Summary of Toxicology Study

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 the human dose multiples up to 1037 fold (in mice), 2073 fold (in rats) and 1731 fold (dogs). *Human dose multiples (fold PCD) are calculated based on the body surface area.* 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. The following results were achieved:

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 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 at necropsy (without microscopic examination) in AF0150-treated mice with **NOAEL**=200 mg/kg (130-fold PCD) (IMUS-037-TOX), but 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.

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

Multiple Dose Toxicity Studies: Two multiple dose toxicity studies (in compliance with GLP) were conducted in rats and dogs with daily intravenous injections of AF0150 at the human dose multiples up to 518 fold (in rats) and 433 fold (in dogs), for 29-30 days, 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 signs of toxicity, changes in body weight and food consumption, or ophthalmic lesion associated with AF0150 treatment in either species. The following results were achieved:

1. Transient and reversible clinical signs, such as vomiting, pale mucous membrane 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 in multiple tissues in rats (IMUS-013-TOX), but not in dogs, at all AF0150 dose levels. This effect was not reversed at the end of 15-day recovery period. The organs/tissues rich in monocytes/macrophages 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). Although increases rather than decreases in these parameters are generally related to organ acute toxicity (increased cell membrane permeability), these may also suggest that AF0150 treatment decreased liver function without significantly changing cell membrane permeability which could lead to lower liver enzymes 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.

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

Conclusion and Comments: 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 those studies appeared to be adequate. Although some transient AF0150-related toxicity was observed in individual species, there was no significant dose- or time-dependency (incidental and sporadic occurrence) in most studies and high **NOAEL**s were achieved, as listed in Overall Summary (Table 11, page) However, "species-specific" pathological findings such as vacuolated macrophages in rats and cecal inflammation in mice were considered toxicologically significant. Since the nature and mechanisms of these toxic reactions are not fully understood, one may not rule out the possibility that a similar effect may occur in human subjects. Closely monitoring monocyte/macrophage function is recommended. Patients with cecum/appendix inflammation history should also be evaluated for possible toxicity of AF0150 to the cecum.

Table 1. **List of Organs/Tissues for Histopathological Examination**

ORGANS	Report No. (IMUS-) and Study Type					
	011-TOX	012-TOX	013-TOX	039-TOX	027-TOX	014-TOX
	Rats/Single*	Dogs/Single	Rats/Multi*	Dogs/Single	Dogs/Multi	Dogs/Multi
Adrenals	X	X	X	X	X	X
Aorta	X	X	X	X	X	X
Bone Marrow Smear	X	X	X(NE)	X (NE)	X	X (NE)
Brain	X	X	X	X	X	X
Cecum	X	X	X	X	X	X
Cervix			X			
Colon	X	X	X			X
Diaphragm						
Duodenum	X	X	X	X	X	X
Epidydimides	X	X	X	X		X
Esophagus	X	X	X	X	X	X
Eyes with Lens	X	X	X	X	X	X
Optic nerve						
Femur	X	X	X	X	X	X
Gallbladder		X		X	X	X
Harderian Gland						
Heart	X	X	X	X	X	X
Ileum	X	X	X	X	X	X
Injection Site	X	X	X	X	X	X
Jejunum	X	X	X	X	X	X
Joint						
Kidneys	X	X	X	X	X	X
Lacrimal Glands	X		X	X		
Larynx						
Liver	X	X	X	X	X	X
Lung	X	X	X	X	X	X
Lymph Nodes	X	X	X	X	X	X
Mammary Glands	X	X	X	X	X	X
Ovaries	X	X	X	X	X	X
Parathyroid Gland	X	X	X	X	X	X
Pancreas	X	X	X	X	X	X
Pituitary	X	X	X	X	X	X
Prostate	X	X	X	X	X	X
Rectum	X	X	X	X		X
Salivary Glands	X	X	X	X	X	X
Sciatic Nerve	X	X	X	X		X
Skeletal Muscles	X	X	X	X		X
Seminal Vesicles	X		X			
Skin	X	X	X	X		X
Spinal cord	X	X	X	X	X	X
Spleen	X	X	X	X	X	X
Sternum	X	X	X	X		X
Stomach	X	X	X	X	X	X
Testes	X	X	X	X	X	X

Thymus	X	X	X	X	X	X
Thyroid	X	X	X	X	X	X
Tongue						
Trachea	X	X	X	X	X	X
Urinary bladder	X	X	X	X	X	X
Uterus	X	X	X	X	X	X
Vagina	X	X	X			X
All gross lesions	X	X	X	X		X

* Single or multiple dose toxicity studies
NE: Prepared but not examined.

