number and location of all fetuses, early and late resorptions, and the total number of implantation sites were collected.

Fetal Observations

All fetuses were examined for sex, weight, external and visceral malformation. The skeleton was examined by stereomicroscopy. External, visceral and skeletal findings were recorded as developmental variations (alterations in anatomic structure without significant biological effect, representing slight deviations from normal) or malformations (structural anomalies that alter general body conformity, disrupt or interfere with body function, or are generally thought to be incompatible with life). [*the number or percentage of fetuses at each group allocated to soft tissue or skeletal examinations was not specified*].

Results

Clinical Signs and Food Consumption: One animal in the 50 mg/kg/day group had a spontaneous abortion on gestation day 27 without remarkable observations at necropsy. All other animals survived to the scheduled necropsy on gestation day 29. AF0150 did not result in significant toxic effects at any time point or dose level. Food consumption slightly increased in the 100 mg/kg/day AF0150 group during gestation days 19-20 and 21-24 (statistically significant as compared to the control group). No food consumption change was noted in the other dose groups and time points.

Body Weights and Gravid Uterine Weights: AF0150 had no significant effects on mean body weights, body weight gains, gravid uterine weights, net body weights and net body weight gains at any dose level.

Maternal Macroscopic Examination: At the scheduled necropsy (gestation day 29), 3, 3, 4 and 1 animals in the control, 50, 100 and 200 mg/kg/day groups, respectively, had an accessory spleen. One animal in each of the control and 100 mg/kg/day groups had cystic oviducts. One animals in the 50 mg/kg/day group had white purulent material in the right uterine horn. One female in the 200 mg/kg/day group had white amniotic fluid. No other AF0150-related internal findings were noted at any dose level.

Gestation Day 29 Laparohysterectomy: There were no significant differences in postimplantation loss, viable litter size, fetal body weights, fetal sex ratios, the numbers of corpora lutea and implantation sites between the AF0150-treated animals at all dose levels and the control group. [*Placenta examination was not indicated*].

Fetal Morphology: Malformation findings in the litters and fetuses available for morphological evaluation were summarized in Table 2. An increased frequency of malformations was noted in some fetuses from the high AF0150 dose groups (100 and 200 mg/kg/day). The **NOAEL** was 50 mg/kg/day (HED: 16.2 mg/kg/day and HDM: 130-fold of PCD).

Table 2. Fetal Morphological Examination

	AF0150 (mg/kg/day), *n=22 Rabbits*			
	0	**50**	**100**	**200**
Number of Litters	17	19	18	19
Number of Fetuses	105	116	108	136
Malformation (Fetuses/Litters)				
External	0/0	0/0	3/2	1/1
Soft Tissue	0/0	0/0	0/0	1/1
Skeletal	2/2	1/1	2/2	8/5
Total Malformation				
Fetuses/Litters	2/2	1/1	4/3	8/5
% Total Fetuses*	1.9	0.9	3.7	5.9
% Total Litters	11.8	5.3	16.7	26.3

* p = 0.12 with multi-group chi-square analysis

External and Visceral Malformations: up to 3 fetuses in the 100 and/or 200 mg/kg/day group had external malformations such as microphthalmia, spina bifida, mandibular agnathia, astomia and an open left eyelid. The visceral malformations were shown in one fetus of the 200 mg/kg/day group, including hydrocephaly with increased cavitation of both lateral ventricles and the third ventricle.

Fetal Skeletal Malformations: The malformations were noted at slightly high incidence in the 200 mg/kg/day group, shown as vertebral anomalies with or without associated rib anomalies. These malformation consisted primarily of extra arches or fused arches (smaller or larger than normal, absent or malpositioned); extra centra or fused centra (absent or malpositioned); and extra ribs or fused ribs (thickened, absent or forked). One fetus in each of the 100 and 200 mg/kg/day groups had only 5 sternebrae. One Fetus in each of the control and 200 mg/kg/day groups had skull anomalies that included an absent zygomatic arch, and/or fused frontal and/or posterior nasal portions. Two fetuses in the 200 mg/kg/day group had rib anomalies (in each case, fused and/or forked ribs). Fused sternebrae were noted in one fetus of the 200 mg/kg/day group.

Discussion and Comments

Pregnant rabbits received IV injection of AF0150 at the doses of 50, 100 and 200 mg/kg/day from gestation days 7 through 20 and the animals were sacrificed on gestation day 29. A slight increase in fetal malformation incidence was observed at the high dose levels, particularly in the 200 mg/kg/day group, as compared to the control group. These malformations included external, visceral (soft tissue) and skeletal anomalies. Percentage of fetuses with malformations was 1.9% in control rabbits, 3.7% and 5.9% in rabbits treated with 100 and 200 mg/kg/day AF0150, respectively. One animal in the 50 mg/kg/day group had a spontaneous abortion on gestation day 27. The **NOAEL** for malformations was 50 mg/kg/day (HED: 16.2 mg/kg/day and HDM: 130-fold of PCD).

AF10150 treatment did not affect post-implantation loss, live litter size, mean fetal body weights, fetal sex ratios, fetal developmental variation, the mean number of corpora lutea and implantation sites, as compared to the control group.

No maternal toxicity (clinical signs, body weigh and food consumption changes, and macroscopic examination) was observed at any AF0150 dose level. The highest AF0150 dose (200 mg/kg/day) did not result in minimal maternal toxicity, suggesting that the dose selection was not appropriate. The **NOAEL** for maternal toxicity was 200 mg/kg/day.

Report Number: IMUS-025-TOX
An Intravenous Study of the Effects of AF0150 on Pre- and Postnatal Development, Including Maternal Function in Rats

Report Location:	Vol.028, p001-389; Vol.029, p001-449; Vol.030, p001-434
Report date:	November 12, 1998
Study Facility:	[redacted]
In-life phase:	April 7 – September 3, 1997
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ16053 (400 mg/vial)
AF0150 Dosage (HMD at BSA):	50, 100, 200 mg/kg/day (65-260 fold PCD)

Specific Aim

To assess effects of AF0150 IV injection on the pregnant/lactating female and on development of the conceptus and the offspring (through sexual maturity)

Methods

Animal Preparation: Crl:CD(SD)BR female rats (125, sexually mature and virgin, 70 days old) were obtained from [redacted] Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation for 11 days, animals (about 12 weeks old) meeting good health and acceptable body weight requirements (minimum 220 g) were paired with resident male rats (the same strain, source and sexually mature) [*the ratio was not specified*] for breeding. The gestation day 0 was defined as the day that positive evidence of mating was identified by the presence of a copulatory plug or sperm in a vaginal smear. The female rats on gestation day 0 were randomly assigned to 4 groups, 25/group (body weight of 214-270 g). All animals were individually housed through the study, except during mating with the resident male rats.

AF0150 Preparation and Administration: AF0150 (400 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 40 mg/kg, and used within 30 minutes after reconstitution. The solutions were not used if they turned clear and could not be restored (by shaking) to an opaque appearance. On the first day of dosing, AF0150 dose was verified by osmolality measurement.

AF0150 solution and saline (0.9% sodium chloride for injection, as a negative control) were administered daily by IV injection via a lateral caudal vein (dilating with warm water, if necessary) from gestation day 6 through lactation day 20. The AF0150 dosages were 50, 100 and 200 mg/kg/day for assigned groups, and 5 ml/kg/day of saline for control group (Table 1) based on the most recent body weights. All animals were treated at approximately the same time each day. No AF0150 injections or other treatments were performed in the F1 generation animals.

Table 1. Prenatal and Postnatal Development Study Design in Rats

Group	AF0150 Dose* (mg/kg/day)	Number of Female Rats	IV Volume (ml/kg/day)
1 (Control)	(Saline)	25	5.0
2 (AF0150)	50	25	1.25
3 (AF0150)	100	25	2.5
4 (AF0150)	200	25	5.0

* AF0150 was reconstituted in SWFI to final concentration of 40 mg/ml. Dosing periods were from gestation days 6 through lactation day 20. Dams were sacrificed on lactation day 21. Twenty-five offspring per sex were used for the developmental study (followed fertility study procedure). The remaining offspring were sacrificed on PND 21.

Maternal (Fo) Observations

Clinical Signs: All animals were observed twice a day for mortality and moribundity, and detailed clinical signs were recorded daily till scheduled sacrifice day (Lactation day 21).

Food consumption: Individual maternal food consumption was recorded on gestation days 0, 6, 9, 12, 15, 18 and 20, and on lactation days 1, 4, 7, 14 and 21; reported as g/animal/day and g/kg/day for each corresponding body weight change.

Body Weights: Maternal body weights were recorded on gestation days 0, 6, 9, 12, 15, 18 and 20, and on lactation day 1, 4, 7 14 and 21. Group mean body weight was calculated for each time point. Mean body weight changes were calculated for each corresponding interval and also for gestation days 0-20 and for lactation days 1-21.

Pregnancy Duration and Parturition: all surviving animals were allowed to delivery naturally and rear their offspring to weaning. The pregnancy duration was defined as days from gestation day 0 to initiation of parturition. The day of complete delivery was designated PND 0 [*PND was not defined, maybe Post-Natal Day*]. The number, sex and malformation of stillborn and live pups in each litter were recorded at the end of parturition.

Necropsy: all animals, including those that were scheduled for termination (on lactation day 21), unscheduled death, and those failed to delivery or had total litter loss, were subjected to necropsy. The thoracic, abdominal and pelvic cavities and contents were examined. The numbers of former implantation sites or corpora lutea (for unscheduled death) were recorded.

Offspring (F1) Observations

Clinical Signs: each litter was examined daily for appearance, behavior, survival and death. Each pup received a detail physical examination on PND 1, 4, 4, 14 and 21 and weekly thereafter till necropsy. Pup sexes were determined on PND 0, 4 and 21. Eight pups per litter (4 each sex when possible) were randomly selected, weighed and sacrificed on PND 4. Litter parameters were processed as described in Table 2.

Table 2. Calculation of Litter Parameters

Postimplantation Survival Index (% Per Litter) =	$\frac{\Sigma \text{ (No. of viable pups per litter/ No. of implantations per litter)}}{\text{No. of litters per group}}$	x 100
Mean Live Litter Size =	$\frac{\text{Total no. of viable pups PND 0}}{\text{No. of litters with viable pups PND 0}}$	
Stillbirth Index (% Per Litter) =	$\frac{\Sigma \text{ (No. of nonviable pups at birth per litter/ No. of pups born per litter)}}{\text{No. of litters per group}}$	x 100
Live Birth Index (% Per Litter) =	$\frac{\Sigma \text{ (No. of pups born alive per litter/ Total no. of pups born per litter)}}{\text{No. of litters per group}}$	x 100
Postnatal Survival Between Birth and PND 0 or PND 4 (% Per Litter) =	$\frac{\Sigma \text{ (Viable pups per litter on PND 0 or PND 4/No. of pups born per litter)}}{\text{No. of litters per group}}$	x 100
Postnatal Survival for All Other Intervals (% Per Litter) =	$\frac{\Sigma \text{ (Viable pups per litter at end of interval N/Viable pups per litter at start of interval N)}}{\text{No. of litters per group}}$	x 100
Where N = PND 0-1, 1-4, 4-7, 7-14, 14-21 or 4-21		
Gestation Survival Index (%) =	$\frac{\text{No. of live litters}}{\text{No. of pregnant females}}$	x 100

Body Weights: individual body weight was recorded on PND 1, 4, 7, 14 and 21, and weekly thereafter till necropsy.

