the purpose of this mass balance study was to evaluate the metabolite profile of [^{14}C]BIBW2992BS in the Göttengen minipig.
Two male and 2 female minipigs received an oral dose of 2.46 mg/kg of [^{14}C]BIBW2992BS (Applicant-**Figure 28**), and samples of feces, urine, and plasma were obtained and analyzed for the presence of radioactive compounds. Spot samples of bile were collected at 6- and 24-hours post-dose by ultrasonographically-guided puncture of the gallbladder in anesthetized animals.

Figure 28: Structure of BIBW2992BS and Location (*) of the ^{14}C Radiolabel



The specific activity of the compound was 0.711 MBq/umol; thus, a dose of 2.46 mg/kg BIBW2992BS produced an exposure of approximately 23-29 MBq/animal (~ 2MBq/kg). As indicated in Applicant-**Table 27**, mass balance was achieved in this study. Only a small portion (<1%) of the radioactivity was recovered from the cage wash.

Table 27: Cumulative Excretion Balance After Oral Dosing of [^{14}C]BIBW2992BS to Male and Female Minipigs

sample	interval [h]	101	102	151	152	mean	CV (%)
urine	0 - 8	0.49	1.16	0.02	0.73	0.60	79.3
	8 - 24	0.42	2.41	0.22	N.C.	1.02	119.1
	24 - 48	0.16	0.62	0.08	1.08	0.49	95.4
	48 - 72	0.16	0.24	0.10	0.00	0.13	80.9
	72 - 96	0.05	0.17	0.11	0.15	0.12	44.1
	96 - 120	0.04	0.11	0.04	0.10	0.07	52.1
	120 - 144	0.03	0.07	0.01	0.04	0.04	66.7
	144 - 168	0.02	0.05	0.01	0.03	0.03	62.1
subtotal		1.4	4.8	0.6	2.1	2.2	81.9
faeces	0 - 24	0.3	1.0	0.2	0.5	0.5	71.8
	24 - 48	67.7	19.8	52.0	4.6	36.0	80.3
	48 - 72	22.6	57.9	36.1	50.0	41.6	37.5
	72 - 96	1.9	10.6	6.7	26.1	11.3	92.9
	96 - 120	0.9	1.4	1.4	3.9	1.9	72.9
	120 - 144	0.4	1.5	0.4	0.9	0.8	68.5
	144 - 168	0.6	1.7	0.4	0.4	0.1	80.6
subtotal		94.2	93.9	97.1	86.5	92.9	4.9
bile	6	NOS	1.0	1.1	NOS	1.0	12.9
	24	NOS	NOS	NOS	0.06	0.06	N.A.
subtotal		N.A.	1	1.1	0.1	0.7	75.1
total (cw inclusive)		95.6	99.5	98.2	88.9	95.7	4.9

data source file: NCPK_tables..xls

After oral dosing, the majority (93%) of the radioactive dose was recovered in feces over the 168 hour collection interval. Unchanged parent constitutes a large quantity of the eliminated dose in both feces and bile.

As there was no IV dose arm, it is unclear how much of the unchanged parent present in feces represents unabsorbed dose. Only ~ 2% was recovered in urine. Excretion was slow, with the majority of the dose being excreted within 96 hours. There was no gender effect on the excretion pattern observed. A summary of the metabolite excretion pattern in male and/or female minipigs is given in **Table 28** and Applicant-**Figure 29**.

The structures of the various metabolites were derived by LC-MS/MS and they are summarized in Applicant-Figure 30**.** BBW2992BS was found adduct to protein by virtue of its chemical reactivity. The process likely proceeds via Michael addition, and is presumably independent of enzymatic activities. In addition, BIBW2992BS was found to undergo a number of Phase I modifications, including oxidative *N*-demethylation, *N*-oxidation, and oxidative *N*- and *O*-dealkylation, as indicated in Applicant Figure 30.

Figure 29: Average Cumulative Recovery After Single Oral Administration of [^{14}C]BIBW2992BS to Male and Female Minipigs

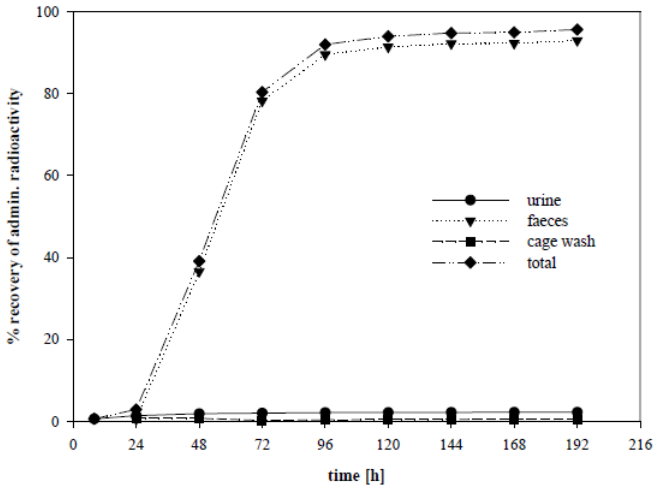


Figure 30: Proposed Metabolic Scheme of [^{14}C]BIBW2992BS in Male and Female Minipigs

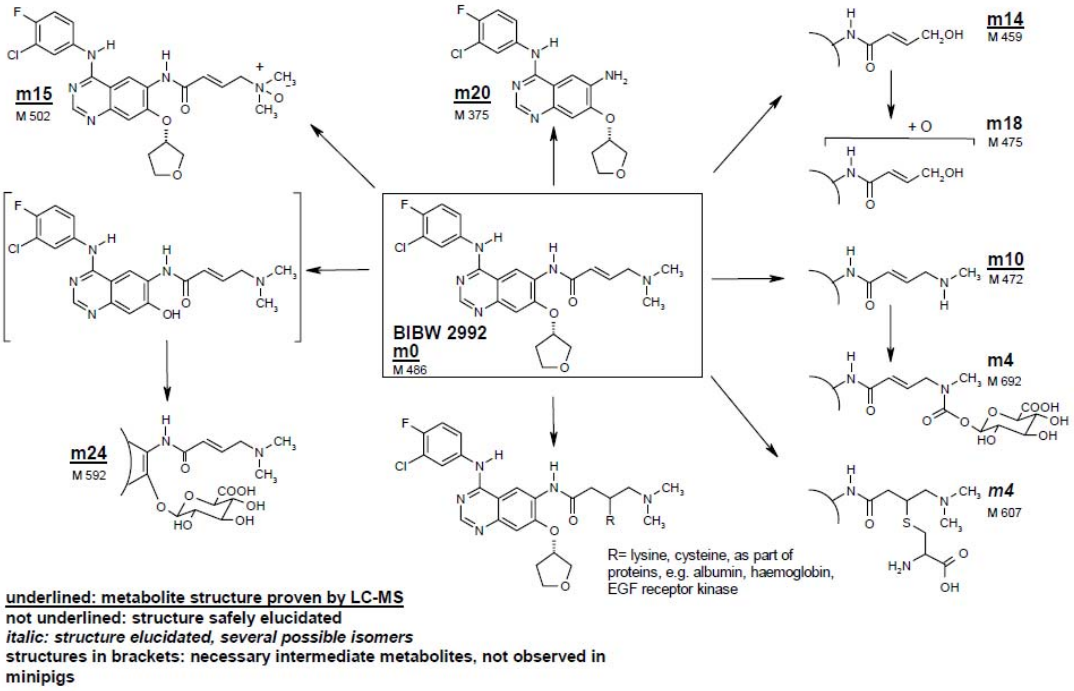


Table 28: Metabolite Pattern (%dose) in Male and/or Female Minipigs Summarized by Matrix

Metabolite	Urine	Bile	Feces
M20	0.01	--	--
M16	--	0.02	--
M14	0.05	0.11	1.44
M10	0.33	0.273	13.75
M18	0.05	0.10	--
M0	0.45	0.827	68.85
M15	1.1	0.19	--
M24	0.08	--	--
M26	--	--	2.72
M17	0.11	--	--
M4	0.26	--	3.59
M27	--	--	3.33

Individual organ radioactivity was also examined in one male and one female minipig at the 168 hour post-dose timepoint. As evident from Applicant-**Table 29**, exposure was widespread; however, the majority of the dose was recovered from the liver and spleen. Whether this is a reflection of the tight RBC binding observed in this species (Applicant-Table 30), is unclear. The carcass was not flushed of blood prior to collection of the organs; thus, the radioactivity measured could reflect the relative residual blood cell

volume present in the organ, rather than a selective distribution of the compound to these particular tissues (i.e. a target-mediated disposition).
Autoradiography of the eyes demonstrated binding to melanin (Applicant-**Figure 31**).

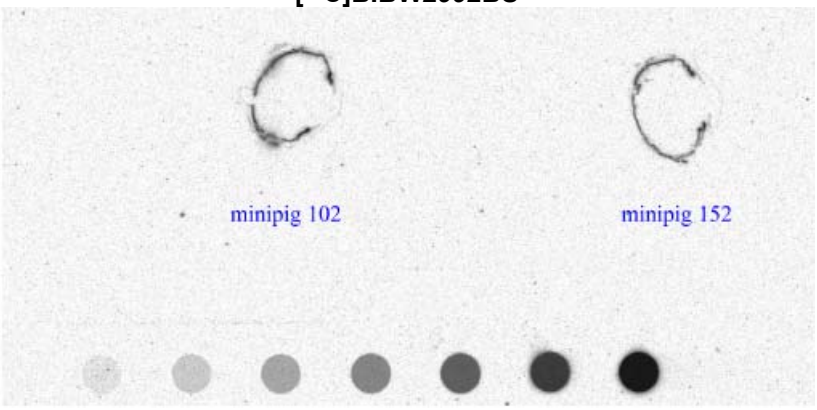
Table 29: Radioactivity in Organs and Tissues of Minipigs After Single Oral Administration of [^{14}C]BIBW2992

	102		152	
Organs and tisues	recovery % of dose	Concentration nmol/kg	recovery % of dose	Concentration nmol/kg
epidydimis	0.006	152	NOS	NOS
adrenal	0.0002	125	0.0002	107
kidney	0.06	366	0.02	297
skin	-	54.3	-	59.8
bone marrow	-	162	-	235
fat	-	69.0	-	44.5
heart	0.03	267	0.02	167
lungs	0.04	392	0.04	343
spleen	0.06	619	0.05	1020
liver	0.34	1180	0.27	953
thyroid	0.0006	380	0.0003	258
testes	0.1	1460	NOS	NOS
ovar	NOS	NOS	0.0006	57.6
muscle	-	202	-	104

Table 30: Distribution of Radioactivity Between Blood Cells and Plasma (Expressed as Ratio of Cc:Cp) After a Single PO Dose of [^{14}C]BIBW2992 in the minipig

[h]	101	102	151	152
6 h	5.346	4.943	5.550	4.262
24 h	14.096	13.008	9.904	11.175
72 h	23.382	22.038	19.460	14.688
96 h	30.786	25.108	24.604	24.189
168 h	29.819	41.094	14.835	25.376

Figure 31: Ocular Accumulation in the Minipig After Single Oral Administration of [^{14}C]BIBW2992BS



Pharmacokinetics of BIBW 2992 MA2 After Oral or Intravenous Administration in the Minipig (A142_05RB)

The pharmacokinetic profile and absolute bioavailability of BIBW2992BS was assessed in the minipig following a single IV dose of 2 mg/kg followed by a single oral dose of 2 mg/kg. Pharmacokinetic parameters are summarized in Applicant-**Table 31**). BIBW2992BS exhibits low oral bioavailability and a relatively high clearance, high volume of distribution but a relatively long terminal elimination half-life (Applicant-**Figure 32**), likely the result of its tendency to partition into RBCs. There was no effect of gender on exposure. No attempt to assess dose-linearity was made, as the study was conducted at a single dose-level.

Figure 32: Concentration-Time Profile of BIBW2992BS after Oral or IV Administration in Minipigs

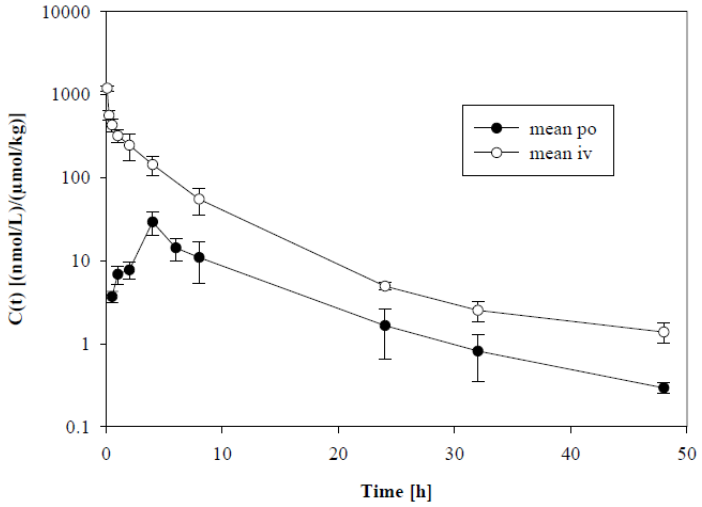


Table 31: Mean pharmacokinetic parameters of BIBW2992BS PO and IV Administration to Minipigs

Parameter	Unit	p.o.	i.v.
Dose	[µmol/kg]	4.12	4.12
Dose	[mg/kg]	2	2
t(max)	[h]	4	0.083
C(max)	[nmol/L]	29.1	1190
AUC(0-inf)	[nmol·h/L]	214	2000
Total plasma clearance	[mL/min/kg]	NA	35.4
Total mean residence time	[h]	11.3	5.90
Mean absorption time	[h]	5.38	NA
Terminal half life	[h]	10.8	13.8
Volume of distribution at steady state	[L/kg]	NA	12.4
Bioavailability	(%)	11.2	NA

NA = not applicable

Excretion, Distribution in Blood and Pharmacokinetics of Radioactivity and Parent Compound After Oral or Intraduodenal Administration of [^{14}C]BIBW 2992 MA2 to Female Rabbits

The purpose of this study was to evaluate the ADME profile of [^{14}C]BIBW2992BS in the Himalayan rabbit after administration by the PO (4 mg/kg) and ID (1.95 mg/kg) routes. The following matrices were sampled: blood, plasma, urine, feces and bile (one timepoint, at 4 hr post-dose). The specific activity of the compound was 0.391 MBq/µmol; thus, a dose of 1.95 mg/kg yielded a total radioactive dose of between 4.26-4.88 MBq per animal. The study achieved mass balance, as over 95% of the administered radioactive dose was accounted for in the matrices collected.
The observed PO pharmacokinetic parameters are listed in Applicant-**Table 32**. As this was a single dose-level study, there was no attempt to assess dose-linearity.
The bulk of the radioactivity was retrieved from the feces. As no IV arm was assessed, it is not possible to ascertain whether the bulk is unabsorbed parent; however, this is possible since in a PK study conducted in another nonrodent species (the minipig), absolute bioavailability was low. Similar to the minipig, biliary excretion accounted for a large proportion of the radioactivity at the 4-hour timepoint (Applicant-**Table 33** and Applicant-**Table 34**); thus, the large concentration observed in the feces can be partially accounted for by excretion by the biliary route.
Similar to the rat and the minipig, strong partitioning to RBCs was observed in the rabbit (Applicant-**Table 35**).

Table 32: Selected PK Parameters in Female Himalayan Rabbits Following a Single PO Dose (2 mg/kg) of [^{14}C]BIBW2992BS

Parameter	Unit	Radioactiovity		Parent
		blood	plasma	compound
C(max)	[nmol/L]	126	76.5	34.0
t(max)*	[h]	1	1	1
t(1/2)	[h]	142	156	2.60
AUC(0-inf)	[(nmol·h)/L]	4060	1600	178
MRT(tot)	[h]	172	165	4.31
CL/F	[mL/(min*kg)]	17.5	42.2	467
AUC(0-24h)	[(nmol·h)/L]	999	552	188

*: median values

Table 33: Urinary and Fecal Excretion (% Radioactive Dose) Following a Single PO Dose to Female Rabbits

Sample	Time point	151	152	153	N	mean	CV	SD
URINE	0 - 8 h	0.3	0.2	0.5	3	0.3	45.8	0.2
	8 h - 24 h	0.0	NS	0.3	2	0.2	141.4	0.2
	24 h - 48 h	0.3	0.1	0.1	3	0.2	69.3	0.1
	48 h - 72 h	0.1	0.1	0.1	3	0.1	0.0	0.0
	72 h - 96 h	0.1	0.1	0.1	3	0.1	0.0	0.0
Subtotal		0.8	0.5	1.1	3	0.8	37.5	0.3
FAECES	0 - 24 h	44.9	37.5	49.0	3	43.8	13.3	5.8
	24 h - 48 h	7.9	21.2	41.9	3	23.7	72.4	17.1
	48 h - 72 h	4.5	7.3	6.5	3	6.1	23.6	1.4
	72 h - 96 h	15.6	20.3	1.6	3	12.5	77.8	9.7
	96 h - 168 h	21.7	6.3	NS	2	14.0	77.8	10.9
Subtotal		94.6	92.6	99.0	3	95.4	3.4	3.3
CAGE WASH	0 - 24 h	0.0	0.0	0.1	3	0.0	173.2	0.1
	24 h - 48 h	0.0	0.0	0.1	3	0.0	173.2	0.1
	48 h - 72 h	0.0	0.0	0.0	3	0.0	0.0	0.0
	72 h - 96 h	0.0	0.0	0.0	3	0.0	0.0	0.0
Subtotal		0.0	0.0	0.2	3	0.1	173.2	0.1
Total		95.4	93.1	100.3	3	96.3	3.8	3.7

NS no sample

Table 34: Biliary Excretion (% Radioactive Dose at 4 hrs) Following a Single ID Dose to Female Rabbits

Sample	Time point	152	153	N	mean	CV	SD
BILE	4 h	24.3	21.3	2	22.8	9.3	2.1

Table 35: Distribution of Radioactivity Between Blood Cells and Plasma (Expressed as Ratio of Cc:Cp) After a Single PO Dose of [14C]BIBW2992 in the Female Rabbit

time[h]	151	152	153	mean	SD	CV (%)
1h	2.13	2.25	2.95	2.45	0.44	18.1
4 h	3.43	2.39	2.53	2.78	0.57	20.4
24 h	4.64	3.87	5.28	4.60	0.71	15.3
96 h	6.19	7.41	8.60	7.40	1.21	16.3

Absorption, Distribution and Excretion of [^{14}C]BIBW2992BS After Oral Administration to Mice (a227-10rb)

The ADME profile of [^{14}C]BIBW2992BS was assessed in mice following a single oral dose of 8.5 mg/kg. The specific activity of the drug was approximately 0.31 MBq/µmol; thus, the administered radioactive dose was approximately 0.8 MBq per animal. As demonstrated in Applicant-
Table 36, the bulk of the radiolabel was eliminated in the feces. A small portion was eliminated in urine (~ 1%). Although some of the radiolabel was excreted in bile (~10%), it was lower than was observed in the rabbit and the minipig. As with the rabbit and minipig, however, no IV arm was conducted to assess the amount of unabsorbed parent that remained following PO administration; thus, it is unclear whether the

radiolabel concentration represents unabsorbed parent or metabolite, as the analytical method (liquid scintillation) was not able to differentiate the radioactive species.

Table 36: Mean Excretion (% Radioactive Dose) after PO Administration of 8.5 mg/kg [^{14}C]BIBW2992BS to Male and Female Mice

Route of excretion	Time period [h]	male*	female*
urinary	0 - 24	1.3	1.0
	0 - 96	**1.3**	**1.0**
faecal	0 - 24	89.8	76.7
	0 - 48	94.7	94.7
	0 - 96	**95.2**	**95.5**
(cage wash)	0 - 96	0.3	0.3
Total	**0 - 96**	**96.8**	**96.8**
biliary excretion	**0 - 6**	**males****	**females****
		9.1	**11.1**

* Pool of 5 mice
** Mean of 4 individual mice

BIBW 2992 MA2: Whole Body Autoradiography After Intravenous Administration of [^{14}C]BIBW2992 MA2 in Male Albino and Male Pigmented Rats and Oral Administration in Male Albino Rats (A058_03UB)

Whole body autoradiography (WBA) was performed after IV or oral administration of 4 or 8 mg/kg [^{14}C]BIBW2992BS, respectively, in male albino rats, and following IV administration of 4 mg/kg BIBW2992BS to male pigmented rats. The specific activity of [^{14}C]BIBW2992BS was 1.02MBq/mg, and this was mixed 1.5:1.0 with cold BIBW2992BS in the formulation of the test article.

In albino rats, [^{14}C]BIBW2992BS distributed rapidly following IV administration, to all tissues except the CNS. At early timepoints, the highest concentrations by this method were observed in the kidney, adrenals and brown fat. At later timepoints, the spleen, pituitary, and accessory sex organs of the male were found to have the highest concentrations, as indicated in Applicant-**Table 37**. A similar pattern was observed at the higher dose level. It is not clear that this is a target-mediated distribution, however, as the spleen (by virtue of RBC sequestration) and the kidney (by virtue of perfusion) have high RBC content. Because the drug partitions strongly to RBCs, tissues with high RBC content may exhibit apparently high drug levels.

In the pigmented rat, the highest concentrations were observed in the retina, indicative of melanin binding (Applicant-**Table 38**); drug accumulation in other tissues was similar to that observed in the albino rat (Applicant-**Figure 33** and Applicant-**Figure 34**).

Table 37: Concentration-Time Profile Radioactivity Distribution in Tissues of Male ALBINO Rats Following IV Administration of [^{14}C]BIBW2992BS

tissues	0.083 h			4 h			24 h			96 h		
	N	mean	CV (%)	N	mean	CV (%)	N	mean	CV (%)	N	mean	CV (%)
brain	6	81	18	6	64	9	6	30	14	6	15	19
pituitary	1	29978	n.c.	2	33175	9	1	6065	n.c.	1	132	n.c.
pineal gland	0	n.d.		1	14308	n.c.	0	n.d.		0	n.d.	
Harder's gland	2	3673	4	3	11839	1	2	6969	12	2	482	3
salivary gland	7	16487	6	7	9841	15	9	1330	7	5	403	8
brown fat	4	41881	10	3	8579	2	2	2170	0	2	537	3
thymus	8	3975	4	8	7878	4	3	1611	3	3	311	6
lungs	10	25590	18	9	12289	11	10	2146	7	9	620	7
heart muscle	8	28037	9	10	4637	6	8	1582	6	9	689	4
blood (heart)	13	3105	8	12	2306	7	8	2045	7	5	740	9
liver	15	24427	4	16	12345	5	16	2850	4	15	1210	3
spleen	2	10668	6	3	30149	4	3	4045	7	3	1792	5
pancreas	8	24607	4	8	7065	5	5	1425	6	4	479	7
kidney total	2	45697	7	2	21324	0	2	7368	2	2	2903	2
kidney cortex	10	58982	8	9	11796	5	8	4206	6	9	1730	10
kidney medulla	8	38718	11	7	27228	10	7	11847	5	7	5164	9
kidney pelvis	2	30596	31	2	34434	2	2	7444	6	2	2173	3
adrenal total	1	54190	n.c.	1	15865	n.c.	1	3662	n.c.	1	1650	n.c.
adrenal cortex	3	60688	2	2	17688	2	2	4074	1	2	1809	12
adrenal medulla	0	n.d.		1	10524	n.c.	1	1840	n.c.	1	902	n.c.
fat	10	757	24	8	154	21	8	51	38	6	11	46
testis	8	197	16	9	362	6	6	421	5	6	219	7
epididymis	3	1032	20	2	1322	30	3	994	30	3	334	7
skin (total)	15	2519	25	14	2741	6	13	673	15	12	278	8
bone marrow	8	5755	10	8	10480	12	4	1084	10	4	238	9
access. genital gl.	1	12583	n.c.	5	16288	13	1	4315	n.c.	0	n.d.	
tongue	4	14513	13	2	4574	5	6	962	6	5	435	6
muscle (cranial)	10	9836	29	15	3667	5	16	938	7	16	450	8
muscle (caudal)	12	9039	13	16	3796	11	16	1004	9	16	475	6

n.c. = not calculated; n.d. = not detected
File: JBARG096.XLS

Table 38: Concentration-Time Profile Radioactivity Distribution in Tissues of Male PIGMENTED Rats Following IV Administration of [^{14}C]BIBW2992BS

tissues	4 h			24 h			96 h		
	N	mean	CV (%)	N	mean	CV (%)	N	mean	CV (%)
retina	2	50224	4	3	77688	12	2	51940	12
brain	6	88	12	6	44	29	6	36	31
pituitary	1	40490	n.c.	1	19196	n.c.	1	1211	n.c.
Harder's gland	2	11235	3	2	4946	6	2	383	8
salivary gland	7	11891	15	8	1630	3	8	418	4
brown fat	6	9229	8	3	2162	11	2	377	10
thymus	6	9410	4	5	2551	3	4	691	7
lungs	11	8513	20	12	2247	4	9	838	8
heart muscle	12	5121	6	9	1660	7	11	649	4
blood (heart)	11	2402	7	10	2218	4	12	884	3
liver	16	11662	6	16	3942	2	16	1200	3
spleen	2	33007	3	2	7491	1	3	2897	8
pancreas	6	9028	5	4	1632	2	5	488	7
kidney total	2	22509	3	2	8009	2	1	2918	n.c.
kidney cortex	10	12221	6	6	4601	6	3	1718	2
kidney medulla	8	30938	8	6	13515	3	4	5008	3
kidney pelvis	2	37186	1	2	7649	1	1	3109	n.c.
adrenal total	1	24284	n.c.	1	4415	n.c.	0	n.d.	
adrenal cortex	2	29171	2	2	4169	2	0	n.d.	
adrenal medulla	1	22363	n.c.	1	2573	n.c.	0	n.d.	
fat	8	276	31	8	76	36	13	10	45
testis	6	380	4	8	413	5	8	221	9
epididymis	2	1858	5	3	930	27	4	238	15
skin (total)	16	3646	8	11	3360	19	16	966	96
bone marrow	9	10092	6	10	2032	19	9	512	13
Access. genital gl.	2	14922	7	4	4070	11	1	3214	n.c.
tongue	5	4953	3	4	1107	3	4	439	7
muscle (cranial)	16	3866	6	16	932	11	16	485	10
muscle (caudal)	16	3570	11	16	1036	7	16	475	9

n.c. = not calculated; n.d. = not detected
File: JBARG096.XLS

Figure 33: Whole Body Autoradiogram of a Male ALBINO Rat 96 h After IV Administration of 4 mg/kg of [^{14}C]BIBW2992BS.

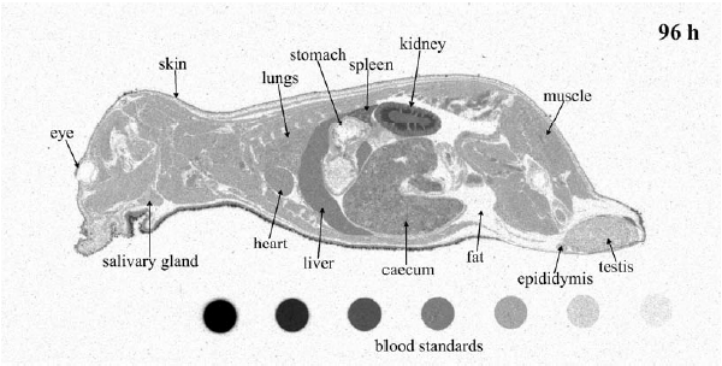
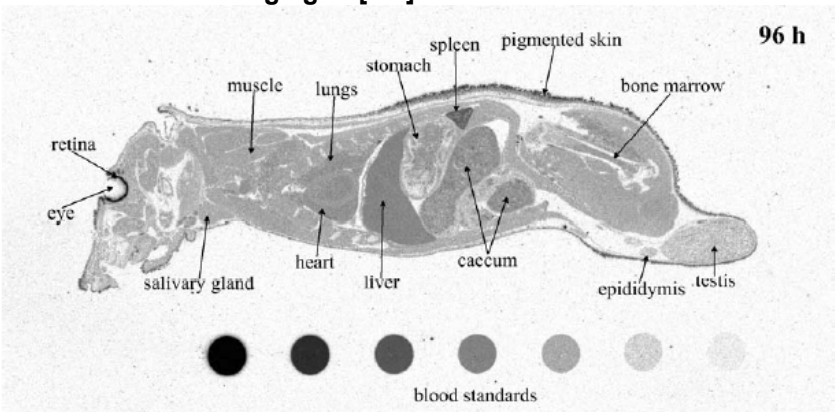


Figure 34: Whole body Autoradiogram of a Male PIGMENTED Rat 96 h After IV Administration of 4 mg/kg of [^{14}C]BIBW2992BS.



Metabolism of BIBW 2992 MA2 in Rats and Covalent Binding of BIBW 2992 MA2 to Blood Constituents (A224_02TE)

The purpose of this study was to evaluate the PK and routes of clearance of [^{14}C]BIBW2992BS in male and female rats after IV (4 mg/kg) and ID (8 mg/kg) dosing, and to structurally identify the metabolites of [^{14}C]BIBW2992BS. In addition, the binding of BIBW2992BS and/or its metabolites to blood and blood proteins was also evaluated. Portions of this study have been separately reported under Study A102-02RB (see **Section 5.2.1,** above for a description of the PK and routes of elimination). Incubation of [^{14}C]BIBW2992BS with protein and hemoglobin demonstrates that the molecule forms covalent attachments via Michael Addition to cysteine-bound SH groups (Applicant-Figure 35;

Table 39). The high level of drug-associated radioactivity associated with hemoglobin partially explains the observed partitioning to RBCs. Covalent binding to other plasma proteins was also demonstrated, and was dependent on temperature and incubation time. By LC-TOF, the Applicant demonstrated that BIBW2992 bound to lysine residue 190 of HSA.

Figure 35: Covalent Binding of [^{14}C]BIBW 2992 MA2 to Human Plasma Proteins

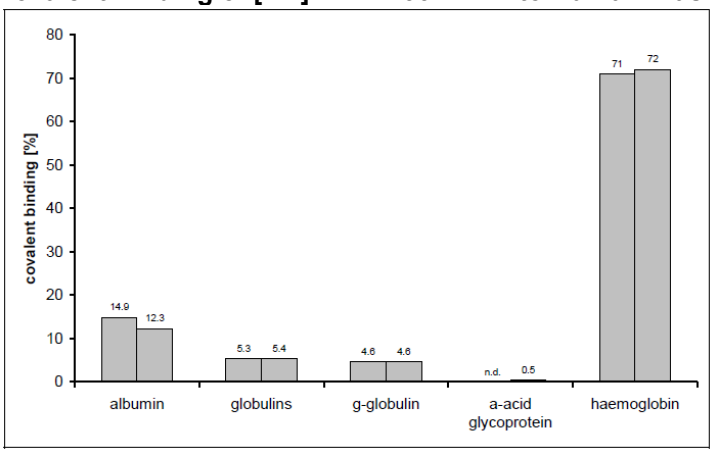


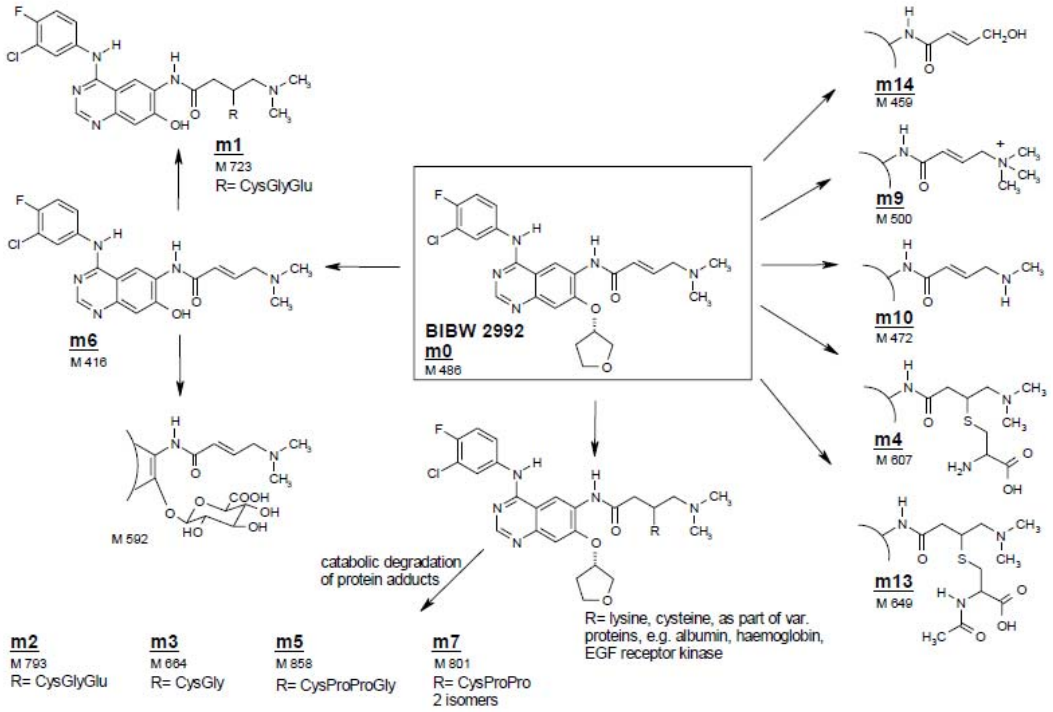
Table 39: BIBW2992 Metabolites Identified in Rats

Metabolite	Presumed Reaction or Structure
M3	Michael adduct; R = CysGly
M4	Michael Adduct with cysteine
M5	Michael adduct ; R = CysProProGly
M6	*O*-desalkyl-BIBW2992
M7	Michael adduct; R = CysProPro
M8	Unknown
M9	*N*-methylated quarternary ammonium BIBW2992
M10	*N*-desmethyl BIBW2992
M11	Unknown
M12	Unknown
M13	Michael adduct with N-acetyl-cysteine
M14	*N*-dealkylated (dimethyl-amino) BIBW2992
unspecified	Glucuronide of *O*-dealkylated BIBW2992 (by MS-TOF)

BIBW2992 is primarily eliminated as parent via bile and feces. Relatively little of the administered dose was eliminated in urine (approximately 13% over 24 hrs.; Applicant-**Table 40**).

The majority of BIBW2992 metabolites arose via nonspecific (i.e. non-enzymatic) conjugation to endogenous proteins (Applicant-**Figure 36**). The Applicant proposes that some of the biliary metabolites were potentially catabolic degradation products of BIBW2992 protein adducts that were subsequently removed prior to elimination. Other metabolic products were formed by dealkylation reactions or mixed deaklylation-conjugation reactions. The Applicant claims that there was no apparent effect of gender on metabolism.

Figure 36: Proposed Metabolic Scheme of [^{14}C]BIBW2992BS in Male and Female Rat



underlined: metabolite structure proven by LC-MS, comparison with reference compounds or unequivocal other data
not underlined: structure safely elucidated
italic: structure elucidated, several possible isomers
structures without code: metabolites detected by LC-MS but not observed by radioactivity detection
structures in brackets: necessary intermediate metabolites, not observed in rats

Table 40: Biliary and Urinary Excretion of BIBW2992BS and its Metabolites in the Rat

metabolite designation	relative excret. (% of dose) bile (0 - 6 h) and urine (0 - 24 h)	
	i.v. dosing	i.d. dosing
m1	1.5	0.9
m2	4.9	1.8
m4	5.0	2.4
m5	2.4	0.8
m13	1.7	1.0
m14	1.3	0.5
m9	1.2	0.2
m0	13	6.5
sum	28	13
minor metabolites	1.9	0.6

data rounded to two significant digits

[14C]BIBW 2992 MA2: Species Comparison of in Vitro Plasma Protein Binding and in Vitro Distribution into Blood Cells.