29. SPECIAL TOXICOLOGY

Report Number: IMUS-028-TOX
Acute Intravenous/Perivenous/Intra-arterial Tolerance Study with AF0150 in Rabbits

Report Location:	Vol.018, p270-336
Report date:	October 8, 1997
Study Facility:	[redacted]
In-life phase:	February 4-18, 1997
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010
AF0150 Dosage (HDM):	2 or 20 mg/kg (5.2-52 fold PCD)

Specific Aim

To assess local irritation with a single intravenous, perivenous or intra-arterial injection of AF0150 to rabbits

Methods

Animal Preparation: Albino rabbits (both sexes), Hra:(NZW)SPF strain, were obtained from [redacted] and were 14 weeks old with body weight of 2.2-2.5 kg at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for 7 days before initiation of treatment, and those meeting health and body weights were randomly assigned into 3 groups (6/group, 3 each sex) as seen in table 1.

AF0150 Preparation and Administration: AF0150 (200 mg fill vial) was reconstituted with 10 ml of sterile water to a concentration of 20 mg/ml and used within 30 minutes of preparation. Administration procedures were grouped as in Table 1. Before dosing, the hair surrounding the marginal ear vein and central ear artery of both ears was gently clipped with an electric clipper. AF0150 (left ear) or saline (right ear) was injected either directly into the marginal ear vein (intravenous, 1 ml/kg), adjacent to the marginal ear vein (perivenous, 0.1ml/kg), or directly into the central ear artery (intra-arterial, 1 ml/kg) of the ears. The injection sites on each animal were marked with indelible ink for identification at necropsy. The dose and frequency of administration were based on the expected or possible human exposures.

Observation Parameters: clinical signs were monitored before and 4 hours after injection on Day 1 and once a day thereafter. Body weights were recorded on Day 1 and at each scheduled termination day (Days 4 and 15). Irritation (erythema and edema) was observed on Days 1 (4 hours post dosing), 2, 3, 4, 8 and 15, and scored based on the following scales (Table 2).

At each scheduled sacrifice (3 animals on each of Days 4 and 15), all animals were subjected to necropsy with macroscopic examination of injection sites. Three sections (distal, middle and

proximal portions) of ear vein and artery (including around skin and cartilage) at the injection site were preserved for histopathology examination.

Table 1. Local Irritation Study Procedure

Group	Route of Injection	Treatment		Dose Volume	Number of Animals*
		Right Ear	Left Ear		
1	Intravenous	Vehicle (Saline)	AF0150	1.0 mL/kg	6
2	Perivenous			0.1 mL/injection	6
3	Intraarterial			1.0 mL/kg	6

* Three animals/group were sacrificed on Day 4 and three animals/group were sacrificed on Day 15.

Table 2. Irritation Scales

Erythema (Redness)	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness to slight eschar formation, injuries in depth)	4
Edema (Swelling)	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges are well-defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4

Results

Intravenous Injection: no erythema or edema reactions were observed in both AF0150 and saline-injected ears. A hematoma was noted in a few injection sites found in both AF0150 and saline-injected sites without significant differences (2 of 6 sites). Minimal focal hemorrhage or thrombus or intimal hyperplasia were found in some injection sites from either saline or AF0150 treatments but without a significant difference.

Perivenous Injection: slight erythema reaction was noted in 4 of 6 AF0150-injected sites but not in saline-treated ears at 4 hours post injection on Day 1. There were more hematoma reactions in the AF0150-injected ears with longer duration (up to Day 4) than saline-injected ears. Histopathology showed minimal to slight focal or multiple hemorrhage in some sites from either saline or AF0150 treatments but without a significant difference.