Offspring Development: Each dam and litter remained together till weaning on PND 21. Fifty pups (25 each sex) between 6-10 days old were randomly selected from each of 4 groups (saline, 50, 100, and 200 mg/kg/day AF0150) for assessment of developmental landmarks and

reproductive performance. The remaining pups were sacrificed and necropsied on PND 21. The following parameters were observed in the selected pups:

1. Balanopreputial Separation: male pups were observed daily for balanopreputial separation beginning on PND 40.

2. Vaginal Perforation: female pups were observed daily for vaginal perforation beginning on PND 30.

3. Auditory Startle Test: 10 pups/sex/group on PND 21 and 60 (±5 days) received an auditory response test in the sound chamber using an automatic Auditory Startle Response System. The responses or movements of animals to the tested noise bursts were monitored by weight-sensitive platforms on the bottom surface of the sound chamber. Mean peak amplitude (grams), latency to peak (mSec), response duration (mSec) and average response (grams) on each block (1-5) of 10 trials were recorded.

4. Motor Activity: 10 pups/sex/group on PND 13, 17, 21 and 60 (±2 days) were subjected to motor activity test using the [redacted] Animal Activity System. The animals were placed in a clear plastic rectangular cage surrounded with series of infrared photobeams. Both fine (interrupting one or two adjacent photobeams) and ambulatory (interrupting 3 or more consecutive photobeams) motor activities were collected.

5. [redacted]Maze Swimming Trials: learning and memory ability of pups (10 pups/sex/group) were evaluated using the maze swimming test in a water-filled, 8-unit T-maze system. The first test was conducted between PND 20 and 24, and the second test was between PND 60 and 64. The mean number of errors and the mean escape time for each trail were considered measures of maze acquisition.

6. Reproductive Performance and Fertility of Offspring: the F1 animals at 87-91 days old were paired on a 1:1 basis within each treatment group but avoiding sibling pairing. Positive evidence of mating was confirmed by the presence of a copulatory plug or sperm in a vaginal smear, and gestation day 0 was defined as the day that positive mating was identified. Clinical signs, estrous cycles, body weights and gravid uterine weights were observed. On gestation day 20, all maternal animals were subjected to complete necropsy and fetuses (F2) were examined for malformation and variations. Female/male mating index and fertility index, post-implantation loss/liter were calculated, as described in the fertility study procedure.

Results

Maternal Observations

1. Clinical Signs: one animal in the 200 mg/kg/day group died on gestation day 9 without a known cause. All other animals survived to the scheduled necropsy. There were no AF0150-related toxic observations at any dose level.

2. Body weights: mean body weight and body weight gains were not significantly different between AF0150 treatment and the control during gestation and lactation, except for a slight increase ($p<0.05$) in body weight gain during lactation day 14-21 in the 50 and 200 mg/kg/day groups.

3. Food Consumption: there were slight decreases ($p<0.05$) in food consumption in the 50 mg/kg/day group during gestation days 6-9, and in the 200 mg/kg/day group during gestation days 9-12 and lactation days 7-14. All other AF0150 dose groups had no significant changes on food consumption at any time point.

4. Pregnancy rate, Gestation duration and Parturition: AF0-150 had no significant effects on pregnancy rates at any dose level. The duration of gestation and parturition outcomes were summarized in Table 3. Animals in the 200 mg/kg/day group tend to have lower survival rate as compared to the control group, although there was not statistical significance. However, the live birth index decreased in the 200 mg/kg/day group with $p<0.05$ ac compared to the control group.

5. Necropsy: There were not necropsy findings on those animals with unscheduled death (one animal in the 200 mg/kg/day on gestation day 9), failure to delivery (two in the control and one in the 200 mg/kg/day group) and total liter loss (one in the control and 2 in the 200 mg/kg/day). All animals at the scheduled necropsy (on lactation day 21) had no remarkable findings on complete macroscopic examination.

Offspring Observations

1. Litter and Postnatal Survival (Table 3): in the 200 mg/kg/day group there was a decrease in the live birth index (by 7% with $p<0.05$), postimplantation survival index (by 6.8%), mean live litter size (by 8%), live pup numbers (by 8.8%) and gestation survival index (by 5%, but without statistical significance). The number of pup loss during the postnatal period (PND0-21) was higher (2-fold more than in control) in the 200 mg/kg/day than the other groups, which was considered to be related to maternal exposure to AF0150.

2. Pup Body Weight: there were no significant changes on pup body weights during the observation period (PND0-21).

3. Pup Necropsy: pups that died during the postnatal period (PND0-21) had no remarkable necropsy findings, except for one in the 50 mg/kg/day group was limited to situs inversus. For those pups scheduled for necropsy, a few pups (3, 3, 4 in the 50, 100 and 200 mg/kg/day, respectively) had dilated renal pelves. One pup in the 100 mg/kg/day group had a distended ureter.

Table 3. Gestation Outcomes and Postnatal Survival

Observations	AF0150 (mg/kg/day)			
	0	50	100	200
Number of Dams	23	25	25	23
Pregnancy Rate (%)	92	100	100	96
Gestation Days	21.8±0.42	21.7±0.54	21.8±0.37	21.7±0.45
Gestation Survival Index (% Live Litter)	100	100	100	95.7
Post-implantation Survival Index (% Per Litter)	94.2±6.73	95.7±4.91	92.7±10.16	87.6±21.58
Stillbirth Index (% Per Litter)	0.3±1.48	0.2±1.12	2.4±9.39	7.7±21.17
Live Birth Index (PND 0)	99.7±1.49	99.8±1.11	97.6±9.38	92.3±21.17*
Live Litter Size (PND 0)	15.1±2.17	15.4±1.85	15.3±2.08	13.9±3.65
Live Pups No.	317	379	382	289
Total Postnatal Death (PND0-21)	26	4	13	38
Postnatal Missing and cannibalized (PND0-21)	9	3	0	26

* $p<0.05$, as compared to the control group (0 mg/kg/day AF0150).

Offspring (F1) Development

1. Balanopreputal Separation and Vaginal Perforation: All male pups had balanopreputial separation by PND 52 and all female pups had vaginal opening by PND 37 except one in the control group who did not have vaginal patency till PND 49 (due to a filamentous attachment).

2. Auditory Startle Test: There were no differences in auditory tests (peak amplitude, latency to peak, response duration and average response) between pups from AF0150-treated dams and pups from control dams.

3. Motor Activity: Pups from the AF0150-treated groups had no differences in motor activity (total and ambulatory) as compared to the control.

4. Maze Swimming Trial: At both testing intervals, PND 20-24 and 60-64, pups from the AF0105-treated animals had no significant changes in swimming ability, leaning and memory as compared to those in the control group.

5. Reproductive performance and Fertility of Offspring: A routine fertility procedure was followed to test the reproductive function of both male and female pups.

i. No remarkable clinical signs and body weight changes were observed in both male and female offspring from all groups before, during and after gestation.

ii. There were no significant differences in estrous cycle, fertility index and mating index in both sexes between AF0150- and control groups.

iii. Gestation Day 20 laparohysterectomy, fetal (F2) development and morphology were not remarkable in any group, except for a filamentous tail noted in one fetus from the 200 mg/kg/day group and a short tail in one fetus from the 50 mg/kg/day group. These malformation were considered to be spontaneous based on the historical control data of this laboratory [redacted]

iv. At necropsy on gestation day 20, one female from the 50 mg/kg/day group and 2 females from the 200 mg/kg/day group had dilated renal pelves, distended ureters, calculi in both kidneys, and another two females from the 200 mg/kg/days had a cystic ovary. One male from the control, 2 males from the 50 mg/kg/day group and 1 male from the 200 mg/kg/day group had small and/or soft testes.

Discussion and Comments

This prenatal/postnatal study was conducted in rats given daily IV injections of AF0150 at 0 (saline), 50, 100 and 200 mg/kg/day from gestation day 6 through lactation day 20. Observations included maternal toxicity (clinical signs, body weight, food consumption and necropsy), gestation, parturition, lactation, neonate/offspring survival and development (growth, behavior, motor activity, reproductive performance and fertility).

AF0150 did not significantly affect maternal toxicity, pregnancy rate and gestation duration at any dose level.

However, at the dose of 200 mg/kg/day, AF0150 decreased the live birth index (by 7% with $p<0.05$), postimplantation survival index (by 6.8%), mean live litter size (by 8%), live pup numbers (by 8.8%) and gestation survival index (by 5%, without statistical significance). AF0150 increased stillbirths from 0.3% (in the control group) to 7.7% (in the 200 mg/kg/day group) per litter (about 25-fold higher). There was also approximately 2-fold increase in total postnatal death and postnatal missing/cannibalized during the postnatal days 0-21 in the 200 mg/kg/day group. The **NOAEL** for neonate toxicity was 100 mg/kg/day (HED = 16 mg/kg/day and HDM = 130-fold).

The offspring from the AF0150-treated animals had no differences in physical and functional development as well as in behavior from those from the control group. Fertility study on the

offspring from the AF0150-treated groups showed no significant effects on fertility and mating indices in either sex, nor on fetal development and morphology, as compared to the offspring from the control group.

This study suggests that exposure to AF0150 at the high dose (200 mg/kg/day) during pregnancy and lactation increases the possibility of pre- and postnatal toxicity.

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Summary of Reproductive Toxicity Study

Reproductive toxicity of AF0150 following intravenous injection was assessed in rats (fertility, teratology, pre-/post-natal toxicity) and in rabbits (teratology). Dose levels of 50-200 mg/kg/day were selected in both species, which corresponded to 65-259 fold the planned clinical dose (PCD) in rats and 130-518 fold the PCD in rabbits. *Human dose multiples (fold the PCD) are calculated based on the body surface area.*

Fertility and Early Embryonic Development (Segment I): The study was conducted in rats (25/sex) via IV injection of AF0150, from 2 weeks (females) and 4 weeks (males) pre-mating till gestation day 7 (female). Animals were sacrificed on female gestation day 13. The dose levels of AF0150 were 50, 100 and 200 mg/kg/day, which correspond to 65, 130 and 259 fold the planned clinical dose (PCD), based on body surface area conversion. Slight decreases in male fertility (by 5%, without statistical significance) and mean epididymal sperm numbers (by 15%, $p<0.05$) were observed in the high dose groups (200 mg/kg/day), with the **NOAEL** of 100 mg/kg/day (HED: 16.2 mg/kg/day and HDM: 130-fold the PCD) for both findings. AF0150 had no significant effects on other reproductive performance (mating index, fertility index, pre-coital interval, and female estrous cycle) and male spermatogenesis (testicular sperm counts, sperm production rate, sperm motility and morphology) at any dose level as compared to saline. No remarkable findings were observed in females upon necropsy, on gestation day 13, including pre- and post-implantation losses, numbers of viable embryos, implantation sites and corpora lutea. However, the high AF0150 dose, 200 mg/kg/day, did not result in maternal toxic signs, including changes on body weights, organ weights (brain, testis, ovary and pituitary) and food consumption. Minimal maternal toxicity should be generally expected in the high-dose group to confirm that appropriate doses were selected for the reproductive toxicity studies.

Teratology and Embryo/Fetal Development (Segment II): Two studies were conducted in rats (25 females) and rabbits (25 females) following IV injection of AF0150 at 50, 100, 200 mg/kg/day. The human dose multiples (HDM) were 65-259 fold for rats and 130-518 fold for rabbits. The animals were dosed daily during the organogenesis period, from gestation day 6 through 17 (sacrificed on gestation day 20) for rats, and gestation day 7 through 20 (sacrificed on gestation day 29) for rabbits. There were no significant differences in fetal development, viability, body weights, sex ratio, corpora lutea number, implantation sites and post-implantation loss between AF0150-treated and control groups in either species. However, the fetal malformation incidence slightly increased in the 100 and 200 mg/kg/day groups in rabbits but not in rats as compared to the control group. These malformations included external, visceral (soft tissue) and skeletal anomalies. Percentage of fetuses with malformations was 1.9% in control rabbits, 3.7% and 5.9% in rabbits treated with 100 and 200 mg/kg/day AF0150, respectively. The **NOAEL** was therefore determined at 50 mg/kg/day. No maternal toxicity (clinical signs, body weight and food consumption changes, and macroscopic examination) was observed in either species at any AF0150 dose level, suggesting that a higher dose should have been used to produce minimal maternal toxicity. The **NOAEL** for maternal toxicity was 200 mg/kg/day (HDM: 259-fold in rats and 518-fold in rabbits).