The Applicant evaluated the extent of plasma protein binding of [^{14}C]BIB2992BS in plasma from humans, rats, minipigs and the nude mouse, by equilibrium dialysis at concentrations ranging between 50-500 nM. There were no significant differences observed across species (Applicant-**Table 41**); though binding was reportedly slightly lower in females than males. Binding was independent of concentration (i.e. not saturable) over the concentration range tested.

Table 41: Mean Binding of [^{14}C]BIBW2992BS to Plasma Proteins

species	N	f_B mean (%)	SD
human	18	95.0	0.5
minipig (Göttingen)	18	92.9	0.4
rat (CrlGlxBrlHan:WI)	17	92.6	0.4
mouse (Hsd:NMRI nu/nu)	3	94.3	0.4

Metabolism of BIBW 2992 MA2 in Female Rabbits (A277/08TE)

The purpose of this study was to evaluate the metabolism of [^{14}C]BIBW2992BS in the rabbit, a species used in the evaluation of reproductive toxicity. Young adult female rabbits received 4 µmol/kg by the ID or PO routes (~ 4-5 MBq/subject). The specific activity of the material was 0.734 MBq/µmol. The study achieved mass balance, as over 95% of the radiolabel was accounted for in the matrices collected.
The majority (~ 95%) of the excreted radiation was detected in the feces. Bile samples collected during the first 4 hours after dosing contained ~23% of the radioactive dose, indicating that a significant proportion of the excreted material was eliminated in bile. Around 0.8% of the radioactive dose was eliminated in the urine.

Applicant-**Figure 37** and Applicant-**Table 42** summarize the proposed metabolites and their relative abundances, respectively, in the rabbit. The major circulating species observed in rabbits was parent (M0). Other metabolites were formed either by nonenzymatic (Michael) adduction to endogenous proteins, adduction to glutathione, or by dealkylation with or without conjugation to other nucleophilic compounds (*e.g.* glutathione, cysteine, *N*-acetylcysteine, etc.).

Figure 37: Proposed Metabolic Scheme of [^{14}C]BIBW2992BS in the Female Rabbit

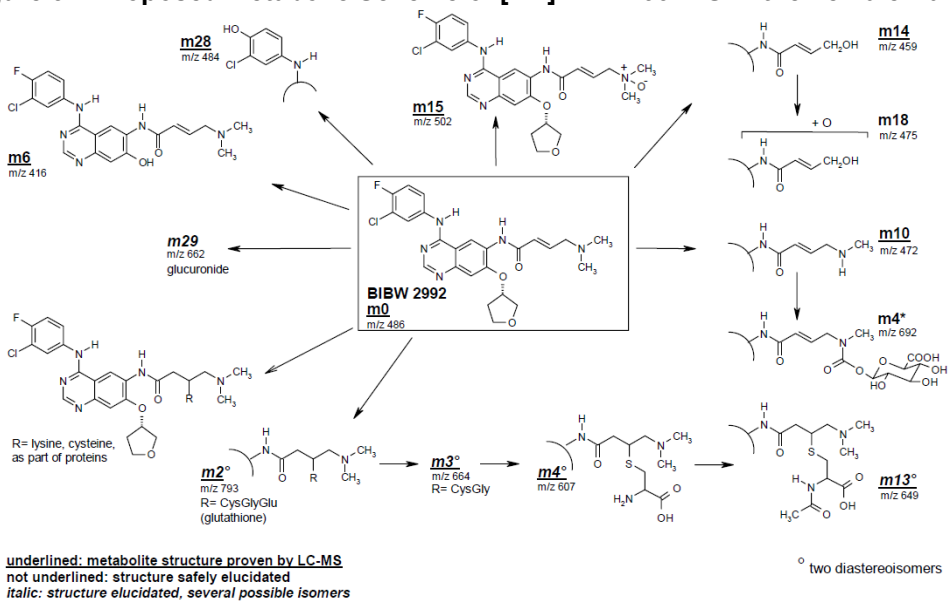


Table 42: Rabbit Metabolites

RT (mean) [min.]	metabolite	conc. of radioactivity [nmol/g] 1 h	4 h	relative amounts (% of sample radioact) 1 h	4 h
7.6	m28	15.7	14.3	1.8	1.6
8.8	m22	13.0	20.5	1.5	2.3
10.0	m6	42.7	96.3	5.0	10.8
10.6	m2°	228.6	132.9	26.6	14.9
12.0	m29	23.2	90.1	2.7	10.1
13.5	m3°	88.6	106.1	10.3	11.9
15.4	m4°	15.5	66.9	1.8	7.5
16.8	m4*	55.9	111.5	6.5	12.5
21.8	m10	43.9	43.7	5.1	4.9
31.8	m0	333.1	191.8	38.7	21.5
sum		860.1	874.2	100.0	98.0
total in sample		860.0	892.0	100.0	100.0
not assigned		-0.1	17.8	0.0	2.0

° = wo diastereoisomers with identical mass (in some chromatograms unresolved)
* = carbamoyl glucuronide conjugate of m10
data source file: metabolite_summary.xls

Excretion, Distribution in Blood and Pharmacokinetics of Radioactivity and Parent Compound After Oral or Intraduodenal Administration of [^{14}C]BIBW 2992 MA2 to Female Rabbits (A035/08JS)

This report contains the kinetic and excretion assessment from Study A277/08TE. Applicant-**Table 43** summarizes the kinetic parameters resulting from PO administration, as described in 5.2.9, above.

As illustrated in Applicant-**Figure 38**, the bulk of the radioactivity was eliminated in feces; relatively little was eliminated in the urine. Similar to other species, the AUC in blood was greater than plasma, presumably due to the observed adduction to hemoglobin observed in other species.
As illustrated in Applicant-**Figure 39**, the parent was cleared rapidly from plasma after PO dosing, but radioactivity in plasma and blood persisted, presumably in a covalently protein-bound state. The composition of these protein adducts (i.e. protein or metabolite) was not elucidated.

Table 43: Mean Kinetic Parameters in the Rabbit after PO Administration of 4 µmol/kg [^{14}C]BIBW2992BS

Parameter	Unit	Radioactiovity		Parent
		blood	plasma	compound
C(max)	[nmol/L]	126	76.5	34.0
t(max)*	[h]	1	1	1
t(1/2)	[h]	142	156	2.60
AUC(0-inf)	[(nmol·h)/L]	4060	1600	178
MRT(tot)	[h]	172	165	4.31
CL/F	[mL/(min*kg)]	17.5	42.2	467
AUC(0-24h)	[(nmol·h)/L]	999	552	188

*: median values

Figure 38: Cumulative recovery in feces and urine as a percentage of the oral dose

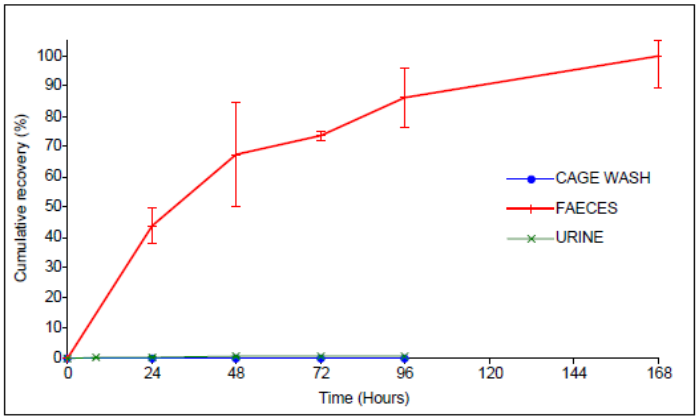
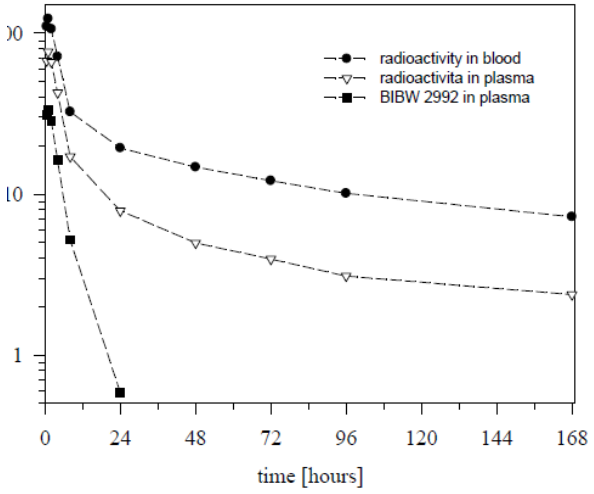


Figure 39: Average concentration of radioactivity and parent compound in blood and plasma after PO dosing



Metabolism of BIBW 2992 in Mice A017-10SKE (B4381)

The purpose of this study was to evaluate the metabolism of [^{14}C]BIBW2992BS in the mouse, a species used in the evaluation of BIBW2992BS toxicity Male and female CD1 mice received single doses of [^{14}C]BIBW2992BS (~8.5 mg/kg) by the PO or ID (intraduodenal) routes. The specific activity of the test article was 0.9425 MBq/µmol. Mass balance was achieved in this study, as > 95% of the administered dose was recovered.

As with the other species tested, the majority (~95%) of BIBW2992BS-associated radioactivity was eliminated in feces (Applicant-**Table 44**). Only 1.2% of the total drug-related radioactivity was excreted in urine. By the ID route, biliary excretion was 9% at 6 hrs post-dose administration, indicating that a portion of the fecal drug-associated radioactivity likely derived from bile. Since nearly 60% of the total dose was recovered in feces, however, it is unclear how much of the difference was unabsorbed drug. Applicant-**Figure 40** summarizes the proposed metabolic scheme of BIBW2992BS in the mouse. As with all other species, metabolism was considered a minor route of elimination compared with the excretion of the unchanged parent. The most abundant metabolites were glutathione adducts. Other compounds included drug-related protein conjugates, as has been demonstrated in other species. There was no effect of gender on metabolism or elimination of drug.

Figure 40: Proposed Metabolic Scheme of [1^{4}C]BIBW2992BS in the Mouse

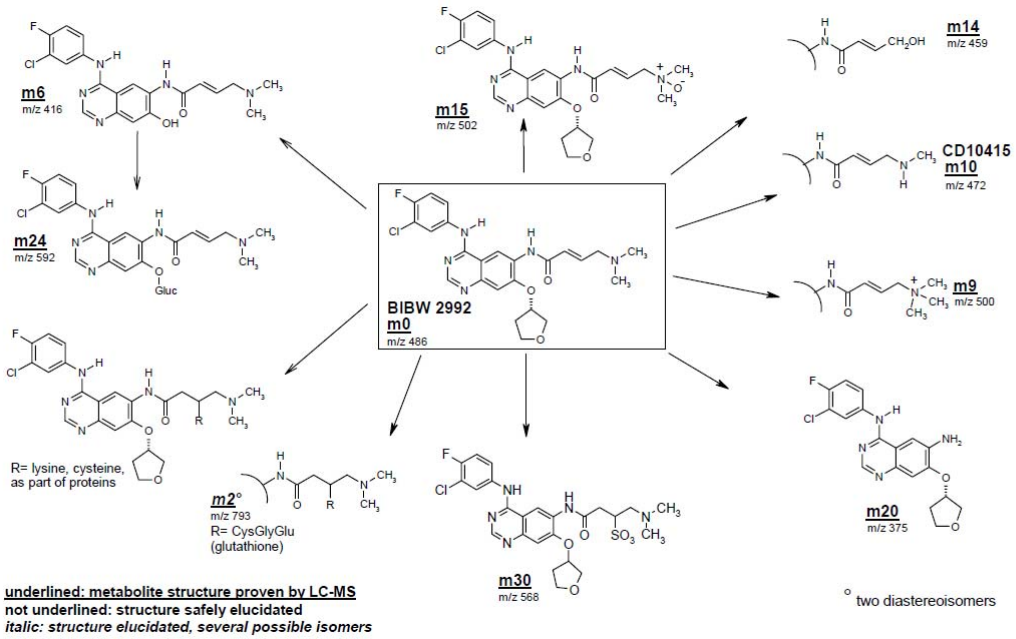


Table 44: Elimination of [^{14}C]BIBW2992BS in Urine, Feces and Bile

compound code		% of dose administered urine p.o. 0 - 24 h male	urine p.o. 0 - 24 h female	faeces p.o. 0 - 48 h male	faeces p.o. 0 - 48 h female	bile i.d. 0 - 6 h male	bile i.d. 0 - 6 h female
m22			0.01				
m23						0.03	0.15
m32						0.12	0.39
m24	M592(1)	0.02	0.03				
m2(1)	M793(1)					0.23	0.41
m6	M416(1)	0.03	0.03	1.60	0.51	0.25	0.39
m15	M502(1)	0.04	0.05				
m30	M568(1)			31.40	32.47	0.55	0.25
m33		0.03	0.05				
m31		0.02	0.04	1.73	1.43	0.07	0.08
m9	M500(1)	0.05	0.01			0.08	0.14
m14	M459(1)	0.02	0.01			0.03	0.02
m10	M472(1)	0.05	0.03			0.20	0.44
m20	M375(1)					0.06	0.05
m26						0.01	0.04
m0	BIBW2992	1.04	0.73	59.97	60.30	7.48	6.44
total		**1.3**	**1.0**	**94.7**	**94.7**	**9.1**	**8.8**

empty cells: not detected
data source file: A01710SKE_calculation of metabolite amounts in excreta.xls

Tissue Distribution and Excretion After Multiple Oral Dosing of [^{14}C]BIBW 2992 MA2 in the Male Rat (A198_09RB)

The purpose of this study was to evaluate the excretion and tissue distribution of [^{14}C]BIBW2992BS in male Wistar rats after 13 daily doses of 6.17 µmol/kg; 3 mg/kg (Applicant-**Table 45**).

Table 45: Design of the Repeat-Dose ADME Study in Rats

Group	Animal No.	N	Time of sacrifice after the first dose
1	101 - 103	3	24 h (Day 2)
2	201 - 203	3	144 h (Day 7)
3	301 - 303	3	192 h (Day 9)
4	401 - 403	3	240 h (Day 11)
5	501 - 503	3	312 h (Day 14)

Note that steady state was not achieved over the dosing interval. Mass balance was also not achieved in this study; total recovery over the 312 hour sampling interval was 91.3%. This is acceptable given the duration of the dosing period, and the fact that a single-dose study did achieve mass balance; however, quantitative conclusions cannot be drawn given the relatively poor recovery.

Table 46: Rat Excretion Data

Route of excretion	Time period [h]	Recovery % of daily dose
Urinary (average daily recovery)	0 - 24	0.3
	all	0.7
Faecal (average daily recovery)	0 - 24	66.8
	all	85.0
Total recovery	**288 - 312**	**91.3**
Gastro-intestinal tract	312	11.7

As in all other studies conducted to ascertain the disposition of BIBW2992BS, the majority of drug was recovered in feces. Very little was recovered from urine. This is consistent with data from single dose studies in all species.
The Applicant evaluated tissue concentrations from animals euthanized at different timeoints over the dosing interval to assess the potential for drug accumulation. This is important given the reactivity of the molecule. As demonstrated in Applicant-**Table 47**, BIBW2992BS tissue levels were generally low; however, accumulation (last dose vs. first dose) was demonstrated, as was expected by virtue of its nonspecific (nonenzymatic) reactivity.

The highest levels were detected in the spleen (which clears drug-bound RBCs) and the organs of excretion (liver and kidneys). In addition, the adrenal, pituitary, and thymus exhibit high levels of binding. The lowest levels were detected in brain, plasma and fat. Levels in blood and plasma also accumulated over the dosing interval (Applicant-**Figure 41** and Applicant-**Figure 42**)

Table 47: Average Concentrations of Drug Related Radioactivity (in ng-eq/g) in Tissue After Multiple Oral Dosing (3 mg/kg) in Male Rats

Sample	mean G1	mean G2	mean G3	mean G4	mean G5	Accumulation factor
	ng-eg/g					
LIVER	140	1170	1130	1590	1990	14.2
EPIDIDYMIS	23	229	200	344	439	18.8
ADRENAL GLANDS	115	1040	976	1240	1630	14.2
LYMPH NODE	112	655	531	788	943	8.4
SUBMANDIBULAR GLAND	43	278	244	379	485	11.3
SKIN	19	254	245	396	453	23.8
PLASMA	1	5	7	8	9	7.9
BLOOD	51	482	403	594	825	16.1
BONE MARROW	71	448	395	547	842	11.9
BRAIN	4	19	18	25	29	6.7
FAT	15	73	60	89	103	7.0
BROWN FAT	45	349	329	557	614	13.6
HEART	39	321	289	428	567	14.6
LUNGS	80	519	500	711	871	10.9
SPLEEN	157	1530	1360	2260	3190	20.3
KIDNEYS	232	1600	1560	2200	2750	11.9
PITUITARY	168	1380	737	1590	1700	10.1
THYROID	65	550	387	710	1030	15.9
THYMUS	52	303	267	415	601	11.5
TESTES	15	126	98	160	200	13.4
PANCREAS	40	290	268	387	501	12.5
MUSCLE	23	170	168	243	339	14.5

source data: DEBRA (project reference: T016/09CS; project name: A198/09RB)

Figure 41: Concentration of BIBW2992BS in Blood following Repeated Oral Administration

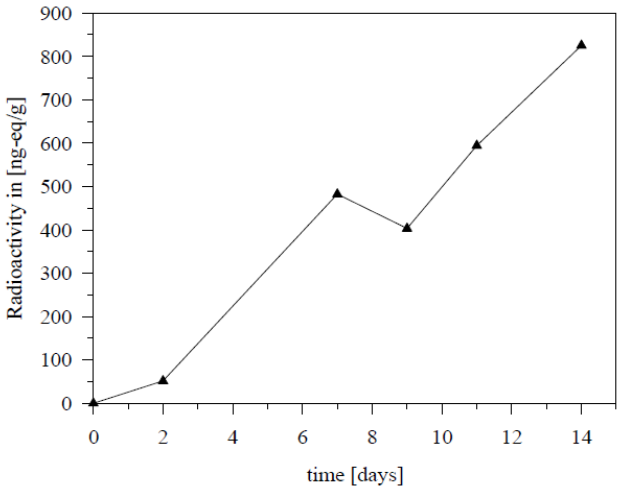
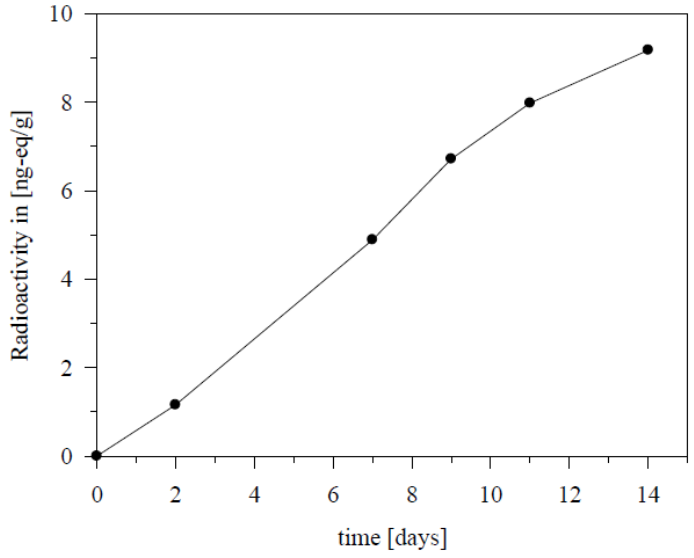


Figure 42: Concentration of BIBW2992BS in Plasma following Repeated Oral Administration



The Effect of BIBW2992 MA2 and Known Model CYP Inducers on Hepatic Levels of Cytochrome P450 and Related Parameters in Male Wistar Rats After Administration for 4 Days

The purpose of this study was to evaluate the potential for BIBW2992BS to induce CYP enzyme levels in comparison to other known CYP inducers, including β-nahthoflavone, phenobarbital, dexamethasone, and clofibric acid. There was no increase in CYP protein levels (Applicant-**Table 48**) or CYP activity. There was also no increase in liver weight in BIBW2992BS-treated animals.

Table 48: Mean Hepatic Microsomal Protein Concentrations

Treatment group	nmol P450/mg protein	nmol P450/g liver
control 10 mL vehicle/kg/day	0.232	3.56
BIBW 2992 MA2 4 mg/kg/day	0.188	3.67
BIBW 2992 MA2 8.5 mg/kg/day	0.254	3.10
ß-naphthoflavone 20 mg/kg/day	0.620	12.9
phenobarbital 120 mg/kg/day	1.27	46.4
dexamethasone 100 mg/kg/day	1.72	56.1
clofibric acid 200 mg/kg/day	0.537	22.8

Transfer of BIBW 2992 to Milk After Oral (gavage) Administration of [^{14}C]BIBW 2992 to Lactating Rats (A240-11RB)

The purpose of this study was to evaluate the potential transfer of BIBW2992BS into milk in lactating rats. Lactating female rats received a single oral dose of 4 mg/kg [^{14}C]BIBW2992BS (dosed as the dimaleinate salt) on Day 11 of lactation. The concentration of radioactivity was measured in plasma and milk for up to 48 hours post-dose. As indicated in Applicant-Figure 43, BIBW2992 readily transferred to milk, with peak milk exposures of about 80-150-fold above those achieved in plasma, at about 6 hours post-dose (Applicant-**Table 49**, Applicant-**Table 50** and, graphically, Applicant-**Figure 43**). Concentrations rapidly declined and achieved concentrations similar to plasma levels (which were BLQ) by 24 hours.

Table 49: Pharmacokinetic parameters of BIBW2992 in milk after single oral administration of 4 mg/kg [14C]BIBW2992 to rats

Parameter	Unit	151	152	153	154	155	N	mean	SD	CV (%)
C(max)	nmol/L	2540	1420	1920	1870	3080	5	2170	648	29.9
t(max)	h	6	6	6	1	6	5	NC	NC	NC
t(1/2)	h	6.30	9.63	8.03	7.50	10.9	5	8.47	1.80	21.2
AUC(0-inf)	nmol·h/L	35900	18500	27800	27300	41600	5	30200	8860	29.3
AUC(0-1)	nmol·h/L	615	144	705	935	655	5	611	289	47.3
AUC(0-6)	nmol·h/L	10000	4410	9030	9860	11600	5	8990	2730	30.3
AUC(0-24)	nmol·h/L	34100	17700	26900	26000	40100	5	28900	8540	29.5
AUC(0-48)	nmol·h/L	35800	18400	27700	27200	41300	5	30100	8820	29.3

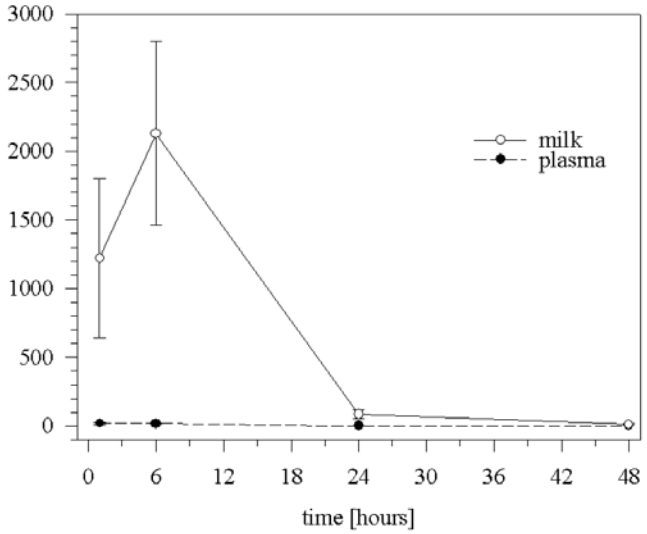
NC not calculated

Table 50: Pharmacokinetic parameters of BIBW2992 in plasma after single oral administration of 4 mg/kg [14C]BIBW2992 to rats

Parameter	Unit	151	152	153	154	155	N	mean	SD	CV (%)
C(max)	nmol/L	12.1	8.27	22.3	25.7	18.7	5	17.4	7.18	41.2
t(max)	h	6	1	1	1	6	5	NC	NC	NC
AUC(0-1)	nmol·h/L	5.70	4.14	11.2	12.9	5.05	5	7.78	3.94	50.7
AUC(0-6)	nmol·h/L	64.5	45.0	114	111	77.1	5	82.3	29.9	36.3
AUC(0-24)	nmol·h/L	173	118	284	232	245	5	211	65.4	31.1

NC not calculated

Figure 43: Concentrations of radioactivity in milk and plasma after single oral administration of 4 mg/kg [^{14}C] BIBW2992 to lactating rats



6 General Toxicology

6.1 Single-Dose Toxicity

Two single-dose toxicity studies were reviewed by Dr. Wei Chen as a part of BIBW 2992 IND 67969 (paper) submission: 02B182: Single dose toxicity study in mice by oral (gavage) administration, and 02B181: Single dose toxicity study in rats by oral (gavage) administration. Both reviews are included here:

Study title: **Single Dose Toxicity Study in Mice by Oral (gavage) Administration**
Key study findings:
Single dose MTD was 300 mg/kg.
Adverse clinical and lethal effects were seen at the dose of 1200 mg/kg.
The gastrointestinal tract was the target organ system.
Study no: 02B182
Volume #13, and page #313-334
Conducting laboratory and location:
Department of Non-Clinical Drug Safety of the Test Facility
Boehringer Ingelheim Pharma GmbH & Co. KG
Biberach, Germany
Date of study initiation: October 15, 2002
GLP compliance: yes
QA report: yes (x) no ( )
Drug, lot #, radiolabel, and % purity: BIBW2992MA2
Batch number: 8260090

Purity: 95.08%
Formulation/vehicle: demineralized water
Dosing:
Species/strain: Crl:NMRI (SPF)mice
#/sex/group or time point (main study): Group 1: 3 /sex/group
Group 2: 3 males
Group 3: 3 females
Satellite groups used for toxicokinetics or recovery: no
Age: 53-58 days
Weight: male: 26.5-34.2 g
Female: 27.5-34.3 g
Doses in administered units: 300, 600, 1200 mg/kg
Route, form, volume, and infusion rate: oral

Group	Dose level of BIBW2992MA2 (mg/kg)	Concentration of BIBW2992MA2 in dose formulation	Dose Volume (mL/kg)
1	300	15	20
2	600	30	20
3	1200	60	20

Observations and times: for 14 days after single dose treatment
Clinical signs: twice per day
Body weights: day 1, 2, 8 and 15.
Food consumption: twice per day by visual inspection
Ophthalmoscopy, EKG, Hematology, Clinical chemistry, Urinalysis, Organs weight, Histopathology, and Toxicokinetics evaluations were not performed.
Gross pathology: performed on all animals, surviving animals on Day 15.

Results:
Mortality:

group	Dose (mg/kg	Sex	Number/Group	No. Of Decedents	Day of Study
1	300	M	3	0	-
		F	3	0	-
2	600	M	3	0	-
		F	-	-	-
3	1200	M	-	-	-
		F	3	1	2-7

* killed in moribund state

Clinical signs:
300 m/kg: no adverse clinical signs were observed.
600 mg/kg (male only): no adverse clinical signs were observed.
1200 mg/kg: No adverse clinical signs were observed in surviving animals. One animal killed in moribund state exhibited adverse clinical signs including reduced activity, abdominal breathing, cold on touch, closed eyes, increased abdominal girth and reddish-yellowish crust around the oronasal region.
Body weights: unremarkable

Food consumption: no changes by visual inspection
Gross pathology:
300 mg/kg: no remarkable changes
600 mg/kg (male only): One animal showed 2 areas of dark-brown discoloration at one liver lobe, a reddened area and partial exfoliation of the epithelial layer in the stomach and a reddened area in the distal part of the duodenum.
1200 mg/kg (female only): one animal showed macroscopic changes including whitish discoloration and erosions of the gastric mucosa, thinned wall of the caecum and hardened caecum contents, distended gastro-intestinal tract filled with gas or with yellowish-brownish fluid.

Study title: Single Dose Toxicity Study in Rats by Oral (gavage) Administration
Key study findings:
Single-dose MTD in rat is 300 mg/kg.
No dose-related clinical signs or macroscopic finding were seen at the oral dose of 300 mg/kg.
Lethal dose was 600 mg/kg.
Macroscopic changes were mostly seen in gastrointestinal tract.
Study no: 02B181
Volume 13#, and page 293-321
Conducting laboratory and location:
Department of Non-Clinical Drug Safety of the test facility
Boehringer Ingelhelheim Pharma GmbH & Co. KG
Biberach, Germany
Date of study initiation: November 5, 2002
GLP compliance: yes
QA report: yes (x) no ( )
Drug, lot #, radiolabel, and % purity: BIBW2992MA2
Batch number: 8260090
Purity: 95.08%
Formulation/vehicle: demineralized water
Dosing:
Species/strain: rat, CrlGlxBrlHan:WI
#/sex/group or time point (main study): Group 1: 3 /sex/group
Group 2: 3 males
Group 3: 3 females
Satellite groups used for toxicokinetics or recovery: no
Age: 53-68 days
Weight: male: 178-239 g
Female: 136-161 g
Doses in administered units: 300, 600, 1200 mg/kg
Route: oral

Group	Dose level of BIBW2992MA2 (mg/kg)	Concentration of BIBW2992MA2 in dose formulation	Dose Volume (mL/kg)
1	300	15	20
2	600	30	20
3	1200	60	20

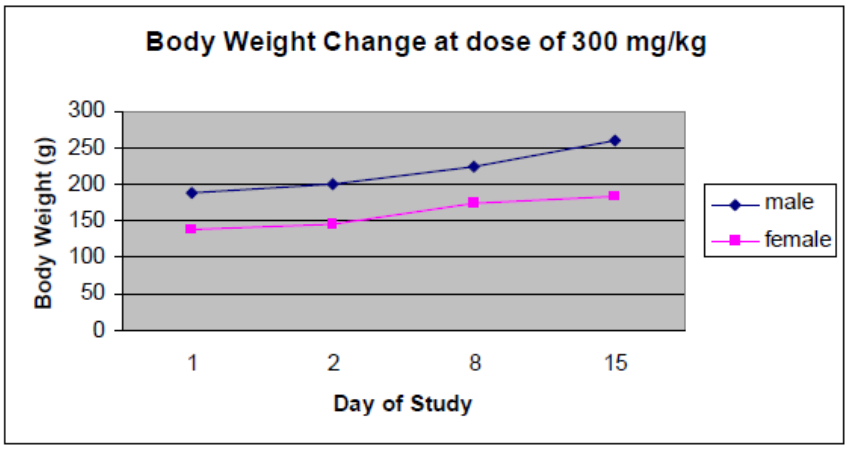
Observations and times: 14 days after single dose treatment
Clinical signs: twice per day
Body weights: day 1, 2, 8 and 15
Food consumption: twice per day by visual inspection
Ophthalmoscopy, EKG, Hematology, Clinical chemistry and Urinalysis: not performed
Gross pathology: performed on all animals, surviving animals on Day 15.
Organs weighed, Histopathology and Toxicokinetics: not performed

Results:
Mortality:

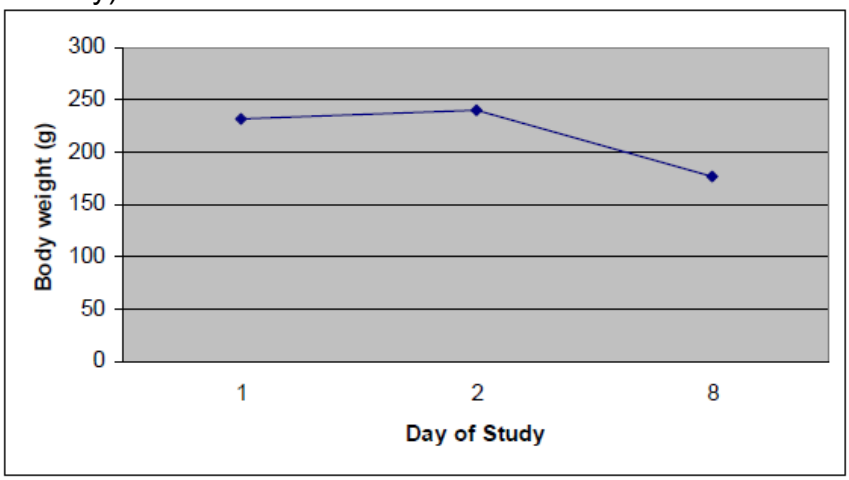
group	Dose (mg/kg	Sex	Number/Group	No. Of Decedents	Day of Study
1	300	M	3	0	-
		F	3	0	-
2	600	M	3	3*	8-15
		F	-	-	-
3	1200	M	-	-	-
		F	3	3*	2-7

* two animals each killed in moribund state

Clinical signs:
300 mg/kg: one animal had reduced activity just after dosing.
600 mg/kg: Piloerection; emaciation; cold on touch and soiled anogenital region were observed at Day 8 and 9.
1200 mg/kg: Piloerection; emaciation; cold on touch and soiled anogenital region; stalked gait; watery feces; reddish around oro-nasal region; reddish crusts at forepaws were observed.
Body weights:
300 mg/kg:



600 mg/kg (male only):



1200 mg/kg (female only): no significant body weight change between day 1 and day 2. Later, no evaluation was possible due to the death of the animals.
Food consumption: no changes by visual inspection
Gross pathology:
300 mg/kg: no treatment-related macroscopic changes were observed.
600 mg/kg (male only): major macroscopic changes included very dry subcutis, focal reddish discoloration of the glandular stomach, necrotic gastric mucosa, ochre-yellow pulpy or reddish-brown liquid stomach contents, thickened duodenal wall, duodenum/jejunum filled with reddish-brown contents, ileum filled with gas or ochre-yellow pulp, distended caecum filled with brownish pulp and colon empty or with pulpy contents.
1200 mg/kg (female only): typical changes were very dry subcuties, reddened gastric mucosa, knobby mucosa of the non-glandular stomach, whitish-slimy gastric contents, and entire gastrointestinal tract filled with gas, intestines containing slimy liquid, ileum and jejunum filled with red-brownish liquid.

6.2 Repeat-Dose Toxicity

Study title: 2-week Oral (gavage) Dose Range-finding Toxicity Study in Wistar Rats
Key study findings:
Mortality was observed at 32 mg/kg treatment group.
Target organs were hemolymphopoietic system, GI, kidney and liver, starting at the dose of 16 mg/kg.
Most of changes were resolved at the end of the recovery period except for renal papillary necrosis.