Intra-arterial Injection: no erythema or edema reactions were observed in both AF0150- and saline-treated ears. There were more hematoma reactions in both groups but without significant differences between AF0150 and saline treatments. Minimal focal hemorrhage (1/3 AF0150 sites and 2/3 saline sites) was noted on Day 15 by microscopic examination.

Clinical Signs and Body Weights: unremarkable.

Discussion and Comments

Perivenous, but not intravenous or intra-arterial, injection of AF0150 resulted in slight local irritation (erythema) in rabbits, mostly seen at 4 hours post injection. **NOAEL** was < 2 mg/kg. This suggests that caution should be exercised during the dosing procedure in clinical application with AF0150.

Report Number: IMUS-038-TOX
Acute Intramuscular Tolerance Study with AF0150 in Rabbits

Report Location:	Vol.018, p337-392
Report date:	February 5, 1999
Study Facility:	[redacted]
In-life phase:	May 26-June 9, 1998
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010 (200 mg Fill)
AF0150 Dosage (HDM):	1 mg/kg (2.6 fold PCD)

Specific Aim

To assess local tolerance with a single intramuscular injection of AF0150 to rabbits

Methods

Animal Preparation: Albino rabbits (both sexes), Hra:(NZW)SPF strain, were obtained from [redacted] and were 14 weeks old with body weight of 2.2-2.5 kg at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for 7 days before initiation of treatment, and 9 animals meeting health and body weight requirements were randomly selected for intramuscular injection (thigh muscle).

AF0150 Preparation and Administration: AF0150 (200 mg fill vial) was reconstituted with 10 ml of sterile water to a concentration of 20 mg/ml and used within 30 minutes of preparation. Before dosing, the hair surrounding the intended injection site of the hind legs was gently clipped with an electric clipper. AF0150 (left leg) or saline (right leg) was injected directly into the thigh muscle with 0.5 ml (1 mg AF0150) per injection sites. The injection sites on each animal were marked with indelible ink for identification at necropsy.

Observation Parameters: clinical signs were monitored before and 4 hours after injection on Day 1 and once a day thereafter. Body weights were recorded on Day 1 and at each scheduled termination day. Irritation (erythema and edema) was observed on Days 1 (4 hours post dosing), 2, 3, 4, 8 and 15, and scored based on the following scales (Table 2).

Table 2. Irritation Scales

Erythema (Redness)	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness to slight eschar formation, injuries in depth)	4
Edema (Swelling)	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges are well-defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4

At each scheduled sacrifice (3 animals on each of Days 4, 8 and 15), animals were subjected to necropsy with macroscopic examination of injection sites. Tissues taken from the injection area (3 sections, *but not specified*) were preserved for histopathologic examination.

Results

Local Irritation: no erythema or edema reactions were observed at any of nine AF0150- or saline-injected sites. Histopathology showed that there was minimal focal hemorrhage, intramuscular inflammation and degeneration/necrosis in some animals given either AF0150 or saline. These changes were attributed to trauma and mechanical injury from the injection procedure.

Clinical Signs and Body Weight: all animals survived till the scheduled termination without remarkable clinical signs or body weight changes.

Discussion and Comments

Intramuscular injection of AF0150 (0.5 ml, or 1 mg) did not lead to significant local irritation in rabbits.

Report Number: IMUS-021-TOX
Antigenicity Study of AF0150 in Guinea Pig

Report Location:	Vol.019, p001-051
Report date:	March 25, 1997
Study Facility:	[redacted]
In-life phase:	November 26, 1996
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010 (200 mg Fill)
AF0150 Dosage (HMD at BSA):	20 mg/kg for IV challenge; 2 mg SC for induction

Specific Aim

To assess antigenicity of AF0150 in guinea pig utilizing active systemic anaphylaxis (ASA) and passive cutaneous anaphylaxis (PCA) methods

Methods

Animal Preparation: albino guinea pigs, Crl:(HA)BR strain, were obtained from [redacted] and were 5-10 weeks old at initiation of treatment with body weight of 350-389 g (for ASA) and 470-645 g (for PCA). Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for 5 days before initiation of treatment.