Prenatal and Postnatal Development (Segment III): This study was conducted in rats (25 females) following daily IV injection of AF0150 at 50, 100 and 200 mg/kg/day (HDM: 65-259 fold) from gestation day 6 through lactation day 20. AF0150, at all dose levels, had no significant maternal toxicity (clinical signs, body weight, food consumption and necropsy), pregnancy rate, gestation duration and parturition process. However, at the dose of 200 mg/kg/day, AF0150 decreased the live birth index (by 7% with $p<0.05$), and tended to decrease (no statistical significance but p values were not provided) postimplantation survival index (by 6.8%), mean live litter size (by 8%), live pup numbers (by 8.8%) and gestation survival index (by 5%). Stillbirths were increased from 0.3% (in the control group) to 7.7% (in the 200 mg/kg/day group) per litter (about 25-fold higher). There was also approximately 2-fold increase in total postnatal death and postnatal missing/cannibalized during the postnatal days 0-21 in the 200 mg/kg/day group. The **NOAEL** for neonate toxicity was 100 mg/kg/day (HED = 16 mg/kg/day and HDM = 130-fold). The offspring from AF0150-treated animals had no differences in physical and functional development as well as in behavior, from those from the control group. A fertility study on the offspring from the AF0150-treated groups showed no significant effects on fertility and mating indices in either sexes, nor on fetal development and morphology, as compared to the offspring from the control group.

Conclusion and Recommendation: Intravenous injection of AF0150 at the human dose multiples of 65-259 fold in rat had no significant adverse effects on fertility, teratology, embryonic (early and later) and fetal development. At the dose of 200 mg/kg/day in rabbits (518-fold the PCD), AF0150 slightly increased incidence of external, visceral and skeletal malformations. Also at this dose in rats (259-fold the PCD), lower neonate live birth and higher stillbirths were noted than in control group. In all 4 reproductive toxicity studies, the highest dose, 200 mg/kg/day, did not result in minimal maternal toxicity, suggesting that dose selection was not optimized. The pregnancy category C is recommended in the labeling.

30. GENETIC TOXICOLOGY

Report Number: IMUS-015-TOX
Mutagenicity Test with AF0150 in the *Salmonella-Escherichia Coli/Mammalian-Microsome* Reverse Mutation Assay Preincubation Method with Confirmatory Assay (Amended Final Report)

Report Location:	Vol.031, p338-378
Report date:	January 25, 1996
Study Facility:	[redacted]
In-life phase:	September 28-November 21, 1995
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ15031 (400 mg/vial)
AF0150 Dosage (HMD at BSA):	1-20 mg/plate

Specific Aim

To assess mutagenicity of AF0150 using *in vitro* reverse mutation assays (Ame's Test)

Methods

Bacterial strains and Materials: *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and *Escherichia Coli* (WP2uvrA) were used to detect point mutation of G-C and A-T, respectively. Overlay (top) agar for revertant selection contained 0.7% agar, 0.5% NaCl, 0.05 mM each of histidine and biotin (for selection of histidine revertants, *Salmonella typhimurium* strains) or 0.05 mM tryptophan (for selection of tryptophan revertants, *Escherichia Coli* strain). Rat liver S9 was obtained commercially.

Preliminary Test (Dose Range-Finding): TA98, TA100 and Wp2uvrA were incubated with AF0150 at the dose range from 0.4 to 20 mg/plate (total 8 doses) in the absence and presence of S9 activation. No cytotoxicity (decreased number of revertant colonies, or thinning/disppearance of the bacterial background lawn) was observed at all dose levels. The maximal dose, 20 mg/plate, was selected for mutagenicity assay.

AF0150 and Positive Control Preparations: AF0150 (400 mg fill) was reconstituted with 10 ml sterile deionized water to a final concentration of 40 mg/ml. For low dose groups (0.4-2 mg/plate), 4 mg/ml AF0150 stock solution was made by dilution of 40 mg/ml. All AF0150 stock solutions were used within 30 minutes after reconstitution. Positive control chemicals included 2-aminoanthracene, 2-nitrofluorene, sodium azide, ICR-191, 4-nitroquinoline-N-oxide [*Preparation methods were not indicated*].

Mutagenicity Assay: The five bacterial strains were pre-incubated in culture tubes (tightly capped) with AF0150 at the doses of 1-20 mg per plate in the absence and presence of rat liver S9 for 2 hours at 37°C. The overlay (top) agar (selection agar medium) containing

histidine/biotin or tryptophan was then added to the tubes. The agar and preincubation mixtures were overlaid onto petri dishes containing minimal bottom agar, in triplicate for all dose levels and controls (negative and positive). Following incubation of the petri dishes for 48 hours at 37°C, the number of revertant colonies on each petri dish was counted manually (for AF0150 and negative control plates) or automatically (with automated colony counter for positive control plates).

Results

AF0150 at the doses of 1-20 mg/plate had no significant cytotoxicity and did not increase revertants in all 5 bacterial strains covered both C-C and A-T point mutations in the absence and presence of a rat liver S9. Positive control compounds (2-aminoanthracene, 2-nitrofluorene, sodium azide, ICR-191, 4-nitroquinoline-N-oxide) significantly increased reverse mutation in all bacterial strains.

In addition, bubbles were observed in the top agar at doses of 4 mg AF0150 per plate and above in both the presence and absence of S9 mix in dose range finding study.

Discussion and Comments

AF150 at the dose of 1-20 mg/plate did not result in significant increase in bacterial reverse mutation. However, there was also no evidence of significant cytotoxicity at the highest dose, 20 mg/plate in terms of changes on revertant numbers or bacterial background lawn. This suggests that the dose selection may not be sufficient.

Report Number: IMUS-031-TOX
Chromosomal Aberrations in Cultured Human Peripheral Blood Lymphocytes with AF0150

Report Location:	Vol.032, p001-028
Report date:	October 22, 1997
Study Facility:	[redacted]
In-life phase:	August 7-September 12, 1997
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ16054 (400 mg/vial)
AF0150 Dosage (HMD at BSA):	2-5 mg/ml

Specific Aim

To assess the ability of AF0150 to induced gross chromosome damages in culture human lymphocytes in the presence or absence activation system (liver S9)

Methods

AF0150 and Positive Control Preparations: AF0150 (400 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 40 mg/ml. For low dose groups (0.4-2 mg/plate), 4 mg/ml AF0150 stock solution was made by dilution of 40 mg/ml. All AF0150 stock solutions were used within 30 minutes after reconstitution. Mitomycin C (MMC) and cyclophosphamide (CP) were dissolved in water and used as positive controls. AF0150 vehicle (water) was used as negative control.

Cell Culture and Treatment: Blood sample (vein) was taken from healthy adult male into a heparinized tube. Whole blood was cultured in RPMI 1640 medium containing 15% fetal bovine serum (FBS), antibiotics, L-glutamine and 1% PHA-M (phytohemagglutinin M) for 2 days before treatment with AF0150 or control compounds. For the initial assays, the culture were treated for 3 hours in the presence or absence metabolic activation (S9), washed and then incubated in fresh culture medium until harvested at 22 hours after inititation of treatment. For confirmatory assay without S9, the culture was treated for 19.3 hours, washed and then incubated in fresh culture medium until harvested at 22 hours of initiation of treatment. For confirmatory assays with S9, the culture was treated for 3 hours in the medium without FBS, washed and then incubated in fresh complete culture medium. In all the assays, Colcemid (0.1 ug/ml) was added to the cell culture at 2 hours prior to cell harvesting for a metaphase-arresting.

Cell Harvest, Staining and Analysis: The cells were centrifuged, treated with hypotonic KCl (75 mM) and fixed with fixative solution (methanol:acetic acid), followed by slide preparation and Giemsa staining. Cells with 46 centromeres were analyzed with microscopy for different types of chromosomal aberrations. Percentages of mitotic cells (mitotic index) and polyploid and endoreduplication were also measured.

Results/Discussion

In both initial and confirmatory assays, AF0150 at the doses of 2-5 mg/ml decreased in some degree the mitotic index as compared to negative control group (Table 1). At the 5 mg/ml group in the initial assay without S9 activation the mitotic index decreased by 47%, suggesting the dose selection was appropriate.

Human blood lymphocytes treated with AF0150 at the doses of 2, 3, 4, 5 mg/ml had no significant increases in chromosomal aberrations, polypoidy, or endoreduplication in the two independent assays with or without metabolic activation. Positive control compounds, mitomycin (without metabolic activation) and cyclophosphamide (metabolic activation) induced statistically significant increases in cells with chromosomal aberrations.

In conclusion, AF0150 treatment *in vitro* did not induce chromosomal aberrations in cultured human whole blood lymphocytes.

Table 1. Mitotic Index (MI, %) and Chromosomal Aberration in PCE (CA-PCE, %) of AF0150-Treated Human Lymphocyte *In vitro*

Treatment	Initial Assay				Confirmatory Assay			
	-S9		+S9		-S9		+S9	
	MI	CA-PCE	MI	CA-PCE	MI	CA-PCE	MI	CA-PCE
2 mg/ml *	5.4	0.5	4.1	0	2.6	0.5	5.4	0.5
3 mg/ml	4.5	1.0	3.9	0	2.8	0	2.9	1.0
4 mg/ml	6.5	0	4.8	0.5	2.6	0.5	4.2	1.0
5 mg/ml	3.4	0	3.3	1.0	2.9	0	4.1	0
CP†			1.6	32			3.6	12
MMC†	0.8	62			1.8	34		
H2O	6.4	1.5	4.5	0	3.5	0	4.2	0

* AF0150 concentration in cultured human lymphocytes.

† CP (cyclophosphamide, with S9 activation) and MMC (mitomycin C, without S9 activation) were positive controls.

Report Number: IMUS-030-TOX

Mutagenicity Test on AF0150 in the *In vivo* Mouse Micronucleus Assay

Report Location: Vol.032, p029-051
Report date: October 22, 1997
Study Facility: [redacted]
In-life phase: August 5-20, 1997
GLP Compliance: Yes (with QA Statement)
AF0150 Lot number: ZZ16054 (400 mg/vial)
AF0150 Dosage (HMD at BSA): 200-800 mg/kg (130-518 fold PCD), single dose.

Specific Aim

To assess the ability of AF0150 to induced chromosome damage *in vivo.*

Methods

Animal Preparations: young adult male and female mice, Crl:CD-1 (ICR) BR strain, were obtained from [redacted]. Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation for at least 7 days, animals were randomly assigned into 5 groups (6 animals/sex/group) and received designed treatments, as seen in Table 1. The animals were 8 weeks old with body weights of 29.2-34.1 (males) and 22.2-28.6 (females) at initiation of treatment.

AF0150 and Positive Control Preparations: AF0150 (400 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 40 mg/ml and was used within 30 minutes after reconstitution.

Animals received IV injection [*site was not specified*] of a single dose of AF0150 or saline (negative control), and oral administration of 80 mg/kg cyclophosphamide (positive control).

Table 1. Micronucleus Study Design in Mice

Treatment	Dosing Vol. (ml/kg)	Route of Dosing	Harvest Time, 24 hr		Harvest Time, 48 hr	
			Male	Female	Male	Female
200 mg/kg AF0150	5.0	IV	6	6		
400 mg/kg AF0150	10	IV	6	6	6	6
800 mg/kg AF0150	20	IV	6	6	6	6
Saline	20	IV	6	6		
Cyclophosphamide	10	PO	6	6		

Observations: Toxic signs were observed at least daily for all animals throughout study. The animals were sacrificed at 24 and 48 hours after dosing (Table 1). Bone marrow was collected from the hind limb and transferred to centrifuge tubes containing 3-5 ml bovine serum. The cells were centrifuged, prepared for slides, fixed in methanol and stained with May-Grunwald and Giemsa. The slides were scored for polychromatic erythrocyte (PCE), normochromatic erythrocyte (NCE) and micronucleated PCE. The micronucleus frequency, expressed as percentage of micronuleated cells, was determined by analyzing the number of micronucleated PCEs from 2000 PCEs per animal.