Study no: 02B126
Volume 12 page97 to Volume 13 page 261
Conducting laboratory and location:

Department of Non-Clinical Drug Safety of the test facility
Boehringer Ingelheim Pharma GmbH & Co. KG
Biberach, Germany
Date of study initiation: August 22, 2002
GLP compliance: no
QA report: yes ( ) no (x)
Drug, lot #, radiolabel, and % purity: BIBW2992MA2
Batch no. 8260090
Purity: 94%
Formulation/vehicle: demineralized water
Methods (unique aspects)**:** troponin T on day 15 and 29
Dosing:
Species/strain: rat, CrlGlxBrlHan: WI
#/sex/group or time point (main study): main study: 5/sex/group
recovery study: 5/sex/group (mid-and high-dose group)
Satellite groups used for toxicokinetics: 4/sex/group
Age: male: 8 weeks
Female: 8-9 weeks
Weight: male: 204-241 g
Female: 158-185 g
Doses in administered units: 0, 8, 16, 32 mg/kg BIBW2992
Route, form, volume, and infusion rate: oral

Group		Control (Group 1)	Low-dose (Group 2)	Mid-dose (Group 3)		High-dose (Group 4)	
				Main study	Recovery	Main study	Recovery
Number of Animals	-male	5	5	5	5	5	5
	-female	5	5	5	5	5	5
Dose mg/kg BIBW2992BS		0 (vehicle)	8	16	16	32	32
Dose mg/kg BIBW2992MA2		0 (vehicle)	12.6	25.2	25.2	50.3	50.3
Dose volume		10 mL/kg					
Dosing day		1-14					

Results:
Mortality:

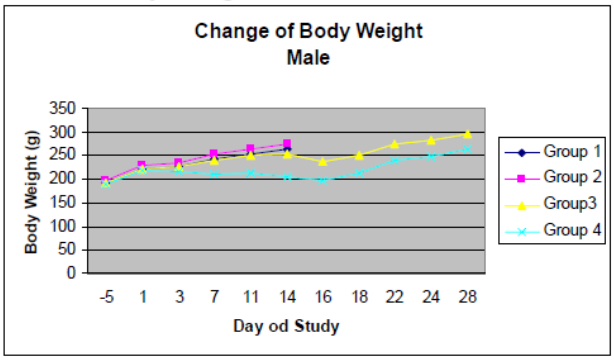
Group	Group 1		Group 2		Group 3		Group 4	
Sex	M	F	M	F	M	F	M	F
No. of Animals	5	5	5	5	5	5	10	1
No. of Death	0	0	0	0	0	0	4*	3

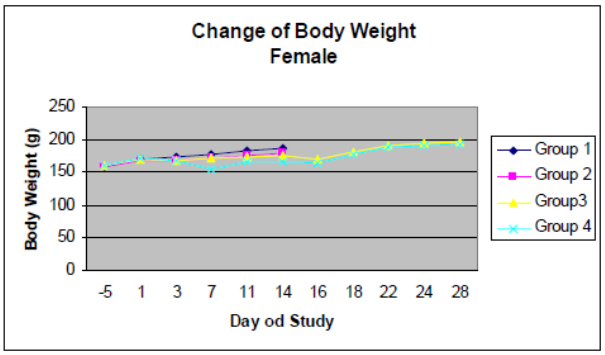
* sacrificed for humane reasons

Clinical signs:

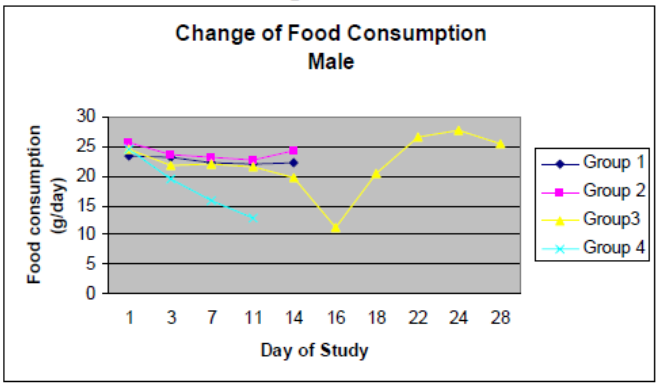
Group	Group 1		Group 2		Group 3		Group 4		Group 3 Recovery		Group 4 Recovery	
Observation period	Day 1-14								Day 16-28			
sex	m	f	m	f	m	f	m	f	m	f	m	f
Number of animals	5	5	5	5	10	10	10	10	5	5	5	4
Exitus	0	0	0	0	0	0	0	1	0	0	0	0
Diarrhea and/or smeary anal region **Day of observation**	0	0	0	0	3 **11-14**	2 **9-14**	9 **4-14**	9 **4-14**	1 **16**	0	2 **16**	1 **16**
Alterations of upper lip and/or snout and/or eyelids	0	0	0	0	2	5	7	4	2	3	5	3

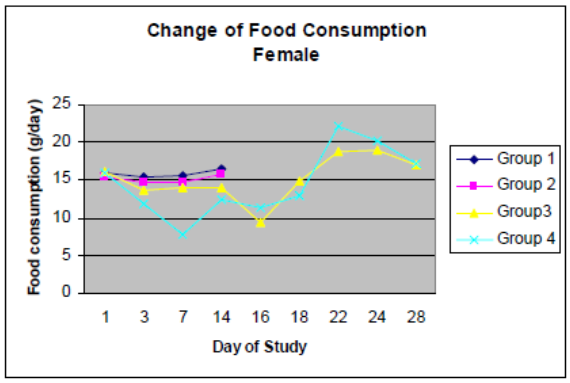
Body weights:





Food consumption:





Hematology:

Parameter	Day	Sex	control 0	Low-dose 8 mg/kg		Mid-dose 16 mg/kg		High-dose 32 mg/kg	
			mean	mean	Δ %	mean	Δ %	mean	Δ %
RBC	15	m	7.504	7.476	0	7.423	1	8.664	15
		f	7.012	7.288	3	7.256	3	7.168	2
	29	m				7.318		7.310	
		f				6.726		6.79	
WBC	15	m	5.97	5.51	-8	7.91	33	8.59	44
		f	3.71	3.9	5	5.92*	59	6.11*	65
	29	m				7.04		6.07	
		f				5.06		3.17	
Lymphocyte	15	m	4.841	4.337	-10	5.400	12	4.592	-5
		f	2.981	3.08	3	3.552	19	3.302	11
	29	m				5.271		4.400	
		f				3.433		2.379	
Lymphocyte %	15	m	80.58	77.9	-3	68.86*	-15	49.82*	-38
		f	79.82	78.94	-1	59.2*	-26	53.32*	-33
	29	m				75.16		72.84	
		f				68.68		75.18	
Neutrophilic cell	15	m	0.868	0.941	8	2.088*	140	3.483*	301
		f	0.566	0.643	14	2.086*	268	2.478*	338
	29	m				1.415		1.400	
		f				1.423		0.660	
Neurophilic cell %	15	m	15.08	17.86	18	25.87*	72	44.00*	192
		f	15.52	16.5	6	35.96*	132	41.45*	167
	29	m				19.92		22.86	
		f				27.10		20.78	

Δ % percentage deviation from control
* $p \leq 0.05$

Clinical chemistry:
Enzymes: Increased enzyme activities in ALT, AST, ALP, GLDH and aldolase were observed on one animal in group 4.
Substrates: BUN concentration were dose-dependently increased in group 3 and group 4 on day 15, by 18% and 99% in males and by 24% and 58% in females. After the recovery period, urea concentrations returned to normal ranges.
Electrolytes: unremarkable
Proteins: In group 4, on day 15, absolute albumin concentration decreased (male up to -30%; female up to -22%). Absolute globulin concentration increased (male up to 20%; female up to 24%), all normalized after the recovery time.
Urinalysis: unremarkable
Organ weights: The weight of prostate, thymus and spleen were slightly or moderate reduced in mid- and high-dose group. There was a trend towards normalization of changed organ weight during the recovery period.

Gross pathology: Changes in the intestinal tract (e.g. liquid contents, discoloration etc.) and the facial integument (e.g. reddish or brownish discoloration or thickening of the lips, muzzle or nostrils) were noted in high-dose group.
Histopathology: Changes in the kidneys, spleen, lymph nodes (mesenteric and axillary), intestinal tract and parotid salivary glands were observed in high-dose group.

02B194 Four-week Oral (gavage) Toxicity Study in Rats With a 2-week Recovery Period (Reviewed by Dr. Wei Chen at the time of IND submission)

Key study findings

- Mortality occurred at 18 mg/kg.
- The toxicological main target organs were the gastrointestinal tract and the kidneys, starting at 18 mg/kg group.
- A dose-dependent neutrophilia was seen in both sexes at 8.5 and 18 mg/kg, and it was recovered at the end of recovery period
- A diminished erythropoiesis in the bone marrow started at 8.5 mg/kg.

Conducting laboratory and location:
Department of Non-Clinical Drug Safety of the test facility
Boehringer Ingelhelheim Pharma GmbH & Co. KG
Biberach, Germany
Date of study initiation: January 16, 2003
GLP compliance: yes
QA report: yes (x) no ( )
Drug, lot #, radiolabel, and % purity: BIBW2992MA2
Batch No.: 8260090
Purity: 95.08%
Formulation/vehicle: demineralized water
Methods (unique aspects)**:** immunotox
Dosing: 0, 4, 8.5 and 18 mg/kg BIBW2992
0, 6.29, 13.36, 28.3 mg/kg
Species/strain: rat, CrlGlxBrlHan: WI
#/sex/group or time point: main study: 10/sex/group recovery study: 10/sex/group in control and high dose group
Satellite groups used for toxicokinetics: 5/sex/group
Age: male: 62-69 days
Female: 69-89 days
Weight: male: 254-294 g
Female: 172-228 g
Route: oral

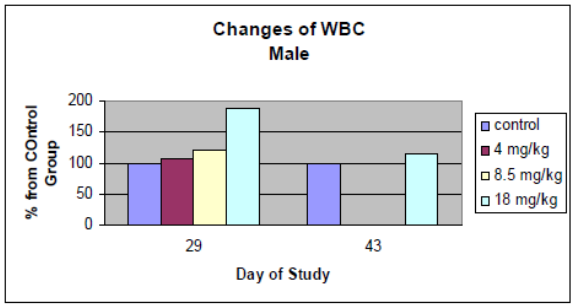
Group	Control (Group 1)		Low-dose (Group 2)	Mid-dose (Group 3)	High-dose (Group 4)	
	Main Study	Recovery			Main study	Recovery
Number of Animals -male - female	10 10	10 10	10 10	10 10	10 10	10 10
Dose mg/kg BIBW2992BS (equivalent to)	0	0	4	8.5	18	18
Dose mg/kg BIBW2992MA2	0	0	6.29	13.36	28.3	28.3
Dose volume	10 mL/kg					
Dosing day	1-28					

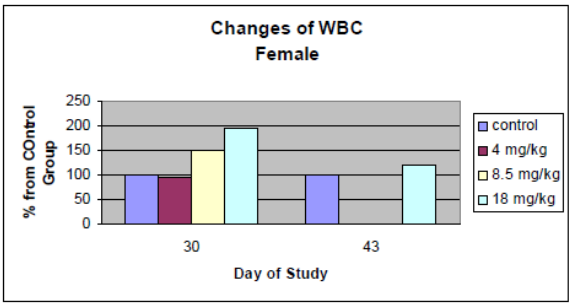
Results:

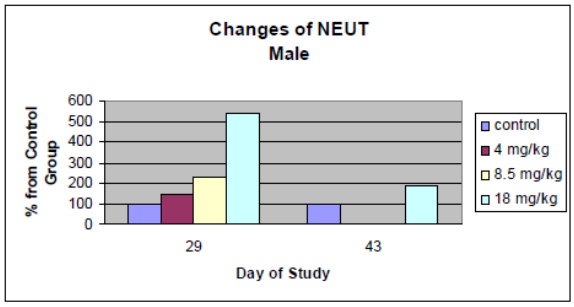
Mortality:
No animal died during the recovery period and no unscheduled deaths occurred in the control, low- and mid-dose groups.

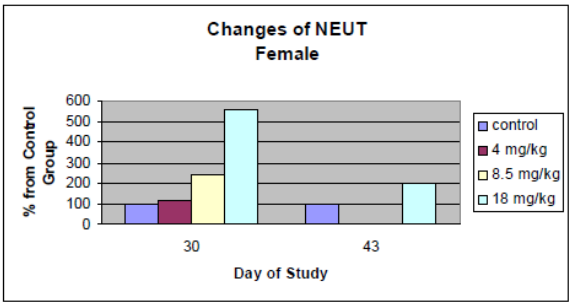
Day	Group 4 (18 mg/kg)-total animals **25**			
	male		female	
	found dead	moribund sacrifice	found dead	moribund sacrifice
17	-	4	-	1
19	1	-	-	-
20	-	1	-	-
24	-	2	1	-
26	-	-	1	-
27	-	3	1	-
28	1	-	-	-
Total	**12**		**4**	

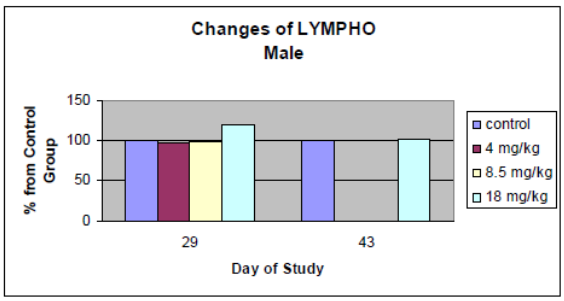
Clinical signs: No adverse clinical signs were observed at 4 mg/kg.
One male at 8.5 mg/kg group had slightly reddened, thickened lips.
Main clinical changes at 18 mg/kg group were soft or liquid feces (seen from day 6); reddened, thickened lips (seen from day 8), entire snout region thickened including red discoloration and/or moistened and reddish, partially moistened fur at the bend of the forelegs (seen from day 13). These clinical signs were more pronounced in males than in females. At the end of the recovery period, the clinical changes were clearly reduced in incidence and severity.
Body weights: No statistically significant body weight differences were seen at 8.5 and 4 mg/kg as compared with the control group.
Hematology:

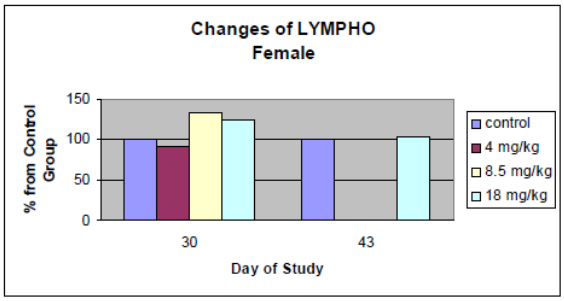


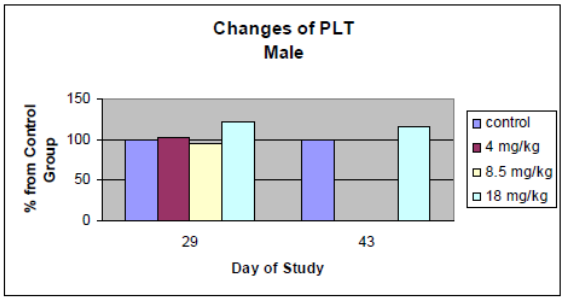


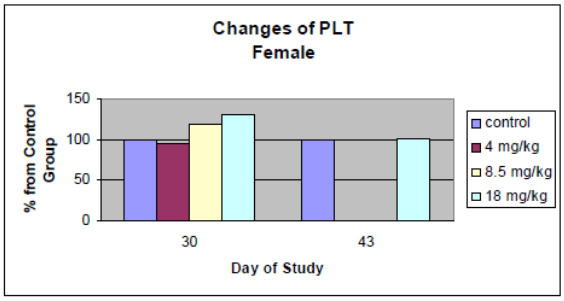












No significant changes in other hematology parameters.

Bone marrow parameter:
4 mg/kg: an increased percentage of reticulum cells were found only in females (+94%).
18 mg/kg: lower percentage of macroblasts, normoblasts, and erythrocyte mitoses observed in both sexes. The percentage of almost all neutrophilic cell types was moderately increased in both sexes (these changes were observed already at 8.5 mg/kg in female). The percentage of plasma cells and macrophages was increased in both sexes (already increase at 4 and 8.5 mg/kg in males). The percentage of lymphocytes was decreased in males and females (also 8.5 mg/kg). All these changes were recovered at day 43.

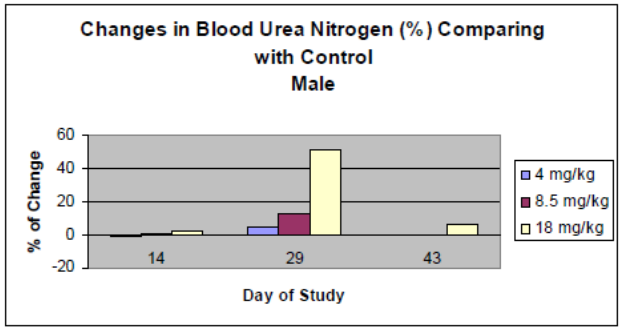
Change of the erythrocyte precursor group:

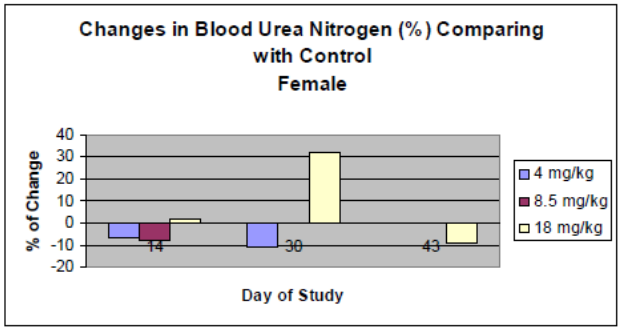
Changes in Bone marrow (Δ%)					
Parameter	Day	Sex	Dose level BIBW2992BS (mg/kg)		
			4	8.5	18
G-Erythrocytes (%)	29	M	5	-4	-31*
	30	f	-15	-23*	-30*
	43	M	-	-	6
	43	f	-	-	-4
Macroblasts (%)	29	M	26	-7	-62*
	30	f	-26*	-22	-31*
	43	M	-	-	-9
	43	f	-	-	38
Normoblasts (%)	29	M	3	-2	-27*
	30	f	-14*	-23*	-30
	43	M	-	-	8
	43	f	-	-	-7
Ery. Mitos (%)	29	M	-10	-29*	-40
	30	f	-6	-10	-49
	43	M	-	-	28
	43	f	-	-	-8
G-Granulocytes (%)	29	M	-11*	-4	20*
	30	F	6	23*	35*
	43	M	-	-	3
	43	F	-	-	4
N.myeloblasts (%)	29	M	-8	62	169*
	30	F	133	233*	265
	43	M	-	-	74*
	43	F	-	-	60
N.promyelocytes (%)	29	M	-16	18	167*
	30	F	-9	20	90*
	43	M	-	-	92
	43	F	-	-	11
N.mylocytes (%)	29	M	2	16	117*
	30	F	56*	66*	128*
	43	M	-	-	14
	43	F	-	-	5
N.metamyelocytes (%)	29	M	-21*	-21*	-6
	30	F	8	39*	64*
	43	M	-	-	-1
	43	F	-	-	5
G-Rest (%)	29	M	27*	25*	18
	30	F	18*	1	-6
	43	M	-	-	-11
	43	F	-	-	0
Lymphocytes (%)	29	M	13	-28*	-67*
	30	F	-16	-37*	-46*
	43	M	-	-	2
	43	F	-	-	-31*
Monocytes (%)	29	M	26	83*	159*
	30	F	-4	14	-11
	43	M	308*	323*	-1
	43	F	16	13	-10
Plasma cells (%)	29	M	-	-	305*
	30	F	-	-	104*
	43	M	309*	475*	45
	43	F	59	50	75*
Macrophages (%)	29	M	-	-	845*
	30	F	-	-	202*
	43	M	34	79*	61
	43	F	94*	64*	71
Reticulum cells (%)	29	M	-	-	80*
	30	F	-	-	56*
	43	M	65	100	-36*
	43	F	511	85	48*
Unclassified cells (%)	29	M	-	-	447*
	30	F	-	-	534
	43	M			67
	43	F			363

Δ%=percentage deviation from control group
*= p≤ 0.05
-= not available

Clinical chemistry:
Enzymes: A minor increase in the activity of aspartate amino transferase (+85%) was observed at 18 mg/kg in males.

Substrates:





Electrolytes: unremarkable

Proteins: Minor increases in the plasma globulin concentration (related to increases in the β-and γ-globulin fraction), and minor decreases in the albumin concentration were observed at 18 mg/kg.

Urinalysis:
A dose-dependent reduction in urine volume was observed during urine collection on day 22/23.
NAG activity (+42%in males; +77% in females), creatinine (+200% in males; +69% in females), and total microprotein concentration (+914% in males; +237% in females) were strongly increased at 18 mg/kg. All were returned to normal value on day 37.
The number of WBC in urinary sediment and the amount of protein in urine were significantly high at 18 mg/kg in males

Organ weights:
Drug treatment-related changes were observed for prostate, ovaries, axillary lymph node, thymus and slightly in spleen. A dose-dependent significant decrease of absolute and relative weight was seen for prostate and ovaries.

Parameter	Day	Sex	Changes from Control (%)		
			4 mg/kg	8.5 mg/kg	18 mg/kg
Prostate (g)	29	M	-8	-19*	-43*
Prostate (% b.w.)	29	M	-13*	-19*	-33*
Prostate (g)	43	M	n.a.	n.a.	-26*
Prostate (% b.w.)	43	M	n.a.	n.a.	-21*
Ovaries (g)	30	F	-16*	-21*	-32*
Ovaries (% b.w.)	30	F	-14*	-19*	-25*
Ovaries (g)	43	F	n.a.	n.a.	-11
Ovaries (% b.w.)	43	F	n.a.	n.a.	-11

Parameter	Day	Sex	Changes from Control (%)		
			4 mg/kg	8.5 mg/kg	18 mg/kg
Ln. axillaris(g)	29	M	15	12	57*
Ln.axillaries (% b.w.)	29	M	9	12	87*
Ln. axillaris(g)	43	M	n.a.	n.a.	21
Ln.axillaries (% b.w.)	43	M	n.a.	n.a.	34*
Ln. axillaris(g)	29	F	-17	21	34
Ln.axillaries (% b.w.)	29	F	-15	23	49*
Ln. axillaris(g)	43	F	n.a.	n.a.	33*
Ln.axillaries (% b.w.)	43	F	n.a.	n.a.	33*

n.a. not available
* $p \leq 0.05$ (t-test with pooled variances, two sided)

Gross pathology:
Premature decedents (18 mg/kg)

Sex	Male	Female*
Number of animals	11	2
Body as a whole		
Exsiccosis	8/11	2/2
Emaciation	6/11	1/2
Skin		
Alopecia, focal	9/11	2/2
Scabs/crusts	10/11	2/2
Intestine		
Content fluid/soft	5/11	1/2
dilatation	5/11	0/2
Thymus		
Reduced in size	11/11	2/2
Spleen		
Reduced in size	4/11	1/2

*Animal No.467 was excluded (evaluation of most organs was not possible due to autolysis/cannibalism)

Animal sacrifice at term: Noteworthy macroscopic findings

Group	Mid-dose (8.5 mg/kg)		High-Dose (18 mg/kg)		High-dose Recovery	
Sex	m	f	m	f	m	f
Number of Animals	10	10	15	13	9	11
Skin						
Alopecia, focal	0/10	0/10	10/15	7/13	1/9	5/11
Discoloration, focal, red/brownish	1/10	6/10	5/15	11/13	2/9	4/11
Scabs/crusts	0/10	0/10	10/15	6/13	0/9	2/11
Swelling/thickening	0/10	1/10	2/15	4/13	0/9	0/11
Stomach						
Discoloration, mucosa, (multi)focal, red/brown/black	0/10	0/10	4/15	1/12	0/9	0/11
Discoloration, mucosa, diffuse red	0/10	0/10	2/15	1/12	0/9	0/11
Discoloration, content, red/black	0/10	0/10	2/15	0/12	1/9	0/11
Intestine						
Content fluid/soft	0/10	0/10	5/15	1/12	0/9	0/11
Content yellow	0/10	0/10	1/15	1/12	0/9	0/11
Content red/black	0/10	0/10	2/15	1/12	0/9	0/11
Dilatation	0/10	0/10	6/15	0/12	0/9	0/11
Reddening	0/10	0/10	4/15	1/12	1/9	0/11
Thymus						
Reduced in size	0/10	0/10	12/15	3/12	0/9	0/11
Spleen						
Reduced in size	0/10	0/10	4/15	1/12	0/9	0/11
Lymph nodes						
Enlargement	1/10	1/10	13/15	1/12	4/9	8/11
Kidney						
Discoloration, cortex, whitish (light), (multi)focal	0/10	0/10	4/15	0/12	0/9	0/11
Discoloration, dark, diffuse	0/10	0/10	1/15	2/12	0/9	0/11
Discoloration, hilus, yellowish white	0/10	0/10	0/15	0/12	0/9	1/11
Male accessory sexual glands (seminal vesicle and/or prostate) Reduced in size	0/10	-	2/15	-	1/9	-

Histopathology:
Premature decedents:

	High-dose premature decedents	
BIBW2992BS	18 mg/kg	
Gender	male	Female*
Number of Animals	11	2
Kidney Papillary nerosis Basophilic tubules, focal Dilated tubules	 7 6 4	 1 2 1
Facilal skin Purulent/granulomatous folliculitis, pustular dematitis	 11	 2
Stomach, nongrandular Erosion/ulcer Atrophic epithelium	 6 5	 1 2
Stomach, glandular Erosion/ulcer	 3	 0
Small intestine (duodenum, jejunum, ileum)		
Atrophy (stunting)of villi, fusion of villi	8	1
Large intestine (cecum, colon, rwectum) Atrophy (flattening of epithelial cells)	 9	 1
Spleen Atrophy	 8	 1
Thymus Atrophy	 11	 2

Animal sacrificed at term:

	Mid-dose		High-dose		HD recovery	
BIBW2992BS	8.5 mg/kg		18 mg/kg		18 mg/kg	
Gender	M	F	M	F	M	F
Number of Animals	10	10	15	12	9	11
Kidney						
Papillary nerosis	1	0	11	2	0	3
Basophilic tubules, focal	1	0	8	1	0	2
Dilated tubules/collecting ducts	1	1	7	1	0	1
PAS-positive droplets in tubular epithelia	0	1	9	11	1	10
Facilal skin						
Purulent/granulomatous folliculitis, pustular dematitis	1	6	15	12	2	8
Skin (different areas)						
Atrophy, epithelium	6	4	13	6	1	0
Stomach, nongrandular						
Erosion/ulcer	0	0	6	3	0	0
Atrophic epithelium	1	0	7	2	0	0
Submucosal edema	0	0	4	3	0	1
Stomach, glandular						
Atrophy, mucosa	0	0	4	0	0	0
Erosion/ulcer	0	0	3	0	0	0
Small intestine (duodenum, jejunum, ileum)						
Atrophy (stunting)of villi, fusion of villi	1	2	9	8	0	0
Edema mucosa	0	0	4	5	0	0
hyperemia	0	0	3	1	0	0
Large intestine (cecum, colon, rwectum)						
Atrophy (flattening of epithelial cells)	3	4	11	6	0	1
Dilatation of crypts	0	0	0	2	0	0
Spleen						
Atropy	0	0	11	4	0	0
Thymus						
Atrophy	0	0	12	2	0	0
Bone marrow						
Myelopoiesis increased	-	-	4	1	-	-
Lymph nodes (head, cervical and/or axillary region)						
Hyperplasia	-	-	13	10	4	8
Testes (seminiferous tubules)						
Apoptosis, increased	0	-	9	-	0	-
Prostate						
Atrophy	0	-	13	-	0	-
Seminal Vesicle						
Atrophy	0	-	13	-	1	-
Uterus						
Atrophy, epithelium	-	5	-	9	-	0
Vagina						
Atrophy, epithelium	-	0	-	11	-	0

Immunotoxicology:

Flow cytometry of peripheral blood cells:

At the end of the treatment period, drug-related decrease in the percentage of analyzed cells, reflecting the lower percentage of mononuclear WBC due to the relative increase of granulocytes. The percentage of monocytes was increased and that of B-lymphocytes was decreased at 18 mg/kg. No recovery was observed at the end of recovery period.

Flow cytometry of spleen cells:
At the end of treatment period, the number of analyzed cells was significantly lower at 18 mg/kg. The percentage of B-lymphocytes was significantly lower in all treatment groups. At the end of the recovery period, the percentage of T-lymphocytes was significantly higher at 18 mg/kg with a concomitant decrease in B-lymphocytes. Furthermore, the percentage of double positive T-lymphocytes and natural killer cells was significantly lower in drug-treated group comparing with control group.

Toxicokinetic parameters are presented in Applicant-provided Table below:

parameter	day	gender	4 mg/kg	8.5 mg/kg	18 mg/kg
C_{max} [nmol/l]	1	m	59.7	220	510
	1	f	95.9	182	536
	27	m	86.8	222	655
	27	f	103	225	508
AUC_{0-24h} [nmol·h/l]	1	m	574	1770	5090
	1	f	621	1430	4590
	27	m	772	2030	6980
	27	f	558	1630	4920

Study title: 03B087 BIBW 2992 MA2: 13-week oral (gavage) toxicity study in Wistar rats followed by a 6-week recovery period U04-1776 (GLP, except DMA and Troponin T analysis)

Summary
BIBW 2992 MA2, batch 8360110, was dissolved in demineralized water and administered daily for at least 91 consecutive days (13 weeks) by oral gavage (10 mL/kg) to groups of 10 male and 10 female CrlGlxBrlHan:WI rats at daily doses of 0 (Control), 2, 5, and 10 mg/kg. An additional 10 animals per gender received either 0 or 10 mg/kg for 13 weeks and were kept for a 6-week recovery period following cessation of treatment. Furthermore, five satellite animals per group per gender were used to measure BIBW 2992 BS plasma concentrations on Day 1 (start of treatment), Day 25 and Day 88 by HPLC-MS/MS. The rats were housed in groups of up to 5 animals per cage under standardized climate conditions. On Day 1, the male animals were 7 to 8 weeks old, females were 9 to 10 weeks old. Body weight ranges were 172 to 238 g in males and 164 to 196 g in females, respectively.

2 mg/kg	No adverse effects considered
5 mg/kg	WBC increased, body weight decreased, wavy/rough fur in 8/20 animals Folliculitis on the face, tail or dorsal areas in 8/10 male and 1/10 females
10 mg/kg	Three animals sacrificed due to overall poor condition, starting on D39; Two of the prematurely sacrificed animals had gross pathology changes of the kidney such as enlargement or discoloration

Study Summary:

- Clinical signs: wavy/rough or dull fur (starting on D 29), loss of hair in the neck, shoulder or genital region, scaly skin on paws and tail and reddish, swollen and/or encrusted muzzle. The findings were more frequent in males than in females.
- The dose dependent, not fully recovered, decrease in body weight in males, specifically in the 10 mg/kg group.
- White blood cell count was increased at mid and high dose levels in both genders (~5 - 50%), mainly neutrophilic cells (~60 - 100%) . Changes in clinical chemistry comprised increase in aldolase activity (max. 2.2 fold), increase in GLDH activity in females (~60%) and increase in gamma-globulins (~130 - 170%). Troponin T values were not significantly changed.
- Urine volume was reduced prominently in males (32% - 53%). Increased protein concentrations in the urine were accompanied by the presence of WBCs and/or erythrocytes (blood).
- Increased weight of the axillary lymph nodes in both genders.
- Gross pathology revealed the skin as one target organ with skin alterations especially at the muzzle. Two of the prematurely sacrificed animals had enlarged and/or or discolored kidneys
- Minimal to severe folliculitis was observed in all animals necropsied at the end of treatment. This was most prominent in the facial area (muzzle) and on the tail, there were also ulcers, scabs and minimal to slight inflammatory infiltration of the adjacent dermis. In the recovery group, an abscess-forming and/or granulomatous inflammation around destroyed hair follicles, especially in the area of the muzzle was noted.
- Renal gross pathology findings were: unilateral or bilateral necrosis of the renal papilla (grade 1 to grade 3 in 6/19 animals). Cutaneous lesions (14/18 animals) and renal papillary necrosis (4/18 animals) were found in the recovery animals.

Mean Toxicokinetic Parameter in Rats—13 week study

parameter	day	gender	2 mg/kg	5 mg/kg	10 mg/kg
C_{max} [nmol/l]	1	m	9.3	52.3	198
	1	f	23.5	87.7	247
	88	m	41.0	152	344
	88	f	27.9	137	253
AUC_{0-24h} [nmol·h/l]	1	m	82.0	378	1590
	1	f	112	508	2030
	88	m	264	1250	3320
	88	f	126	965	2290

Study title: **BIBW 2992 MA2: 26-week oral (gavage) toxicity study in rats with an 8-week recovery period**

Study no.:	04B227/ U06-1217
Study report location:	E submission, tab 4.2.3.2 (Toxicology)
Conducting laboratory and location:	Boehringer Ingelheim Pharma GmbH & Co. KG; Birkendorfer Str. 65; 88397 Biberach an der Riss; Germany
Date of study initiation:	12 October 2004
GLP compliance:	Yes (Germany)
QA statement:	Yes
Drug, lot #, and % purity:	BIBW 2992 MA2, 8430191; 99.1%

Key Study Findings

- Adverse effects consisted of skin lesions, decrease in body weight gain (males), changes in WBC counts; gross/histopahology findings of the skin (folliculitis) and the kidneys (papillary necrosis); findings observed in mid and high dose groups
- Difference in exposures observed at all doses tested, with higher exposure noted in males than females
- Repeat dosing resulted in more pronounced accumulation in males than in females

1.5 mg/kg defined as NOAEL

3 mg/kg (Mid dose)

- wavy/rough fur, hair loss, scaly skin, swollen and /or encrusted muzzle, all signs more pronounced in males than females
- histopathologic observations in skin consistent with folliculitis, inflammatory infiltration; in spleen extramedullary hematopoiesis and reactive hyperplasia, increased histiocytosis and plasmocytosis of regional lymph nodes

6 mg/kg (High dose)

- marginally reduced body weight gain in males
- increased WBC count due to increased neutrophil cell count
- slightly increased platelet count at the study end in males
- slightly decreased albumin count in males;
- increase in globulin numbers which resulted in reduced A/G ratio
- urine volume reduced in males, accompanied with slightly increased total protein concentration and WBC presence
- histopathologic observations occurred at lower dose, and become more pronounced and observed in higher incidence in higher dosed animals
- kidney papillary necrosis in 8/20 males and 1/20 females; still present in recovery animals
- inflammatory changes of nasal cavity in both sexes
- some skin alterations were still present in recovery animals

Methods

Doses: 0, 1.5, 3, and 6 mg/kg of BIBW 2992 BS;
0, 2.22, 4.43, and 8.87 mg/kg of BIBW 2992 MA2

Frequency of dosing: Once daily

Route of administration: Oral gavage

Dose volume: 10 mL/kg

Formulation/Vehicle: BIBW 2992 in demineralized water/demineralized water

Species/Strain: Crl:WI (Han) Wistar rats (SPF quality)

Number/Sex/Group: 20/sex/group; 124 males and 124 female (including two of each sex as sentinel animals)

Age: F 69-75; M 55-61 days

Weight: F 160-201 g; M 199-241 g

Satellite groups: Toxicology group, recovery group and sentinel control

Unique study design:

Group No.	Daily dose [mg/kg]		Animal No.					
			Males			Females		
	BIBW 2992 BS	BIBW 2992 MA2	Main study	Recovery	Kinetic	Main study	Recovery	Kinetic
Group 1	0	0	101-120	121-130	131-136	151-170	171-180	181-186
Group 2	1.5	2.22	201-220		221-226	251-270		271-276
Group 3	3	4.43	301-320		321-326	351-370		371-376
Group 4	6	8.87	401-420	421-430	431-436	451-470	471-480	481-486

Deviation from study protocol: None that undermined the validity of the study

Observations and Results

Mortality

Two animal deaths are noted in the study, male No 422 and female No 353. Neither death was considered to be test-item related deaths. The review of the pathology signs of these animals is listed below:

Animal No: 422=discoloration and reduced size kidneys, discoloration and swelling of brain; muzzle discoloration and irregular shape of skin with hemorrhages; spleen reduced in size. Accidental trauma was a cause of death listed within the histopathology section of the review.

Animal No: 353=acute liver congestion; proestrus, acute kidney congestion with diluted pelvis; acute lung congestion; lymphoid hyperplasia (H) and vascular mineralisation of lungs; focal hypertrophy of pancreas. Histopathology observations for this animal noted an acute cardiovascular failure as cause of acute congestion of the lung, liver and adrenal gland.

Clinical Signs

Clinical signs noted in all dose groups focused on fur, with growing intensity at higher doses.

- Control animals had loss of hair, and hairless patches on individuals, while low dose group show fur-related signs later in the study, between D 120 and 140. Swelling of paws was accompanied wavy/rough fur in the low dose group.