AF0150 and BSA Preparation: AF0150 (200 mg fill vial) was reconstituted with 10 ml of sterile water to a concentration of 20 mg/ml and used within 30 minutes of preparation. 10 mg/ml of BSA was prepared by adding saline to 0.1 g BSA to a total volume of 10 ml. Saline (0.9% NaCl) was used as negative control. For induction where applied, AF0150 was mixed with FCA (Freund's Complete Adjuvant) at the ratio of 1:1 (v/v).

Procedure for Active Systemic Anaphylaxis (ASA) (Table 1): 25 guinea pigs (male) were assigned into 5 groups (5/group) and received saline, BSA or AF0150 by subcutaneous injection (dorsal area) on Days 1, 8 and 15 for induction. On Day 29, 2 ml blood samples were taken via toenail of the hind foot from each group except group 2, and the serums were stored at –20°C and saved for the PCA study. On Day 33, all animals received a bolus IV injection of the respective material into the dorsal metatarsal vein for challenge. Clinical signs were observed daily except that on the injection days observation were recorded at pre-dosing, 15 minutes and 1 hour post dosing. Body weights were measured on Days 1 and 33. No pathology and necropsy were performed. The ASA reactions were determined according to the scales shown in Table 1.

Table 1. Active Systemic Anaphylaxis (ASA) Procedure

Group	Treatment			
	Induction (Subcutaneous)		Challenge (Intravenous)	
	Formulation	Dose Volume (Dose Level)	Formulation	Dose Volume (Dose Level)
1	Saline	0.1 mL/animal	Saline	1 mL/kg
2	Saline	0.1 mL/animal	AF0150	1 mL/kg
3	AF0150	0.1 mL/animal	AF0150	1 mL/kg
4	AF0150/FCA (1:1 v/v dilution)	0.2 mL/animal	AF0150	1 mL/kg
5	BSA (10 mg/mL)	0.1 mL (1 mg BSA)/animal	BSA (10 mg/mL)	0.5 mL (5 mg BSA)/animal

FCA Freund's Complete Adjuvant. BSA Bovine Serum Albumin.

Scales of ASA Reactions

0 - No symptoms
1 - Very slight reaction (intermittent scratching or retching)
2 - Tremors, sneezing, dyspnea, plus scratching or retching
3 - Lacrimation, coughing, cyanosis, impaired locomotor activity, plus any of the above listed clinical signs
4 - Convulsions, all or some of the above clinical signs but not death
5 - Death (or moribund condition) within 15 minutes of challenge injection

Procedure for Passive Cutaneous Anaphylaxis (PCA) (Table 2): 20 male guinea pigs were assigned into 4 groups (5 animals/group) and treated as shown in Table 2. Different dilutions of serum samples collected in ASA procedure were intradermally injected into each animal with 0.05 ml per injection site and at least 1 cm apart between sites. At approximately 4.5 hours following serum injection, the animals in Groups 6, 7, and 8 received 1 ml/kg of Evan's Blue/AF0150 mixture (0.1% Evan's Blue and 20 mg/ml AF0150) by IV injection, and the animals in Group 9 received 0.5 ml/animal of Evan's Blue/BSA mixture (0.1% Evan's Blue and 10 mg/ml BSA). Approximately 30 minutes after IV injection, the diameter of blue dye staining area at each intradermal injection site was measured. Clinical signs, body weight and pathology were not observed.

Table 2. Passive Cutaneous Anaphylaxis (PCA) Procedure

Group	Treatment	Dilutions Administered
6	Group 1 serum	1:1, 1:10, 1:100, 1:1,000, and 1:10,000
7	Group 3 serum	1:1, 1:10, 1:100, 1:1,000, and 1:10,000
8	Group 4 serum	1:1, 1:10, 1:100, 1:1,000, and 1:10,000
9	Group 5 serum	1:100, 1:1,000, and 1:10,000

The serums were collected from animals sensitized with AF0150 or BSA or saline in ASA study. PCA Reactions were measured based on the diameter of blue dye staining area at serum injection sites.