Results/Discussion

Treatments with AF0150, saline or CP had no significant toxic signs in all animals. As seen in Table 2, AF0150 at all doses was no cytotoxic to bone marrow in terms of PCE:NCE ratio. There was no significant difference in micronucleated PCEs between AF0150- and saline-treated mice. The positive control, cyclophosphamide, induced statistically significant increases in micronucleated PCEs in both sexes as compared to saline control group.

Table 2. Micronucleus Assay Summary

TREATMENT	DOSE	HARVEST TIME (HR)	% MICRONUCLEATED PCEs MEAN OF 2000 PER ANIMAL ± S.E. MALES	FEMALES	TOTAL	RATIO PCE:NCE MEAN ± S.E. MALES	FEMALES
CONTROLS							
VEHICLE	0.9% saline	24 hr	0.08 ± 0.03	0.04 ± 0.03	0.06 ± 0.02	0.59 ± 0.04	0.79 ± 0.05
		48 hr	0.08 ± 0.03	0.04 ± 0.03	0.06 ± 0.02	0.46 ± 0.02	0.52 ± 0.06
POSITIVE	CP 80.0 mg/kg	24 hr	3.52 ± 0.19*	2.28 ± 0.29*	2.90 ± 0.26*	0.71 ± 0.07	0.68 ± 0.09
TEST ARTICLE	200 mg/kg	24 hr	0.05 ± 0.03	0.07 ± 0.01	0.06 ± 0.02	0.74 ± 0.09	0.76 ± 0.05
	400 mg/kg	24 hr	0.15 ± 0.04	0.08 ± 0.04	0.12 ± 0.03	0.69 ± 0.04	0.52 ± 0.04
	800 mg/kg	24 hr	0.05 ± 0.02	0.10 ± 0.04	0.08 ± 0.02	0.64 ± 0.06	0.72 ± 0.11
		48 hr	0.03 ± 0.01	0.04 ± 0.03	0.04 ± 0.02	0.50 ± 0.08	0.43 ± 0.02

* Significantly greater than the corresponding vehicle control, $p<0.01$.
CP = Cyclophosphamide
PCE = Polychromatic erythrocyte
NCE = Normochromatic erythrocyte

Report Number: IMUS-032-TOX

Mutagenicity Test on AF0150 in the L5178Y TK+/- Mouse Lymphoma Forward Mutation Assay with a Confirmatory Assay

Report Location: Vol.032, p052-079
Report date: December 31, 1997
Study Facility: [redacted]
In-life phase: August 4-September 17, 1997
GLP Compliance: Yes (with QA Statement)
AF0150 Lot number: ZZ16054 (400 mg/vial)
AF0150 Dosage (HMD at BSA): 1-5 mg/ml

Specific Aim

To assess mutagenicity of AF0150 using *in vitro* forward mutation assay

Methods

Cell Culture: The mouse lymphoma L5178Y cell line, heterozygous at the TK locus, was used for this assay. The cells were cultured in growth medium (RPMI 1640 medium containing 10% heat-inactivated horse serum, Pluronic F68, L-glutamine, sodium pyruvate, and antibiotics) in a shaking incubator at 37°C. Treatment medium was Fisher's medium containing above supplements with 5% horse serum. Cloning medium was Fisher's medium containing 20% horse

serum and 0.24% agar but no Pluronic F68. Selection medium was the cloning medium supplied with 3 ug/ml TFT.

AF0150 and Positive Control Preparations: AF0150 (400 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 40 mg/ml and used within 30 minutes after reconstitution. Methyl methanesulfonate (MMS, without S9 activation) and methylcholanthrene (MCA, with S9 activation) were used as positive controls. AF0150 vehicle, SWFI, was used as negative control.

Treatment: The tested compounds were added to $6x10^6$ cells in 10 ml treatment medium per tube. The final concentrations were 1, 2, 3, 4, 5 mg/ml for AF0150; 5 and 10 ng/ml for MMS; and 2 and 4 ug/ml for MCA in the presence or absence of metabolic activation (S9). After treatment for 4 hours, cells were washed and incubated in 20 ml of growth medium for a 2-day expression period.

Cloning and Mutant Selection: A total of $3x10^6$ cells were then suspended in the cloning medium or selection medium and a certain number of cells were seeded in 100-mm dishes followed by incubation at 37°C for 10-14 days for cloning and mutant analysis. The mutant frequency was calculated as the ratio of the total number of mutant colonies found in each selection dish to the total number of cells seeded, adjusted by the absolute selection cloning efficiency (cultures from cells in cloning medium).

Results/Discussion

AF0150 at 5 mg/ml in the assays without activation and 4 and 5 mg/ml in the assays with activation induced moderate cytotoxicity in terms of decreases in cell growth by more than 50%, suggesting the dose selection was appropriate. The average cloning efficiencies for the vehicle controls was 93.0-101.7% without activation and 91.4-110.0% with S9 metabolic activation. The positive controls, MMS (nonactivation) and MCA (activation), induced significant increases in mutant frequency by more than 5-fold as compared to vehicle control groups.

Incubation of cells with AF0150 at concentrations from 1-5 mg/ml did not increase mutant frequency in the presence and absence of metabolic activation from two separate assays. However, as seen in Tables 1 and 2, the relative growth rate in all AF0150 dose groups tended to decrease as compared to vehicle control groups, corresponding to lower mutant frequency than in vehicle control group. This suggests that the AF0150-induced inhibition of cell growth could result in underestimating mutation potential, although positive controls showed more significant inhibition of cell growth.

Table 1. Growth Rate and Forward Mutation Frequency without S9 Activation

Trial 1

TEST CONDITION:	TOTAL MUTANT COLONIES	TOTAL VIABLE COLONIES	CLONING EFFICIENCY[b]	RELATIVE GROWTH (%)[c]	MUTANT FREQUENCY (10E-6 UNITS)[d]
NONACTIVATION CONTROLS[a]			AVG VEHICLE CONTROL		
VEHICLE CONTROL	205	590	98.3	100.0	69.5
VEHICLE CONTROL	160	565	94.2	100.0	56.6
VEHICLE CONTROL	200	519	86.5 93.0	100.0	77.1
MMS 5 ml/ml	799	485	80.8	58.1	329.5*
MMS 10 ml/ml	576	293	48.8	26.0	393.2*
TEST COMPOUND			RELATIVE TO VEHICLE CONTROL (%)		
1000 µg/ml	170	639	114.5	107.7	53.2
2000 µg/ml	145	584	104.7	115.1	49.7
3000 µg/ml	127	655	117.4	108.8	38.8
4000 µg/ml	128	572	102.5	74.8	44.8
5000 µg/ml	133	500	89.6	48.7	53.2

Trial 2

TEST CONDITION:	TOTAL MUTANT COLONIES	TOTAL VIABLE COLONIES	CLONING EFFICIENCY[b]	RELATIVE GROWTH (%)[c]	MUTANT FREQUENCY (10E-6 UNITS)[d]
NONACTIVATION CONTROLS[a]			AVG VEHICLE CONTROL		
VEHICLE CONTROL	139	604	100.7	100.0	46.0
VEHICLE CONTROL	121	631	105.2	100.0	38.4
VEHICLE CONTROL	114	596	99.3 101.7	100.0	38.3
MMS 10 ml/ml	702	332	55.3	36.3	422.9*
TEST COMPOUND			RELATIVE TO VEHICLE CONTROL (%)		
1000 µg/ml	92	597	97.8	85.1	30.8
2000 µg/ml	139	528	86.5	65.6	52.7
3000 µg/ml	87	465	76.2	65.5	37.4
4000 µg/ml	117	592	97.0	39.9	39.5
5000 µg/ml	176	502	82.3	28.1	70.1

b. Cloning Efficiency (%) = Total Viable Colony Counts/Numbers of Cells Seeded

c. Relative Growth (%) = (Relative Suspension Growth x Relative Cloning Efficiency)/100

d. Mutant Frequency = (Total Mutant Colonies/Total Viable Colonies) x $2x10^4$, expressed as in unit of 10^6.

Table 2. Growth Rate and Forward Mutation Frequency with S9 Activation

Trial 1

TEST CONDITION	TOTAL MUTANT COLONIES	TOTAL VIABLE COLONIES	CLONING EFFICIENCY[b]	RELATIVE GROWTH (%)[c]	MUTANT FREQUENCY (10E-6 UNITS)[d]
S9 ACTIVATION IN:			AVG VEHICLE CONTROL		
VEHICLE CONTROL	162	535	89.2	100.0	68.0
VEHICLE CONTROL	147	523	87.2	100.0	56.2
VEHICLE CONTROL	156	586	97.7 91.4	100.0	53.2
MCA 2 µg/ml	714	515	85.8	65.9	277.3
TEST COMPOUND			RELATIVE TO VEHICLE CONTROL (%)		
1000 µg/ml	132	454	82.8	71.2	58.1
2000 µg/ml	93	437	79.7	72.3	42.6
3000 µg/ml	112	433	79.0	62.4	51.7
4000 µg/ml	146	472	86.1	77.1	61.9
5000 µg/ml	139	446	81.3	71.6	62.3

Trial 2

TEST CONDITION	TOTAL MUTANT COLONIES	TOTAL VIABLE COLONIES	CLONING EFFICIENCY[b]	RELATIVE GROWTH (%)[c]	MUTANT FREQUENCY (10E-6 UNITS)[d]
S9 ACTIVATION INDUCED:			AVG VEHICLE CONTROL		
VEHICLE CONTROL	259	786	131.0	100.0	65.9
VEHICLE CONTROL	165	534	89.0 110.0	100.0	61.8
MCA 2 µg/ml	726	342	57.0	53.3	424.6
MCA 4 µg/ml	1081	465	77.5	41.3	464.9
TEST COMPOUND			RELATIVE TO VEHICLE CONTROL (%)		
1000 µg/ml	184	699	105.9	82.8	52.6
2000 µg/ml	114	695	105.3	78.9	32.8
3000 µg/ml	104	492	74.5	84.9	42.3
4000 µg/ml	91	521	78.9	88.3	34.9
5000 µg/ml	129	573	86.8	66.8	45.0

b. Cloning Efficiency (%) = Total Viable Colony Counts/Numbers of Cells Seeded

c. Relative Growth (%) = (Relative Suspension Growth x Relative Cloning Efficiency)/100

d. Mutant Frequency = (Total Mutant Colonies/Total Viable Colonies) x $2x10^{-4}$, expressed as in unit of 10^{-6}.

Genotoxicity Study Summary

The standard genotoxicity battery was used to test the potential genotoxicity of AF0150, including a bacterial reverse mutation test, a mammalian cell DNA damage (chromosomal aberration assay with human lymphocytes and forward mutation with mouse lymphoma tk cells), and an *in vivo* mouse micronucleus assay. The AF0150 dose selection and treatments (in sealed culture tubes and flasks) were appropriate based on cytotoxicity or maximal solubility of the product. Appropriate negative and positive controls were included in all *in vitro* and *in vivo* studies, and the expected results were produced from the positive control compounds. All studies were conducted in the presence and absence of metabolic activation (using induced rat liver S9).

AF0150 at doses up to 20 mg/plate had no significant increase in bacterial reverse mutation in all bacterial strains detecting C-G and A-T point mutation. At doses up to 5 mg/ml, AF150 did not induce significant chromosomal aberrations and forward mutation as compared to vehicle control group. Single IV injection of AF0150 at the dose up to 800 mg/kg (518-fold PCD based on body surface area conversion) did not increase micronucleated polychromatic erythrocytes in mouse bone marrow.