- Mid-dose group had swelling of the muzzle, swollen/encrusted paws with individual animal loss of hair, and wavy /rough fur. The onset of signs were slightly sooner than in the low dose group, between D100 and D110.
- The high dose group animals show the same signs exhibited in the previous groups, with onset at D35 and higher intensity than in the previous groups. The clinical signs reversed in recovery animals.

Body Weights

During the pretest and during the experimental period, body weight was recorded once weekly.

- When compared to control animals, body weight loss was only present in the high-dose male group; reversed in the recovery period.

Feed Consumption

Determination of food consumption was done on the same day that body weight was recorded. The food was weighed before it was placed in the cage and then when the animals were weighed.

- A slight decrease in food consumption was noted in the high-dose males when compared to the control group during both administration and recovery periods.

Ophthalmoscopy

A slit lamp ophthalmological examination of bulbus, conjunctiva, sclera, cornea, uvea, and lens was performed on Day -5 (all study animals) and on Days 84 and 169 (control and the high dose groups). Additionally, the recovery groups of animals were examined on Day 234. The eye fundus was examined using a fundus camera. Pupillary dilatation was induced approximately 10 minutes before examination using ca 1-2 drops of a mydriatic agent (Mydriaticum Stulln®, Pharma Stulln GmbH, 92551 Stulln/Germany).

- all findings such as opacities on corneal surface or in lens were considered incidental and spontaneous in nature by the Applicant

The reviewer disagrees with the Applicant-proposed explanation for ocular findings in this study as similar findings were also observed in mini pig toxicity studies and keratitis has been described clinically. Therefore, the eyes are potentially organs of toxicities.

Hematology

Methods applied for hematological determinations are listed in the Applicant-provided table, below:

Parameter	Abbreviation	Units	Method	Instrument (No.)/calculation
Hemoglobin concentration	HGB	g/dL	Photometric	ADVIA 120 (500740)
Red blood cell count	RBC	10^6/µL	Laser technology	ADVIA 120 (500740)
Hematocrit	HCT	Vol%	Laser technology	ADVIA 120 (500740)
Mean corpuscular hemoglobin	MCH	pg	Laser technology	ADVIA 120 (500740)
Mean corpuscular hemo-globin concentration	MCHC	g/dL	Laser technology	ADVIA 120 (500740)
Mean corpuscular volume	MCV	fL	Laser technology	ADVIA 120 (500740)
Reticulocyte count	Retic	‰	Laser technology	ADVIA 120 (500740)
Normoblasts	Normobl	cells/100 WBC	Counted from smear	Wright's stain: Ames Hema-Tek
White blood cell count	WBC	10^3/µL	Laser technology	ADVIA 120 (500740)
Differential blood count	#	10^3/µL %	Laser technology	ADVIA 120 (500740)
Morphological parameters	§	$	Laser technology	ADVIA 120 (500740)
Platelet count	PLT	10^3/µL	Laser technology	ADVIA 120 (500740)
Prothrombin time	PT	sec	Coagulometer	STA compact (519054)

Basophilic cells (% values), eosinophilic cells (% values), neutrophilic cells (abs. + % values), lymphocytes (abs. + % values), monocytes (% values), large unstained cells (LUC, % values)
§ Microcytes, macrocytes, hypochromasia, hyperchromasia, anisocytosis, polychromasia, atypic lymphocytes
$ Score: no, slight, moderate, strong

- Increase in white cell counts (~45%) and platelets are noted in the high-dose males, which were reversed in recovery period; protrombin time was not affected with the change in platelet counts.
- A dose-dependent increase in neutrophils in males and females was observed, which was reversed only in recovery females

Clinical Chemistry

Methods applied for clinical chemistry determinations are listed in the Applicant-provided table, below:

Parameter	Abbreviation	Units	Method	Instrument (number)
Aspartate aminotransferase	AST	U/L	UV-test (R)	Hitachi 917 (405657)
Alanine aminotransferase	ALT	U/L	UV-test (R)	Hitachi 917 (405657)
Glutamate dehydrogenase	GLDH	U/L	Optimised kinetic (R)	Hitachi 917 (405657)
Alkaline phosphatase	ALP	U/L	Optimised kinetic (R)	Hitachi 917 (405657)
γ-Glutamyl-transferase	G-GT	U/L	Colorimetric test (R)	Hitachi 917 (405657)
Total bilirubin	Tot-Bili	µmol/L	DPD-method (R)	Hitachi 917 (405657)
Glucose	Glucose	mmol/L	Hexokinase (R)	Hitachi 917 (405657)
Total cholesterol	Tot-Chol	mmol/L	CHOD-PAP-method (R)	Hitachi 917 (405657)
Triglycerides (total glycerol)	Triglyc	mmol/L	Enzymatic/lipase (R)	Hitachi 917 (405657)
Urea nitrogen (BUN)	Urea	mmol/L	Oxoglutarate/glutamate (R)	Hitachi 917 (405657)
Creatinine	Creat	µmol/L	M. Jaffé/kinetic (R)(P)	Hitachi 917 (405657)
Total protein	Tot-Prot	g/L	Biuret method (R)	Hitachi 917 (405657)
Protein fractions	#	g/L, %	Microelectrophoresis	Olympus Hite 320 (543657)
Sodium	Na	mmol/L	Ionic selective electrode	Hitachi 917 (405657)
Potassium	K	mmol/L	Ionic selective electrode	Hitachi 917 (405657)
Calcium	Ca	mmol/L	o-Cresolphthalein (R)	Hitachi 917 (405657)
Magnesium	Mg	mmol/L	Xylidyl blue (R)	Hitachi 917 (405657)
Chloride	Cl	mmol/L	Ionic selective electrode	Hitachi 917 (405657)
Inorganic phosphate	Inorg-P	mmol/L	Molybdenum blue (R)	Hitachi 917 (405657)

(R) Roche Diagnostics reagents
(P) Without protein precipitation
(#) Albumin (Alb), Globulin (Glob), α-Globulin (A-Glob), β-Globulin (B-Glob), γ-Globulin (G-Glob), A/G ratio

- Changes in ALT and G-GT were seen in males on Days 86 and 183

- Lower, dose-dependent decrease in total cholesterol concentrations in males was observed
- Decrease in albumin and increase in globulins was seen in high-dose males on D 86 and 183, which normalized in recovery period
- Troponin T analysis did not show any changes in the test animals, when compared to control animals

Urinalysis

Methods applied for urinalysis determinations are listed in the Applicant-provided table, below:

Urine parameter	Abbreviation	Instrument (number)
Volume	Vol	Manual determination
Specific gravity	Spec.grav	Clinitek Atlas (404912)
pH	pH	Clinitek Atlas (404912)
Colour	Colour	Clinitek Atlas (404912)
Turbidity	Turbid	Clinitek Atlas (404912)
Red blood cells	Blood	Clinitek Atlas (404912)
White blood cells	WBC	Clinitek Atlas (404912)
Ketone bodies	Keto	Clinitek Atlas (404912)
Bilirubin	Bili	Clinitek Atlas (404912)
Glucose	Glucose	Clinitek Atlas (404912)
Urobilinogen	Ubili	Clinitek Atlas (404912)
Nitrite	Nitrite	Clinitek Atlas (404912)
Protein	Prot	Clinitek Atlas (404912)
Urine sediment parameter	Abbreviation	Instrument
Red blood cells	RBC[1]	Microscope
White blood cells	WBC[1]	Microscope
Casts	Casts[1]	Microscope
Epithelial cells	Epi[1]	Microscope
Bacteria	Bact[1]	Microscope
Inorganic material	Inorg[1]	Microscope
Other constituents	Other[2]	Microscope

Urine sediment parameters were rated as follows:
1 = no (negative), few (weakly positive), some (moderately positive), many (strongly positive)
2 = negative, sperm, kidney cells, yeast, hyaline cast, granular cast, helminthic eggs, trichomonas, amoeba

Urine volume on D 21 was reduced in high-dose males for app. 20% when compared to the control group, this trend persisted to D 171, when the volume was ~40% less than the control animals urine volume. The urine volume was normal in the recovery animals.

- total protein increased about 50%, together with WBC, in the high-dose males, which recovered in 6-weeks post treatment

Gross Pathology

- Gross pathology observations included findings in two prematurely-dead animals. Mid-dosed female had acute, moderate congestion of the liver, while high-dose male had a focal, subcutaneous hemorrhage of the dorsal cranial region and a focal grey discoloration of the brain tissue.
- Skin findings were present in all treated animals, with dose-related increases in categories such us thickening of the lips of muzzle, scaly and red skin of tail. Rough and/or wavy hair coat category was observed in majority of animals across the doses tested, as seen in the following table:

Daily dose of BIBW 2992 BS [mg/kg]	Group 1 0		Group 2 1.5		Group 3 3		Group 4 6	
Gender	M	F	M	F	M	F	M	F
Number of animals examined	20	20	20	20	20	19	20	20
Muzzle, thickening of the lips	0	0	1	0	14	12	20	18
Muzzle with discoloration	0	0	0	0	0	0	6	1
Rough and/or wavy hair coat*	0	0	20	20	20	18	19	20
Scaly skin of tail**	0	0	0	0	2	0	12	3
Red skin of tail**	0	0	0	0	1	0	3	2

* See Single Finding List: skin, hair, irregular-shaped
** See Single Finding List: skin, tail, [free text] or discoloration

Organ Weights

The list of harvested tissues is listed in the Applicant-provided table, below:

Adrenals	Ovaries
Brain	Pituitary gland (following fixation)
Heart	Prostate (following fixation)
Kidneys	Spleen
Liver	Testes
Lung (before instillation)	Thymus
Lymph node (draining = mesenteric lymph node, Ln. mes.)	Thyroid glands $
Lymph node (distant = axillary lymph node, Ln. axil.)	

$ including parathyroid glands

- organ weights of the high-dose given animals were only affected with the treatment. The increases were noted in axilliary lymph nodes, kidney and spleen, while liver weight decreased in high-dose males. High-dose females had increase in absolute and relative mesenteric lymph node weight.
- weight increase of kidney persisted in the recovery males, while all other changes ameliorated.

Histopathology

The list of harvested tissues is listed in the Applicant-provided table, below:

Adrenal glands	Ovaries
Aorta	Pancreas
Bone (sternum)	Parotid salivary glands
Bone marrow (sternum)	Peripheral (sciatic) nerve
Brain	Peyer's patches
Cecum	Pituitary gland
Cervix uteri	Prostate
Colon	Rectum
Duodenum	Seminal vesicles
Epididymides	Skeletal muscle
Esophagus	Skin (abdominal region and lateral shoulder)
Extraorbital lacrimal gland	Spinal cord (cervical, thoracic, lumbar)
Eyes	Spleen
Harderian gland	Stomach
Heart	Sublingual salivary glands
Ileum	Submandibular salivary glands
Jejunum	Testes
Kidneys	Thymus
Knee joint (with femur)	Thyroid and parathyroid glands
Larynx	Tongue
Liver	Trachea
Lungs	Ureters
Lymph nodes, axillary	Urinary bladder
Lymph nodes, mesenteric	Uterus
Mammary gland (only females)	Vagina
Muzzle (and nasal cavity)	Macroscopic changes observed at necropsy
Optic nerves	

Adequate Battery Yes
Peer Review Yes
Histological Findings

- kidney (necrosis of papilla and tubulary changes), skin (folliculitis), and regional lymph nodes lesions were slightly increased in mid-dosed animals, while all of the lesions were more frequently observed and of greater intensity in high-dose group animals, the greatest increases were specifically seen in kidneys of male animals.

	Group 1		Group 2		Group 3		Group 4	
Daily dose of BIBW 2992 BS [mg/kg]	0		1.5		3		6	
Gender	M	F	M	F	M	F	M	F
Number of animals	20	20	20	20	20	19	20	20
Kidney								
Necrosis of papilla	0	0	0	0	0	0	8	1
Basophil. tubules, cortical	2	2	1	0	5	1	13	1
Interstitial cell infiltrates	2	1	1	0	4	0	12	1
Muzzle								
Folliculitis	0	0	5	1	7	0	16	12
Inflamm. infiltration, dermis/epidermis	0	1	2	0	2	1	12	4
Abscess	0	0	0	0	0	0	5	0
Hair follicles reduced in number	0	0	0	0	3	0	2	0
Degeneration of hair	0	0	0	0	1	1	3	0
Acanthosis, focal	0	0	0	0	0	0	7	2
Scab formation, focal	0	1	1	0	1	0	11	2
Apoptosis (hair follicles, epidermis)	0	0	0	0	7	0	17	9
Skin, abdominal region and/or shoulder								
Folliculitis	0	1	2	2	5	1	15	7
Inflammatory infiltration of dermis	0	0	0	1	0	0	5	2
Atrophy of hair follicles	0	0	0	0	0	1	13	4
Degeneration of hair	0	0	1	0	2	1	10	6
Scab formation	0	1	1	0	0	0	3	2
Skin, tail*								
Folliculitis	-	-	-	-	2	-	15	4
Apoptosis (hair follicle, epidermis)	-	-	-	-	2	-	13	2
Hyperkeratosis, slight	-	-	-	-	-	-	4	4
Hyperkeratosis, moderate	-	-	-	-	2	-	12	2
Scab formation, inflamm. infiltr., abscess	-	-	-	-	-	-	3	-
Lymph nodes, axillary								
Hyperplasia, lymphoid	7	5	8	4	14	12	17	8
Histiocytosis	2	0	0	0	5	2	9	3
Plasmocytosis	0	0	0	0	2	0	8	3
Lymph nodes, cervical region**								
Plasmocytosis	2	-	-	-	-	-	16	7
Hyperplasia, lymphoid	2	2	-	-	-	-	18	11
Nasal cavity								
Inflammatory infiltration	3	6	2	7	4	8	13	14
Erosion, focal	0	2	0	3	1	4	4	10
Intraluminal crusts with inflamm. cells	1	4	2	6	4	3	7	11
Spleen								
Hematopoiesis, increased	1	2	2	1	3	6	12	12

* The tail is not a protocol organ and therefore not regularly available. Note: the total number of histological samples of the skin of the tail is identical with the number of animals with [skin, tail, hyperkeratosis]

** The lymph nodes of the cervical region (probably mandibular lymph nodes) are not a protocol organ. If present in the slides of the salivary glands and if alterations of these lymph nodes were detected, the findings were recorded.

Note: Lessions of abdominal region, shoulder and tail are summarized under skin in the BIO2100 tables.

- Necrosis of the papilla of kidneys was prominent in high-dose males, which occurred bilaterally in 6 cases, and unilaterally in two males and a single female. The incidence of focal basophilic tubules was increased in the male animals at 6 mg/kg, while females were not affected. Both of these conditions were rated minimal to slight, and frequently associated with subacute or chronic interstitial cell infiltration.
- Dose-related folliculitis of the skin and muzzle was the most prominent microscopic finding which was accompanied by an increased rate of the apoptotic epithelial cells. The majority of high-dose group animals (93%) had skin lesions around the muzzle and at the tail areas, less prominent in the abdominal and shoulder regions. Lesions were more prominent and more severe in males than females, and were accompanied with inflammation that ranged from mild subacute to focal granulocytic purulent infiltration, destructive for the hair follicles and granulomatous, in some cases reaching a foreign body granuloma-like reactions. These were accompanied by an unspecified inflammatory infiltration of other dermal layers, forming subcutaneous abscesses predominantly at the muzzle area.
- Focal acanthosis and scab formation of the superficial epidermis and in fundibular regions was also noted.
- The hair follicles were often atrophic, degenerative and/or reduced in number, with hyperkeratosis and dilatation in the distal hair follicles. These lesions were less prominent and frequent in mid-dose group animals, with the majority of observations noted in males. Low-dose group animals showed low-grade lesions of the same nature.
- Dose-related changes in the axillary and cervical region lymph nodes related to the inflammatory skin lesion were noted in mid- and high-dose animals with follicular lymphoid hyperplasia, increased histiocytosis (macrophages in the paracortex) and medullary cords plasmocytosis.
- A dose-related incidence of unspecific nasal infection was noted by the examination of the rostral part of the nasal cavity of all dosed animals, including control animals. The intensity of infiltration of the nasal mucosa, sometimes together with focal erosion and/or intraluminal secretory-exsudative crusts, occurred more frequently in the high-dose animals. Since all animals with these findings were infected, these findings could be considered as secondary to the possible nasal infections, but important since the sequalae of toxicities would increase parallel to the increase in the BIBW 2992 dose.
- A dose related increase in erythropoiesis and/or an increase in extramedullary hematopoiesis occurred in the red pulp of the spleen.
- Small foci of foam cell accumulation in the lung parenchyma was more pronounced in the high-dosed animals (13/40) than in the other treatment groups (low 7/40; mid group 3/40), including control animals (7/40). Cholesterol clefts within these foam cell accumulations or cholesterol granuloma were not seen in the control, but in all treatment groups (1 in each, low and mid-dose, and 4 in high dose animals).

The Applicant considered lung findings to be of minor toxicological relevance, if at all; however, the reviewer's assessment is that these toxicities may correlate with the lung toxicities seen in patients, therefore the associations to the drug-treatment should not be excluded.

Recovery observations
Toxicities observed in the recovery animals are listed in the following table:

	Group 1		Group 4	
Daily dose of BIBW 2992 BS [mg/kg]	0		6	
Gender	M	F	M	F
Number of animals	10	10	10	9
Kidney				
Necrosis of papilla, re-epithelialization	0	0	1	0
Basophil. tubules, cortical	0	0	6	0
Interstitial cell infiltrates	1	0	2	0
Muzzle				
Folliculitis	0	0	6	3
Hair follicles reduced in number	0	0	2	0
Inflamm. infiltration, dermis/epidermis	0	0	2	2
Abscess	0	0	2	1
Acanthosis, focal	0	0	0	1
Skin, abdominal region and/or shoulder				
Folliculitis	0	0	7	2
Atrophy of hair follicles, sporadic	0	0	9	5
Degeneration of hair	0	0	0	2
Inflammatory infiltration of dermis	0	0	1	0
Skin, tail*				
Hyperkeratosis, slight	-	-	9	1
Lymph nodes, axillary				
Histiocytosis	2	1	2	1
Plasmocytosis	0	0	1	0
Spleen				
Hematopoeisis, increased	1	2	2	3
Nasal cavity				
Inflammatory infiltration	2	5	5	6
Erosion, focal	0	1	0	2
Intraluminal debris with inflamm. cells	2	3	5	2
Lung				
Foam cell accumulation	2	2	4	2

* The tail is not a protocol organ; the total number of [skin, tail, hyperkeratosis] is ide
samples examined histopathologically

(excerpted from the Applicant's submission)

In addition to the findings listed in the above table, additional findings were noted:

- Necrosis of the papilla in the kidney was observed in one male of the high-dose recovery group. In another high-dose recovery male, the surface of the necrotic tip of the papilla was partially covered by new epithelial cell layers, the finding that was considered to be a sigh of healing.

- Subacute to chronic, partly granulomatous folliculitis and sporadic atrophy of hair follicles were still present in the recovery animals; while the findings in the axillary lymph nodes, nasal cavity and spleen were mostly recovered.
- Lung focal foam cell accumulations were found in the both, recovery and the control groups at the end of the 8-week period, suggesting that these findings are incidental and of minor toxicological relevance.

Toxicokinetics

Toxicokinetics analysis were done on the following dates:

Day	Date	Group	Sampling time points postdose
-4	05 November 2004	1-4	Pre-treatment
1	09 November 2004	1-4	1, 2, 4, 8, 24 h
23	01 December 2004	1-4	2 h
91	07 February 2005	1-4	2 h
177	04 May 2005	1-4	1, 2, 4, 8, 24 h

- Systemic exposure to BIBW 2992 MA2 was demonstrated with a wide range plasma concentrations partially due to apparent significant accumulation of the test article, with males showing significantly higher maximum plasma levels and systemic exposure than that of the females, which was clearly noted in the toxicological observations. The parameters measured were listed below:

Parameter	Day	Gender	Daily dose of BIBW 2992 BS [mg/kg]		
			1.5	3	6
C(max) [nmol/L]	1	m	7.36	15.7	56.4
	1	f	6.64	20.6	60.4
	177	m	37.9	102	345
	177	f	17.2	49.6	159
AUC(0-24h) [nmol·h/L]	1	m	62.2	174	568
	1	f	51.6	143	473
	177	m	303	873	3500
	177	f	97.7	355	1390

n= 6 per group per gender

Study title: **02b144 BIBW 2992 MA2: 2-week oral (gavage) dose range study in Göettingen minipigs with a 2-week recovery period U03-1780 (batch No: 8260090)**

This study was reviewed by Dr. Wei Cheng under IND 67969. The findings of this study were similar to the findings in the 52-week study. A short summary of the study is presented in this review.

BIBW 2992 MA2 was dissolved in demineralized water and administered by oral gavage once daily for 14 or 15 days to Göttingen minipigs. At start of treatment the animals weighed 9.6 to 18.6 kg and were 4-7 months of age. Two animals/sex/group were used with an additional 2 animals per sex in the high-dose group. They received doses of 0,

2.0, 4.5 and 10.0 mg/kg body weight BIBW 2992 MA2 (doses expressed as free base) in groups 1, 2, 3 and 4, respectively.

Key study findings:

- Loose stool/diarrhea in one mid-dose group animal (D 11,13) and in the majority of the high-dose group (all animals affected by D 8), recovered within 2-4 days after cessation of treatment. Weight loss was observed in most animals of the high-dose group.
- Recovered increase in percentage and number of neutrophilic cells in the blood in the high-dose animals was observed.
- In males on D12, blood urea nitrogen concentration of the high-dose group was 3.5 times higher than that of the control group. Similar, but less pronounced increases were seen in females. Values had returned to normal at the end of the recovery period.
- The macroscopic examination revealed drug treatment-related findings (red discoloration of the mucosa, fluid contents of intestines) in the gastrointestinal tract of one female of the mid-dose group, and all high-dose group.
- The histopathological examination shows drug treatment-related findings in the digestive tract, the submandibular salivary glands, trachea, eyes, skin and the vagina. Changes were most pronounced in the high-dose group and less frequent and/or severe in the mid-dose group.
- A few changes were also present in the low-dose group. Most prominent was an atrophy of the gastric epithelium/mucosa, villous atrophy of the small intestine and atrophic changes in other parts of the digestive tract. Atrophy was also seen in the corneal epithelium of the eye and vaginal tissue. In addition, glycogen depletion of hepatocytes and reduction of parts of the submandibular gland was noted in low-dose group.

Study title: BIBW 2992 MA2: 4-week oral (gavage) toxicity study in Göettingen minipigs with a 2-week recovery period (batch No: 8260090)

This study was reviewed by Dr. Wei Cheng under IND 67969. The findings of this study were similar to the findings in the 52-week study. A short summary of the study is presented in this review.

Four animals per sex and per group of Göttingen minipigs were given 1, 2.45, and 6 mg/kg of BIBW 2992 BS daily for 28 days. An additional two animals per sex were included in the control high dose (6 mg/kg) groups as recovery animals. Standard daily observations and additional measurements, including PK, cardiovascular and ophtalmological examinations were obtained for all animals during the study.

Results:

GI related toxicities, although recoverable, were characteristic of BIBW 2992 oral application seen following its administration in previous animal studies. Specifically: soft or loose stool was observed in two mid-dosed animals and four (50%) high-dosed

animals starting at D11 and observed until the end of the dosing period. There were no effects on body weight gain, except for one high-dosed male.
Treatment at doses of 2.45 and 6 mg/kg caused a slight to moderate, dose- and time-depended, reversible increase in heart rate which correlated with a shortening in QT-interval on D 1, 10 and 24 at 3.5 hours post administration.

A dose-dependent but small increase in the number and percentage of neutrophils in mid and high-dose group animals was noted. No other changes in blood parameters were found.

Atrophy of the GI tract epithelial surface was present in all dose groups, in the minimal to slight graduation. The most pronounced changes were noted in high-dose group animals, with moderate increases in the mid-dose group animals (compared to controls). The esophagus, larynx and trachea (epithelia atrophy) were affected in the mid dose group (2.45 mg/kg) , while these organs in addition to mucinous glands, seminal vesicles and in a single case the ocular cornea were found to be atrophic in the high-dose group. Although the findings were slight in severity, and reversible in the scope, all were attributed to BIBW 2992. Toxicokinetic parameters observed are presented below:

parameter	day	gender	1 mg/kg	2.45 mg/kg	6 mg/kg
C_{max} [nmol/l]	1	m	29.0	57.3	157
	1	f	16.5	87.0	174
	26	m	19.1	35.4	73.7
	26	f	16.7	44.4	187
AUC_{0-24h} [nmol·h/l]	1	m	141	373	1180
	1	f	81.9	486	1490
	26	m	109	268	848
	26	f	92.6	299	1600

Study title: **BIBW 2992 MA2: 13-week oral (gavage) toxicity study in Göettingen minipigs 03B090**

Summary
BIBW 2992 MA2 was dissolved in demineralized water and administered by oral gavage once daily for at least 91 days to Göttingen minipigs. At start of treatment animals were 5-7 months old and weights ranged from 11.3 to 17.0 kg and. The animals were housed in groups of two per pen. They received daily doses of 0, 0.5, 2.0 and 7.0/5.5 mg/kg BIBW 2992 BS in control, low, mid and high dose groups, respectively. Four animals per sex per group were used with an additional 2 animals per sex per BIBW-treatment group included as recovery animals. In the high-dose group, due to observed clinical effects, the dose was reduced from 7.0 to 5.5 mg/kg on Day 32, but erroneously increased again to 7.0 mg/kg on Day 43, 44 and 46-77.

0.5 mg/kg no adverse effects observed

2 mg/kg Mucosal discoloration, fluid contents and/or meteorism of the jejunum and ileum in two females;
Atrophic epithelia of GI, upper respiratory tract, mucinous glands and male genital tract
Hematopoiesis increased in 4/8 animals
All above changes have reversed

7/5.5 mg/kg Loose stool lasting for several days, coinciding with increased dose in 50% of the animals
Elevated neutrophils (absolute and relative), higher number of WBC, increase in BUN; decreases in the albumin/globulin ratio on Days 15, 28, and 91
Atrophic epithelia of GI, upper respiratory tract, mucinous glands, male genital tract and slight atrophy of the corneal epithelium of the eyes
All above changes reversed at the end of 6 week recovery period, except for sublingual gland hyperthrophy in one and a small increase of hematopoiesis in three animals.

NOAEL=0.5 mg/kg; C_{max}=3.52 M and 2.88 nmol/L F; $AUC_{(0-24)}$=25.3 and 15.4 nmolh/L respectively. Toxicokinetic data are presented below:

parameter	week	gender	dosage [mg/kg/day]			
			low dose 0.5	mid dose 2	high dose 5.5	7
C_{max} [nmol/l]	1	m	7.7	24.1	NA	183
		f	2.0	30.8	NA	251
	4	m	2.6	34.0	NA	173
		f	4.1	24.5	NA	198
	13	m	3.5	44.2	98.9	NA
		f	2.9	34.8	105	NA
AUC_{0-24h} [nmol·h/l]	1	m	34.0	158	NA	1630
		f	10.6	204	NA	1760
	4	m	23.3	259	NA	2140
		f	27.4	195	NA	1840
	13	m	25.3	315	976	NA
		f	15.4	262	1020	NA

NA = not applicable

Study title: BIBW 2992 MA2: 52-week oral (gavage) toxicity study in Göettingen minipigs (Pivotal study)

Study no.:	05B018A1
Study report location:	Nonclinical section:
Conducting laboratory and location:	Boehringer Ingelheim Pharma GmbH & Co. KG; Birkendorfer Str. 65; 88397 Biberach an der Riss; Germany
Date of study initiation:	27 January 2005
GLP compliance:	Yes (German)
QA statement:	Yes
Drug, lot #, and % purity:	BIBW 2992 MA2, 8430191, 98.9%

Key Study Findings

0.5 mg/kg (Low dose group)

- Microscopic findings in the GI tract: the minimal to slight atrophy of the superficial squamous epithelium of esophagus and stomach. Only the superficial epithelial layers were affected, and the Applicant considered this finding as a "sequel of the pharmacodynamic activity" of BIBW 2992 "with no toxicological relevance".

1.5 mg/kg (Mid dose group)

- Atrophy of the upper digestive tract, the laryngeal mucous glands and the corneal epithelium of the eye

5.0 mg/kg (High dose group)

- soft or liquid feces periods
- increases in the neutrophilic cell count and in the blood urea nitrogen (BUN) concentration
- decrease in the serum albumin/globulin ration
- higher severity in histopathologic alterations of the upper digestive tract, the laryngeal mucous glands and the cornea were observed

NOAEL: 0.5 mg/kg
Upper GI tract and corneal eye considered targets of BIBW 2992 toxicity in minipigs.

Methods

Doses:	Free base: 0.5; 1.5 and 5 mg/kg BIBW 2992 MA2: 0.739; 2.217 and 7.39 mg/kg (1.000 g free base corresponds to 1.478 g BIBW 2992 MA2)
Frequency of dosing:	Once daily for 7 days per week for at least 364 days
Route of administration:	Oral gavage
Dose volume:	2 mL/kg
Formulation/Vehicle:	
Species/Strain:	Gottingen minipig
Number/Sex/Group:	4/sex/group (24 males and 24 females total)

Age: App 6 months
Weight: Approximately 11 to 16 kg

Unique study design:

Table 2.5.1: 1 BIBW 2992 MA2: 52-week oral (gavage) toxicity study in Göttingen minipigs. Study design, groups and animal numbers

Group No.	Dose of BIBW 2992 MA2 [mg/kg]	Dose of BIBW 2992 BS [mg/kg]	Males		Females	
			Main study	Recovery	Main study	Recovery
1	0 (vehicle)	0	101-104	105-108	151-154	155-158
2	0.739	0.5	201-204	-	251-254	-
3	2.217	1.5	301-304	-	351-354	-
4	7.390	5.0	401-404	405-408	451-454	455-458

Deviation from study protocol: Minimal, did not undermine the study validity

Observations and Results

Mortality

Mortality and general health condition were inspected at least twice daily, except once daily during the pretest period, on weekends and on non-working days.

- there were no premature mortalities on this study.

Clinical Signs

The overall appearance and behavior of each animal was inspected at least twice daily, with the same schedule as the mortality observations.

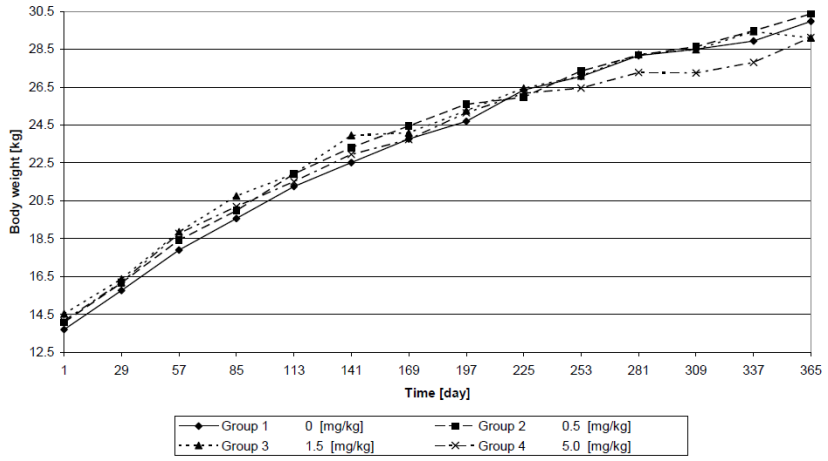
- High-dosed group of animals experienced brief periods of soft or liquid stools.
- Bite and scratch wounds in males and blood-filled prepuce were observed, and attributed to antagonistic behavior in males across all doses tested.

Body Weights

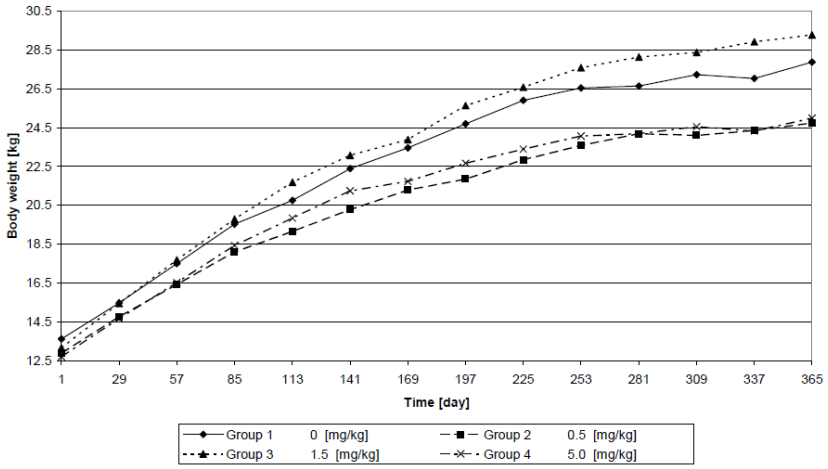
Body weight was recorded weekly during the treatment period prior to dosing, and prior to necropsy.

- Body weight of BIBW 2992-treated females did not change when compared to the control animals during the study, as visible in the figure below.
- Low and high-dosed males weighed 0.7 to 1.3 kg less than that of the mid- and control group males. The change in weight was visible during the course of study, and remained low compared to mid and control group males until the end of the dosing period.

Figure 44: Male and Female Body Weights (52-Week Minipig)



Mean absolute body weight -females



Mean absolute body weight -males

Feed Consumption

Remains of food, if any, were weighed and recorded daily.

- No changes in food remains were observed.

Ophthalmoscopy

Ophthalmological examinations were done using a slit lamp and a fundus camera, once in the pretest period, and once at the end of the study (D351). Three additional observations were conducted for control and recovery group animals (Days 79, 169, 267, and 401), and one additional observation was conducted for low and mid dose group animals (D 277).

- Opacities of the subcapsular lens were observed in 3 animals, one in the low dose group and two in the high dose group, on D 267/277 and on D 351 and 401. The findings increased in severity during the study and persisted in the recovery group. The Applicant states that the low incidence and the lack of dose-dependency make this finding incidental in these animals.

The reviewer's opinion is that the origin of these opacities is not clearly defined in this study. The lack of similar findings in the control animals implies that the opacities might actually be the result of BIBW 2992 application. It is also hard to rule out the lack of dose-dependency based on lack of this finding in four mid-dose animals.

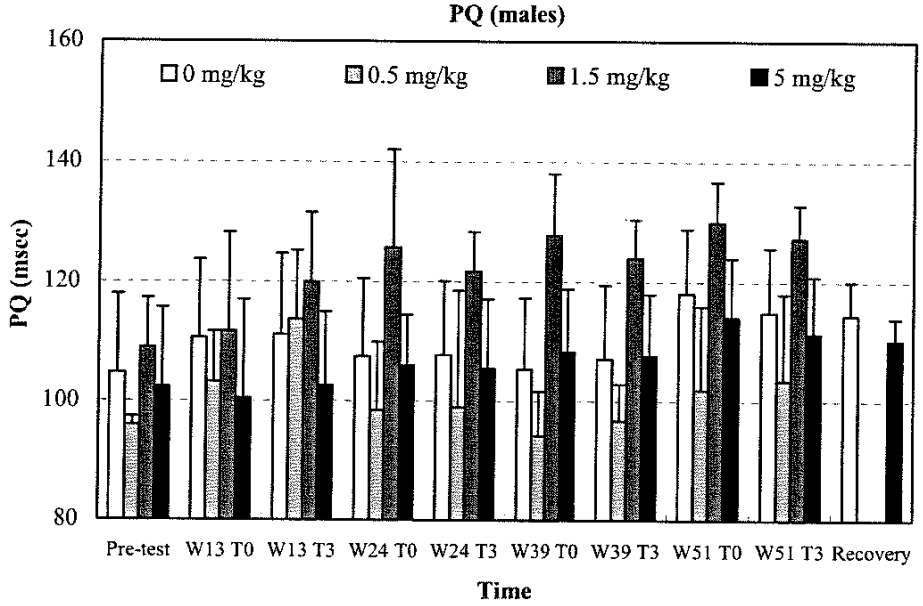
ECG

ECG recordings were performed during the pre-test, during Weeks 13, 24, 39 and 51 and at the end of recovery period, with a nine-channel ECG recorder. Heart rate (HR), PQ, QRS and QT intervals were evaluated

An increase in PQ duration was observed with heart rate decrease in all groups without relation to dose

- The incidence of first degree atrioventricular block (AVBI: PQ > 130 msec) was not dose-related and was rather correlated with individual decreases of heart rate.