Results

Active Systemic Anaphylaxis (ASA):

During Induction Phase, subcutaneous injection of AF0150 mixed with FCA, but not AF0150 alone, induced subcutaneous masses and/or scab formation at the injection sites. One of 5 animals injected with BSA (Group 5) showed a generalized erythema reaction on Days 11 and 12. One of 5 animals given AF0150 (Group 3) was hypothermic to touch on Day 19. Soft/watery stools were noted in most animals but without association with AF0150 treatment. High bacterial level (pseudomonas) found in the drinking water might be attributed to these effects.

During Challenge Phase, one of 5 animals pretreated with AF0150 showed retching after IV challenge with AF0150, which is frequently associated with an anaphylactic reaction. However, no other sings of anaphylaxis were observed in this animal and the PCA reaction to this animal's serum was negative. All animals induced with AF0150/FCA appeared normal to IV challenge with AF0150. In the positive control group (induction with BSA), 2 of 5 animals had grade 5 anaphylactic reactions following challenge with BSA. Clinical signs included pawing at nose/mouth, cyanosis, convulsions, gasping, retching and death (within 4 minute following IV challenge). A grade 3 anaphylactic reactions (ear scratching, pawing at nose/mouth, hypoactivity, staggered gait, dyspnea, and cyanosis) were observed in the rest of animals in this group. IV challenge of saline-induced animals with AF0150 did not lead to remarkable observations. All animals gained body weight, particularly for those animals treated with AF0150 alone during induction [*no explanation was provided*].

Passive Cutaneous Anaphylaxis (PCA): intradermal injection of the serums collected from saline-, AF0150- or AF0150/FCA-induced guinea pigs during the ASA induction period had no measurable area of blue dye staining at the injection sites in any of guinea pigs. However, the serum prepared from BSA-induced animals resulted in a positive PCA reactions at 9 of the 10 injection sites with 1:100 serum dilutions and at one site with 1:1000 serum dilution.

Discussion and Comments

AF0150 alone or co-administrated with FCA did not lead to significant active systemic anaphylaxis and passive cutaneous anaphylaxis in guinea pigs. One of 5 animals given AF0150, but not AF0150/FCA, had retching, which was considered an isolated incidence. The reviewer agrees with the sponsor's explanation that this was not associated with clinical signs, there was a lack of a similar reaction in the other AF0150-treated animals and the PCA reaction was negative.

Report Number: IMUS-029-TOX
Dermal Sensitization Study of AF0150 in Guinea Pig-Maximization Test

Report Location:	Vol.019, p052-129
Report date:	April 17, 1997
Study Facility:	[redacted]
In-life phase:	January 22-February 24, 1997
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010 (200 mg Fill)
AF0150 Dosage (HMD at BSA):	Patch (Filter paper saturated with 20 mg/ml AF0150)

Specific Aim

To assess the contact sensitization potential of AF0150 via intradermal injection or topical application (Magnusson and Kligman Maximization Assay)

Methods

Animal Preparation: 34 albino guinea pigs, Crl:(HA)BR strain, were obtained from [redacted] and were 4-8 weeks old at initiation of treatment with body weight of 400-501g. Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for 5 days before initiation of treatment. The animals were divided into 3 groups: irritation screening group (4); AF0150 group (20) and control group (10).

AF0150 Preparation: AF0150 (200 mg fill vial) was reconstituted with 10 ml of sterile water to a concentration of 20 mg/ml and used within 30 minutes of preparation. Where applied, the AF0150 solution was mixed with FCA at 1:1 (v/v).

Irritation Screening Study: various concentrations (20, 15, 10, 5 mg/ml) AF0150 were saturated on [redacted] Filter papers. The resulted paper patches were placed on the shaved back skin (right and left sites) of each animal followed by covering with [redacted] tape and [redacted] tape. Each animal received two different concentrations of AF0150. The patches were

removed after 24 hours and dermal reactions at the application sites were observed at 24 and 48 hours after patch removal.