AF0150 is concluded to be negative in the following genotoxicity assays:

1. *In vitro* bacterial reverse mutation assay
2. *In vitro* chromosomal aberration assay in culture human lymphocytes
3. *In vitro* mouse lymphoma tk forward mutation assay
4. *In vivo* micronucleus assay in mice

33. OVERALL SUMMARY

AF0150 (*Imavist™*) is a perflexane-phospholipid microbubble-based ultrasound contrast agent. Intravenous administration of AF0150 increased ultrasound reflexivity of blood and thus improved delineation of left ventricle endocardial border and visibility of segmental wall motion. The clinical indication is proposed to opacify the left ventricle and assess cardiac function in patients with suboptimal echocardiograms. *In vivo* Animal and *in vitro* studies including general pharmacology, pharmacokinetics/toxicokinetics, safety pharmacology and toxicology were conducted to address the efficacy and safety of AF0150 for the intended clinical indication. *Human dose multiples (fold the PCD) in al studies are calculated based on the body surface area conversion.*

General Pharmacology Studies (Table 1)

Efficacy and efficacy-related studies (all non-GLP) of AF0150 microbubble agent were conducted *in vivo* and *in vitro* to test the effects of AF0150 on Doppler signal of carotid artery blood flow and echocardiographic imaging of left ventricular cavity. The following observations were included in these studies: dose-response, administration modes (IV bolus or infusion), AF0150 reconstitution conditions, ultrasound power settings, and external pressure. The major results are summarized in Table 1.

Dose-response of Doppler Signal and Echocardiography: IV injection (bolus and infusion) of AF0150 enhanced the Doppler signal of carotid artery blood flow in rabbits and swine in a dose-dependent manner. AF0150 also dose-dependently increased the contrast of echocardiographic images (closed chest) of left ventricular myocardium in swine and dog with the optimal dose of approximately 0.5 mg/kg. Left ventricular cavity imaging was enhanced, but without significant dose-dependence in swine (0.13-1.3 mg/kg) and dog (0.03-0.6 mg/kg). In the swine study, AF0150 demonstrated effects on both fundamental and second harmonic ultrasound imaging of the LV cavity and myocardium. In the dog model, AF0150 at the doses of 0.3 and 0.6 mg/kg was effective for determination of LV cavity opacification, normal myocardial perfusion, and myocardial perfusion defects.

AF0150 Administration Modes: IV bolus and infusion were assessed in rabbits and swine for comparison of AF0150-induced Doppler signal enhancement and cardiac imaging intensity. Dose-dependent increases in Doppler signal enhancement was observed with either administration mode. In the swine study, optimal videointensity was achieved with both fundamental and second harmonic imaging after the 1-minute infusion of 0.66 mg/kg AF0150.

AF0150 Reconstitution Conditions: Two AF0150 fill sizes (100 mg vs. 200 mg per vial), reconstituted concentrations (10 mg/ml vs. 20 mg/ml), microbubble stability (time post reconstitution and vial inversion), physical conditions (pretreated water, application of pressure to reconstituted AF0150) and microbubble sizes were tested in terms of effects on ultrasound signal enhancement. Studies in swine and rabbits demonstrated that fill sizes and reconstituted AF0150 concentrations had no significant effects on AF0150 Doppler signal and myocardium

imaging enhancement. Pretreatment of SWFI with different temperature (15° to 30°C) and gas tension (460 to 1060 mm Hg) prior to reconstitution of AF0150 had no significant effect on the Doppler signal. At 15°C for all gas tensions and at 30°C for the low gas tension (460 mm Hg), however, Doppler signal slight decreased. Optimal efficacy in rabbits was obtained within the first 30 minutes following AF0150 reconstitution and within the first 60 seconds after vial inversion. Following reconstitution of AF0150 and separation into two microbubble populations by creaming method (large bubbles float to the top of the suspension), the small microbubbles (<3 um diameter, 97-99%) contributed more to the Doppler peak signal (initial intense response), while the large microbubbles (>3 um in diameter, 19-24%) contributed more to Doppler persistent signal.

Effects of Ultrasound Settings on AF0150 Microbubble: Exposure of AF0150 microbubbles to increasing ultrasound power (mechanical index, MI) or time in an *in vitro* simulated circulation system decreased microbubble count with a slight shift. This suggests that ultrasound exposure destroys microbubbles in a power-and time-dependent manner. Application of ultrasound burst (continuous and triggered) to the heart (closed-chest) decreased AF0150-induced Doppler signal (peak and persistence) enhancement of carotid artery blood flow in rabbits with increasing ultrasound power levels. Triggered ultrasound application had less decrease than the continuous ultrasound. Echocardiography studies in closed-chest swine with three different ultrasound imaging equipment showed that application of increasing ultrasound power or frame rate diminished signal detection of LV cavity imaging at AF0150 doses of 0.125 mg/kg (0.95 fold the PCD) and 0.250 mg/kg (1.9 fold the PCD).

Effects of External Pressure on AF0150 Microbubbles: the effects of external hydrostatic pressures (application of high pressure to reconstituted AF0150 prior to injection, and phenylephrine-induced hypertension) and high O2 inhalation were studied on AF0150 microbubble stability in rabbits and swine. External pressure application to reconstituted AF0150 prior to injection tended to decrease the Doppler signal (both peak and persistence) in rabbits in a pressure level-dependent manner. At high pressure (1090 mmHg), the peak Doppler signal significantly decreased. Hypertension and high LV pressure induced by phenylephrine (alpha1 agonist) had no significant effect on AF0150-induced Doppler signal and LV cavity imaging enhancement in rabbits and swine. Inhalation of higher than 50% O2 concentration decreased AF0150-induced enhancement of Doppler signal and echocardiography.

Conclusion and Comments for General Pharmacology Studies

Conclusion:

IV administration of AF0150 microbubbles enhanced ultrasound signals (Doppler signal of carotid artery blood flow and left ventricle imaging) in animal models (rabbits and swine).

Comments

i. The number of animal used in several major studies were not high enough for statistical analyses. However, results from those studies showed similar tendencies. These studies include EB-98-18 (2 swine); EB-98-19 (2 swine); EB-98-20 (3 swine); EB-98-16 (2 rabbit).
ii. Measurement of Doppler signals of carotid artery blood flow was used in most studies, in order to optimize conditions of AF0150 application. This was different from the clinical indication for left ventricular cavity imaging. No explanation was provided to correlated the two efficacy endpoints.
iii. SEM (Standard Error of Mean) instead of SD (Standard Deviation) was used for data analysis in all study reports. SD, but not SEM, should be used for demonstration of variation of samples.

Safety Pharmacology Studies (Table 2)

Studies regarding safety assessment of AF0150 in hemodynamic (pulmonary and cardiovascular systems), neurological, renal and gastrointestinal systems were conducted in rats, rabbits, dogs and monkeys. Most of these studies were non-GLP studies. The major results from these studies are summarized in Table 2 and **NOAEL**s from each study was listed in Table 10.

Cardiovascular Safety Assessment: Cardiovascular safety of an intravenous administration of AF10150 was assessed in anesthetized rabbits (7 non-GLP studies) and dogs (3 non-GLP studies and 1 GLP study), and non-anesthetized monkeys (1 GLP study) and rats (1 GLP study). Hemodynamic parameters, such as arterial blood pressure, heart rate, cardiac output and pulmonary arterial pressure (PAP), and ECG were included in those studies, with observation times up to 4 hours post AF0150 dosing. The hemodynamics were measured through arterial catheterization.

In anesthetized rabbits, AF0150 at doses up to 20 mg/kg (52-fold PCD) had no significant effects on heart rate and mean arterial pressure (MAP) during the 1- hour post dosing observation (EB-95-19). In rabbits treated with a cardiac stress agent dipyridamole (EB-98-06), AF0150 at both tested doses (2 and 20 mg/kg) transiently (about 20 minutes) increased (by 18%) dipyridamole-induced tachycardia without change on MAP. The mechanism of this effect remains unclear. Neither change was noted in rabbits that pre- and co-treated with cardiac stress agents, adenosine, arbutamine and dobutamine (EB-98-05/07/08), as summarized in Table 9.

There were no studies to address effects of AF0150 in any animal model receiving physical stress (such as treadmill) on the cardiovascular or any other system in this NDA submission. It is conceivable that AF0150 will be used in patients receiving either pharmacological stress agents or physical stress tests in a clinical setting. Therefore, appropriate evaluation of AF0150 under the stress treatment should be made in both preclinical and clinical settings.

In the thromboxane-induced pulmonary hypertensive rabbit model (EB-98-13), AF150 at doses up to 10 mg/kg (26-fold PCD) had no effects on HR and MAP. In an experimental ischemic myocardium model in rabbits, with concurrent 99mTc-Sestamibi cardiac imaging (EB-97-09),

AF0150 at 2 mg/kg (5.2-fold PCD) did not change HR and MAP, nor did it affect myocardial ischemic/infarction areas during a 30-minute post dosing observation period.

In anesthetized dogs, heart rate, MAP and cardiac output were not different between pre dosing and 1 minute post dosing at AF0150 dose up to 0.6 mg/kg (2.6-fold PCD). The studies were conducted with and without sustained application of ultrasound (EB-95-25), or 10 minutes post dosing at AF0150 doses up to 1.6 mg/kg (6.9-fold PCD) (EB-95-27). Intravenous infusion of AF0150 at 20 mg/kg (52-fold PCD) with ultrasound application did not affect heart rate and MAP (EB-97-13) during a 4-hour post dosing observation period.

In conscious monkeys (IMUS-016-TOX), animals were pre-catheterized under anesthesia and allowed to recover from the surgical procedure. The heart rate (HR), cardiac output (CO), pulmonary arterial pressure (PAP), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), left ventricular pressure (LVP) and derived contractility (dp/dt) were measured at 2-60 minutes after AF0150 dosing up to 40 mg/kg (104-fold PCD). It appeared that there were no differences in the measured parameters between AF0150 and control groups during a 1-hour observation period.

Electrocardiograph (ECG) examination was performed in two GLP studies, one in anesthetized dogs and anther in non-anesthetized cynomolgus monkeys. Six limb leads of ECG were recorded before and after AF0150 or saline administration. Time-dependent increase in the uncorrected QT intervals was observed in anesthetized dogs (IMUS-035-TOX) following IV infusion of AF0150 at 20 mg/kg (86-fold PCD, 1 mg/kg/min for 20 minutes) during a 30-minute observation period, as compared to pre-dosing records. However, the corrected QT (QTc) for the heart rate, using both Bazett's and Fredericia's formula, revealed no significant changes. The QTc intervals from the non-anesthetized monkey study (IMUS-016-TOX) were unremarkable during a 60-minute post dosing observation period at AF0150 doses of 10, 20 and 40 mg/kg (26-40 fold PCD). Other ECG parameters were not remarkable in either studies.

Pulmonary Safety Assessment: Pulmonary artery pressure (PAP) measurements were included in some of the above cardiovascular safety studies, in anesthetized rabbits and dogs, and in non-anesthetized monkeys. PAP was recorded before and after bolus IV injection of AF0150 or saline through pulmonary arterial catheterization.

In a dog study (EB-95-27), AF0150 at doses up to 1.6 mg/kg (6.9-fold PCD) did not affect PAP during a 10-minute post dosing observation period, as compared to pre dosing (baseline). Another dog study (EB-95-25) with a concurrent application of ultrasound showed no difference in PAP from pre-dosing during a 1-minute post dosing observation period at AF0150 doses up to 0.6 mg/kg (2.6-fold PCD). In a rabbit study (EB-98-13), AF0150 at doses up to 10 mg/kg (26-fold PCD) did not change the pharmacologically-induced pulmonary hypertension during a 10-minute post dosing observation period. In a monkey study (IMUS-016-TOX), due to non-anesthesia and surgical procedure, PAP was highly variable in both AF0150- and saline- treated animals. It appeared that AF0150 at doses up to 40 mg/kg (104-fold PCD) did not induce

significant changes on PAP during a 60-minute post dosing observation period, when compared with baseline and saline control.