Figure 45: Variations of PQ interval duration in male minipigs



Hematology

Blood specimens were taken during the pretest period, and on Days 45, 85, 175, 268, and 359. Recovery animals had additional sampling on Day 401. The list of hematology parameters that were analyzed is presented below:

Parameter	Abbreviation	Units	Method	Instrument (no.)/calculation
Hemoglobin concentration	HGB	g/dL	Photometric	ADVIA 120 (500740)
Red blood cell count	RBC	10^6/µL	Laser technology	ADVIA 120 (500740)
Hematocrit	HCT	Vol%	Laser technology	ADVIA 120 (500740)
Mean corpuscular hemoglobin	MCH	pg	Laser technology	ADVIA 120 (500740)
Mean corpuscular hemoglobin concentration	MCHC	g/dL	Laser technology	ADVIA 120 (500740)
Mean corpuscular volume	MCV	fL	Laser technology	ADVIA 120 (500740)
Reticulocyte count	Retic	‰	Laser technology	ADVIA 120 (500740)
Normoblasts	Normobl	cells/100 WBC	Counted from smear	Wright's stain: Ames Hema-Tek
White blood cell count	WBC	10^3/µL	Laser technology	ADVIA 120 (500740)
Differential blood count	#	10^3/µL, %	Laser technology	ADVIA 120 (500740)
Morphological parameters	§	$	Laser technology	ADVIA 120 (500740)
Platelet count	PLT	10^3/µL	Laser technology	ADVIA 120 (500740)
Activated partial thromboplastin time	APTT	sec	Coagulometer	STAcompact (720215 and 519054)
Prothrombin time	PT	sec	Coagulometer	STAcompact (720215)

#: Basophilic cells (% values), eosinophilic cells (% values), neutrophilic cells (abs. + % values), lymphocytes (abs. + % values), monocytes (% values), large unstained cells (LUC, % values)
§: Microcytes, macrocytes, hypochromasia, hyperchromasia, anisocytosis, polychromasia, atypic lymphocytes
$: Score: no, slight, moderate, strong

- A slightly elevated mean number of neutrophils (above $4X10^3$/µL) was noted for high-dose males on Days 85 and 268. Increased neutrophil counts were also observed on Day 10 for low-, and mid-dose group males; though one mid-dose male (No: 304) had particularly high individual values. In contrast, 6/8 males of the high-dose group had elevated neutrophil counts. At the end of the recovery period, the neutrophil counts of control and high-dose groups were almost identical.

Parameter [unit]	Day		Daily dose of BIBW 2992 BS [mg/kg]			
			0	0.5	1.5	5.0
		Group	1	2	3	4
		Gender	mean	mean	mean	mean
Neutrophils* [10^3/µL]	-10	M	2.986	4.688	5.440	2.733
	45	M	1.764	3.003↑	1.724	2.859↑
	85	M	1.847	2.542	1.930	4.456↑
	178	M	1.432	2.034	1.860	2.450↑
	268	M	2.143	3.323	2.040	4.448↑
	359	M	2.350	2.662	2.090	3.385
	401	M	1.464	n.a.	n.a.	1.514

* No relevant changes were seen in females
Group 1 and 4, n=8, except on Day 401 (n=4) / Group 2 and 3, n=4
↑ statistically significant increase ($p<0.05$)
M males
n.a. not available

Clinical Chemistry

Methods used for clinical chemistry analyses are shown below:

Parameter	Abbreviation	Units	Method	Instrument (number)
Aspartate aminotransferase	AST	U/L	UV-test (R)	Hitachi 917 (405657)
Alanine aminotransferase	ALT	U/L	UV-test (R)	Hitachi 917 (405657)
Alkaline phosphatase	ALP	U/L	Colorimetric assay (R)	Hitachi 917 (405657)
γ-Glutamyl-transferase	G-GT	U/L	Enzymatic Colorimetric assay (R)	Hitachi 917 (405657)
Glutamate dehydrogenase	GLDH	U/L	Optimised kinetic (R)	Hitachi 917 (405657)
Lactate dehydrogenase	LDH	U/L	Colorimetric test (R)	Hitachi 917 (405657)
Creatine kinase	CK	U/L	Optimised kinetic (R)	Hitachi 917 (405657)
Total bilirubin	Tot-Bili	µmol/L	DPD-method (R)	Hitachi 917 (405657)
Glucose	Glucose	mmol/L	Hexokinase (R)	Hitachi 917 (405657)
Total cholesterol	Tot-Chol	mmol/L	CHOD-PAP-method (R)	Hitachi 917 (405657)
Triglycerides (total glycerol)	Triglyc	mmol/L	Enzymatic colorimetric assay (R)	Hitachi 917 (405657)
Urea nitrogen (BUN)	Urea	mmol/L	Kinetic-UV-assay (R)	Hitachi 917 (405657)
Creatinine	Creat	µmol/L	M. Jaffé/kinetic (R)(P)	Hitachi 917 (405657)
Sodium	Na	mmol/L	Ionic selective electrode	Hitachi 917 (405657)
Potassium	K	mmol/L	Ionic selective electrode	Hitachi 917 (405657)
Calcium	Ca	mmol/L	o-Cresolphthalein (R)	Hitachi 917 (405657)
Magnesium	Mg	mmol/L	Xylidyl blue (R)	Hitachi 917 (405657)
Chloride	Cl	mmol/L	Ionic selective electrode	Hitachi 917 (405657)
Inorganic phosphate	Inorg-P	mmol/L	Molybdenum blue (R)	Hitachi 917 (405657)
Total protein	Tot-Prot	g/L	Biuret method (R)	Hitachi 917 (405657)
Protein fractions	#	g/L, %	Microelectrophoresis	Olympus Hite 320 (543657)

(R): (b) (4) reagents
(P): Without protein precipitation
(#): Albumin (Alb), Globulin (Glob), α-Globulin (A-Glob), β-Globulin (B-Glob), γ-Globulin (G-Glob), A/G ratio

- A minimal and reversible increase in BUN was seen in males of the high-dose group, especially on Days 268 and 359.
- In males and females of high-dose group the A/G ratio was decreased on almost all sampling days of the treatment period. This decrease was a composite result of a slightly lower serum albumin concentration and higher serum globulin concentrations as compared to the control values. The relative difference to mean concurrent control values was up to -19% in males and -26% in females. This was less pronounced at the end of the recovery period, although the difference still existed

Parameter [unit]	Day		Daily dose of BIBW 2992 BS [mg/kg]			
			0	0.5	1.5	5.0
		Group	1	2	3	4
		Gender	mean	mean	mean	mean
BUN* [mmol/L]	-10	M	2.074	2.345	2.180	2.224
	45	M	2.378	2.695	2.400	3.100
	85	M	2.290	2.603	2.020	3.184↑
	178	M	3.159	3.640	2.800	4.325↑
	268	M	3.574	4.053	3.573	5.095↑
	359	M	3.463	4.363	3.123	5.118↑
	401	M	3.193	n.a.	n.a.	3.823
Albumin/ Globulin ratio	-10	M	1.743	1.710	1.726	1.570
	-10	F	1.542	1.546	1.552	1.521
	45	M	1.591	1.644	1.507	1.327↓
	45	F	1.507	1.568	1.445	1.332↓
	85	M	1.795	1.801	1.909	1.532↓
	85	F	1.734	1.699	1.536	1.414↓
	178	M	1.538	1.589	1.679	1.348
	178	F	1.401	1.414	1.262	1.164↓
	268	M	1.637	1.598	1.754	1.448
	268	F	1.614	1.542	1.364↓	1.188↓
	359	M	1.451	1.467	1.505	1.174↓
	359	F	1.259	1.256	1.183	0.985↓
	401	M	1.470	n.a.	n.a.	1.216
	401	F	1.311	n.a.	n.a.	1.147↓

* No relevant changes were seen in females
Group 1 and 4: n=8, except on Day 401 (n=4) / Group 2 and 3: n=4
↑, ↓ increase, decrease (p>0.05)
M= males, F=females
n.a. not available

Urinalysis

Parameters assessed in urine obtained on the day of necropsy by puncture of the urinary bladder are shown below:

Urine parameter	Abbreviation	Instrument (number)
Specific gravity	Spec.grav	Clinitek Atlas (404912)
pH	pH	Clinitek Atlas (404912)
Colour	Colour	Clinitek Atlas (404912)
Turbidity	Turbid	Clinitek Atlas (404912)
Red blood cells	Blood	Clinitek Atlas (404912)
White blood cells	WBC	Clinitek Atlas (404912)
Ketone bodies	Keto	Clinitek Atlas (404912)
Bilirubin	Bili	Clinitek Atlas (404912)
Glucose	Glucose	Clinitek Atlas (404912)
Urobilinogen	Ubili	Clinitek Atlas (404912)
Nitrite	Nitrite	Clinitek Atlas (404912)
Protein	Prot	Clinitek Atlas (404912)
Urine sediment parameter	**Abbreviation**	**Instrument**
Red blood cells	RBC[1]	Microscope
White blood cells	WBC[1]	Microscope
Hyaline casts	Casts Hyal.[1]	Microscope
Granulated casts	Casts Gran.[1]	Microscope
Erythrocyte casts	Casts Ery.[1]	Microscope
Leukocyte casts	Casts Leuko.[1]	Microscope
Epithelial cells	Epi[1]	Microscope
Kidney cells	Kidney Epith[1]	Microscope
Bacteria	Bact[1]	Microscope
Inorganic material	Inorg[1]	Microscope

Urine sediment parameters were rated as follows:
[1] 0= no, 1= few (1-5/range of vision), 2= some (6-12/ range of vision), 3= many (13-19/range of vision), 4= massive (>20/range of vision)

- No changes in the urinalysis parameters were seen.

Gross Pathology

The macroscopic examination was done after animals were sacrificed.

- Aplasia or size reduction of the gall bladder was observed in several control, mid, and high-dose animals at the end of treatment period. In one high-dose female, aplasia was confirmed by histopathology at the end of recovery period.

Although the Applicant specifies aplasia as a rare species-specific spontaneous lesion, since the finding only persisted in the high-dose animal,it might be considered a possible result of the continuous use of BIBW 2992 over a year period.

Organ Weights

The weight of the following organs was recorded:

Adrenals	Lymph node (large axillary)
Brain	Ovaries
Epididymides	Pituitary gland
Heart	Spleen
Kidneys	Testes
Liver	Thyroid glands
Lungs (before instillation)	

(Excerpted from the Applicant's submission).

- No drug-related changes of absolute or relative organ weights were observed. The significantly lower weights of the ovaries at low and mid-dose treated females at the end of treatment period (Day 365) were not detectable in the low-dosed animals. Compared with unusually high weight of the ovaries in the control group females, this finding was considered as result of the variations in weight due to the individual stages of the estrous cycle.

Histopathology

Organs were collected from all animals and fixed in 4% neutral buffered formaldehyde solutions, except eyes with optical nerve, testes and epididymides which were kept in modified Davidson's fixative for 24 hours prior fixing in 4% neutral buffered formaldehyde. Lungs were filled with fixative at necropsy prior to immersion fixation.

Adrenal glands	Ovaries
Aorta	Oviducts
Bone (sternum)	Pancreas
Bone marrow (sternum)	Parotid salivary glands
Brain	Peripheral (sciatic) nerve
Cecum	Peyer's patches
Cervix uteri	Pituitary gland
Colon	Prostate
Duodenum	Rectum
Epididymides	Seminal vesicles
Esophagus	Skeletal muscle
Eyes	Skin
Gall bladder	Spinal cord (cervical, thoracic, lumbar)
Heart	Spleen
Ileum	Stomach
Jejunum	Sublingual salivary glands
Kidneys	Submandibular salivary glands
Knee joint (with femur)	Testes
Larynx	Thymus
Liver	Thyroid/parathyroid glands*
Lungs	Tongue
Lymph node, mesenteric	Trachea
Lymph node, large axillary	Ureters
Macroscopic changes	Urinary bladder
Mammary gland (only females)	Uterus
Optic nerves	Vagina

*Parathyroid glands were collected if detected.

Histopathology examinations revealed test item-related findings of minimal to slight severity on superficial epithelia of the upper digestive tract (esophagus and gastric pars proventricularis), laryngeal glands, and eyes. All findings were attributed to BIBW 2992 administration.

Adequate Battery: Yes
Peer Review: Yes
Histological Findings:

Daily dose of BIBW 2992 BS	**0 mg/kg**		**0.5 mg/kg**		**1.5 mg/kg**		**5.0 mg/kg**	
Gender (M = male, F = female)	M	F	M	F	M	F	M	F
Number of animals examined	4	4	4	4	4	4	4	4
Esophagus								
Atrophy of epithelium	0	0	2	0	2	0	2	3
minimal (+)					*		**	**
slight (++)			**		*			*
Vacuolation of stratum corneum	0	0	2	0	0	0	2	3
minimal (+)			*				*	*
slight (++)			*				*	**
Stomach - pars proventricularis								
Atrophy of squamous epithelium	0	0	4	0	3	0	4	4
minimal (+)			*					*
slight (++)			***		**		***	**
moderate (+++)					*		*	*
Vacuolation of squamous epithelium (surface)	0	0	3	0	2	0	2	2
minimal (+)			**		**		*	
slight (++)			*				*	**
Larynx								
Atrophy of mucous glands	0	0	0	0	2	3	1	2
minimal (+)					*	**		
slight (++)					*	*	*	**
Eye								
Atrophy of corneal epithelium	0	0	0	0	1	2	4	4
minimal (+)					*	**	***	****
slight (++)							*	

* each asterisk represents one affected animal

- The squamous epithelium of the esophagus and of the gastric pars proventricularis was atrophic at all dose levels of BIBW 2992. Low and mid-dose group males were more affected with the changes, while the toxicity equally affected both sexes at the high-dose level of 5 mg/kg. Additionally, vacuolation of the stratum corneum was the deepest epithelial cell layer in which this GI- finding was observed. These findings persisted in two high-dose males at the end of 6-week recovery period; changes were, thus, considered only partially recoverable upon discontinuation of the BIBW 2992 therapy.
- Glandular atrophy of the larynx was observed in 5/8 (62.5%) animals of the mid-dose group and 3/8 (37.5%) animals of the high-dose group.
- Atrophy of the corneal epithelium of the eyes was noted in one male and two females at the mid-dose (3/8, 37.5%), in addition to 7/8 animals in the high-dose group. Reduced thickness of squamous epithelium together with a reduction of the number of the cellular layers was stated as a cause of ocular atrophy.
- The Applicant states that "the opacities in the way of the local swelling of the subcapsular ocular lens was observed at the ophthalmological examination at the approximately the same grade in the control animals as in the BIBW 2992 tested animals, without a dose-related relationship, and concluded to be similar to the background findings of these experimental animals."

All histopathology changes observed in the animals, except the GI and eye-related ones were designated as background, spontaneous, or accidental pathological findings.

Toxicokinetics

Blood for toxicokinetic analysis was collected on the following time points:

Day	Date	Sampling time points
1	14 February 2005	pre-treatment*, 2, 4, 8 and 24 h post dose
22	07 March 2005	4 h post dose
88	12 May 2005	4 h post dose
183	15 August 2005	2, 4, 8 and 24 h post dose
267	07 November 2005	4 h post dose
361	09 February 2006	2, 4, 8 and 24 h post dose

*sample taken on Day -10

The following toxicokinetic data was collected from this toxicity study:

	Day	Group	2	3	4
		Sex	0.5 mg/kg	1.5 mg/kg	5 mg/kg
C(max) [nmol/L]	1	M	3.37	30.4	92.8
	1	F	5.27	12.6	81.3
	183	M	3.54	19.4	122
	183	F	4.96	8.11	108
	361	M	2.35	17.0	98.7
	361	F	1.34	6.05	80.5
AUC(0-24h) [nmol·h/L]	1	M	26.1	194	792
	1	F	35.6	91.0	729
	183	M	31.8	174	1300
	183	F	41.7	77.7	1170
	361	M	26.2	165	1180
	361	F	19.5	82.9	966

Group 1 and 4: n= 8/group and sex, Group 2 and 3: n=4/group and sex

- Systemic exposure to BIBW 2992 BS increased close to dose-proportionally between 0.5 and 1.5 mg/kg, and more than dose-proportionally between 1.5 and 5 mg/kg. There were no major differences in plasma concentrations among males and females treated after repeated treatments.
- Animal No. 303 had unusually high plasma concentrations throughout the study (3 to 6 times over the values of other males of this group), and corresponded to about half of the average C_{max} for the high-dose group. The values of that animal were excluded in the overall calculations.

7 Genetic Toxicology

7.1 *In Vitro* Reverse Mutation Assay in Bacterial Cells (Ames)

Modified from review by Dr. Chen under IND 67969
Study title: Mutagenicity Study using the S. typhimurium/mammalian-microsome Assay (Ames test)

Study no.:	03B095
Study report location:	e-file, tab 4.2.3.3. Genotoxicity
Conducting laboratory and location:	Department of Non-Cliniical Drug Safety of the test facility Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Date of study initiation:	September 22, 2003
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	BIBW 2992 MA2; 8360110; 97.8%

Key Study Findings

- Revertant frequencies for all dose of BIBW2992MA2, in 4 tester strains (TA1535,

TA1537, TA100 and TA10298) with and without S9 were not increased comparing with the negative control in the standard plate test.

- There was a reproducible mutagenic response in the frameshift strain *S. typhimurium* TA98 using plate incorporation.

Methods

Strains:	Salmonella typhimurium: TA1535; TA1537; TA98; TA100; TA102
Concentrations in definitive study:	5, 10, 20, 30, 40, 60, 100, 200, 300, 1000 µg/plate (BIBW2992BS)
Basis of concentration selection:	solubility and bacterial-toxicity
Incubation & sampling time:	2 days (TA102: 3 days)

No. of replicates: triplicate; 6 plate for vehicle control
Counting method: using automatic colony counter IPI Analyzer 982B

Criteria for positive results: The diagnostic mutagens (positive controls) induced a distinct increase in the number of revertants, reflecting also the activity of the metabolizing system.

Results:

Compound (µg/plate)	*S. typhimurium* (TA98) Mean revertants/Plate			
	Without activation		With activation	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Negative Control (Dist.Water)	40	40	43	49
BIBW2992BS				
10	56	56	72	71
20	-	61	-	95
30	81	-	93	-
40	-	71	-	101
60	-	78	-	105
100	67	69	49	88
Potitive control				
2-NF				
10	532	764	-	-
2-AA				
4	-	-	1006	1295

7.2 *In Vitro* Chromosomal Aberration Assays in Mammalian Cells

Modified from review by Dr. Chen under IND 67969

Study title: 03B096 BIBW 2992 MA2: Mutagenicity study for chromosomal aberrations in human lymphocytes *in vitro*

Study no.:	U03 1863
Study report location:	e-file, tab 4.2.3.3. Genotoxicity
Conducting laboratory and location:	Boehringer Ingelheim Pharma GmbH & Co. KG Germany
Date of study initiation:	August 8, 2003
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	BIBW 2992 MA2 (dimaleate), 8360110, 97.8%

Key Study Findings

- BIBW2992MA2 was negative for inducing chromosomal aberrations in human lymphocytes with and without metabolic activation at nontoxic concentrations
- At the highly toxic concentration of 20 µg/mL in the nonactivation system and delayed harvest (96 hrs) the increase of aberrant cells numbers is noted (7.5% compared with 2.5% in control)

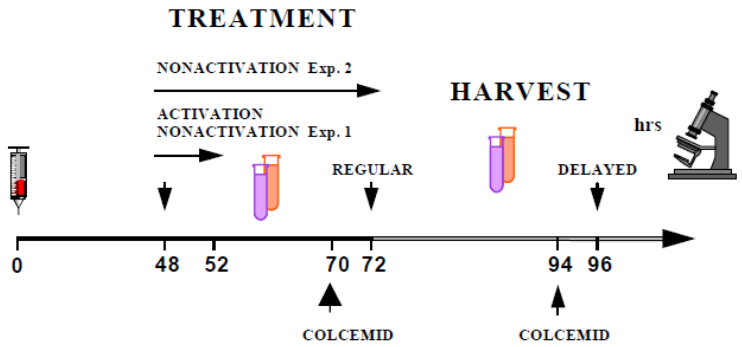
Methods

Cell line:	Rat, LS9 (lymphocyte cultures) with added 0.25mL heparinized whole blood
Concentrations in definitive study:	Nonactivation: 4 hr exposure: 1, 3, 10 and 30 ug/mL 24 hr exposure: 1, 3, and 10 ug/mL 96 hr exposure: 10 and 20 ug/mL Activation: 4 hr exposure: 1, 3, 10 and 30 ug/mL
Basis of concentration selection:	Mutagenicity range finding studies in bacteria with BIBW 2992 MA2; 30 uL of test solution (BIBW 2992 MA2 in DMSO) was added to 3 mL blood culture
Negative control:	DMSO and vehicle control
Positive control:	Adriamycin (ADR 0.05 ug/mL, nonactivation); Cyclophosphamide/CP (7ug/mL, activation)
Formulation/Vehicle:	BIBW 2992 MA2 dissolved in DMSO; 30uL of this test solution was added to 3 mL blood culture

Incubation & sampling time:

Treatment Schedule (hours)

Test	Culture Initiation	Treatment	Colcemid	Harvesting Regular	Delayed
- S9	0	48 - 52	70	72	-
- S9	0	48 - 72	70 (94)	72	96
+ S9	0	48 - 52	70	72	-



Study validity was based on the tested sensitivity of the cell cultures for induction of chromosomal aberrations and the metabolic capacity of the liver enzymes by documenting the actions of the use of adriamycin for reactions of nonactivated positive control and cyclophoshamide for reactions with metabolic activation. Cytotoxicity, the mitotic index (MI) and the cellular morphology of cultures exposed to at least three different concentrations of afatinib were also compared with the DMSO as a vehicle control.

Results

Mitotic inhibition and/or morphological changes

- BIBW 2992 MA2 (free base) did not precipitate in the blood cultures up to 1000 ug/mL when exposed for 4 hr; the mean mitotic index for non activated vehicle control cultures ranged from 149 – 151, and 146 for activation induces cultures

Absence of metabolic activation

- Resulted in a increase in number of polyploidy cells at toxic concentrations of 20 µg/mL
- Highly toxic concentration of 30 µg/mL (63% of the control value) caused a concentration dependent decline of the mitotic index in the absence of metabolic activation after 4 h exposure
- When compared with negative control, there was no indication that BIBW 2992 induced structural chromosomal aberration when applied in concentrations of up to 10 µg/mL.

Structural Chromosome Aberrations (% Aberrant Cells excl. Gaps)
without Metabolic Activation

Compound (µg/mL)	Cult. No.	Treatment 4 hrs Harvest: 72. hr	Treatment 24 hrs 72. hr	Treatment 24 hrs 96. hr
Controls:				
Negative DMSO	A	0	1.0	2.0
	B	1.0	0	3.0
	Mean	**0.5**	**0.5**	**2.5**
Positive ADR 0.05	A	14.0	19.0	26.0
	B	18.0	19.0	25.0
	Mean	**16.0*** *P<0.01%*	**19.0*** *P<0.01%*	**25.5*** *P<0.01%*
BIBW 2992 free base **1**	A	0	2.0	
	B	2.0	1.0	
	Mean	**1.0** *P=100%*	**1.5** *P=62.3%*	**nd**
3	A	2.0	5.0	
	B	0	2.0	
	Mean	**1.0** *P=100%*	**3.5 t** *P=6.8%*	**nd**
10	A	2.0	4.0	1.0
	B	0	2.0	2.0
	Mean	**1.0** *P=100%*	**3.0 t** *P=12.2%*	**1.5** *P=72.4%*
20 t	A			8.1
	B			7.0
	Mean	**nd**	**ne**	**7.5*** *P=3.21%*
30 t	A			
	B			
	Mean	**ne**	**ne**	**ne**

t: toxicity (mitotic inhibition and/or cellular toxicity)
nd: not done na: not analyzed ne: not evaluable
*: Significantly different from the vehicle control (P≤5%)
Historical negative control values (% aberrant cells excl. gaps): 0.8 (range 0-4)

7.3 *In Vivo* Clastogenicity Assay in Rodent (Micronucleus Assay)

Modified from review by Dr. Chen under IND 67969
Study title: **Oral (gavage) mutagenicity study using micronucleus analysis in rat bone marrow**

Study no.:	03B026 (part of the 4-week oral toxicity study No: 02B194)
Study report location:	e-file, tab 4.2.3.3. Genotoxicity
Conducting laboratory and location:	Department of Non-Clinical Drug Safety of Boehringer Ingelheim Pharma GmbH & Co. KG Biberach, Germany
Date of study initiation:	January 16, 2003
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	BIBW 2992 MA2; 8260090; 95.08%

Key Study Findings

- The mean percentage of polychromatic erythrocytes ranged from 33.6-41.5 % (males) and 36.8-43.4 % (females) in BIBW 2992 treated groups
- Historical control ranges were similar (18.3-42.5% M; 26.3-46.5% F) to the negative vehicle values (40.9% M; 41.5% F), therefore, BIBW 2992 is considered to have low potential for inducing myelotoxicity or mutagenicity *in vivo* up to the dose level of 18 mg/kg given once-daily oral gavage application.

Methods

Doses in definitive study:	0, 4, 8.5, 18 mg/kg BIBW 2992 base (6.3, 13.4 and 28.3 BIBW 2992 MA2)
Frequency of dosing:	Once daily
Route of administration:	gavage
Dose volume:	10 mL/kg body weight
Formulation/Vehicle:	Demineralized water
Species/Strain:	Rat, CrlGlxBrlHan:WI
Number/Sex/Group:	5/sex/group, 4 males for mid and high dose group
Satellite groups:	None for this experiment
Basis of dose selection:	Results of 2-week dose-range finding study No: 02B126
Negative control:	Demineralized water
Positive control:	Not mentioned

Study Validity

- The bone marrow slides were coded in random order and then read “blind” using light microscopy (1000x magnification). A total of 2000 polychromatic erythrocytes per animal were scored for the incidence of micronucleated cells. The unit of scoring is the micronucleated cells, not the micronucleus.

Criteria for positive results: The criterion for a positive result is a statistically significant, dose-dependent increase in the frequency of micronucleated polychromatic erythrocytes in treated animals as compared with the negative vehicle. Additional, historical control frequencies obtained in similar experiments using this rat strain are taken into consideration.

Observations and Results

Results of counts of micronucleated polychromatic erythrocytes (MNE) and percentages of polychromatic erythrocytes (PCE) were presented in the following table:

	Negative Control		BIBW 2992 MA2 (mg/kg)						Historical values	
	Dem H2O		4		8.5		18			
Sex	M	F	M	F	M	F	M	F	M	F
PCE(%)	40.9	41.5	41.5	43.4	40.4	39.8	33.6	36.8	30.27	37.71
MNE (%)	0.17	0.23	0.16	0.22	0.12	0.19	0.16	0.22	0.15	0.15

Based on the above presented results, oral use of BIBW 2992 up to 18 mg/kg, did not induced structural or numerical damage to the chromosome complement when given to rats continuously for four weeks.

7.4 Other Genetic Toxicity Studies

03B198 *In vivo* Comet assay in liver, kidney and jejunum of rats after oral dosing (gavage)-summary

Based on positive Ames test findings and GI-tract-related toxicities exhibited in rat and minipig studies following oral administration of BIBW 2992, an additional *in vivo* Comet assay using cells from the the liver, kidney, and jejunum of treated rats was performed to further explore the mutagenic potential of BIBW. The results of the Comet assay indicate that BIBW 2992 does not induce DNA damage in any tissue tested from rats treated with up to 200 mg/kg BIBW 2992.

The Comet assay is generally used to detect DNA damage in any tissue at the individual cell level by using alkaline single cell gel electrophoresis. DNA damage in form of fragmented DNA of any tissue is visualized by fluorescence staining and microscopy.

BIBW 2992 (free base) doses of 0 (vehicle control), 2, 16, and 200 mg/kg were given to 4 male animals in two intervals of 24 hours. An additional positive control group of animals was given 110 mg/kg methyl methanesulfonate (MMS) as a single dose. Four hours after the second 24 hr treatment, all animals were necropsied and single cell suspensions from liver, kidney and jejunum were further lysed and analyzed after migration by electrophoresis, and slides preparations. 150 cells were analyzed per organ and animal.

All animals completed the study, although the dose of 200 mg/kg resulted in decreased motor activity and piloerection in some animals. Similar amounts of DNA damage by Olive Tail Moment (OTM) measurement were recorded for cells from the liver (0.75), kidney (0.76) and jejunum (0.7) cells. There were no increases in the OTM numbers in tissues from animals treated at, mid, or high dose tissues when compared with cells from control animals. The MMS positive control samples showed clear increases of comet-positive cells.

Figure 46: Afatinib Comet Assay Results

Liver

Test Article (mg/kg)		Sampling time (hrs) post 2nd dose	Animal No. Male	Anal. cells	Olive Tail Moment Mean	SD	Group Mean	Group SD
Neg. Control: Demin. water		4	101	150	**0.60**	0.49	**0.75**	0.89
			102	150	**0.73**	0.68		
			103	150	**0.77**	0.63		
			104	150	**0.92**	1.43		
BIBW 2992		4	201	150	**0.82**	1.04	**0.78**	0.80
base	salt		202	150	**0.77**	0.73		
2	3		203	150	**0.80**	0.78		
			204	150	**0.71**	0.56		
16	24.2	4	301	150	**1.21**	3.35	**0.81**	1.82
			302	150	**0.62**	0.52		
			303	150	**0.60**	0.70		
			304	150	**0.80**	1.02		
200	302.2	4	401	150	**0.61**	0.55	**0.64**	0.69
			402	150	**0.66**	0.78		
			403	150	**0.70**	0.87		
			404	150	**0.60**	0.51		
Pos. Control: MMS 110 (1x)		4	501	150	**3.93**	4.31	**3.97**	4.78
			502	150	**4.01**	5.23		

Kidney

Test Article (mg/kg)		Sampling time (hrs) post 2nd dose	Animal No. Male	Anal. cells	Olive Tail Moment Mean	SD	Group Mean	Group SD
Neg. Control: Demin. water		4	101	150	**0.74**	0.75	**0.76**	1.13
			102	150	**0.64**	0.79		
			103	150	**0.90**	1.77		
			104	150	**0.77**	0.90		
BIBW 2992		4	201	150	**0.68**	0.64	**0.69**	0.88
base	salt		202	150	**0.64**	0.61		
2	3		203	150	**0.60**	0.72		
			204	150	**0.84**	1.34		
16	24.2	4	301	150	**0.65**	0.63	**0.71**	0.75
			302	150	**0.66**	0.67		
			303	150	**0.75**	0.93		
			304	150	**0.79**	0.76		
200	302.2	4	401	150	**0.61**	0.66	**0.71**	0.81
			402	150	**0.80**	1.05		
			403	150	**0.71**	0.73		
			404	150	**0.71**	0.73		
Pos. Control: MMS 110 (1x)		4	501	150	**6.34**	5.29	**6.56**	5.89
			502	150	**6.79**	6.44		

Jejunum

Test Article (mg/kg)		Sampling time (hrs) post 2nd dose	Animal No. Male	Anal. cells	Olive Tail Moment Mean	SD	Group Mean	Group SD
Neg. Control: Demin. water		4	101	150	**0.75**	0.73	**0.70**	0.75
			102	150	**0.72**	0.86		
			103	150	**0.65**	0.57		
			104	150	**0.68**	0.80		
BIBW 2992		4	201	150	**0.63**	0.55	**0.63**	0.61
base	salt		202	150	**0.60**	0.57		
2	3		203	150	**0.57**	0.53		
			204	150	**0.73**	0.76		
16	24.2	4	301	150	**0.83**	0.86	**0.74**	0.73
			302	150	**0.73**	0.65		
			303	150	**0.82**	0.81		
			304	150	**0.57**	0.53		
200	302.2	4	401	150	**0.72**	0.79	**0.67**	0.73
			402	150	**0.67**	0.64		
			403	150	**0.63**	0.70		
			404	150	**0.65**	0.80		
Pos. Control: MMS 110 (1x)		4	501	150	**8.62**	6.42	**8.34**	6.64
			502	150	**8.06**	6.87		

8240164 BIBW 2992: induction of lac Z mutations in tissues of orally treated MutaTM Mice Summary

The MutaTM Mouse (lacZ/gale) assay is one of the *in vivo* genotoxicity assays used for detecting the induction of point mutations and small deletions in variety of tissues that interact at the site of the contact with tested chemicals.

In this experiment, six male MutaTM Mice were treated with 24, 47, and 70 mg/kg/day of BIBW 2992 for 28 consecutive days. An additional six animals were given reverse

osmosis water as the vehicle control. Mid dose animals showed signs of piloerection and loss of body weight. All high-dose animals exhibited hunched posture and piloerection on Day 12 of the experiment. From Day 16 until the end of the treatment period, four out of six high-dose group animals died or were euthanized due to poor overall condition. All high-dose males exhibited marked loss of weigh.

Due to mortality at the 70 mg/kg dose level (high-dose group), and in line with OECD 2010 recommendations, the two lower dose groups were examined for mutation frequency. DNA was extracted from liver, duodenum and skin from all surviving animals, on Day 31. Aliquots of isolated DNA were introduced into competent E. coli cells with specific mutations to allow incorporation into Stratagene Transpack bacteriophage lambda vector. Plating of the cells was performed on phenylgalactose plates so that only lac Z- mutants cells (the ones with DNA mutations induced by BIBW 2992) could grow. Concurrent packaging of positive control DNA (from other independent studies) confirmed the correct functioning of the packaging reactions on each occasion when mutation frequency data were generated. At least 200 000 pfu per tissue for each animal was generated, to a total yield in excess of 1 million pfu for each tested concentrations. Vehicle control values were comparable with the historical control data for each tissue.

There were no statistically significant increases in mutation frequency in samples of liver, duodenum and skin by using ANOVA at the 5% level on data obtained by transformed and untransformed cells. Therefore it is concluded that dose levels of BIBW 2992 of 24 or 47 mg/kg/day do not induce mutations in the liver, duodenum, or skin of MutaTM Mice.

Table 51: MutaMouse Results

Table 7: Summary of liver results

Group	Treatment (mg/kg/day)	Number of animals	Mutation frequency ($x10^{-6}$)	Standard Deviation
1	Vehicle	6	34.87	7.2
2	BIBW 2992 (24)	6	46.55	12.1
3	BIBW 2992 (47)	6	40.84	14.3

Table 8: Summary of duodenum results

Group	Treatment (mg/kg/day)	Number of animals	Mutation frequency ($x10^{-6}$)	Standard Deviation
1	Vehicle	6	46.67	14.0
2	BIBW 2992 (24)	6	46.14	25.1
3	BIBW 2992 (47)	5	25.27	13.1

Table 9: Summary of skin results

Group	Treatment (mg/kg/day)	Number of animals	Mutation frequency ($x10^{-6}$)	Standard Deviation
1	Vehicle	6	75.25	35.3
2	BIBW 2992 (24)	6	56.95	29.5
3	BIBW 2992 (47)	6	43.41	13.1

Study No: 09b130 (U09-2371-01) [redacted] (b) (4) (impurity of BIBW 2992): Mutagenicity study using the S. typhimurium/mammalian-microsome assay (Ames test) GLP study

The investigation [redacted] (b) (4) was used to see whether it induces mutations in the Salmonella *typhimurion* strains TA 1535, TA 100, TA 102 strains which are sensitive to base-pair substitutions. TA 1537 and TA 98 strains are sensitive to framshift mutations in presence and absence of a metabolic activation system (S9 mix:Aroclor 1254-induced rat liver microsomal fraction and co-factors). [redacted] (b) (4) was dissolved in dimethylsulfoxide and added to bacterial cultures in triplicate at final concentrations of 30 to 3000 µg/plate.