Induction: by intradermal injection in duplicate on a clipped area of the shoulder, animals in the test group received 0.1 ml of AF0150, or AF0150/FCA (1:1), or FCA (1:1 with sterile water), and control animals received 0.1 ml FCA (1:1 with sterile water) or sterile water. On day 7, a 10% (w/w) sodium lauryl sulfate (SLS) suspension was applied topically on the injection sites and allowed to remain for one day. The injection sites were then wiped and cleaned, and 2x4-cm pieces of [redacted] filter paper saturated with AF0150 (20 mg/ml) or sterile water were placed over the injection sites of both control and test animals. The patches were covered with [redacted] tapes. Forty-eight hours later, the patches were removed and the induction/injection sites were rinsed with tap water and dried with disposable paper towels.

Challenge: 2 weeks after the above induction, [redacted] filter papers saturated with AF10150 (20 mg/ml) or sterile water were placed on the either side of induction areas (after re-shaving), respectively. The patches were covered with [redacted] tapes, and removed at 48 hours later, followed by rinsed with tap water, and dried.

Observation Parameters: dermal reactions were examined at 24 hours and 48 hours after patch removal and scored according to the scale described in Table 1. Clinical sings were observed daily (except on Day 9) and body weights were recorded.

Table 1. Dermal Reaction Scales

0 = No reaction
1 = Scattered mild redness
2 = Moderate and diffuse redness
3 = Intense redness and swelling

Results

Irritation Screening Study: there were no dermal reactions associated with any AF0150 concentrations.

Dermal Reaction to AF0150 Challenge: no dermal reactions were noted in AF0150-induced and control animals challenged with AF0150. However, there were no concurrent positive control groups included in this study. It is difficulty to judge the true negative results. The sponsor provided a separate report of a positive control study (using 2,4-dinitrochlorobenzene) in an Appendix. The study was conducted within 6 month of AF0150 study and followed the same procedure. A strong positive dermal reaction was found in guinea pigs induced and then challenged with 2,4-dinitrochlorobenzene.

Clinical Signs and Body Weight: no AF0150 treatment-related clinical signs were found. All animals showed body weigh gain during the study.

Discussion and Comments

AF0150 alone or co-administrated with FCA had no significant dermal reactions using the guinea pig maximization test (Magnusson and Kligman Assay). However, with lack of a concurrent positive control in the same study (although positive control study was conducted separately at different time), it is difficulty to conclude that AF0150 has no contact hypersensitivity in guinea pigs.

The absolute AF0150 amount on each filter paper (i.e., mg AF0150) for screening, induction and challenge was not provided.

Report Number: BC-95-17-Amended
***In vitro* Activation of Complement (C3) by AF0150 in Human Plasma**

Report Location:	Vol. 010, p001-009
Report date:	February 2, 1996
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	Not specified
GLP Compliance:	Not specified
AF0150 Lot number:	ZZ15036
AF0150 Dosage (HMD):	0.5 mg/ml

This study was submitted to IND and reviewed by Dr. Nakissa Sadrieh.

Specific Aim

To assess whether AF0150 *in vitro* activates complement in human plasma (by measuring the conversion of C3 to C3a)

Methods

Plasma was prepared from the blood of 4 human volunteers (2 each sex, [*age and medical history were not indicated*]) by centrifugation (blood was stored in heparin-containing tubes with 10 units heparin per ml blood). AF0150 (20 mg/ml in SWFI) was added to the plasma (within 15 minutes from the time of blood collection) at a final concentration of 0.5 mg/ml. The mixture was incubated in a 37°C water bath for 10 minutes and the reaction was stopped by addition of an equal volume of 20 mM EDTA. Zymosan (2.5 ug/ml at final concentration) and saline (1:40 to plasma) instead of AF0150 were used as a positive and negative controls, respectively. C3a concentration was measured with a [redacted]

Results

As seen in table 1, AF0150 slightly increase C3a concentration following *in vitro* incubation with human plasma as compared to the negative control (saline-plasma incubation), although the difference was not statistically significant.

Table 1. Conversion of Human Plasma C3 *in vitro*

Incubation with	C3a Concentration (ng/ml)[#]
Saline	151 ± 26
AF0150 (0.5 mg/ml)	507 ± 295*
Zymosan (2.5 ug/ml)	26358 ± 2949**

[#] Mean±SEM, n= 4 per group

* $p>0.05$ and ** $p<0.05$ as compared with saline-incubated plasma.