No pulmonary functions were tested with any animal model in this NDA submission. Blood gas analysis (arterial PaO2, PaCO2, pH, base access) was performed in anesthetized rabbits (EB-98-05, EB-98-06, EB-98-08, EB-98-13) and anesthetized dog (EB-95-27, PaO2 only). There were no significant effects on blood gases following IV injection of AF0150 at doses up to 52-fold (in rabbits) and 6.9-fold (in dog) as compared to pre dosing (baseline) or saline control animals. The observation period was 60 minutes post AF0150 dosing. However, the blood samples were collected from the anesthetized animals that had undergone tracheotomy (although no mechanical ventilation was applied). Unfortunately, blood gases were not analyzed in the study where non-anesthetized monkeys were used.

A study in rats (IMUS-046-TOX) showed that bolus IV injection of AF0150 at doses up to 100 mg/kg (130-fold) did not significant affect respiratory rate and body temperature during the 15-minute observation period.

AF0150 toxicity was not investigated in any animal model with acute or chronic pulmonary disorders.

Microcirculation Study: No microcirculation study was submitted in this NDA, and the sponsor was requested during a T-Con held on March 30, 2000 to conduct such a study. A study protocol (using *ex vivo* mesentery microcirculation model in rats) was submitted by fax from the sponsor on March 31, 2000 for comments. The protocol appeared to be adequate, however, the following comments and suggests were forwarded to the sponsor on April 10, 2000:

i. To ensure that enough microbubbles can be observed, AF0150 dose may need to be increased to approximately 10-fold of PCD.
ii. To ensure enough microbubbles are delivered into the mesenteric circulation, direct injection of AF0150 into the mesentery artery may be considered as an alternative administration route.
iii. A positive control (such as solid microspheres) needs to be included to validate the assay system.
iv. Coadministration or pretreatment of the animals with pharmacological stress agents is suggested to test microbubble behavior in the presence of vasoconstriction and vasodilation.
v. Please consider addressing possible effects of blood lipids and atherosclerotic lesions on microbubble behavior. In order to mimic hyperlipidemia, would it be possible to study the effects of high blood lipids by using an intravenous or intra-arterial injection of cholesterol in the proposed assay system?

Neurological and Behavioral Effects: Three studies were conducted in rats to evaluate neurotoxicity of AF0150 following intravenous (IMUS-043-TOX) and intra-arterial (PSM-98-01 and IMUS-043-TOX) administration. Two of the studies, IMUS-042-TOX and IMUS-043-TOX, were in compliance with GLP.

The gross behaviors (IMUS-043-TOX) evaluated using the Irwin test (Primary Observation Test) in male rats treated with AF0150 at doses up to 100 mg/kg (130-fold PCD) were not significantly different from the saline control group during a 2.5-hour post dosing observation period. The positive control drug chlorpromazine resulted in moderate to severe effects on behavior and physiology. However, only male rats were included in the study, and the observation time period may not have been long enough to elicit measurable behavioral effects (particularly animal handling may have transiently changed behavioral and physiological activities at early time points).

To evaluate potential risks of AF0150 to patients who have a cardiac right-to-left shunt, two rat studies (IMUS-043-TOX and PSM-98-01) were conducted with an intra-arterial injection of AF0150 to mimic cardiac right-to-left shunt. Functional Observational Battery test, Spontaneous Locomotor Activity Test, and brain histopathological examination were performed in rats intra-arterially (via carotid artery catheter) injected with saline or AF0150 at doses of 4 and 16 mg/kg (20-fold PCD). There were no significant differences in the behavioral tests between AF0150 and saline control groups during a 8-day observation period. However, multifocal infarction and ischemic lesions were observed in the kidneys, brain, testes and other organs in both control and AF0150-treated animals, which was consistent with the neurological signs from clinical observations. Thromboarteritis or thrombosis was shown in the catheterized carotid artery of all animals. This might explain the embolization and thus multiorgan infarction. However, the AF0150-treated animals tended to have a slightly higher incidence of renal ischemic pathology than the saline control animals. Also, brain infarction was identified in the two unscheduled deaths in the AF0150 (16 mg/kg) groups but not in the two unscheduled deaths of the control group.

Renal Toxicity: One study was conducted in male rats in compliance with GLP (IMUS-044-TOX). The saline-loaded rats were treated with an IV bolus injection of AF0150 at doses of 0, 4, 40 and 100 mg/kg (130-fold PCD) followed by measurements of urinary volume and electrolytes at different time points up to 24 hours post dosing. Urinary volume, pH, and urinary Na+, K+ and Cl- excretion significantly decreased during the first 3 hours post dosing at all dose levels. The electrolytes (Na+, K+ and Cl-) returned to control levels after 3 hours post dosing. The AF0150-induced urinary changes had no dose-dependency, and the **NOAEL** was below 4 mg/kg (5-fold PCD), which corresponds to a HED (human dose equivalence) of 0.65 mg/kg.

In single dose toxicity study (IMUS-039-TOX with dogs) and multiple dose toxicity studies (IMUS-014-TOX with dogs; IMUS-013-TOX with rats), there were no remarkable findings in urinalysis and BUN levels. However, in these studies urinary electrolytes and detailed urinary volumes were not measured in the urinalysis, and a volume challenge was not included. Therefore, AF0150-induced acute renal toxicity, particularly functional changes, may need to be further addressed. This would include evaluation of glomerular filtration and tubular re-absorption and secretion. The use of AF0150 in the patients with decreased renal function is not recommended unless the data from the clinical studies show otherwise. The reader is referred to the medical officer's review.

Gastrointestinal Toxicity: One GLP study was conducted in male rats to assess gastrointestinal transit function using a charcoal meal test. The animals were given a charcoal suspension by oral gavage 5 minutes following IV bolus injection of AF0150 at doses up to 100 mg/kg (130-fold PCD). There were no differences in charcoal transit in the gastrointestinal tracts between AF0150 and saline control groups during the 30-minute observation period. The positive control group (treated with morphine) showed a complete inhibition of charcoal emptying from the stomach. Effects of AF0150 on digestion and absorption were not tested in this NDA submission.

Hematology: Transient decreases in blood platelets and WBC were observed in both rabbits (EB-95-19) and dogs (EB-97-13) following IV administration of AF0150 at doses of 20 mg/kg (52-fold PCD for rabbits and 86-fold PCD for dogs). The decreases in the rabbit study lasted for about 30 minutes post dosing, but in the dog study with concurrent application of ultrasound power, the platelet and WBC counts returned to baseline within 4 and 2 hours post dosing, respectively. There were no changes in other hematology parameters.

Effects of IV infusion vs IV Bolus AF0150 and the Role of the Mechanical Index: There were no appropriate studies to compare IV bolus vs. IV infusion of AF0150 with concurrent application of ultrasound at various power settings (mechanical index, MI), for the safety assessment AF0150 in animals. Comparison of administration modes (bolus/infusion) with ultrasound application was performed in General Pharmacology Studies for efficacy evaluation purposes. Most safety pharmacology studies and all toxicology studies were conducted with IV bolus injections of AF0150. Two safety studies, EB-97-13 and IMUS-035-TOX, used IV infusion of AF0150 at 1 mg/kg/min (in 20 minutes, total 20 mg/kg) in dogs to assess cardiovascular safety. No concurrent IV bolus injection was included for comparison. The observations included heart rate, arterial blood pressure, hematology, coagulation, hemolysis, and ECG. Ultrasound was applied over the heart at the power levels of 1-1.1 MI (in the study EB-97-13) and in a standard cardiac imaging setting (for the study IMUS-035-TOX, where MI was not specified) both before and during AF0150 infusion. There were no significant effects on the observed parameters.

Conclusion and Comments for Safety Pharmacology Studies

Conclusion:

i. AF0150 had no significant effects on arterial blood pressure, heart rate, cardiac output and pulmonary arterial pressure following IV administration at the doses up to 20 mg/kg (52-fold PCD) in anesthetized rabbits, 1.6 mg/kg (6.9-fold PCD) in anesthetized dogs, and 40 mg/kg (104-fold PCD) in non-anesthetized monkeys. Concurrent application of ultrasound over the heart (closed-chest) appeared not to affect hemodynamics.
ii. AF0150 had no significant effects on blood gases (including arterial PaO2, PaCO2, pH, base access) following IV injection at doses up to 52-fold in anesthetized rabbits and 6.9-fold in anesthetized dogs during a 60-min post dosing observation period.

iii. AF0150 had no significant effects on ECG including QTc in anesthetized dogs dosed at 20 mg/kg (86-fold PCD) and observed for 30-min post dose. Similarly, in non-anesthetized monkeys at AF0150 doses up to 40 mg/kg (104 fold PCD) with a 60-min post-dosing observation period, AF0150 did not affect ECG parameters.
iv. AF0150 significantly decreased rat renal function following IV injection at doses from 4-100 mg/kg (5-130 fold PCD). The **NOAEL** for effects on renal function was less than 4 mg/kg (5-fold PCD).
v. AF0150 had no significant neurological and behavioral toxicity following intravenous or intra-arterial injection at doses up to 16 mg/kg (20-fold PDC) in rats, but AF0150 tended to increase kidney and brain ischemia upon injection in the arterial system.
vi. AF0150 had no significant effects on gastrointestinal charcoal transit following IV bolus doses up to 100 mg/kg (130-fold PCD) in rats during a 30-min post-dose observation period.
vii. AF0150 transiently (30 min to 4 hours) decreased blood platelets and WBC in anesthetized rabbits and dogs following IV administration of 20 mg/kg (52-fold PCD for rabbits and 86-fold PCD for dogs).

Comments:

i. Study with AF0150 using thromboxane-induced pulmonary hypertension rabbit model (EB-98-13) is considered adequate. However, it was an acute study (10-minute observation) with anesthesia. A chronic or subacute compromised pulmonary circulation disorder model (e.g. chronic or subacute pulmonary embolism, COPD) are still necessary to further assess potential impact of AF0150.
ii. Effect of AF0150 microbubbles on blood gases in non-anesthetized animals (dogs or monkeys) needs to be evaluated.
iii. Renal function needs to be further evaluated at a lower AF0150 dose range in order to achieve **NOAEL**. Animal species besides rat may be considered, such as dog or monkey.
iv. AF0150 microbubble behavior and effects on blood flow and capillary endothelial cells need to be evaluated with a microcirculation study in animals. Pathological conditions (such as atherosclerosis, hypertension, hyperlipidemia) should be considered. The AF0150 dosage should be high enough (10-fold or more) to ensure the visibility of microbubbles. A positive control (such as solid microspheres) needs to be included to validate the assay system. Coadministration or pretreatment of the animals with pharmacological stress agents is suggested to test microbubble behavior in the presence of vasoconstriction and vasodilation. In order to address possible effects of blood lipids on microbubble behavior, the microcirculation study may be conducted in a hyperlipidemic animal models (intravenous or intra-arterial injection of cholesterol). This comment has been conveyed to the sponsor, but the study report has not yet been submitted for review.
v. Only male rats were used in the neurotoxicity study, and the observation period was relatively short to elicit behavioral effects. Animal handling (such as injection procedure) may transiently change behavioral and physiological activities. The sponsor needs to comment on these issues.
vi. Safety of AF150 by IV bolus *vs* IV infusion with and without concurrent ultrasound application was not evaluated. The sponsor will need to address this deficiency.

vii. AF0150 was not studied in any animal model receiving physical stress (such as treadmill) on the cardiovascular or any other system. It is conceivable that AF0150 will be used with either pharmacological or physical stress tests in a clinical setting. Therefore, appropriate risk assessment of AF0150 under the stress treatments should be made in both preclinical and clinical setting.

Pharmacokinetics/Toxicokinetics (Table 3)

Only one pharmacokinetic study (GLP) was conducted in rats to determine the kinetic profile of perfluohexane (PFH, one of the major components in AF0150 microbubbles) in expired air and blood. The animals received a single bolus intravenous injection of AF0150 at 20 mg/kg (26-fold PCD) followed by a 48-hour observation period, during which PFH in expired air and blood (from femoral vein) was measured.