[redacted] (b) (4) precipitates at concentrations ≥ 1000 µg/plate. A strain-dependent toxicity was observed starting primarily at concentrations ≥ 1000 µg/plate.

[redacted] (b) (4) did not increase the number of reverent colonies in different tester strains of S. typhimurium compared to the negative control when tested up to insoluble and bacteriotoxic concentrations. Metabolic activation by S9 mix did not alter the mutation frequency of these bacterial strains. All values obtained were within the historical background data range. These data were verified in an independent test using the preincubation technique, and along with the results of vehicle control, showed spontaneous revertants in different tester strains at frequencies similar to those known from the literature and in the Applicants's laboratory. Incubation with the positive control articles NaN3, 9-AA, 2-NF, MMC and 2-AA resulted in the expected activity with and without metabolic activation.

(excerpted from Applicant's submission)

Experiment 1 (Plate test)

µg/plate	Mean Revertants/Plate *S. typhimurium*				
	TA 1535	TA 1537	TA 98	TA 100	TA 102
Negative Control					
DMSO	8	4	18	110	344
(b) (4)					
30	7	5	18	129	355
100	10	4	14	118	339
300	8	3	20	113	336
1000	8 P	3 P	20 P	115 P	347 P
3000	5 PT	3 P	14 P	90 P	284 P
Positive Controls					
NaN_3 5	1085	-	-	1237	-
9-AA 50	-	284	-	-	-
2-NF 10	-	-	695	-	-
MMC 0.5	-	-	-	-	1652

Experiment 2 (Preincubation)

µg/plate	Mean Revertants/Plate *S. typhimurium*				
	TA 1535	TA 1537	TA 98	TA 100	TA 102
Negative Control					
DMSO	5	3	19	68	271
(b) (4)					
30	6	6	20	67	271
100	7	7	19	68	296
300	7	4	19	66	260
1000	5 P	5 P	23 P	68 P	217 P
3000	2 PT	2 PT	16 PT	58 PT	213 PT
Positive Controls					
NaN_3 5	1069	-	-	1129	-
9-AA 50	-	255	-	-	-
2-NF 10	-	-	557	-	-
MMC 0.5	-	-	-	-	1781
P: Precipitation T: Toxicity -: Not tested Underlined values are regarded as increased					
Historical Range	5 - 22	3 - 28	16 - 68	57 - 197	252 - 465

Based on the presented data, (b) (4) did not cause base-pair substitutions or framshift mutations in different S. typhimurium strains in the presence and absence of metabolic activation when tested up to insoluble and bacteriotoxic concentrations. Based on these outcomes, (b) (4) was classified as an "Ames negative" BIBW 2992 impurity.

Study No: u10-1793-01 (done under GLP standards (b) (4) **In vitro Mammalian chromosome aberration test in human lymphocytes with** (b) (4)

(b) (4) was investigated for its potential to induce structural chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 homogenate. The chromosomes were prepared 24 h after the start of the treatment with the test item in the interval of 4 hours with and without metabolic activation (experiment I) and 24 h without metabolic activation with and without a recovery period of 24 h (experiment II). Two parallel cultures were evaluated for at least 100 metaphases scored for structural chromosome aberrations in concentrations of 2.5, 5, and 10 mM of (b) (4)

Table 2: Experiment I - Summary of Aberration Rates.

Dose Group	Concen-tration [mM]	Treatment Time	Fixation Interval	mean % aberrant cells incl. Gaps	mean % aberrant cells excl. Gaps
without metabolic activation					
C	0	4 h	24 h	**3.5**	**1.5**
5	2.5	4 h	24 h	**3.0**	**0.0**
6	5	4 h	24 h	**1.5**	**0.5**
7	10	4 h	24 h	**1.0**	**0.0**
EMS	900 µg/mL	4 h	24 h	**16.0**	**15.0**
with metabolic activation					
C	0	4 h	24 h	**3.0**	**1.5**
5	2.5	4 h	24 h	**3.5**	**1.0**
6	5	4 h	24 h	**1.0**	**0.0**
7	10	4 h	24 h	**4.0**	**2.0**
CPA	5 µg/mL	4 h	24 h	**11.0**	**9.0**

200 cells evaluated for each concentration
C: Negative Control (Culture Medium)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

Number of polyploidy cells and mitotic index:4 h treatment, 24 h fixation period

Table 3: Experiment II - Summary of Aberration Rates.

Dose Group	Concen-tration [mM]	Treatment Time	Fixation Interval	mean % aberrant cells incl. Gaps	mean % aberrant cells excl. Gaps
without metabolic activation					
C	0	24 h	24 h	**3.5**	**1.0**
7	2.5	24 h	24 h	**1.5**	**0.5**
8	5	24 h	24 h	**2.0**	**0.0**
9	10	24 h	24 h	**4.0**	**2.0**
EMS	400 µg/mL	24 h	24 h	**17.0**	**13.0**
without metabolic activation, with recovery period					
C	0	24 h	48 h	**2.5**	**0.5**
7	2.5	24 h	48 h	**3.0**	**1.5**
8	5	24 h	48 h	**2.5**	**1.5**
9	10	24 h	48 h	**1.5**	**0.5**

200 cells evaluated for each concentration
C: Negative Control (Culture Medium)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)

Number of polyploidy cells and mitotic index: 24 h treatment, 24 h fixation period, additionally with a recovery period of 24 h

No biologically relevant decrease of the relative mitotic index (by the decrease of below 70% of the relative mitotic index) was noted at any concentration of [redacted] (b) (4) evaluated.

The number of aberrant cells found in the dose groups treated with the test item did not show a biologically relevant increase when compared to the corresponding negative control.

In experiment II without metabolic activation, the aberration rates of the negative control and all the dose groups treated with the [redacted] (b) (4) were within the historical control data of the testing facility at all times tested.

Additionally, no increase in the frequency of polyploidy cells was found after the treatment with [redacted] (b) (4).

In conclusion, [redacted] (b) (4) was rated as non-clastogenic in the chromosome aberration test in human lymphocyte test *in vitro*.

Study No: 09B131 (U10-1285-01) [redacted] (b) (4) (impurity of BIBW 2992): Mutagenicity study using the S. thyphimarium /mammalian-microsome assay (Ames test)

Two batches of [redacted] (b) (4) were tested in repeat pre-incubation experiments to assess its potential to induce mutations in the Ames assay: PR4HAE3388-A2 (sum of impurities [redacted] (b) (4)) and PR4HAE03559PA1 (sum of impurities [redacted] (b) (4)). In the fist set of tabulated results, the batch with the lower sum of impurity was used.

Test substance: [redacted] (b) (4)
Method: **Exp. 2 / Preincubation**
Strain: ***S. typhimurium* TA 98**
Evaluation: **17 September 2009**
Investigator: [redacted] (b) (4)

µg/plate	his$^+$-Revertants/plate			
	Nonactivation	**Mean**	**S9 Activation**	**Mean**
Neg. Control: DMSO	22 28 15 11 20 15	**19**	25 30 15 20 21 20	**22**
30	15 23 21	**20**	30 32 18	**27**
100	20 23 17	**20**	14 28 25	**22**
300	23 29 22	**25**	17 23 19	**20**
1000	50 46 46	**47**	16 23 34	**24**
3000 PT	3 m 12 m 2 m	**6**	12 12 10	**11**
Pos. Control 2-NF 10	505 555 610	**557**	- - -	/
Pos. Control 2-AA 4	- - -	/	1501 1171 1199	**1290**

P: Precipitation C: Contamination m: Manual -: Not done
T: Toxicity (Reduction of Revertants and/or Background lawn) /: No Mean

Test substance: (b) (4)
Method: **Exp. 3 / Preincubation**
Strain: ***S. typhimurium* TA 98**
Evaluation: **21 January 2010**
Investigator: (b) (4)

µg/plate	his⁺-Revertants/plate			
	Nonactivation	**Mean**	**S9 Activation**	**Mean**
Neg. Control: DMSO	13 17 18 16 21 12	**16**	27 22 19 23 25 23	**23**
100	26 18 18	**21**	24 28 33	**28**
300	29 29 21	**26**	26 20 23	**23**
600	33 37 24	**31**	21 24 30	**25**
1000	31 42 47	**40**	22 23 19	**21**
2000	21 29 34	**28**	21 14 22	**19**
3000 PT	18 20 16	**18**	14 12 14	**13**
Pos. Control 2-NF 10	540 494 555	**530**	- - -	/
Pos. Control 2-AA 4	- - -	/	1738 1734 1427	**1633**

P: Precipitation C: Contamination m: Manual -: Not done
T: Toxicity (Reduction of Revertants and/or Background lawn) /: No Mean

Table 8: 2 Mutagenic activity of (b) (4) (batch PR4HAE3388-A2) in *S. typhimurium*
Exp. 2 / Preincubation (Summary)

Without metabolic activation

µg/plate	Mean Revertants/Plate *S. typhimurium*				
	TA 1535	TA 1537	TA 98	TA 100	TA 102
Negative Control					
DMSO	5	3	19	68	271
(b) (4)					
30	5	4	20	66	271
100	4	4	20	73	303
300	5	5	25	62	255
1000	6	5	47	39 T	59 T
3000	0 PT	1 PT	6 PT	2 PT	0 T
Positive Controls					
NaN_3 5	1069	-	-	1129	-
9-AA 50	-	255	-	-	-
2-NF 10	-	-	557	-	-
MMC 0.5	-	-	-	-	1781

With metabolic activation

µg/plate	Mean Revertants/Plate *S. typhimurium*				
	TA 1535	TA 1537	TA 98	TA 100	TA 102
Negative Control					
DMSO	8	6	22	87	360
(b) (4)					
30	9	2	27	83	369
100	7	6	22	91	372
300	4	4	20	86	348
1000	10	2	24	66 T	270 T
3000	1 PT	1 PT	11 PT	10 PT	100 T
Positive Controls					
2-AA 4	79	91	1290	340	-
2-AA 10	-	-	-	-	532
Historical Range	5 - 22	3 - 29	16 - 68	57 - 197	252 - 531

P: Precipitation T: Toxicity -: Not tested Underlined values are regarded as increased

Table 8: 3 Mutagenic activity [redacted] (b) (4) (batch PR4HAE03559PA1) in *S. typhimurium* Exp. 3 / Preincubation (Summary)

Without metabolic activation

µg/plate	Mean Revertants/Plate *S. typhimurium*				
	TA 1535	TA 1537	TA 98	TA 100	TA 102
Negative Control					
DMSO	6	4	16	59	353
[redacted] (b) (4)					
30	7	6	-	63	342
100	5	6	21	63	332
300	8	4	26	55	308
600	-	-	31	-	-
1000	6 T	4 T	40	53 T	296
2000	-	-	28	-	-
3000	1 PT	1 PT	18 PT	4 PT	252
Positive Controls					
NaN_3 5	1160	-	-	1367	-
9-AA 50	-	243	-	-	-
2-NF 10	-	-	530	-	-
MMC 0.5	-	-	-	-	1748

With metabolic activation

µg/plate	Mean Revertants/Plate *S. typhimurium*				
	TA 1535	TA 1537	TA 98	TA 100	TA 102
Negative Control					
DMSO	8	5	23	77	444
[redacted] (b) (4)					
30	8	5	-	79	460
100	11	4	28	82	440
300	10	4	23	75	438
600	-	-	25	-	-
1000	7 T	4 T	21	73 T	437
2000	-	-	19	-	-
3000	2 PT	2 PT	13 PT	15 PT	429
Positive Controls					
2-AA 4	44	142	1633	703	-
2-AA 10	-	-	-	-	1068

P: Precipitation T: Toxicity -: Not tested Underlined values are regarded as increased

Historical Range	5 - 22	3 - 29	16 - 68	57 - 197	252 - 531

[redacted] (b) (4) precipitated at concentrations higher than or equal to 3000 µg/plate, and induced a strain-dependent bacteriotoxicity at concentrations ≥ 1000 µg/plate. Overall, these data show that [redacted] (b) (4) was mildly positive at a high concentration in a single strain (TA-98) in the absence of metabolic activation. These results were consistent with those of BIBW 2992, the parent compound, alone.

Study report 10b032 (U10-1479-01) [redacted] (b) (4): Exploratory 3-day oral (gavage) toxicity study in male rats (only a short summary of the study was provided)

[redacted] (b) (4) was specified as a impurity of BIBW 2992 and used in this experiment to assess the Maximum Tolerated Dose (MTD) after repeated oral administration to male

rats as a dose range findings study for a planned Comet/Micronucleus test. Male Crl:WI (Han) rats were administered daily doses of [redacted] (b) (4) batch designated as: PR4HAE3570-A4) suspended in 0.5% hydroxyethylcellulose (NatrosolTM 250 HX) and observed for three consecutive days. Doses of 150, 200, 400, 1000 and 2000 mg/kg were tested. One male at the 2000 mg/kg dose level was found dead approximately 5.5 h after treatment on Day 2. Dosing of animals for three days at levels of 150, 200, or 400 mg/kg necessitated sacrifice on Day 5 or 6 due to severe GI side effects. Doses of ≥ 150 mg/kg showed limited signs of toxicity, including rough fur and transiently prone position at Day 2. Clinical signs observed after the cessastion of the treatment on Day 3 included liquid stool, soiled anogenital area, partial palpebral closure, stilted gait, or positive skin tenting starting on Day 4 or 5. Body weight loss or no body weight gain was also observed in all animals in the study. Macroscopic examination at necropsy revealed severely distended stomach and intestine at 1000 mg/kg, and in the prematurely dead animal at 2000 mg/kg. Comparable observations were noted in animals necropsied 2 or 3 days after the cessation of treatment at doses equal or higher than 400 mg/kg. The amount of 2000 mg/kg of [redacted] (b) (4) exceeded the MTD in rats.

Study report 10b033-aml (U10-1792-01-AM1) [redacted] (b) (4) **(impurity of BIBW 2992): Rat bone marrow micronucleus test and detection of DNA damage in liver, stomach and jejunum of orally treated rats using the Comet assay** (GLP-study)

This *in vivo* genotoxicity assay was done as a follow up study in order to assess the overall genotoxic potential of the [redacted] (b) (4) impurity. The liver, stomach, and jejunum were selected as possible target organ tissues and used for the assesessment of [redacted] (b) (4) -mediated induction of micronuclei in DNA damage.
[redacted] (b) (4) was suspended in 0.5% hydroxyethylcellulose and given by oral gavage daily for 3 days to five male rats per group at dose levels of 0 (vehicle control), 200, and 600 mg/kg. Two additional animals were treated with a positive control substance (EMS, 200 mg/kg). Standard Comet assay methodology was employed throughout the experiment.

Stomachs of all [redacted] (b) (4) -treated animals were enlarged and filled with air at the time of necropsy. The percentage of micronucleated PCE in [redacted] (b) (4) -treated rat tissues was 0.13 to 0.15%; negative control was 0.18% and EMS was 0.7%. [redacted] (b) (4) induced myelotoxicity based on the reduction in mean percentage of PCEs (8.7% and 7.3% in rats given 200 and 600 mg/kg, respectively) compared to vehicle control (26.5%) or 200 mg/kg EMS of 16.3%.

In a Comet assay, [redacted] (b) (4) did not increase the mean Percent Tail Intensity (%TI) and mean Tail Moment ™. The mean %TI values in the liver and jejunum were ~0.8 and ~4, respectively, for both dose levels of [redacted] (b) (4) and the negative control. The mean TM values in the liver (for low and high dose) and the jejunum were 0.19 and 0.74 to 0.99, respectively, for both dose levels of [redacted] (b) (4) and the negative control. These values indicate that the impurity did not produce a significant change under the conditions of

the assay. An increase in mean % TI values was, however, seen in the stomach tissue of the 200 mg/kg rats (6.1 vs. ~4.2 in high-dosed or negative control rats). The percentage of "hedgehogs", as an indicator for possible cytotoxicity was also increased in stomach tissue (~20 compared to ~8 in the negative control tissues).

The Applicant's states that '[redacted] (b) (4) itself did not induce any DNA damage in the stomach, but rather cytotoxicty was responsible for the increased values". This rationale is questioned from this reviewer's point of view, since the afatinib-induced toxicities were GI oriented., It is not, however, clear why the % TI increase seen in stomach tissue happened only at the dose of 200 mg/kg, and not at 600 mg/kg.

Study report 09B096 BIBW 2992 MA2 spiked with impurities [redacted] (b) (4) [redacted] (b) (4)): 13-week oral (gavage) toxicity study in rats U10-1345 (GLP study)

Summary
Afatinib spiked with specific impurities [redacted] (b) (4) [redacted] (b) (4)) was suspended in 0.2% Natrosol 250 HX and daily orally administered to the Han Wistar rats (10 per sex per group) at daily doses of 0 (control), 2 (low), 5 (mid) and 8.5 mg/kg (high). Additional four satellite animals per group per gender were used as PK animals. Specific results were presented by the dose level administered:

2 mg/kg Fur changes (hair breakage, rough fur) common
Epidermal ulceration without folliculitis seen in one animal

5 mg/kg Scaly, reddish discolored skin and muzzle, also fur changes as in the low dose
Slightly elevated neutrophil counts in males
Folliculitis (purulent or granulomatous) noted on the muzzle, tail, and digits of all animals, often accompanied with dermatitis, acanthosis, erosions, focal parakeratosis and skabs

8.5 mg/kg Scaly, reddish discolored skin and muzzle, also fur changes as in previously mentioned, with swollen and partly reddish discolored digits in all animals
Decrease in body weight in males
Slightly elevated neutrophil count and increased beta-globulins
Folliculitis present in the skin, the muzzle, tail, digits, and often accompanied by dermatitis, acanthosis, erosions, focal parakeratosis and scabs
Reduction of goblet cells and a minimal blunting of villi observed in the ileum of 8/20 animals
Necrosis of the tip of papilla observed in the kidneys of 2/20 animals
Changes in urine (increased red blood cell, protein, WBC and nitrite), pyelitis and chronic interstitial nephritis was observed in one male

Concentrations					
BIBW 2992 (free base)					Impurities (b) (4)
					Target [% of nominal BIBW 2992 (free base)] (b) (4)
Group no.	Target concentration [mg/mL]		Actual [mg/mL	Actual* [% of nominal]	Actual [% of nominal BIBW 2992 (free base)]
1	0	A, M, E	-	-	-
2	(b) (4)				
3					
4					

1) = A: samples taken before dosing, M: samples taken during dosing, E: samples taken after dosing

- = absent, N.A. = not applicable

* = Calculation based on unrounded actual results

Based on the evaluation of this study, addition of specific impurities to the BIBW 2992 drug substance did not increase any of the previously observed toxicity of the rat.

The proposed commercial specifications for impurities (b) (4) were above ICH Q3A qualification levels. At the proposed specifications of NMT (b) (4) respectively, patients would be exposed to these impurities at levels of (b) (4) (b) (4) daily at the recommended dose of afatinib. The Applicant conducted the 13-week study described above to qualify these impurities. Based on this 13-week study, in which animals received up to (b) (4) of (b) (4), and (b) (4) o (b) (4) without significant increases in toxicity compared to afatinib alone, these impurities have been qualified from pharmacology/toxicology safety perspective at the proposed specifications.

8 Carcinogenicity

The Applicant submitted protocols for carcinogenicity studies in rat and mouse for review by the FDA's Executive Carcinogenicity Assessment Committee; however these

studies are not required to support the development of drugs for patients with advanced cancer and have not been initiated for this development program following discussion with the Agency.

9 Reproductive and Developmental Toxicology

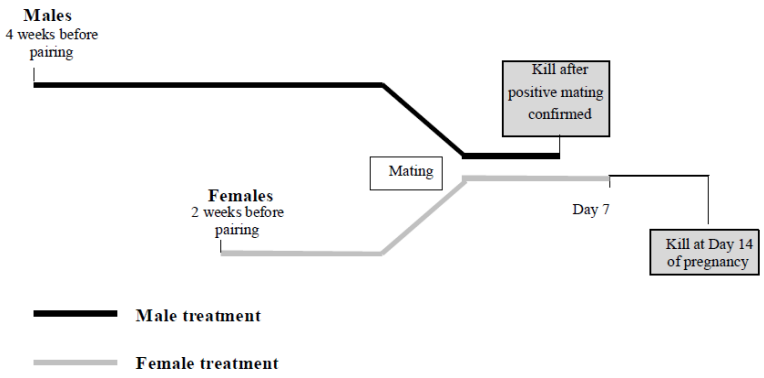
9.1 Fertility and Early Embryonic Development

Study title: **Fertility and early embryonic development study in the Han Wistar rat by oral gavage administration**

Study no.:	U11-2843-01
Study report location:	Electronic file, 4.2.3.5.1 tab, Nonclinical study reports
Conducting laboratory and location:	(b) (4) (b) (4) Test site: Boehringer Ingelheim Pharma GmBH and Co. KG
Date of study initiation:	16 September 2010
GLP compliance:	Yes, GB
QA statement:	Yes, GB
Drug, lot #, and % purity:	BIBW 2992 MA2; 1040023 (1 g of the free base corresponds to 1.478g of the salt form; not including impurity); 98.8%

Methods

Doses:	4, 6, 8 mg/kg/day (free base BIBW 2992 BS)
Frequency of dosing:	M: once daily four weeks F: once daily before pairing, throughout pairing and until D7 after mating
Dose volume:	10 mL/kg
Route of administration:	Gavage
Formulation/Vehicle:	Free base (1 g of the free base corresponds to 1.478g of the salt form; not including impurity)/reverse osmosis water
Species/Strain:	HsdHan:WIST rats
Number/Sex/Group:	22 male and 22 female
Satellite groups:	Additional 6 males and 6 females to each group for toxicokinetic evaluation, 3 males and 3 females constitutes a control group
Study design:	Treated males and treated females were crossed during the study, as presented in the following figure:



(excerpted from the Applicants submission)

Deviation from study protocol: Minimal, not affecting the study results

Observations and Results

Mortality

Visual inspections were noted once daily.

- A high dose male (No:109) was necropsied on Day 19, counted from the first dosing day (D19) due to weigh loss and abnormal/elevated gait, hunched posture, piloerection, encrustations, loose stools, hair loss on dorsal body surface and forelimbs. The animal had enlarged mandibular lymph nodes, empty stomach and cecum and soft fecal formation in the rectum.

Clinical Signs

Visual observations were recorded once daily during the first week, twice daily during week 2 to 4, and once per week from week 5 onward including D2 and D7 of gestation. Detailed observations were also recorded immediately before dosing, immediately after dosing, on completion of dosing, between one and two hours after the dosing, and as late as possible on the working day of the dosing day.

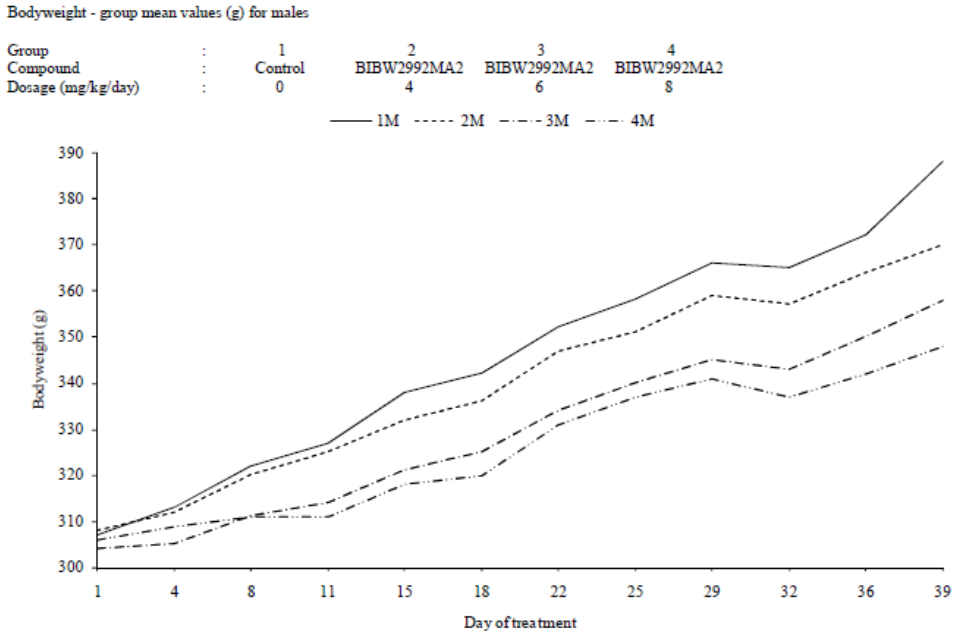
- Loose feces, encrustations around the muzzle and buccal cavity, reddening the muzzle and buccal cavity was noted for males and occasionally for females receiving the mid or high dose of the BIBW 2992.

Body Weight

Weight was recorded on the day of the treatment, and twice-weekly throughout the treatment.

- A dose related, statistically significant reduction in mean body weight gain was noted in males at the mid (mean body weight ~22 g lower) and high (mean body weight ~28 g lower) dose levels when compared to the control animals (Figure 47, excerpted from Applicant's submission). No significant changes in body weight were seen in females throughout the experiment.

Figure 47: Male Body Weight (Rat Fertility)



Feed Consumption

Twice-weekly recordings of the weight of food given, the remaining food, and an estimate of the spilled food were performed throughout the study.

- Mean food consumption was reduced during D1-D21 for males in the high dose group, and D4-D7 for females in the mid and high dose groups. During the gestation period, specifically D0-D3, mean food consumption was significantly reduced in mid and high dose group females when compared to the control females. After D4, food consumption was similar in all female groups.

Toxicokinetics

- Control animal samples were negative for BIBW 2992. The maximum plasma concentration was reached 2-4 hours after dosing in all test article treated animals, with males having up to two fold higher plasma concentrations than females. The exposure increased proportionally with the dose, plasma concentrations were similar on D1 vs. D36 (males) and Gestation D7 (females). Toxicokinetic summary table is presented below:

Summary Table: Mean toxicokinetic parameters (doses are reported as BIBW 2992)

Parameter	Period	Sex	4 mg/kg	6 mg/kg	8 mg/kg
C(max) [nmol/L]	1	m	109	154	184
	1	f	48.2	92.2	107
	1	m & f	78.7	123	145
	2	m	82.2	144	192
	2	f	41.1	70.1	104
	2	m & f	61.6	107	148
AUC(0-24h) [nmol·h/L]	1	m	915	1880	1960
	1	f	372	682	1150
	1	m & f	643	1280	1550
	2	m	824	1760	1980
	2	f	375	863	807
	2	m & f	599	1310	1390

Period 1 = Day 1 of treatment
Period 2 = Day 36 of treatment for males and Day 7 of gestation for females

Necropsy

- Macroscopic examination noted high dose level males having a higher incidence of scabs on the skin.
- low corpora lutea numbers (0.91 times the number in control females) were noted in females at the 8 mg/kg high dose level, which possibly correlated with the slightly lower numbers of live embryos (0.82 times the number in the historical control data).

The reviewer does not agree that the effects on corpora lutea are an effect of the general toxic potential of the test article, and not the outcome of specific reproductive organ toxicity. The Applicant hypothesizes that reduced food consumption during gestation results in lower corpora lutea numbers and, therefore, lower numbers of live embryos, but there is not clear evidence presented to support this idea.

- post implantation loss (2.3 times higher compared with the historical control) was noted in the test-article-administered animals

Fertility Parameters (Mating/Fertility Index, Corpora Lutea, Preimplantation Loss, etc.)

Females

Estrous cycles were examined for 15 days before pairing, by obtaining daily vaginal smears from all females on the study.

- Estrous cycles, mating performance and fertility parameters such us pre-coital interval, percentage mating, conception rate and fertility index (Table 52), excerpted from Applicant's submission) were found not to be affected by the treatment.

Table 52: Female Fertility Parameters

Daily Dose [mg/kg]		0 (Control)	4	6	8
Females	Premating Food Consumption [%[b]]	16 g	98	97	95*
	Gestation Food Consumption [%[b]]	21 g	99	99	96
	Mean No. Estrous Cycles/regular 4-5 day [n/%]	22/100	22/100	20/91	21/95
	Mean No. Days Prior to Mating 1-4 [n/%]	22/100	22/100	21/95	22/100
	No. of Females Sperm-Positive	22	22	22	22
	No. of Pregnant Females	22	22	22	22
	No. Aborted or with Total Resorption of Litter	0	0	0	0
	Mean No. Corpora Lutea	14.5	13.6	13.5	13.2*
	Mean No. Implantations	13.7	12.5	12.7	12.1*
	Mean % Pre implantation Loss	7.2	8.5	5.9	8.9
	Mean No. Live Conceptuses	13.0	11.2	11.7	10.7**
	Mean No. Resorptions	0.7	1.4	1.0	1.5
	Mean % Post implantation Loss	5.1	11.0	7.9	11.8*

Williams' test: * - p<0.05 ** - p<0.01. *Wald's test*: * - p<0.05 (Post-implantation loss)

b – At the end of the phase. For controls, group means are shown. For treated groups, percent differences from controls are shown. Statistical significance is based on actual data (not on the percent differences).

Males

- Slight reduction in the number of mating pairs with high numbers (4-6) of copulation plugs (high dose males 48% vs. control males 68% vs.)
- Increase in the incidence of males with low or no sperm counts in high dose males versus control animals (5/21 animals with no sperm or, ~24% vs. 1/22 animals or ~0.45%).

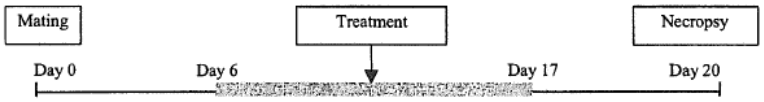
9.2 Embryonic Fetal Development

U07-2064 BIBW 2992 MA2 Preliminary study for effects on embryo-fetal development in Han Wistar rats by oral (gavage) administration (BIBW 2992 batch number 06221 T9/06, 98.7% purity)

Summary

Three groups of 6 female animals received the test article by oral gavage at doses of 8, 12, and 16 mg/kg/day from D6 to D17 after mating to assess the pregnancy outcome in correlation with the dose of the test article. An additional control group received reverse osmosis water throughout the experimental period.

Study design



- Administration of 16mg/kg/day resulted in the death of one female due to GI toxicities of the BIBW 2992. This animal had marked body weight loss and was cold to touch with piloerection, loose feces, partially closed eyelids, a dull eye, hunched posture, and pale skin color. Macroscopically, the cecum was filled with abdominal fluid, and the rectum with soft fecal material.

- Two other females in the same group experienced piloerection after dosing on D14/15 of gestation, one with partially closed eyelids on D14.
- Low mean body weight throughout D7-13, and low food consumption on D6-17, which improved after the cessation of the treatment.

12 mg/kg/day
- Body weight gain was lower during gestation D9-12, probably as a consequence of low food consumption during D6-9;
- One female experienced piloerection after dosing on gestation D15 (1/6; ~16%).

8 mg/kg/day
- Minimally lower mean body weight gain was observed during gestation D 10-12.
- Food consumption was not lower at any day of the study.
- No other adverse effects were noted.

There were no changes in numbers of litters, litter weight or any other changes noted for the offspring in this experiment. There was a slightly higher mean number of live young in all treatment groups when compared with control animals, which reflected the differences in the mean numbers of corpora lutea and/or lower mean percentages of pre-implantation loss compared with controls.

08B025 BIBW 2992 MA2: Dose range finding study for effects on embryo-fetal development in rabbits by oral (gavage) administration (BIBW 2992 batch no: 06221; purity not found)

Summary

Four groups of six Himalayan rabbit (Crl: CHBB (HM)) females were given oral doses of 5.9, 11.8 and 23.6 mg/kg of BIBW 2992 MA2 (equals to 4, 8, and 16 mg/kg of BIBW 2992 BS) by gavage from gestation day (GD) 6-18. A control group of six females received demineralization water, and additional four animals per group were used for toxicokinetic assessment. All females became pregnant in the study.

16 mg/kg/day
- Lethality in all does, on gestational days 15 (one doe), 16 (four animals), and 17 (one doe). The animal that died on GD 16 died early, while the other animals were euthanized in moribund condition.
- Two does reabsorbed all fetuses, while one doe reabsorbed only one fetus.
- GI-related toxicities were observed by the second gestational week, visible by ulceration of the stomach wall, retarded embryo-fetal growth was noted at this dose and was hypothesized to be the related to maternal toxicity, particularly the observed GI toxicities.

8 mg/kg/day
- One doe had complete reabsorption and reddish-brownish stained fur on the anal area with blood in the tray

- Decreased maternal body weight during and after treatment period associated with reduced fetal body mass (small fetuses and 15 runts[4] in 3 of 5 does(~43%; doe No: 303, 305 and 306) by the third gestation week. All remaining fetuses survived with no macroscopic signs of teratogenicity.

Visceral and skeletal malformations were not assessed in this non-GLP experiment. Based on the results of this study, doses of 3.7 mg/kg (low, group 2); 7.4 mg/kg (mid, group 3); and 14.8 mg/kg (high, group 4) of BIBW 2992 MA2 were proposed for the GLP-compliant rabbit embryo-fetal developmental study.

Specific litter data were presented in Table 53 (excerpted from the Applicant's submission):

Table 53: Dose Range Finding EFD Rabbit Caesarean Data

Parameter (means)	G 1 Control	Dose groups BIBW 2992 BS [mg/kg]			Historical data+	Spontaneous incidences from the evaluation study (U05-1804)
		G 2 4 mg/kg	G 3 8 mg/kg	G 4 16 mg/kg		
n litters ##	6	6	5#	6##	158	60
	Means / individual ranges				means / ranges of means	
Corpora lutea	7.8/6-11	8.0/7-10	7.4/7-8	8.2/6-11	7.5/ 6.4-8.4	7.6/7.6-7.8
Implantations	7.0/6-11	7.7/6-9	7.2/6-8	7.3/4-10	7.0/ 6.1-7.8	7.3/7.2-7.4
Viable fetuses	6.2/4-9	7.3/5-9	7.0/5-8	4.5/3-9	6.5/ 5.3-7.4	6.9/6.8-7.2
Dead fetuses	0	0	0	0	0.01/ 0-0.08	<0.1/0-0.1
Sex (%)						
male	50.61/ 0-71.43	48.66/ 28.57-62.50	47.50/ 0-75.00	-	51/48-59	42.93/39.38-48.30
female	49.39/ 28.57-100	51.34/ 37.50-71.43	52.50/ 25.00-100	-	49/41-52	57.07/51.70-60.62
Total resorptions	0.83/0[1]-2	0.33/0-1	0.20/0-1	2.83/ 0[1]-10	0.5/ 0.2-1.2	0.32/0.15-0.45
early resorption	0.83/0[1]-2	0.33/0-1	0.20/0-1	1.17/ 0[1]-6	0.4/ 0.1-1.1	0.28/0.15-0.40
late resorption	0	0	0	1.67/ 0-10	0.1/0-0.1	0.03/0-0.05
Fetal weight (g)	36.14/ 32.57-38.79	34.14/ 30.45-38.91	27.59*↓/ 20.53-33.33	-	38.3/ 35.9-40.3	34.88/34.29-35.30
Pre-implantation loss (%)	10.71/ 0[14.29]-25	4.05/ 0[10]-14.29	2.86/ 0-14.29	10.74/ 0[9.09]-42.86	6.5/ 1.7-13.1	4.46/3.48-5.94
Resorption rate (%)	11.36/ 0[16.67]-33.33	4.63/ 0[11.11]-16.67	3.33/ 0-16.67	37.50/ 0[25]-100	7.3/ 4.2-14.4	4.67/1.91-7.19

* significant difference (p<0.05)
↑ significantly increased; ↓ significantly decreased
\# doe No. 301 with complete resorption excluded
doe No. 404 with intercurrent death and Nos. 401, 402, 403, 405 and 406 killed in extremis included
\+ Control groups from 10 studies before 10 October, 1998
[ ] the lowest number greater than 0
\- data not available

All pregnant animals (prematurely died and abortions included)

[4] Fetuses classified as runt are offspring weighing less than 65% of the control group mean value.