Discussion and Comments

No statistically significant differences in C3 conversion were induced by incubation with AF0150 incubation. This may have been due to the great variation observed. The C3a concentration varied from 161 to 1390 ng/ml in the AF0150 group but 87-208 ng/ml in the saline group. This was very likely due to individual human differences (age, health condition, medication history, etc). Increasing the sample size and/or conducting an *in vivo* study may be considered.

Report Number: CMB-96-14

Effects of AF0150 on Tumor Necrosis Factor-Alpha Levels in Rat and Human Blood *in vitro*

Report Location:	Vol. 010, p010-015
Report date:	November 23, 1998
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	March-June 1996
GLP Compliance:	No
AF0150 Lot number:	ZZ15036
AF0150 Dosage (HMD):	0.5 mg/ml

Specific Aim

To assess whether AF0150 *in vitro* stimulates TNF production by incubation with the whole blood of rat and human.

Methods

Whole blood samples were collected from human (cephalic vein, 4 healthy volunteers) and rats (jugular catheter [*vein?*], 4 SD) and mixed with heparin (20 ug/ml). 1-ml aliquots of the whole blood were incubated with one of the followings: saline (negative control), 0.5 mg/ml AF0150; 1 ug/ml LPS (positive control); 1.25 ug/ml Zymosan (positive control). After incubation at 37°C for 5 hours with constant mixing, the mixtures were centrifuged and the plasma was frozen till assayed for TNF. TNF levels in the plasma were measured with [redacted] in duplicate.

Results

As seen in Table I, both LPS and zymosan strongly stimulated TNF production when incubated with both rat and human blood. AF0150 incubation did not increase TNF level in rat blood samples as compared to saline control. However, data from human blood samples incubated with AF0150 and saline were not collected.

Table I. TNF Plasma Levels in Rat and Human Whole Blood *in vitro*

	TNF Plasma Levels (pg/mL)	
Group	**Rat**	**Human**
Zymosan	25456 ± 11796	4910 ± 2800
LPS	10798 ± 855	6043 ± 3670
Saline	42 ± 11	None Detected
AF0150	39 ± 14	None Detected

Data are Means ± SEM; n=4/group

Discussion and Comments

AF0150 at concentration equivalent to 100-fold clinical dose had no effects on TNF production and/or release from blood cells when *in vitro* incubated with rat whole blood samples. The sponsor claimed the results were consistent with clinical trial (0.125-4 mg/kg).

TNF data from AF0150-indubated human blood were missing without an appropriate explanation. Also, individual TNF values were not provided in the report.

Report Number: IMUS-018-TOX
Acute Intravenous Toxicity Study of AF0150 in Rabbits Preinfused with Adenosine

Report Location: Vol.019, p130-384
Report date: June 17, 1996
Study Facility: [redacted]
In-life phase: October 7-22, 1996
GLP Compliance: Yes (with QA Statement)
AF0150 Lot number: UA16010

AF0150 Dosage (HMD): 2 and 20 mg/kg (5.2-52 fold PCD)

Specific Aim

To evaluate the single dose acute toxicity of AF0150 in rabbits pre-treated with adenosine

Methods

Animal Preparation: Albino rabbits (30 each sex), Hra:(NZW)SPF strain, were obtained from [redacted] and were 4-4.5 months old with body weight of 2.3-3.1 kg (males) and 2.6-3.1 kg (females) at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for 7 days before initiation of treatment, and 25 animals of each sex meeting health and body weight requirements were randomly assigned to 5 groups (5/sex/group), as seen in Table 1. All animals were catheterized in a lateral ear vein for adenosine infusion followed by bolus injection of saline or AF0150.

Adenosine Infusion and AF0150 Administration: Adenosine (3 mg/ml) or saline was infused through a catheter in the ear vein for a total dose of 840 ug/kg (in 6 minutes). Following the infusion, bolus injection of saline or AF0150 (2 or 20 mg/kg) was given through the same line. treatment assignments for each group were shown in Table 1.