PFH elimination profile in expired air revealed that about 90% of the administrated PFH was excreted from the lungs within the first 3 hours post dosing and almost completely eliminated within the first 48 hours. PFH in blood had the same profile as in expired air, with a two-compartment model. Blood PFH level decreased by 78% during the first 2 minutes post dosing, and became non-detectable by 24 hours. The terminal elimination half-life of blood PFH was about 88 minutes, based on the pooled data from individual animals.

No distribution and metabolism studies on AF0150 and/or PFH were submitted with this NDA. The sponsor stated that there were no metabolites of PFH in urine and tissues of rats according to a literature report. However, in the cited literature PFH was administrated to rats by inhalation (2-hours exposure) instead of intravenous injection. Besides, there was no coadministration of other amphiphilic compounds formulated in AF0150 such as DMPC, HES and Poloxamer-188. The different administration routes and the interaction with other compounds may change the metabolic profile. Identification of PFH forms in expired air and blood could have provided valuable information from this study.

The fate of the other AF0150 components, including DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), HES (m-hydroxyethyl starch) and Poloxamer-188 was not investigated. The sponsor provided some literature information and a brief review regarding the pharmacokinetics of DMPC contained in liposomes. In only one clinical study cited by the sponsor, IV injection of 99mTc-liposomes composed of DMPC and DMPG (dimyristoylphosphatidylglycerol) showed clearance of the liposome by macrophages of liver and spleen. Based on the literature report, the sponsor concluded that DMPC is safe because of the nature of DMPC as a phospholipid and its minimal contribution to blood phospholipid pool (400-1000-fold less) after IV administration of 200 mg AF0150. However, DMPC is a semisynthetic phospholipid and not a natural endogenous lipid, and it is not formulated as a liposome in AF0150. Therefore, knowing the pharmacokinetic profile of DMPC is still important for long-term safety assessment.

Poloxamer-188 is a nonionic surfactant with a molecular weight of 8350D. It is an approved drug and has been used orally for stool-wetting and stool-softening. However, the pharmacokinetics and safety via intravenous administration is unknown.

Hydroxyethyl starch (HES) is commonly used for plasma volume expansion, and FDA-approved HES products include Hetastarch and Pentastarch. Literature articles report that HES is taken up by macrophages and parenchymal cells in organs, which frequently resulted in cell vacuolation. It was also found that species-dependent elimination of HES and clearance of HES from spleen and liver was slower in rats than in dogs. These may partially explain why vacuolated macrophages were observed in rats but not in dogs following AF0150 administration.

Conclusion and Comments for Pharmacokinetics Study

Conclusion

Major elimination route of PFH, one of AF0150 major components, was through expired air. Following IV bolus of 20 mg/kg (26-fold) in rats, about 90% of PFH was excreted from the expired air within 3 hours post dosing. PFH in blood decreased by 78% within the first 2 minutes post dosing, and became non-detectable by 24 hours. The terminal blood half-life of PFH was 88 minutes. Kinetics profiles of PFH in both expired air and blood fitted into a two-compartment model.

Comments

i. Due to technical difficulties in accurately measuring blood PFH levels, great variation in blood PFH values were noted. Therefore, the expired air PFH kinetics need to be individually processed in more detail.
ii. More dose groups and at least 2 animal species need to be included in pharmacokinetic studies. The fate of DMPC and Poloxamer-188 following IV administration also needs to be evaluated.
iii. There was great variation in PFH levels of reconstituted AF0150 in each vial (pre-dosing and post-dosing). The relationship between PFH levels and microbubble profile (density and size) was not addressed in this or other pharm/tox studies. The true dosage may be more accurately reflected by bubble counts/kg. Also it was not clear how PFH levels and bubble counts/ml were related to osmolality measurement, as used in most pharm/tox studies for dose verification.

Toxicology (Table 3)

Five acute single dose toxicity studies in mice, rats and dogs, and 2 multiple dose toxicity studies in rats and dogs were conducted in compliance with GLP. Dose selection, animal species, treatment duration (including recovery period) and observation parameters in these studies appeared to be adequate. The major results from these studies are summarized in Table 3. Some

transient AF0150-related toxicity was observed in all tested animal species with high **NOAEL** values, as shown in Table 11.

Acute Single Dose Toxicity Studies: Standard and expanded acute single dose toxicity studies in compliance with GLP were conducted in mice, rats and dogs following intravenous injection of AF0150 at doses up to 1037-fold PCD (in mice), 2073-fold PCD (in rats) and 1731-fold PCD (dogs). All animals survived to the scheduled sacrifice without significant changes on body weight and food consumption associated with AF0150 treatment during the 14-day post dosing observation period. The following results were obtained:

1. AF0150-related transient toxicity was observed in mice, rats and dogs. The transient toxic signs included hypoactivity and dyspnea in mice with **NOAEL**=800 mg/kg (518-fold PCD) (IMUS-037-TOX); reddening of lips, nose ears, paw and tail in rats with **NOAEL**=400 mg/kg (518-fold PCD) (IMUS-010-TOX); slight decrease in platelets, and slight increase in serum triglyceride and alanine aminotransferase (ALT) in dogs with **NOAEL**=200 mg/kg (865-fold PCD) (IMUS-039-TOX). Transient increase in the incidence of vomiting, excessive salivation, hypoactivity, and injected sclera was also noted in AF0150-treated dogs. These reactions occurred within the first 60 minutes post dosing and were resolved within 3 hours (No **NOAEL** was achieved).

2. Cecal inflammation was noted in AF0150-treated mice at necropsy (without microscopic examination) with **NOAEL**=200 mg/kg (130-fold PCD) (IMUS-037-TOX). The cecal lesion was not seen in rats and dogs. AF0150 at all dose levels (50-400 mg/kg) induced macrophage vacuolation in the spleen and mesenteric lymph nodes of rats (IMUS-011-TOX) but not dogs.

3. No functional observations were provided, such as blood gas analysis and ECG. Macroscopic and microscopic examinations, including lung, heart, kidney and brain, were not remarkable. The selected organs/tissues for histopathological examination were listed in Table 1 of the **Summary of Toxicology Studies**.

4. Other observations in hematology, blood chemistry and urinalysis were not significantly different between AF0150 treatment and control groups in the tested species.

Multiple Dose Toxicity Studies: Two multiple dose toxicity studies were conducted in rats and dogs in compliance with GLP following daily intravenous injection of AF0150 at doses up to 518-fold PCD (in rats) and 433-fold PCD (in dogs) for 29-30 days, and followed by a 15-day recovery period. One interim termination time point was applied to both studies. All animals survived to the scheduled termination day without remarkable clinical signs, changes in body weight and food consumption, and ophthalmic lesion associated with AF0150 treatment in both species. The following results were obtained:

1. Transient and reversible clinical signs, such as vomiting, pale mucous membranes hypoactive or uncoordinated behavior, were observed in AF0150-treated dogs with **NOAEL**=25 mg/kg/day (108-fold PCD) (IMUS-014-TOX, IMUS-027-TOX).

2. Vacuolated macrophages were found at all AF0150 dose levels in multiple tissues in rats (IMUS-013-TOX), but not in dogs. This effect was irreversible (no change at the end of a 15-day recovery period). The organs/tissues rich in monocyte/macrophage showed a higher incidence of vacuolated macrophages, such as in spleen and lymph nodes, However, none were noted in bone marrow.

3. AF0150 at high doses (>200 mg/kg/day) induced eosinophil infiltration in mesenteric lymph nodes and perivascular area in the lungs in rats (IMUS-013-TOX), and increased extramedullar hematopoiesis in the spleen. **NOAEL** for both effects was 50 mg/kg/day (HED: 310 mg/kg/day; HDM: 65-fold).

4. Several blood chemistry parameters associated with liver function decreased in AF0150-treated rats on Day 30 (IMUS-013-TOX), including creatinine, total protein, globulin, AST, ALT, alkaline phosphatase, etc, with **NOAEL**= 50 mg/kg/day (65-PCD). Increases rather than decreases in these parameters are generally related to acute organ toxicity (increased cell membrane permeability). However, these findings may also suggest that AF0150 treatment decreased liver function without significantly changing cell membrane permeability, which could lead to lower liver enzyme markers in blood.

5. Observations related to pulmonary and cardiac toxicity such as blood gas analysis and ECG were not provided. Macroscopic and microscopic examinations, including lung, heart, kidney and brain, were not remarkable. The selected organs/tissues for histopathological examination in all studies were listed in Table 1 of the **Summary of Toxicology Studies**.

6. Other observations in hematology, blood chemistry and urinalysis were not significantly different between AF0150 treatment and control groups in the tested species.

Conclusion and Comments for Toxicology Studies

Conclusion

i. IV administration of AF0150 induced transient toxicity in mice, rats and dogs.
ii. There were species-specific macrophage vacuolation in AF0150-treated rats and cecal inflammation in AF0150-treated mice.

Comments

Since the mechanisms of AF0150-induced species-specific pathological findings such as vacuolated macrophages and cecal inflammation are not fully understood, potential impact on monocyte/macrophage function and cecum/appendix physiology in patients receiving this drug can not be ruled out, particularly for those patients with a history of cecum/appendix inflammation.

Special Toxicology (Table 4)

Local Irritation Studies: Two GLP studies were conducted in rabbits following intravenous, perivenous, intra-arterial injections (IMUS-028-TOX) of AF0150 at 2 and 20 mg/kg (5.2-52 fold PCD), and intramuscular injection (IMUS-038-TOX) at 1 mg per animal. Skin reactions and pathology (macroscopic and microscopic examination) of the injection sites were monitored up to 15 days post dose. Perivenous injection at both AF0150 doses resulted in slight local irritation mostly noted at 4 hours post dose. The **NOAEL** was < 2 mg/kg. No significant skin reactions and pathological findings were observed at the intravenous, intra-arterial and intramuscular injection sites.

Immunotoxicity Studies: Two GLP *in vivo* studies were conducted in guinea pigs to test antigenicity and delayed hypersensitivity. AF0150 antigenicity (IMUS-021-TOX) was evaluated by using active systemic anaphylaxis (ASA) and passive cutaneous anaphylaxis (PCA) methods. AF0150 with and without Freund's Complete Adjuvant (FCA) did not induce significant active systemic anaphylactic and passive cutaneous anaphylactic reactions.

The delayed hypersensitivity (IMUS-029-TOX) of AF0150 with and without Freund's Complete Adjuvant (FCA) was tested in guinea pigs using Magnusson and Kligman maximization assay (guinea pig maximization study). No significant dermal reactions were observed. However, no concurrent positive control was included in the study to validate the assay system.

Two non-GLP *in vitro* studies were conduced to test if AF0150 activates complement C3 and stimulates TNFα release from blood cells. AF0150 (0.5 mg/ml) tended to increase C3a level (507±295 ng/ml) as compared to saline (151±26 ng/ml) when incubated with human plasma *in vitro* (BC-95-17). The difference was not statistically different due to great variation. Incubation of AF0150 (0.5 mg/ml) with rat whole blood did not increase plasma TNFα level (CMB-96-14).

Acute Toxicity Studies with Cardiac Stress: Single dose acute toxicity of AF0150 was conduced in rabbits pretreated with each of 4 cardiac stress agents, adenosine (IMUS-018-TOX), dipyridamole (IMUS-019-TOX), dobutamine (IMUS-020-TOX) and arbutamine (IMUS-036-TOX). The four studies were in compliance with GLP and their results are summarized in Table 9. AF0150 at the doses of 0, 2 and 20 mg/kg (up to 52-fold PCD) was given to animals intravenously immediately after IV infusion of each cardiac stress agent followed by a 14-day observation. No significant toxic findings, including clinical signs, hematology, blood chemistry, necropsy and heart histopathology, were observed with relation to AF0150 treatment. However, no stress responses (such as heart rates and other hemodynamic parameters) were monitored before, during and after IV infusion of the cardiac stress agents, suggesting that the animal model was not appropriate to assess risk of AF0150 under cardiac stress scenarios.