Embryo-fetal parameters were summarized in Table 54 (excerpted from the Applicant's submission):

Table 54: Rabbit DRF EFD Litter Data

Findings / Group		G 1	G 2	G 3	G 4	Historical Data+ / %§	Spontaneous incidences from the evaluation study (U05-1804) %§
			Dose [mg/kg]				
		Control	4	8	16		
		n (%)§§	n (%)§§	n (%)§§	no fetuses available#		
Total number of litters		**6**	**6**	**5**	-	**158**	**60**
Number of fetuses		**37**	**44**	**35**	-	**1028**	**416**
Runts		0	0	15 (42.86)§	-	8 (0.78)	3 (0.72)
Flexures of extemities							
forelimbs	unilateral	3 (8.10)	1 (2.27)	5 (14.28)	-	9 (0.88) ++	7 (1.68)
	bilateral	1 (2.70)	4 (9.09)	1 (2.85)	-		
hindlimbs	bilateral	2 (5.40)	5 (11.36)	2 (5.71)	-		0
Variations:							
External:							
Less integument in the region of forelimbs		1 (2.70)	6 (13.63)	2 (5.71)	-	0	1(0.84)§§[from U06-1200 n=118 (less integument of extremities)]
Less integument in the region of hindlimbs		0	4 (9.09)	1 (2.85)	-	0	-

%§ Decimals rounded
(%)§§ Decimals truncated
doe No. 404 with intercurrent death and Nos. 401, 402, 403, 405 and 406 killed in extremis included
- No data
+ These data origin from an internal historical data set from 10 control groups from 10 studies before 1998 (U93-2032, U94-2044, U94-2119, U94-2192, U95-2127, U95-2267, U96-2578, U98-2389, 43S, 97B057). They are filed in the Laboratory of Reproductive Toxicology at Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach.
++ Sum of all positions of flexures

Study title: **BIBW 2992 MA2 study for effects on embryo-fetal development in Han Wistar rats by oral (gavage) administration**

Study no: Boi-0361-073332
Study report location: Electronic submission; 4.2.3.5.1 tab, Nonclinical study reports
Conducting laboratory and location: (b) (4) [redacted]
Boehringer Ingelheim GmbH & Co. KG, Birkendorfer Strasse 65 D-88397 Biberach/Riss Germany
Date of study initiation: 2 May 2007
GLP compliance: Yes
QA statement: Yes
Drug, lot #, and % purity: BIBW 2992 MA2, 06221 (T9/06), 98.7%

Key Study Findings:

16 mg/kg/day

Early termination of one pregnant female (12 fetuses identified at necropsy) on GD12 due to loss of weight (total loss 28 g from the start of the treatment), hunched posture,

piloerection and GI-toxicities (yellow, watery anus discharge accompanied by empty GI tract, and yellow fluid in cecum, duodenum, ileum and jejunum.

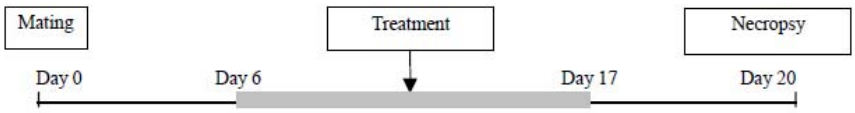
Another female in the same group showed similar GI signs, along with salivation and encrustations around the muzzle and nose, after treatment on GD12.
Slightly lower litter weight, and statistically significant lower fetal and placental weights were noted in the high-dose group when compared to the control group. In addition, a slightly higher incidence of incomplete ossification was also noted in litters of the high-dose group.

8 mg/kg/day
No treatment related clinical or macroscopic signs, except lower body weight gain and reduced food consumption. Partial recovery was noted before the end of the treatment period in the mid group (8 mg/kg/day) although animals did not reach the weight levels of the control group.

Maternal NOAELwas determined to be 8 mg/kg/day (Cmax 112 nmol/L and AUC (0-24h) 727 nmol.h/L D12); while NOAEL for fetal survival was determined to be 16 mg/kg/day (Cmax 342 nmol/L and AUC (0-24h) 3540 nmol.h/L D12)

Methods

Doses:	0, 4, 8 and 16 mg/kg/day of BIBW B2- free base
Frequency of dosing:	Once daily from day 6-17 of gestation
Dose volume:	10 ml/kg
Route of administration:	Oral gavage
Formulation/Vehicle:	BBW 2992 MA2 in RO water/RO water
Species/Strain:	Han Wistar rats/HsdRccHan:WIST
Number/Sex/Group:	22/F/group (four groups)
Satellite groups:	None mentioned
Study design:	As depicted on the following figure:



Deviation from study protocol:	Minimal that did not change validity of the study

Observations and Results

Mortality

- One high-dose female was euthanized on GD 16 due to loss of weight, hunched posture, piloerection and aqueous yellow fluid anus discharge. Macroscopic

evaluation noted GI toxicities, specifically yellowish gelatinous fluid in duodenum, ileum and jejunum. This animal lost 26 g from the beginning of the treatment.

- Another female in the high dose group had salivation, piloerection, loose and/or liquid feces between GD 12 and 14, specifically occurring after the dosing.

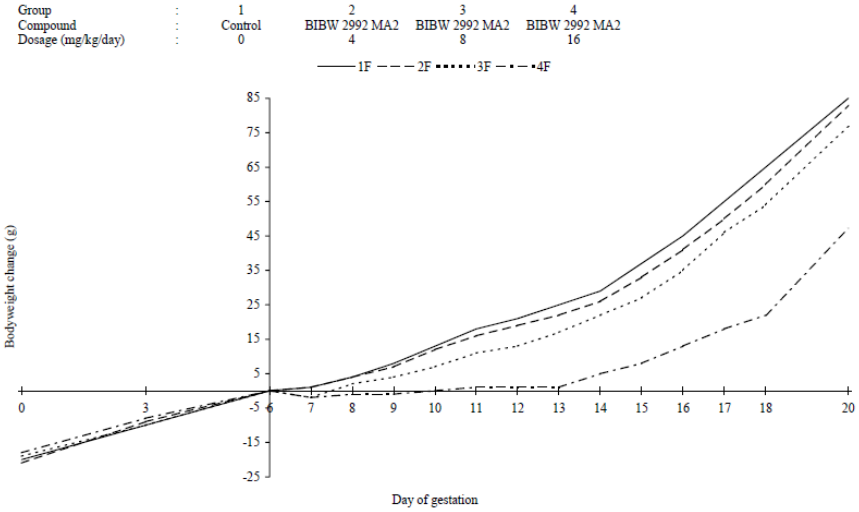
Clinical Signs

- Females in the 16 mg/kg high dose group had muzzle and nose area encrustations, along with piloerection, encrustations around orbits, hunched posture, loose feces, and abnormal coloration, mostly brown, around the nose in some animals.
- No treatment related clinical signs were noted in low and mid-dose groups.

Body Weight

- High and to some extent, mid-dose group females had markedly lower mean body weight gain, predominantly on D 6-13, when compared to the control group, with a trend to recovery by D 20 .
- Low-dose animal weight was found to be unaffected, as seen in Figure 47 (excerpted from Applicant's submission):

Figure 48: Maternal Body Weight (Rat EFD)



Feed Consumption

Values for food consumption coincided with the values obtained for weight gain. Animals given mid and high dose of BIBW 2292 had lower food consumption on GD 6-17 than the control or low dosed animals. On GD 18 and 19, food consumption increased, which was visible by the weight gain, specifically in the high-dose group of animals.

Toxicokinetics

Systemic drug exposure was demonstrated after oral dosing of the test substance. Maternal exposure increased more than proportionally with the dose increase.

Moderate variability in plasma concentration was noted with similar concentrations on D1 and D12 of the treatment

Table 55: Rat EFD Mean Toxicokinetics

(doses are reported as BIBW 2992 BS)

parameter	day	4 mg/kg	8 mg/kg	16 mg/kg
C(max)	1	46.3	116	488
[nmol/L]	12	60.0	112	342
AUC(0-24h)	1	322	953	3340
[nmol·h/L]	12	299	727	3540

Stability and Homogeneity

The concentration results for all individual samples were within the range of 97.9 -99.8% of the nominal concentration value.

Cesarean Section Data (Implantation Sites, Pre- and Post-Implantation Loss, etc.)

- All animals were pregnant, but one high-dose female was euthanized while on treatment, specifically on GD20.
- Litter data as assessed by mean corpora lutea, number of implantations, early, late and total resorptions, live young, sex ratio and pre-and post-implantation loss were similar among all groups.

Table 56: Caesarean Data-Rat EFD

Dose (mg/kg)	Corpora Lutea #	Implantations	Total Resorption	Live Young	Sex Ratio (%M)	% Pre-Implantation Loss	Post-Implantation Loss	Placenta Weight (g)	Litter Size
0	12.4	11.5	0.7	10.7	47	7.7	6.1	0.52	10.73
4	12.7	11.4	0.5	10.9	50.5	9.8	4.2	0.52	10.91
8	13.2	12.3	0.6	11.7	47.4	7.1	4.8	0.49	11.68
16	13.2	11.5	0.9	10.7	45.5	12	8.6	0.45	10.67

Table 57: Fetal Body Weight--Rat EFD

Dose (mg/kg)	Male Fetal Weight (g)	Female Fetal Weight (g)
0	3.63	3.42
4	3.62	3.46
8	3.58	3.36
16	3.36	3.17

Offspring Data

- There was no relationship of the treatment dose to distribution of malformations noted in the fetal pathology

- Cardiovascular abnormalities and complete 14th rib with associated costal cartilage and/or 20 thoracolumbar vertebrae were noted in two fetuses, from different litters of the females given the high dose of the treatment molecule
- An increase in the number of fetuses/litters with left umbilical artery was noted in mid and high dose offspring, however the incidences were similar to the background findings
- Increases in the number of fetuses/litters with hemorrhages around the brain or spinal cord were noted, when compared to the background control data range

(excerpted from Applicant's submission)

Table 58: Litter Data (Rat EFD)

Fetal examinations - minor skeletal abnormalities/variants - fetal %, group incidences

Group	:	1	2	3	4
Compound	:	Control	BIBW 2992 MA2	BIBW 2992 MA2	BIBW 2992 MA2
Dosage (mg/kg/day)	:	0	4	8	16

		Fetuses				Litters			
Group		**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Number examined		118	119	128	113	22	22	22	21
Number affected (% fetal incidence)									
Skeletal abnormalities									
Cranial	bridge of ossification/partially fused maxilla to jugal	5(4.2)	3(2.5)	6(4.7)	10(8.8)	3	3	5	7
Vertebral element abnormality									
Ribs	medially thickened/kinked	15(12.7)	19(16.0)	9(7.0)	8(7.1)	11	11	5	5
Sternebrae	offset alignment	2(1.7)	1(0.8)	1(0.8)	1(0.9)	2	1	1	1
	bipartite ossified	-	-	1(0.8)	-	-	-	1	-
	wide/misshapen 6th	-	4(3.4)	-	3(2.7)	-	2	-	2
Total affected by one or more of the above		22	25	17	17	12	13	11	9
Rib and vertebral configuration									
Cervical rib		8(6.8)	11(9.2)	13(10.2)	5(4.4)	6	10	8	4
Number with 13/14 or 14/14 ribs		52(44.1)	48(40.3)	62(48.4)	44(38.9)	21	17	20	15
Complete 14th rib/with associated costal cartilage		4(3.4)	2(1.7)	3(2.3)	7(6.2)	4	2	3	6
20 thoracolumbar vertebrae		1(0.8)	1(0.8)	3(2.3)	6(5.3)	1	1	2	4
Offset alignment pelvic girdle		2(1.7)	-	1(0.8)	3(2.7)	2	-	1	2

		Fetuses				Litters			
Group		**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Number examined		118	119	128	113	22	22	22	21
Number affected (% fetal incidence)									
Incomplete ossification/unossified									
Cranial centres		34(28.8)	43(36.1)	32(25.0)	24(21.2)	17	16	14	10
Hyoid		-	1(0.8)	-	2(1.8)	-	1	-	2
Vertebrae	thoracic	1(0.8)	3(2.5)	1(0.8)	2(1.8)	1	2	1	2
	lumbar	-	2(1.7)	1(0.8)	-	-	1	1	-
	sacrocaudal	1(0.8)	3(2.5)	4(3.1)	1(0.9)	1	3	2	1
Sternebrae	5th and/or 6th	36(30.5)	29(24.4)	27(21.1)	40(35.4)	12	16	15	16
	other	7(5.9)	4(3.4)	6(4.7)	8(7.1)	6	4	4	5
	total	36(30.5)	30(25.2)	28(21.9)	43(38.1)	12	16	15	17
Pelvic bones		-	-	2(1.6)	-	-	-	2	-
Metacarpals/metatarsals		-	-	5(3.9)	2(1.8)	-	-	4	1
Precocious ossification									
Cervical vertebral centra (>5 ossified)		25(21.2)	21(17.6)	31(24.2)	30(26.5)	11	12	17	12
Additional observations at necropsy									
Left umbilical artery		7(5.9)	4(3.4)	13(10.2)	8(7.1)	7	4	10	7
Shiny skin		-	-	3(2.3)	-	-	-	2	-

Note: Individual fetuses/litters may occur in more than one category.

		Fetuses				Litters			
Group		**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Number examined		118	121	129	111	22	22	22	21
Number affected (% fetal incidence)		22	21	31	36	13	14	19	20
Liver	additional lobe	-	-	1 (0.8)	-	-	-	1	-
	posterior caudate lobe folded/fissured	1 (0.8)	-	2 (1.6)	2 (1.8)	1	-	2	2
Kidney(s)	rudimentary papilla	-	-	-	1 (0.9)	-	-	-	1
Testis(es)	displaced	1 (0.8)	4 (3.3)	2 (1.6)	3 (2.7)	1	4	2	3
Umbilical artery	left	7 (5.9)	6 (5.0)	12 (9.3)	13 (11.7)	5	5	10	10
Haemorrhages	brain/spinal cord	4 (3.4)	-	8 (6.2)	7 (6.3)	4	-	8	7
	eye/surrounding tissue	1 (0.8)	-	1 (0.8)	-	1	-	1	-
	abdominal cavity	2 (1.7)	-	-	2 (1.8)	2	-	-	2
	liver lobe(s)	-	1 (0.8)	1 (0.8)	3 (2.7)	-	1	1	2
	subcutaneous	-	4 (3.3)	3 (2.3)	5 (4.5)	-	3	3	4
Additional observations at necropsy									
	shiny skin	1(0.8)	-	-	-	1	-	-	-

Study title: **BIBW 2992 MA2: Study for effects on embryo-fetal development in rabbits by oral (gavage) administration**

Study no: 08B026 (Document no: U09-1336-01

Study report location: e-file: 4.2.3.5.1 tab, Nonclinical study reports

Conducting laboratory and location: Boehringer Ingelheim Pharma GmbH& Co. KG; Birkendofer Str. 65, 88397 Biberach an der Riss, Germany

Date of study initiation:	24 July 2008
GLP compliance:	Yes
QA statement:	Yes
Drug, lot #, and % purity:	BIBW 2992 dimaleate(1 g BIBW 2992 BS corresponds to 1.478 g BIBW 2992 MA2); 06221; 98.4%

Key Study Findings

- A minimum of 17 females per group were pregnant and evaluated
- Control group had 21 litters, low dose group (2.5 mg/kg) had 18 litters, mid dose (5 mg/kg) had 17 litters, and high dose (10 mg/kg) had 19 litters

10 mg/kg group

- There were 2 deaths in the high dose group during the treatment, 2 additional females needed to be euthanized after the treatment period due to poor condition; 3 other females had complete abortions.
- GI toxicities observed are ulceration in the stomach (8/30), changed filling of the gut (3/30) in connection with changed mass and consistency of feces (14/30 animals).
- Decreased body weight gain and food consumption

5 mg/kg group

- Similar toxicities were seen in this group, as in the high-dose group, demonstrating a dose-relationship
- No maternal death occurred, and one complete abortion was noted
- No ulceration; mass and consistency of feces were changed in 2/21 animals
- No changes in body weight gain and food consumption

2.5 mg/kg group

- No test-article related findings noted

Findings in litters

- No dose-related difference in the mean number of corpora lutea or implantations
- The mean number of total resorptions and the resoprtion rate as well as the mean number of viable and dead fetuses were unaffected
- There was an increase in the mean number of late resorptions in the mid-dose group, which was not observed in the high-dose group. This finding was regarded as incidental and with no relationship to the drug-administration.

Fetal observations

- Four runts[5] (~3.1%) and decreased mean fetal body weight were noted in 10 mg/kg group-fetuses

[5] Runts were defined as a fetus weighing less than 65% of the control mean weight.

- One dead fetus noted in the low dose group was defined as incidental, based on the other findings in the females, and in comparison with the control group fetuses
- A cyst in the liver was noted in a single high-dose group fetus; this finding is not a clearly defined "variation" or "malformation".

Most **variations**[6] seen in the low and mid-dose range groups were comparatively observed in the control group at the same rate, and without the relationship to the dose, those were concluded to be incidental;
Flexure of extremities, less integument in the region of forelimb, an additional vessel at the aortic arch, an additional vessel at the right or left of carotid arteries, thin stomach wall, small testes; lumbar and/or isolated lumbar rib (flying rib), unilateral change in the curvature of the rib, in addition to distally, partly unossified (bilateral) humerus were variations seen in the fetuses of high dose group (10 mg/kg) females.

Retardation-defined findings were observed in mid and high-dose given groups, and consist of observations of small nature of sternebra and of changes in the curvature of ribs (~1.9% in mid; ~1.5% in high-dose fetuses, or ~1.9 and ~5.4 incidence, because of the total number of fetuses in high dose was smaller than of that in mid dose) which was defined as a result of delayed ossification caused by decreased fetal body weight and slow maternal body weight gain

Malformation[7] in the form of ventricular septal defect (VSD) occurred in the high-dose group (3.7%) was the only one attributed to the test article administration. All other malformations noted were equally distributed in all dose groups including control, thus, they were defined as incidental.

Methods

Doses: 0, 5.9; 11.8; and 23.6 mg/kg BIBW 2992 MA2 or
0, 4, 8, and 16 mg/kg BIBW 2992 BS

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[6] Variations were defined by the Applicant: "as a gross morphological alterations that include:
a) strain-specific, frequently observed, minimal anomalies which do not impair viability (cervical ribs, lumbar ribs)
b) All retardations, with or without a reduction in body weight, e.g. dilated renal pelvis or delayed ossification (abnormally low number of ossification centers or a reduced degree of ossification e.g. of the cranial bones). If ossification is delayed at three or more sites, this is classified as a generalized delay of ossification. The missing ossification centers are not listed individually (e. g. fewer than 5 metacarpal bones and/or fewer than two middle phalanges); only the regions are listed (e.g. delayed ossification of the fore-limbs)."

[7] Malformations were defined by the Applicant: "as serious anatomical deviations present at birth, which exceed the normal range of variation for the species. They are result of a developmental disorder and comprise the following:
a) All severe anomalies which are incompatible with life or which impair further development (e.g. Truncus arteriosus persistens, hydronephrosis, hernia, cleft palate).
b) Anomalies which persist, but which do not necessarily affect development (e.g. hemivertebra, bifid ribs, polydactyly, microphthalmia).
c) Minor anomalies which may disappear subsequently during development and the significance of which is still unclear (e.g. cleft vertebra, wavy ribs, fused sternebrae)

Frequency of dosing: Once daily from early to late organogenesis, GD 6-18
Dose volume: 10 mL/kg
Route of administration: Oral gavage
Formulation/Vehicle: BIBW 2992 MA2/reverse osmosis water
Species/Strain: Crl:CHBB (HM) rabbits (Himalayan rabbits)
Number/Sex/Group: As depicted in the Table 2.4.3:1 (Study design section, below)
Satellite groups: Additional four animals per group for TK sampling
Study design:

Group	Females per group	BIBW 2992 MA2 (salt)#		BIBW 2992 BS (free base)		Animal Nos.
		Weighed test item [mg/kg KGW]	Conc. %	[mg/kg KGW]	Conc. %	
1	21	0 (demineralized water)	0	0 (demineralized water)	0	101-121
2	21	3.70	0.037	2.5	0.025	201-221
3	21	7.40	0.074	5	0.050	301-321
4	33	14.80	0.148	10	0.100	401-433

1.000 g free base equals 1.478 g salt
Animals Nos. 408, 409 and 410 had to be killed and were prematurely necropsied 11 September 2008 due to insufficient water supply on 06 and 07 September 2008 and were excluded from further evaluation.

(excerpted from the Applicant's submission)

Deviation from study protocol: Minimal deviations were recorded that did not affect the scientific integrity or validity of this study

Observations and Results

Mortality
At least two daily observations were recorded during the administration period if they fell during the workdays. On weekends and holidays, once daily recording was done, concomitant to the administration time.

Four moribund females (two died during the treatment and two in moribund condition shortly after the cessation of the treatment) were attributed to the test article application. Decreased weight gain and low food consumption in addition to GI toxicities (ulceration of the stomach and changed mass and fecal consistency) were noted before the fatal outcome.

Clinical Signs
At least two daily observations were recorded during administration period if they fell during the workdays. On weekends and holidays, once daily recording was done, concomitant to the administration time.

- A dose related increase in findings related to the consistency of feces was recorded in mid (two animal out of 21) and high (14/30 animals) dose-groups
- One animal (doe No: 111) had an unexplained finding of blood in the tray. Normal development of fetuses was noted in this doe, and abortion or resorption were ruled out.

- There were no other clinical signs noted in this study.

(Excerpted from the Applicant's submission)

Table 59: BIBW 2992 MA2: Clinical Signs (EFD-Rabbits)

Finding	Control	Dose groups (BIBW 2992 BS)		
Groups	G1	G2	G3	G4
Dose [mg/kg]	0	2.5	5	10
n pregnant animals	21	19	19	26
Non-pregnant animals	0	2	2	4
Does with viable fetuses	21	18	17	21 (19 evaluable)
Animals intercurrently died	0	0	0	Nos. 401 (GD 17), 429 (GD 19)
Animals killed in extremis	0	0	0	Nos. 413(TK) (GD 22), 426 (GD 22)
Animals with more than one resorption	0	No. 209	Nos. 319(TK), 321	Nos. 411, 426 (killed in extremis)
Animals with corpora lutea only	0	No. 215	No. 318	0
Animals with complete abortion	0	0	No. 310 (GD 26)	Nos. 403 (GD 28), 406 (GD 24), 421 (GD 28)

TK animal with blood sampling

Body Weight

Recordings on Days 1, 21, and 28 of gestation period were obtained. Daily recordings were done during the treatment period (GD 6-18).

- A decrease in weight gain was observed in the high dose group throughout the study, compared to the control group (Figure 49).

(excerpted from the Applicant's submission)

Figure 49: Maternal Body Weight Gain (Rabbit EFD)

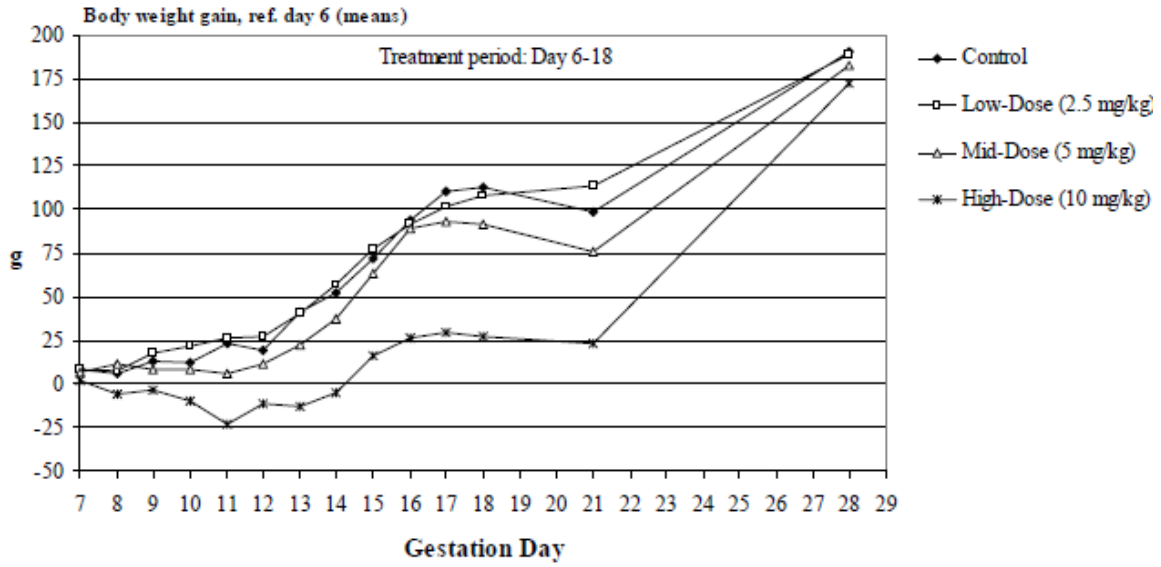


Table 60: Means of Maternal Body Weight Gain (Rabbit EFD)

Dose [mg/kg]	n does	Mean of body weight gain [g] relative to GD 6								
		GD 1	GD 7	GD 8	GD 9	GD 10	GD 15	GD 18	GD 21	GD 28
Control	21	-16.79	7.80	5.99	13.25	12.13	71.49	112.95	98.44	190.90
2.5	18	-22.96	8.37	7.47	17.79	21.89	76.99	107.91	113.90	189.01
5	17	-22.26	6.26	11.67	8.50	8.19	63.59	91.75	75.95	182.92
10	19	-37.77	1.89	-6.29	-3.36	-9.66*↓	16.37*↓	26.83*↓	22.94*↓	172.71

* significant difference (p<0.05)
↓ decreased
GD gestation day
Doses refer to BIBW 2992 BS.

Although the differences in the weight gain are seen in the mid, and to the lowest extend in the low-dose group, the high dose group was the most affected.

Feed Consumption

Food intake was determined weekly on GD 6, 14, 21, and 28.

- Lower food consumption was observed during the treatment phase (Week 2 and 3, as seen in the table below). Consumption was normalized after the treatment phase of the study, as visible from the following table:

(excerpted from the Applicant's submission)

Table 61: Weekly Food Consumption (EFD Rabbits)

Dose [mg/kg]	Week 1 GD 1 - 6	Week 2 GD 6 - 14	Week 3 GD 14 -21	Week 4 GD 21 - 28
Control	591.63 (n=21)	920.30 (n=21)	733.73 (n=21)	653.22 (n=21)
2.5	620.05 (n=18)	961.87 (n=18)	781.85 (n=18)	746.49*↑ (n=18)
5	628.15 (n=17)	906.64 (n=16)	711.48 (n=15)	739.77 (n=15)
10	598.57 (n=19)	725.07*↓ (n=19)	471.83*↓ (n=18)	792.44*↑ (n=19)

* significant difference (p<0.05)
↓ decreased
↑ increased
Doses refer to BIBW 2992 BS.

Toxicokinetics

Blood (~0.3 mL) was collected from PK animals in all groups on GD 13 for the following time points: prior to treatment; 1, 2, 4, 8, and 24 hous after treatment.

- The systemic exposure of all animals on the treatment was seen by plasma concentrations of the test article on GD13 of the study, as seen in the following table:

Table 62: Toxicokinetics (EFD Rabbits)

Dose [mg/kg]	Day of dosing	Mean C(max) [nmol/L]	Mean AUC(0-24h) [(nmol·h)/L]
		BIBW 2992 BS	BIBW 2992 BS
2.5	8+	16.2	67.1
5	8	116	425
10	8	260	1340

+ gestation day 13

(Excerpted from Applicant's submission)

Stability and Homogeneity

Standard procedures of stability and homogeneity testing were performed during the study.

- The formulation analysis show concentrations in the range of 98.7% to 103.2% of the nominal concentration values, with stability of the samples in the range of 98.9 to 99.1% of nominal values.

Necropsy

Necropsy was performed as soon as possible on prematurely morbid animals. For all other animals, *in situ* macroscopic examinations noted embryo-fetal toxicity: fetal weight, sex, number of corpora lutea, number and position of implantation sites, live/dead fetuses and resorptions per animal. Early resorptions were defined as the ones that show scars in the uterus mucosa, with scant placental tissue.

- line listing of the specific necropsy observations is presented below

(excerpted from the Applicant's submission)

Table 63: Maternal Necropsy Findings (EFD Rabbits)

Animal No.	Macroscopy and Histopathology
G1, Control group	
101	cyst (ca. 5 mm diameter) on the right oviduct
108	calcification in the left renal pelvis
109	renal calculus, retention of urine and calcification in the right renal pelvis
113	calcification in the aortic arch
G2, 2.5 mg/kg BIBW 2992 BS	
201	cyst (ca. 3 mm diameter) on the left oviduct
207	cyst (ca. 5 mm diameter) on the left oviduct
208	right uterine horn missing, ovar small, oviduct short
209	calcification in the left renal pelvis
211	the right uterine cavum to the cervix was plugged
212 (TK)	cyst (pinhead size) on the left oviduct
214 (TK)	cyst (ca. 3 mm diameter) on the left oviduct
215++	cyst (ca. 5 mm diameter) on the right oviduct
216+	induration in the fat close to the right uterine horn; histopathology: adipose tissue, uterus, right horn; moderate multifocal inflammation, mixed cellular with areas of slight fibrosis and slight multifocal perivascular mononuclear cell infiltrate.
G3, 5 mg/kg BIBW 2992 BS	
305	cyst (pinhead size) on the left oviduct
306+	calcification in both renal pelves
307	cyst (ca. 4 mm diameter) on the right oviduct
309	right uterine horn no connection to cervix, filled with transparent liquid, cyst (ca. 3 mm diameter) on the lobus caudatus (processus papillaris) of liver
315	calcification in the aortic arch
317	focal induration in the fat of the thoracal and abdominal region; histopathology: adipose tissue, thoracic and abdominal cavities; multiple areas of slight mononuclear cell aggregation with minimal fibrosis, mainly perivascular
G4, 10 mg/kg BIBW 2992 BS	
401$	caecum with dark liquid filling, beginning ulceration in the stomach, uterus filled with blood
403##	ulceration (ca. 10 mm diameter) in the pylorus, surface of gall bladder with whitish spots
411#	cyst (ca. 3 mm diameter) on the left oviduct
413§ (TK)	ulceration (ca. 5 mm diameter) in the pylorus, ulceration (ca. 5 mm diameter) in the fundus, changes in the liver – gall bladder region; histopathology: gall bladder with severe necrosis, extended to surrounding hepatic tissue, with moderate mixed inflammatory cell infiltrates and many bacterial colonies (Giemsa stain positive)
414 (TK)	left ovary rudimentary
420	lobus hepatis caudatus (processus papillaris) of liver darkish discolourated; histopathology: moderate diffuse, more pronounced in periportal areas, greenish pigment accumulation, hepatocelluar and/or sinusoidal
421##	one cyst (ca. 2 mm diameter) each on the oviducts, three ulcerations in the pylorus (ca. 1, 3 and 4 mm diameter)
425	ulceration (ca. 5 mm diameter) in the pylorus
426§#	gastrointestinal tract filled with gas, ulceration (ca. 3 mm diameter) in the pylorus, ulceration (ca. 2 mm diameter) in the fundus, changes in the liver – gall bladder region; histopathology: gall bladder; severe necrosis, extended to surrounding hepatic tissue, with moderate mixed inflammatory cell infiltrates and many bacterial colonies (Giemsa stain positive).
429$	caecum with dark liquid filling, ulceration in the stomach, uterus filled with blood
430	calcification in the aortic arch
431+	one ulceration (pinhead size) in the stomach

+ non pregnant , ++ corpora lutea only , # more than one resorption , ## complete abortion , $ intercurrently died , § animal killed in extremis , TK animals with blood sampling , Nos. 401, 426 and 429 circumanal faecal stained fur

The analysis of the data presented above implies that the use of the test drug at doses ≥ 5 mg/kg results in dose-related histopathologic liver toxicities (one liver cyst in the mid dose-animal No. 309, and several in high dose animals-Nos. 413, 420, 426).

Cesarean Section Data (Implantation Sites, Pre- and Post-Implantation Loss, etc.)

Early reasorptions were defined as the ones that show scars in the uterus mucosa, with scant placental tissue.

- There were no differences in the mean values of corpora lutea and of number of implantations among treated and control groups.
- The numbers of the endpoints of intrauterine death (the mean number of total resorptions; the mean of early resorptions, the rate of the resorption, the mean number of viable and dead fetuses) were similar among the treatment and control animals
- The mid dose group had a significant increase number of mean late resorptions, whereas, no late resorptions were seen in the high dose group (10 mg/kg) surviving does; however, animals with complete resorptions, total abortions, or that died during the study were excluded from the calculations, making calculations at the top dose under-representative of potential reproductive toxicity.

Table 64: Cesarean Parameters (Rabbit EFD)

Parameter (means)	G 1 Control	Dose groups BIBW 2992 BS [mg/kg] G 2 2.5	G 3 5	G 4 10	Historical data+	Spontaneous incidences from the evaluation study [U05-1804]
n litters #	21	18	17	19	158	60
	Means / individual ranges				means / ranges of means	
Corpora lutea	7.4/5-10	6.8/5-10	7.2/5-9	8.0/5-11	7.5/6.4-8.4	7.6/7.6-7.8
Implantations	6.6/3-10	5.9/1-10	6.8/5-9	7.2/5-10	7.0/6.1-7.8	7.3/7.2-7.4
Viable fetuses	6.1/2-9	5.6/1-10	6.2/4-9	6.8/4-9	6.5/5.3-7.4	6.9/6.8-7.2
Dead fetuses	<0.1/0-1	0.1/0-1	0	0	0.01/0-0.08	<0.1/0-0.1
Sex [%]						
male	45.52/0 [14.92]-83.33	39.56/ 0[16.67]-85.71	58.00/ 14.29-85.71	41.19/ 12.50-75.00	51/48-59	42.93/39.38-48.30
female	54.48/ 16.67-100	60.44/ 14.29-100	42.00/ 14.29-85.71	58.81/ 25.00-87.50	49/41-52	57.07/51.70-60.62
Total resorptions	0.43/0-1	0.22/ 0[1]-2	0.59/ 0[1]-2	0.37/ 0[1]-2	0.5/0.2-1.2	0.32/0.15-0.45
early resorption	0.33/0-1	0.11/ 0-1	0.24/ 0-1	0.37/ 0[1]-2	0.4/0.1-1.1	0.28/0.15-0.40
late resorption	0.10/0-1	0.11/ 0-2	0.35*↑/ 0-1	0	0.1/0-0.1	0.03/0-0.05
Fetal weight [g]	37.91/ 31.16-45.36	37.48/ 31.33-45.32	38.50/ 32.18-48.59	34.72*↓/ 26.52-41.35	38.3/35.9-40.3	34.88/34.29-35.30
Pre-implantation loss [%]	11.03/ 0[12.50]-42.86	15.38/ 0[16.67]-80.00	4.83/ 0[12.50]-28.57	9.00/ 0[12.50]-44.44	6.5/1.7-13.1	4.46/3.48-5.94
Resorption rate [%]	7.60/ 0[12.50]-33.33	3.19/ 0[12.50]-25.00	9.09/ 0[12.50]-33.33	4.61/ 0[10.00]-22.22	7.3/4.2-14.4	4.67/1.91-7.19

* significant difference (p<0.05) , ↓ decreased , ↑ increased
\# non-pregnant animals, animals with complete resorptions and total abortion as well as intercurrently died animals and animals killed in extremis excluded from evaluation
\+ These data origin from an internal historical data set from 10 control groups in 10 embryo-fetal studies performed before 1998 (U93-2032, U94-2044, U94-2119, U94-2192, U95-2127, U95-2267, U96-2578, 97B102 [Internal Number], 43S [Internal Number], 97B057 [Internal Number]). They are filed in the Teratology Laboratory at Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach.
[ ] the lowest number greater than 0

(excerpted from the Applicant's submission)

Offspring

External assessment, skeletal and visceral examinations of the fetuses (done radiographically, or with the help of binocular microscope) were recorded.