Microbubble Size: Microbubble profile data (AC-00-08) were submitted in a fax on April 6, 2000 as requested by T-Con on March 30, 2000. The submission included AF0150 microbubble dosages defined as bubble counts/kg body weight for all GLP studies.

As summarized in Table 5 (from report AC-00-08), there did not appear to be significant changes on microbubble size and corresponding counts/ml at 30 and 60 minute post reconstitution, as compared to the results from right after reconstitution. However, there was a significant decrease in total (whole range) bubble counts/ml at 60 minutes post reconstitution. The percentage of bubbles larger than 10 um was less than 0.2% at all time points post reconstitution.

Microbubble size analyses were also included in some pharmacodynamics studies. In study RE-99-47 (reviewed in General Pharmacology), two peaks of microbubble populations were found in reconstituted AF0150: peak one with bubble size of 1-1.5 um and peak two with bubble size of 4-5 um in diameter. After separation of the microbubbles using a "creaming" method, 97-99% microbubbles in the Lower part (small bubbles) were <3 um in diameter, and the remaining were larger than 3 um (but size distribution was not further addressed). In the Upper part (large bubbles), 19-24% microbubbles were >3 um and the remaining were <3 um.

Table 5. Size Distribution and Counts/ml of AF0150 Microbubbles Post Reconstitution

Time (min) Post-Reconstitution	**Microbubble Size (um) and Counts/ml ($x10^6$)**		
	Whole Range	**3-10 um**	**>10um**
0	979±1.17	181±0.158 (18.5%)*	1.51±0.607 (0.15%)
30	912±1.09	173±0.250 (19.0%)	1.36±0.806 (0.15%)
60	858±1.02†	173±0.250 (20.2%)	1.50±0.739 (0.17%)

* data in parenthesis are percentage of bubbles at that size over total bubbles (whole range).
† <0.05 as compared to time 0 with Student's t-test.

Effects of ultrasound power on AF0150 microbubble size were tested using a simulated *in vitro* circulation system circulated with bovine albumin solution (RE-99-46, reviewed in General Pharmacology). Sustained ultrasound exposure caused little change in microbubble size (median diameter range of 5 to 8 um) in the albumin solution, with a slight shift to smaller sizes except at the highest power setting (MI 1.7). At 1.7 MI, bubble size increased from 4.1 to 6.1 um. Microbubble counts (both small and larger bubbles) decreased with increasing time and power levels of ultrasound exposure.

AF0150 Dosage Conversion of mg/kg to bubbles/kg: As per our request during T-Con on March 30, 2000, the sponsor also provided data sheets in the April 6, 2000 fax submission, which recalculated AF0150 dosages based on microbubble counts per kg body weight for all GLP studies. The information in Table 6 was extracted from the data sheets. After reconstitution, the bubble counts per mg of AF0150 were identical, 4.9 bubbles/mg, in all fill sizes. The sponsor converted doses in mg/kg to bubble counts/kg by simply multiplying mg with 4.9 bubbles/mg for all studies. For example, 20 mg/kg for any animal species was $9.8x10^8$ bubbles/kg.

Table 6. AF0150 Microbubble Counts for Dosage Conversion in Animal Studies

AF0150 Fill Size (mg/vial)	Reconstituted Concentration (mg/ml)	Microbubble Counts	
		Counts/ml ($x10^8$)	Counts/mg ($x10^7$)
100	10	4.9	4.9
200	20	9.8	4.9
400	40	19.6	4.9

The mg/kg doses were converted to counts/kg by multiplying 4.9 counts/mg for all studies.

Conclusion and Comments for Special Toxicology Studies

Conclusion

i. Perivenous injections of AF0150 caused local skin pathological reactions.
ii. AF0150 did not cause active systemic anaphylaxis (ASA), passive cutaneous anaphylaxis (PCA), and delayed hypersensitive reactions in guinea pigs. In the *in vitro* studies AF0150 tended to activate complement C3 and did not increase plasma TNFα.
iii. Following a single IV dose of AF0150 up to 20 mg/kg (52-fold of PCD) no significant toxic findings were reported in the rabbits pretreated with cardiac stress agents.
iv. AF0150 microbubble count and size appeared not to be significantly different at 30 and 60 minutes post reconstitution from the results obtained immediately after reconstitution. However, total (whole range) counts/ml at 60 minutes post reconstitution were statistically decreased.

Comments

i. Pretreatment of rabbits with cardiac stress agents did not produce related cardiovascular effects, suggesting that the animal model may not have been appropriate to assess the risk of AF0150 under cardiac stress scenarios.
ii. Detailed information about bubbles larger than 10 um was not provided although the percentage of those bubbles was less than 0.2%. No stability results in terms of bubble sizes and counts were provided and discussed.
iii. Conversion of AF0150 administration doses from mg/kg to microbubble count/kg was processed by simply multiplying mg with 4.9 bubbles/mg for all AF0150 lots (100, 200, and 400 mg fills/vial with reconstituted concentrations of 10, 20 and 40 mg/ml) used in all pharm/tox studies. However, PFH concentrations, which is correlated with microbubble size and count, were significantly different from vial to vial, as indicated in the PK study. This suggests that bubble count/mg may not be constant in different vials.

Reproductive Toxicity (Table 7)

Reproductive toxicity of AF0150 following intravenous injection was assessed in rats (fertility, teratology, pre-/post-natal toxicity) and in rabbits (teratology). Dose levels of 50-200 mg/kg/day were selected in both species, which corresponded to 65-259 fold the planned clinical dose

(PCD) in rats and 130-518 fold the PCD in rabbits. The major results are summarized in Table 7 and **NOAELs** are listed in Table 12.

Fertility and Early Embryonic Development (Segment I): The study was conducted in rats (25/sex) via IV injection of AF0150, from 2 weeks (females) and 4 weeks (males) pre-mating till gestation day 7 (female). Animals were sacrificed on female gestation day 13. The dose levels of AF0150 were 50, 100 and 200 mg/kg/day, which correspond to 65, 130 and 259 fold the planed clinical dose (PCD), based on body surface area conversion. Slight decreases in male fertility (by 5%, without statistical significance) and mean epididymal sperm numbers (by 15%, $p<0.05$) were observed at the high dose groups (200 mg/kg/day), with the **NOAEL** of 100 mg/kg/day (HED: 16.2 mg/kg/day and HDM: 130-fold the PCD) for both findings. AF0150 had no significant effects on other reproductive performance (mating index, fertility index, pre-coital interval, and female estrous cycle) and male spermatogenesis (testicular sperm counts, sperm production rate, sperm motility and morphology) at any dose level as compared to saline. No remarkable findings were observed on female necropsy upon gestation day 13, including pre- and post-implantation losses, numbers of viable embryos, implantation sites and corpora lutea. However, the high AF0150 dose, 200 mg/kg/day, did not result in maternal toxic signs, including changes on body weights, organ weights (brain, testis, ovary and pituitary) and food consumption. Minimal maternal toxicity should be generally expected in the high-dose group to confirm that appropriate doses were selected for the reproductive toxicity studies.

Teratology and Embryo/Fetal Development (Segment II): Two studies were conducted in rats (25 females) and rabbits (25 females) following IV injection of AF0150 at 50, 100, 200 mg/kg/day. The human dose multiples (HDM) were 65-259 fold for rats and 130-518 fold for rabbits. The animals were dosed daily during the organogenesis period, from gestation day 6 through 17 (sacrificed on gestation day 20) for rats, and gestation day 7 through 20 (sacrificed on gestation day 29) for rabbits. There were no significant differences in fetal development, viability, body weights, sex ratio, corpora lutea number, implantation sites and post-implantation loss between AF0150-treated and control groups in either species. However, the fetal malformation incidence slightly increased in the 100 and 200 mg/kg/day groups in rabbits but not in rats as compare to the control group. These malformations included external, visceral (soft tissue) and skeletal anomalies. Percentage of fetuses with malformations was 1.9% in control rabbits, 3.7% and 5.9% in rabbits treated with 100 and 200 mg/kg/day AF0150, respectively. The **NOAEL** was therefore determined at 50 mg/kg/day. No maternal toxicity (clinical signs, body weight and food consumption changes, and macroscopic examination) was observed in either species at any AF0150 dose level, suggesting that a higher dose should have been used to produce minimal maternal toxicity. The **NOAEL** for maternal toxicity was 200 mg/kg/day (HDM: 259-fold in rats and 518-fold in rabbits)

Prenatal and Postnatal Development (Segment III): This study was conducted in rats (25 females) following daily IV injection of AF0150 at 50, 100 and 200 mg/kg/day (HDM: 65-259 fold) from gestation day 6 through lactation day 20. AF0150, at all dose levels, had no significant maternal toxicity (clinical signs, body weight, food consumption and necropsy), pregnancy rate, gestation duration and parturition process. However, in the dose of 200 mg/kg/day, AF0150

decreased the live birth index (by 7% with $p<0.05$), and tends (no statistical significance but p values were not provided) to decrease postimplantation survival index (by 6.8%), mean live litter size (by 8%), live pup numbers (by 8.8%) and gestation survival index (by 5%). Stillbirths were increased from 0.3% (in the control group) to 7.7% (in the 200 mg/kg/day group) per litter (about 25-fold higher). There was also approximately 2-fold increase in total postnatal death and postnatal missing/cannibalized during the postnatal days 0-21 in the 200 mg/kg/day group. The **NOAEL** for neonate toxicity was 100 mg/kg/day (HED = 16 mg/kg/day and HDM = 130-fold). The offspring from AF0150-treated animals had no differences in physical and functional development as well as in behavior, from those from the control group. A fertility study on the offspring from the AF0150-treated groups showed no significant effects on fertility and mating indices in either sex, nor on fetal development and morphology, as compared to the offspring from the control group.

Conclusion and Comments for Reprotoxicity Studies

Conclusion

Intravenous injection of AF0150 at the human dose multiples of 65-259 fold in rats had no significant adverse effects on fertility, teratology, embryonic (early and later) and fetal development. At the dose of 200 mg/kg/day in rabbits (518-fold the PCD), AF0150 slightly increased incidence of external, visceral and skeletal malformations. Also at this dose in rats (259-fold the PCD), lower neonate live birth and higher stillbirths were noted than in control group. In all 4 reproductive toxicity studies, the highest dose, 200 mg/kg/day, did not result in minimal maternal toxicity, suggesting that dose selection was not optimized. The pregnancy category C is recommended in the labeling.

Comments

Pregnancy category C is recommended for AF0150.

Genotoxicity (Table 8)

The standard genotoxicity study battery was used to test the potential genotoxicity of AF0150, including a bacterial reverse mutation test, a mammalian cell DNA damage (chromosomal aberration assay with human lymphocytes and forward mutation with mouse lymphoma tk cells), and an *in vivo* mouse micronucleus assay. The AF0150 dose selection and treatments were appropriate based on cytotoxicity or maximal solubility of the product. Appropriate negative and positive controls were included in all *in vitro* and *in vivo* studies, and the expected results were produced from the positive control compounds. All studies were conducted in the presence and absence of metabolic activation (using induced rat liver S9). The major results of these studies were summarized in Table 8.

AF0150 at doses up to 20 mg/plate did not significantly increase bacterial reverse mutation. At doses up to 5 mg/ml, AF150 did not induce significant chromosomal aberrations and forward

mutations, as compared to a vehicle control group. Single IV injection of AF0150, at doses up to 800 mg/kg (518-fold PCD based on body surface area conversion) did not increase micronucleated polychromatic erythrocytes in mouse bone marrow. AF0150 is concluded to be negative in the following genotoxicity assays:

1. *In vitro* bacterial reverse mutation assay
2. *In vitro* chromosomal aberration assay in culture human lymphocytes
3. *In vitro* mouse lymphoma tk forward mutation assay
4. *In vivo* micronucleus assay in mice

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