- Significantly decreased mean fetal body weight and an increase in the number of runts (`3.1%) occurred in the high dose group. One runt was recorded in the control group; no runts occurred in the mid and low dose groups (Table 3.5:1, below).
- Flexure of extremities occurred in ~7.7% of the 10 mg/kg dose group fetuses vs. ~ 2.8% for low and mid dose group animals.

Findings	Control	2.5 mg/kg	5 mg/kg	10 mg/kg	Historical data
Total number of litters	21	18	17	19	158
Number of fetuses	129	101	106	130	1028
Runts	1			4	8
Flexure of extremities	1	3	3	10	9
Variations/ external					
Forelimb area		1	2	8	
Variations/ visceral					
Aortic arch vasculature (one and two combined)	53	42	43	57	19
Carotid and pulmonary arteries (all findings combined)	37	33	34	49	29
Missing lung lobus	9	14	5	16	26
VSD intensity	1		1	2	
Thin stomach wall				1	
Gallbladder hypoplasia	2	1	5	4	0/16
Unilateral small testis				1	
Variations/skeletal					
Sternebral findings (combined)	2	1	4	4	

Findings	Control	2.5 mg/kg	5 mg/kg	10 mg/kg	Historical data
Rib findings (combined)	9	7	9	27	3
Ossified humerus				1	
Not ossified talus				2	1

Maternal toxicity observed in this study, such us death, poor overall condition, stomach ulceration, different gut mass and filling in addition to the fecal abnormalities, mirrors to the previously observed toxicities reported in dose-range and toxicity studies at doses above 8 mg/kg. The animals treated with 5 mg/kg showed similar toxicological findings, but smaller in intensity, while the low dose group (2.5 mg/kg) had minimal observable toxicity.

9.3 Prenatal and Postnatal Development

Study title: BIBW 2992: **Pre- and Post-natal development study in the Han Wistar rat by oral (gavage) administration**

Study no: U11-2844-01 ([redacted] (b) (4) DDB0103)

Study report location: Electronic file; 4.2.3.5.1 tab, Nonclinical study reports

Conducting laboratory and location: [redacted] (b) (4)

Date of study initiation: 7 October 2010

GLP compliance: Yes

QA statement: Yes

Drug, lot #, and % purity: BIBW 2992, 1040023, 98.8%

Key Study Findings

- C_{max} and AUC increased with the dose to 6 mg/kg, and stayed at the similar level for the high dose of 8 mg/kg, without accumulation of BIBW 2992
- during early lactation, low food consumption resulted in lower body weight gain in the high-dose group when compared to the animals in the control group;
- this lower birth weight and weigh gain of offspring in the mid and high-dose groups was noted, but did not affect any of functional or developmental landmarks
- NOAEL for maternal treatment from D6-20 and for offspring is 8 mg/kg
- NOEL for maternal and offspring effects was 4 mg/kg/day

Methods

Doses: 0, 4, 6, and 8 mg/kg/day

Frequency of dosing: Once daily from Day 6 after mating to Day 20 (D20)

of lactation; F1 exposure is in utero or via the milk

Dose volume: 10 mL/kg

Route of administration: Oral gavage

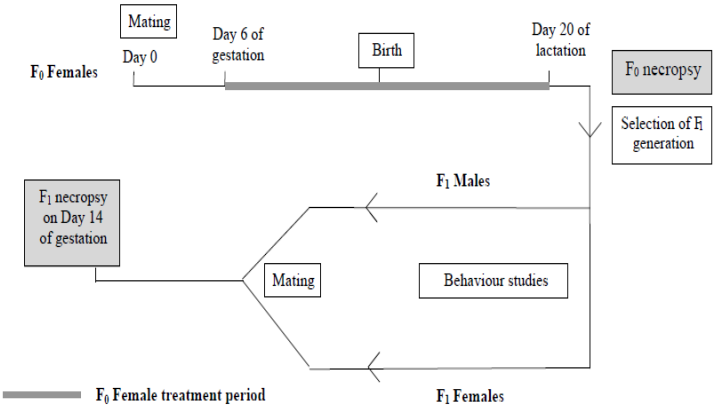
Formulation/Vehicle: BIBW 2992/RO water

Species/Strain: Harlan rat/HsdHan:WIST

Number/Sex/Group: F0 consists of 22/F/group main study, satellite study 3/F/group; F1 generation consists of 20 F plus 20 males/group

Satellite groups: TK group (3 females per group, including control)

Study design:



Deviation from study protocol: Minimal; did not impact the validity of the results

Observations and Results

Tabular overview of results is presented below:

Table 65: PPND Study Data (Rats)

F_o Dam observations**:**

Daily dose:	0 mg/kg	4 mg/kg	6 mg/kg	8 mg/kg
Survival:	21	22	22	22
No. pregnant	22	21	21	22
Body weight (% change compared to control):				
Gestation	*12 g**	83	75	92
Lactation	*10 g**	80	50	40
Feed consumption (%weight change):				
Gestation	*20 g**	95	95	90
Lactation	*50 g**	102	98	100
Toxicokinetics:				
Day 1 C(2h)nmol/L		13.2	80.7	130
Day 20 Lactation C(2h)nmol/L		33.8	63.7	60.7
Day 20 Lactation AUC (0-24h) nmol.h/L		161	364	325
Day 4 Lactation C(4h)nmol/L		22.3	43.5	57.4

F_1 litter observations:				
Daily dose:	0	4 mg/kg	6 mg/kg	8 mg/kg
No. litters evaluated	22	21	21	22
Mean No of implantations	12	12	12.2	12.7
Litter on D1	11.2	10.9	11.7	12.2
Live born pups/litter (mean)	11.2	10.6	11.5	12.1
Postnatal survival (D4, %)	98.1	99.2	97.1	96.7
Postnatal survival (weaning, %)	94.8	100	100	100
Body weight (% change):				
Females	40.3	39.9	36.8	36.7
Males	41.4	40.6	37.8	37.7
Feed consumption (post weaning weight)				
F1 males (g)	259	248	238	250

*italicized numbers are total weight change for controls; other columns reflect percent of total change in column 1

The results indicate that BIBW 2992 exposure only affected birth weight and body weight gain of mid and high-dose F1 offspring, but not the attainment of functional developmental landmarks or sexual maturation, or any of the behavioral performance parameters assessed in the study. Low body weight of the F1 offspring of mid and high-dose treated dams continued throughout the experiment; however, lower weights did not impact F1 mating performance or fertility parameters.

10 Special Toxicology Studies

02B183 BIBW 2992 MA2: Local dermal tolerance after single administration to rabbits U02-1670

Summary
With the exception of the observation of slight erythema on two treatment-exposed patches in one animal, dermal exposure of 4 Chbb:NZW rabbits to 100 mg BIBW 2992 MA2 for up to 4 hours did not cause erythema or edema in any animal at any other time point. Therefore, the test substance is considered to have potential to induce erythema shortly after the exposure, although, overall, to be of non-irritating and non-corrosive potential.

07B008 BIBW 2992 MA2: Acute eye irritation/corrosion study in rabbits U07-1510

Summary
This study was performed on a single female rabbit's one eye, while the other eye was untreated and evaluated as a control. Microscopic, slit lamp and post-mortem histological evaluation was performed during 21 day-study-period.

Changes to the iris, conjunctivae, and lids were observed immediately following application. Marked swelling of the lids (more than half closed) along with hyperaemia of the iris and the conjuctive resulted in washing the test item one hour after administration. Chemosis become less severe by the end of the first day, and improved by second day, but swelling persisted intermittently until D 17. The conjunctivae was hyperemic with observations of diffuse crimson color; hyperemic vessels were observed through Day 17. Iris alteration persisted through Day 12.

Dilated sclera blood vessels with sprouting were noted upon slit lamp examination on Day 12; no findings were noted on other study days.

Red discoloration and conjuctival irritation were noted as histopathological findings of the treated eye.

Based on the findings of this study, BIBW 2992 was classified as an "category 2A" eye irritant according to the "Globally harmonized system of classification and labeling of chemicals (GHS)" released in 2003 by the United Nations.

U11-2433-01: In vitro 3T3 NRU phototoxicity test with BIBW 2992 (GLP study)

Summary

To assess the phototoxic potential of BIBW 2992, an *in vitro* cytotoxicity assay using the BALB/c 3T3 mouse fibroblast cell line was used. The comparison of cytotoxicity of the test item in the presence (+Irr) and in the absence (-Irr) of a non-cytotoxic dose of UVA irradiation is measured. Determination of the cellular uptake by the vital dye Neutral Red (NR) was done one day after the treatment. Reduced cellular NR uptake is regarded as parameter of toxicity. The assay is usually done in duplicate, and in cases of borderline or unclear results, should be repeated under modified conditions. According to the OECR guidlanes, test articles with Photo Irritation Factor (PIF) values of 5 and above are considered "phototoxic".

Five consecutive experiments were done, but only results of three, qualified experiments were used to evaluate phototoxicity potential of BIBW 2992.

	EC 50 -Irr	EC 50 +Irr	Photo Iritation Factor
First Experiment	12.2	2.3	5.3
Second Experiment	19	11	1.9
Third Experiment	18	9	2

The PIF value of more than 5 obtained in the first experiment justified two additional evaluations of the afatinib phototoxicity; however, divergent results in three independent experiments suggest that afatinib has a phototoxic potential based on the median PIF result from these experiments of ~3. Under the conditions of the assay, BIBW 2992 was classified as having "probable phototoxicity".

BIBW 2992 MA2: Single dose toxicity study in rats by oral (gavage) administration U03-1088

Summary
The Applicant assessed the acute toxicity and estimated the Approximate Lethal Dose (ALD) of BIBW 2992 MA2 by administration of the test article in aqueous solution after it was dissolved in demineralized water to six male and six female CrlGlxBrlHan:WI rats. Three females and three males were dosed with 300 mg/kg, additional three males with 600 mg/kg body weight and additional three females with 1200 mg/kg body weight. 20 mL/kg solution of BIBW 2992 was administered to all animals. Animals were followed for 15 days.

Key study findings:
300 mg/kg body weight- NOAEL for all animals
600 mg/kg (males only)-no adverse signs noted until D8 when one animal died. Piloerection, solied anogenital region, reduced body temperature and emaciation was observed on other two animals on D8. All animals died on D9.
1200 mg/kg (females only)-piloerection, reduced body temperature and diarrhea were observed on D1 in different intensities and on different animals on different times. Two animals were necropsied on D2 and D7, respectively. One female was found dead on D4. Microscopically, GI tract was defined as the primary target of toxicities in these animals.

11 Integrated Summary and Safety Evaluation

Afatinib (BIBW 2992) is a small molecule which the Applicant has demonstrated can covalently bind to members of the Epidermal Growth Factor Receptor (EGFR) family including EGFR, HER2, and HER4. This binding inhibits the autophosphorylation that occurs following receptor homo- or heterodimerization in this family thus preventing downstream signaling. Boehringer Ingelheim has submitted data showing the covalent binding of affatinib not only to EGFR, HER2, and HER4, but also to a mutated EGFR L858R/T790M. Inhibition of downstream signaling following incubation with aftatinib was demonstrated both biochemically and in cellular assays. In kinase assays, afatinib was able to inhibit wild type EGFR and HER2 with IC_{50}s of 0.5 and 14 nM, respectively. Afatinib showed similar potency against the L858R mutant EGFR (0.43 nM) as well as strong inhibition of the erlotinib resistant L858R/T790M EGFR double mutant (10 nM). In screening assays afatinib showed low potency for all other kinases tested, with the exception of Lyn. Afatinib was able to inhibit Lyn activity with an IC_{50} as low as 100 nM in some experiments, a concentration that is achievable at the clinically recommended dose, however, this inhibition was inconsistent.

In cellular *in vitro* assays, afatinib activity was demonstrated by inhibition of phosphorylation and proliferation of multiple cell lines. Afatinib-mediated inhibition of

EGFR exon 19 deletion mutants, including an exon 19/T790M double mutant was shown in cell lines ectopically expressing the constructs. Afatinib EC_{50}s for EGFR-signaling dependent survival in this model ranged from 10-100 nM. Incubation with afatinib also resulted in decreased phosphorylation of activated EGFR mutants expressessed in 3T3 cells and inhibition of phosphorylation of wild type EGFR or HER2 as well as proliferation of cell lines expressing these receptors. Prolonged inhibition, consistent with covalent binding of afatinib to the receptor was demonstrated in a time course experiment using the EGFR overexpressing A431 human epidermal carcinoma cell line incubated with either afatinib or other EGFR inhibitors for 1 hour prior to washout. In this experiment EGF treatment of A431 cells did not result in significant EGFR phosphorylation for at least 24 hours after afatinib washout. Following incubation with a reversible inhibitor of EGFR, EGF treatment of A431 cells resulted in high levels of phosphorylation at timepoints as early as 8 hours post-washout. In *in vivo* assays, treatment of nude mice implanted with xenografts derived from the A431 cell line, the SKOV-3 human ovarian carcinoma cell line, the MDA-MB-453 human breast carcinoma cell line, or the NCI-N87 human gastric carcinoma cell line with 20 mg/kg of afatinib inhibited tumor growth or, in some cases, caused tumor regression.

BIBW2992BS is a dimalic acid salt (BIBW2992MA2). Although dosed as a salt, the test article is referred throughout the application by its free-base form, as analytical assays only measure the parent, not the counterion moiety. Doses were generally adjusted for free base content; thus, the nominal doses reflect the actual dose of the free base form.

Metabolism is considered a minor route of clearance for BIBW2992BS. BIBW2992BS is excreted largely in the bile and/or feces, and predominantly as unchanged parent. Small amounts of radioactivity were detected in urine in all species tested (mouse, rat, rabbit, minipig). Conjugates to glutathione and endogenous proteins were the most abundant metabolite species observed. Adduction was presumed to proceed via a non-enzymatic mode (Michael reaction) leading to covalent attachments to endogenous proteins, predominantly hemoglobin and albumin, and to a lesser extent, to globulins. The presence of covalent adducts is of concern for the overall safety of BIBW2992, since adduction of reactive small molecules to foreign proteins has been repeatedly associated with adverse idiosyncratic drug reactions (IDRs), including fatalities, in patients.

Characteristics of IDRs include: delay between the onset of drug treatment and the onset of a reaction; weak or no dose-dependence; and cross-reactivity to other, similar members of a class. These properties – latency, dose-insensitivity, and cross-reactivity – are highly suggestive of an immune-mediated mechanism. Indeed, one molecular mechanism by which idiosyncratic drug reactions are hypothesized to occur involves covalent modification of endogenous proteins by reactive small molecules, resulting in the formation of neoantigens (haptens). Protein-drug conjugates are thought to become targets of immune activation, leading to destruction of the cells harboring the hapten. IDRs have been repeatedly associated with drug-induced hepatic failure, anaphylaxis, autoimmune disease (e.g. SLE), and thrombocytopenia, anemia and agranulocytosis,

among others[8]. Although they occur at low frequencies (1/100-1/100,000), they are not uncommon, particularly for widely prescribed drugs, where they may number in the hundreds or thousands of cases per year. IDRs have led to numerous fatalities, and the withdrawal of several marketing applications. Because of the diverse array of potential adverse outcomes and the low frequency at which each type occurs, it is not possible to prospectively design monitoring or exclusion criteria for IDRs. Although there have been some published associations between particular HLA genotypes and certain IDRs, this does not appear to be universally applicable for all drugs[9].

For BIBW2992BS, the primary burden of bound metabolite appears to be the RBC. Some accumulation was observed in liver and kidney as well as other organs such as the adrenal, pituitary, thyroid and spleen; however, these are considerably lower than the levels exhibited by RBCs, and in the case of the spleen, may reflect sequestered RBCs undergoing degradation. Thus, while IDRs appear to be theoretically possible with this drug – particularly in the liver – it is not clear whether this yet represents a significant clinical risk. Given the dismal prognosis for patients with locally advanced or metastatic non-small cell lung cancer the risk of IDRs may be warranted in by demonstrable therapeutic benefit. Physicians should be apprised of the potential for this compound to elicit clinical IDRs.

Boehringer Ingelheim conducted dedicated safety pharmacology studies with afatinib to evaluate the effects of the drug on CNS, cardiac, respiratory, gastric, and renal function. General behavior and locomotor activity was assessed using the modified Irwin test in two strains of mice, Harlan and OF-1. In these studies afatinib had no effect on general behavior or motility at doses of up to 300 mg/kg. Treatment with afatinib did result in dose dependent effects on gastric emptying and transit in rats. Afatinib treatment also resulted in effects on renal and liver function. A single 300 mg/kg dose of afatinib in rats was associated with a mild sustained enhancement of serum glucose compared to control treated animals along with elevations in serum and urinary enzymes. Consistent with these findings, in a 4-week repeat dose study in rats at the high dose of 18 mg/kg there was a mild decrease in the albumin, and increases in blood urea nitrogen, N-acetyl-b-D-glusosaminidase (NAG), creatinine, and urinary protein. By the end of the experimental period in this study, a dose-dependent reduction in urine volume was observed in males at doses ≥8.5 mg/kg and in both genders at 18 mg/kg.

In vitro assays to assess potential cardiovascular toxicity included studies on isolated guinea pig papillary muscle and the hERG assay. Afatinib did not have an effect on action potential duration in guinea pig papillary muscle at concentrations of up to 10 μM. Afatinib inhibited hERG-mediated potassium current inHEK293 cells with an IC_{50} of 2.4 μM, suggesting low potential for QTc prolongation at clinically relevant concentrations.

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[8] Knowles, Sandra R., Uetrecht, J., Shear, Neil H. 2000. Idiosyncratic drug reactions: the reactive metabolite syndromes. *The Lancet*. 356:1587-1591

[9] Pichler, W. 2002 Pharmacological interactions of drugs with antigen-specific immune receptors: the p-i concept. *Curr. opin. in allergy and clin. Immune. 2:301-305.*

Vital respiratory and cardiovascular function and parameters were assessed by whole body bias flow plethysmography on telemetered rats. At the afatinib dose of 100 mg/kg rats displayed increased arterial blood pressure (an average of 12 mmHg). An increase was also observed at the lower dose of 30 mg/kg and increases were sustained over a 7 hour observation period. In the same study a slightly increased heart rate (~25 beats/min) was noted in the high dose groupover a two hour post-dose interval. No effects were noted on body temperature, respiratory rate, or tidal volume and motility. A similar dose-dependent increase in heart rate with consequent QT-interval shortening was observed in the 4-week oral toxicity study in the minipig. In an additional cardiovascular examination conducted in domestic pigs afatinib was administered as a bolus intravenous injection of up to 2 mg/kg; blood pressure, heart rate and other electrocardiographic parameters were not affected in this study; however, when pigs were given an intravenous infusion of afatinib at doses of 10 and 30 mg/kg, decreased LVdP/dt-max contractility was noted.

Toxicology studies with afatinib evaluated single-dose and multiple daily administration of the small molecule in mice, rats and minipigs. A report of an oral single dose toxicity study in mice noted GI and skin toxicities, characteristic for EGFR inhibitors, and established an approximate lethal dose (ALD) range of 191 to 382 mg/kg in mice of both genders. The Applicant also assessed acute toxicity in Han:Wistar rats; this experiment established a dose of less than 300 mg/kg as a maximum non-lethal dose. The dose of 600 mg/kg resulted in death of one male in the study. Piloerection, solied anogenital region, reduced body temperature, and emaciation were observed one day before death on Day 9. A dose of 1200 mg/kg was tested in females only (n=3) and resulted in piloerection, reduced body temperature and diarrhea by Day 1. All three female rats died between Days 2 and 7. Microscopically, the GI tract was defined as the primary target of toxicity in these animals.

Repeat dose toxicity studies evaluated toxicity due to administration of afatinib in mice, rats, and minipigs. Overall, the toxicity profile observed in these studies is similar to that seen with other EGFR inhibitors. In a mouse 13-week study, following administration of afatinib by oral gavage mortality occurred at doses ≥ 27 mg/kg/day. Treatment-related toxicities were significantly increased in male and female animals at a dose of 18 mg/kg/day. Observed toxicities at 18 mg/kg/day included piloerection, hair loss, elevated neutrophils, increased spleen weights, decreased ovary weights, and histopathological findings in the gastrointestinal tract, skin, cornea, and ovaries.

In a four-week Han:Wistar rat GLP study afatinib was administered daily by oral gavage at doses of 4, 8.5 and 18 mg/kg. Forty percent (12/20 of males and 4/20 females) of the animals administered the high dose of 18 mg/kg (approximately 3.8 and 2.5 times the exposure at the recommended clinical dose in males and females, respectively) died in this study due to afatinib induced GI toxicities. The majority of clinical observations were recorded in high dose group (18 mg/kg) males, and included skin (thickened lips, scratch wounds, reddened skin) and GI (loose stool) toxicities with one male in the mid dose group exhibiting the same toxicities. High dose animals, predominantly males,

also had significantly lower weight, reflecting the significantly reduced food consumption of these animals. A dose-dependent neutrophilia was seen at doses ≥8.5 mg/kg in rats of both genders., Lower percentages of erythrocyte precursor cells and decreases in the percentages of lymphocytes were noted in the mid- and high-dose groups. The percentages of early neutrophilic precusors, plasma cells, macrophages, and reticulum cells were increased in mid and high dose animals Low dose females (4 mg/kg) also had an increase in the percentage of reticulum cells. Immunological tests showed a decrease in the percentage of B-lymphocytes in the spleens of all afatinib-treated animals, with higher percentages of T-lymphocytes. All changes were resolved or resolving by the end of the recovery period.

Gross pathology observations included alopecia, scabs, crusts, red to brownish discoloration of the facial area; dilatation, diffuse reddening, liquid, yellowish/red contents in the intestinal tract; brown/black foci and diffuse red discoloration in the stomach; and diffuse dark brown or focal light discoloration in the kidneys. Gross findings in the mid (8.5 mg/kg) and high (18 mg/kg) dose group animals correlated with observations in microscopic examinations. Renal papillary necrosis was present in 13/27 animals at 18 mg/kg at the end of treatment period and in one male at the mid-dose level. Renal findings were still apparent in the recovery animals. Skin findings of severe folliculitis and dermatitis, and segmental atrophy of the epidermis were present in all high- and some mid-dose animals. Atrophy of squamous epithelium of esophagus or stomach, atrophy of the small-intestinal villi and flattening of the surface epithelial cells of the large intestine, ulcers/erosions of the stomach were also noted in ~30% of high dose animals. Atrophy of the lymphatic tissues of thymus and spleen was noted in roughly half of the high-dosed animals, with higher occurrences noted in males. Hyperplasia of mandibular or axillary lymph nodes was frequent in the high dose animals, as a secondary outcome to the skin inflammation. Dose-dependent decreases in absolute and relative weights of the prostate and ovaries along with a reduction in the size of male accessory sexual glands were observed at the end of the treatment period. These findings correlated with histopathological findings of atrophy of the prostate and/or seminal vesicles in most high-dose males that were resolved or resolving by the end of the 2 week recovery period. Atrophy of vaginal epithelium was noted at the end of the dosing period in 11/12 high-dose females along with atrophy of endometrial epithelium in 5/10 and 9/12 animals treated at the 8.5 and 18 mg/kg dose levels, respectively. Except for the advanced findings of kidney and skin, all other toxicities were ameliorated by the end of two weeks recovery period.

Rats were also employed in 13- and 26-week repeat-dose toxicology studies. Daily oral administration of afatinib in rats showed accumulation of the drug over time and systemic exposure at more than dose–proportional levels. In the 13-week toxicology study at the high dose level of 10 mg/kg, there were 3 premature sacrifices (2 male, 1 female), decreased in weight gain of ≥15% in male and female rats compared to the controls, and necrosis of the renal papilla in 8/22 main-group animals and 4/18 recovery animals. In the 26-week toxicology study, there were no treatment-related mortalities at doses up to the high dose of 6 mg/kg. Renal toxicity (papillary necrosis in 8/20 males and 1/20 females), skin lesions and increased neutrophil cell count were the main

concerns at this dose level. There were also dose-dependent increases in cholesterol clefts within foam cell accumulations in the lungs. Except for more pronounced skin lesion and papillary necrosis of kidney tissue, all other changes noted ameliorated at the end of 8-week recovery period.

The minipig was used as the major non-rodent species for evaluation of afatinib toxicity. Oral repeat-dose toxicology studies of 2, 4, 13, and 52 weeks were conducted in minipigs with doses as low as 0.5 mg/kg and as high as 10 mg/kg. The GI tract was a major target organ in minipigs in all studies conducted regardless of duration as evidenced by observations of diarrhea or soft stool and microscopic findings of increasing degrees of epithelial atrophy of the esophagus, stomach, and duodenum. In the 4-week study at the mid dose level of 2.45 mg/kg and the high dose of 6 mg/kg minipigs presented with atrophy of larynx surface epithelia and atrophy of submandibular and sublingual mucous glands. Epithelial atrophy of the upper respiratory tract was also reported at the 6 mg/kg dose level. An increase in the total number of neutrophils was noted at the end of the dosing period. The toxicities observed in the 4-week study were also seen in 13-, and 52-weeks study at the doses of 2, 5, and 7/5.5 mg/kg with the exception of heart rate increases. Additionally, microscopic observations in animals in longer term studies included atrophy of the surface epithelia of the prostate and seminal vesicles and the cornea of the eye (4/4 animals in the 5 mg/kg dose group in the 52 week study, 7/8 animals in the 7/5.5 mg/kg dose group in 26 week study), activation of erythro- and myelopoiesis in bone marrow, increases in BUN, and decreases in A/G ratio, specifically at the high doses of 5 and 7/5.5 mg/kg. During the 6-week the recovery period, the majority of the toxicities were resolved in both studies. Finally, in all minipig studies there were occasional ocular findings including microscopic findings of corneal atrophy. Keratitis has been reported clinically.

Reproductive and developmental toxicity studies of BIBW 2992 were conducted in rabbits and rats. In rabbit does, oral administration of afatinib in amounts of more than 8 mg/kg resulted in low food intake, loss of weight, and gastro-intestinal toxicities. The dose range finding study conducted to look for effects on embryo-fetal development in rabbits resulted in premature death of pregnant females at doses of 16 mg/kg with no available fetuses for analysis. The dose of 8 mg/kg/day BIBW 2992 resulted in a ~43% incidence of runts, while doses of 4 mg/kg/day or less resulted in no runt fetuses. Historical control incidence of runts in rabbits in comparable experimental trials was less than 1%.

Based on these findings, a subsequent pivotal embryo-fetal development study in rabbits explored doses of 2.5, 5, and 10 mg/kg of BIBW 2992. The high dose of 10 mg/kg resulted in the early death of 4/33 does (~12%). An additional three females at the 10 mg/kg dose level (approximately 0.74 times the exposure based on AUC in patients at the recommended dose) had complete abortions at late timepoints in the study. There was an additional late abortion in a single female at the 5 mg/kg dose level (approximately 0.24 times the human exposure by AUC at the recommended dose). Maternal toxicity in the high dose (10 mg/kg) group consisted primarily of gastro-

intestinal toxicities (ulceration of stomach, change of filling in the gut, changed mass and consistency of stools) and was accompanied by a decrease in food consumption and weight loss; however, mid-dose group (5 mg/kg/day) and low dose group females did not show changes in weight or food consumption.

No differences were recorded in the mean values of corpora lutea, number of implantations, and the numbers of the endpoints of intrauterine deaths among control and treatment groups of the pivotal embryofetal study in rabbits, though does with total litter loss were excluded from this analysis. The number of mean late resorptions significantly increased in the mid-dose group, when compared to the high-dose group where no late resorption was noted. The analysis however, does not include the 44 percent of pre-implantation loss in the high dose group, an increase of approximately 15% compared to the mid-dose group, meaning that some of the fetuses may have been lost in pre-implantation stage. A significant decrease in the mean fetal body weight along with an increase in the number of runts was noted in litters from females treated at the high dose level compared to other groups. Additionally, when grouped together,dose-related increases were observed in visceral and skeletal variations (predominantly delayed ossifications). In summary, treatment of does with doses of ≥5 mg/kg resulted in increased rates of late abortions, lower fetal weights, and increased occurrence of visceral and skeletal variations.

Additional evaluations of reproductive and developmental toxicities of BIBW 2992 were performed in Han Wistar rats. In a dedicated fertility study, male and female rats were both administered BIBW 2992 at dose levels of 4, 6, or 8 mg/kg/day. Males were treated for 4 weeks before mating, females for 2 weeks prior to mating through GD7. Males treated at the high dose of 8 mg/kg/day demonstrated a slight reduction in the number of copulation plugs and an increased incidence of low sperm count compared to control males. Necropsy analysis of females treated at the 8 mg/kg/day dose level showed low numbers of corpora lutea and a slight decrease in the numbers of live embryos.

In a dose range finding embryo-fetal development study in female rats, the high dose of 16 mg/kg/day resulted in maternal clinical signs similar to those observed at high dose levels in the rabbit studies including low food consumption along with lower body weight gain and GI toxicities; however, no numerical changes in any of the cesarean parameters were observed at this dose level. In an additional GLP study using 22 female Han Wistar rats/group, administration of the high dose of 16 mg/kg/day resulted in death of one animal due to similar GI-related toxicities observed in other reviewed studies. The high dose resulted in additional fetal abnormalities such as visceral (cardiovascular abnormalities and increase in the number of umbilical arteries), and skeletal (additional number of ribs and/or additional numbers of thoracolumbar vertebrates). Also, increased numbers of fetuses with hemorrhages around the brain or spinal cord were seen in the high dose group litters, when compared to control or background data.

Han Wistar rats were used for a prenatal and postnatal evaluation of BIBW 2992 at doses of 4, 6, and 8 mg/kg/day. The only observed difference due to test-article administration was lower body weight change, ~3% less in the mid and high doses F1 offspring. All other noted observations were comparable among the treatment groups and control. Despite continuing effects on the ability to gain weight, in utero/neonatal exposure to maternal doses of up to 8 mg/kg/day of BIBW 2992 did not appear to have significant effects on the attainment of developmental milestones or learning and memory in prenatal and postnatal developmental studies in rats.

Afatinib was evaluated in both *in vitro* and *in vivo* assays for its potential genotoxicity. The drug was positive in the Ames assay with an approximate two-fold increase in revertants seen in the presence of aftainib in both the presence and absence of metabolic activation in a single strain. Afatinib was negative in all other assays for genetic toxicity. Though the results of the Ames assay do suggest mild genotoxic potential, after consultation with the Associate Director of Pharmacology/Toxicology, the review team agreed that the weight of evidence from the genetic toxicology assessments suggests that afatinib is not genotoxic. In the absence of metabolic activation only, similar findings were seen following an assessment of the (b) (4) impurity. Again the weight of evidence suggests that this is a non-genotoxic impurity. Specifications for the (b) (4) impurity along with another impurity, (b) (4), were above the qualification threshold described in ICH Q3A. A 13-week rat toxicology study using afatinib spiked with high levels of these and several other impurities was conducted to qualify these impurities. No deaths occurred during this study. Animals received up to (b) (4) without significant increases in toxicity compared to that seen with afatinib in other studies. At the proposed specifications of NMT (b) (4) respectively, patients would be exposed to these impurities at levels of (b) (4) daily at the recommended dose of afatinib, thus, these impurities have been qualified from pharmacology/toxicology safety perspective at the proposed specifications.

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/s/

DENALI D KUFRIN
04/29/2013

SHAWNA L WEIS
04/29/2013

WHITNEY S HELMS
04/29/2013

PHARMACOLOGY/TOXICOLOGY FILING CHECKLIST FOR NDA/BLA or Supplement

NDA/BLA Number: 201292 **Applicant: Boehringer Ingelheim** **Stamp Date: November 15, 2012**

Drug Name: Afatinib (BIBW 2992 MA2) **NDA Type: 505(b)(1)**

On **initial** overview of the NDA/BLA application for filing:

	Content Parameter	Yes	No	Comment
1	Is the pharmacology/toxicology section organized in accord with current regulations and guidelines for format and content in a manner to allow substantive review to begin?	X		
2	Is the pharmacology/toxicology section indexed and paginated in a manner allowing substantive review to begin?	X		
3	Is the pharmacology/toxicology section legible so that substantive review can begin?	X		
4	Are all required (*) and requested IND studies (in accord with 505 b1 and b2 including referenced literature) completed and submitted (carcinogenicity, mutagenicity, teratogenicity, effects on fertility, juvenile studies, acute and repeat dose adult animal studies, animal ADME studies, safety pharmacology, etc)?	X		
5	If the formulation to be marketed is different from the formulation used in the toxicology studies, have studies by the appropriate route been conducted with appropriate formulations? (For other than the oral route, some studies may be by routes different from the clinical route intentionally and by desire of the FDA).	X		
6	Does the route of administration used in the animal studies appear to be the same as the intended human exposure route? If not, has the applicant submitted a rationale to justify the alternative route?	X		
7	Has the applicant submitted a statement(s) that all of the pivotal pharm/tox studies have been performed in accordance with the GLP regulations (21 CFR 58) or an explanation for any significant deviations?	X		
8	Has the applicant submitted all special studies/data requested by the Division during pre-submission discussions?			N/A: There were no pre-submission discussions in which special nonclinical studies were requested.

File name: 5_Pharmacology_Toxicology Filing Checklist for NDA_BLA or Supplement 010908

PHARMACOLOGY/TOXICOLOGY FILING CHECKLIST FOR NDA/BLA or Supplement

	Content Parameter	Yes	No	Comment
9	Are the proposed labeling sections relative to pharmacology/toxicology appropriate (including human dose multiples expressed in either mg/m2 or comparative serum/plasma levels) and in accordance with 201.57?	X		
10	Have any impurity – etc. issues been addressed? (New toxicity studies may not be needed.)	X		No clear issues have been identified at filing. Further impurity issues may be identified as the review of this NDA proceeds.
11	Has the applicant addressed any abuse potential issues in the submission?			NA
12	If this NDA/BLA is to support a Rx to OTC switch, have all relevant studies been submitted?			NA

IS THE PHARMACOLOGY/TOXICOLOGY SECTION OF THE APPLICATION FILEABLE? ___Yes____

If the NDA/BLA is not fileable from the pharmacology/toxicology perspective, state the reasons and provide comments to be sent to the Applicant.
N/A

Please identify and list any potential review issues to be forwarded to the Applicant for the 74-day letter.

There are no pharmacology/toxicology issues for the Applicant for the 74-day letter.

Dubravka Kufrin PhD — December 12, 2012
Reviewing Pharmacologist — Date

Whitney Helms PhD — December 12, 2012
Team Leader/Supervisor — Date

File name: 5_Pharmacology_Toxicology Filing Checklist for NDA_BLA or Supplement 010908

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/s/

DENALI D KUFRIN
12/13/2012

WHITNEY S HELMS
12/17/2012