Table 1. AF0150 Acute Toxicity Study Procedure Adenosine-pretreated Rabbits

Group	Treatment	Number of Animals M/F	Adenoscan®[a] Dosage (µg/kg/min)	AF0150[b] Dosage (mg/kg)
1	Saline[c]-Saline[d]	5/5	-	-
2	Saline[c]-AF0150	5/5	-	20
3	Adenoscan®-Saline[d]	5/5	140	-
4	Adenoscan®-AF0150	5/5	140	2
5	Adenoscan®-AF0150	5/5	140	20

[a] Infused first over 6 minutes for a total dose of 840 µg/kg
[b] Injected immediately after Adenoscan® (or saline) by slow IV push
[c] Saline volume/rate same as Adenoscan® volume/rate
[d] Saline volume/rate same as AF0150 volume/rate used for Group 5

Observation Parameters: clinical signs and mortality were observed twice a day, pre-dosing and up to 4 hours post doing. Body weights were recorded pre-dosing and weekly thereafter. Blood samples were taken from an ear artery or vein at 24 hours and 14 days (necropsy) post dosing for hematology and blood chemistry analyses. Macroscopic examination was performed at necropsy and selected organs/tissues were preserved. Only heart was microscopically examined and all other tissues were saved for possible future examination.

Results

Clinical signs: all animals survived to the scheduled termination (14 days). No treatment-related toxic signs and body weight changes were observed. A few animals given adenosine followed by a bolus injection of saline but not AF0150 showed hypoactivity or soft stool during 14-day observation.

Hematology and Blood Chemistry: there were no significant changes in blood chemistry and hematology parameters in any of animals.

Pathology: No macroscopic changes at necropsy were observed. Microscopic examination of heart (atrial and ventricular epicardium, myocardium and endocardium) was not remarkable. No other organs/tissues were microscopically examined.

Discussion and Comments

The results showed that IV bolus injection of AF0150 (2 and 20 mg/kg) to the adenosine-pretreated rabbits had no significant toxicity. However, adenosine treatment alone did not induce any clinical signs, and CV stress responses (such as heart rates and other hemodynamic parameters) were not monitored before, during and after adenosine infusion. All these suggest that the animal model was not appropriate to evaluate effects of AF150 under adenosine-induced cardiovascular stress.

Report Number: IMUS-019-TOX
Acute Intravenous Toxicity Study of AF0150 in Rabbits Preinfused with IV Persantine (Dipyridamole USP)

Report Location:	Vol.020, p001-257
Report date:	June 17, 1996
Study Facility:	[redacted]
In-life phase:	October 15-30, 1996
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010
AF0150 Dosage (HMD):	2 and 20 mg/kg (5.2-52 fold PCD)

Specific Aim

To evaluate the single dose acute toxicity of AF0150 in rabbits pre-treated with Persantine (dipyridamole)

Methods

Animal Preparation: Albino rabbits (30 each sex), Hra:(NZW)SPF strain, were obtained from [redacted] and were 4.5-5 months old with body weight of 2.4-3.1 kg

(males) and 2.6-3.1 kg (females) at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for 15 days before initiation of treatment, and 25 animals of each sex meeting health and body weight requirements were randomly assigned to 5 groups (5/sex/group), as seen in Table 1. All animals were catheterized in a lateral ear vein for Persantine infusion followed by bolus injection of saline or AF0150.

Persantine Infusion and AF0150 Administration: Persantine (5 mg/ml) or saline was infused through a catheter in the ear vein for a total dose of 568 ug/kg (in 4 minutes). Following the infusion, bolus injection of saline or AF0150 (2 or 20 mg/kg) was given through the same line. Treatment assignments for each group were shown in Table 1.

Observation Parameters: clinical signs and mortality were observed twice a day, pre-dosing and up to 4 hours post dosing. Body weights were recorded pre-dosing and weekly thereafter. Blood samples were taken from an ear artery or vein at 24 hours and 14 days (necropsy) post dosing for hematology and blood chemistry analyses. Macroscopic examination was performed at necropsy and selected organs/tissues were preserved. Only the heart was microscopically examined and all other tissues were saved for possible future examination.

Table 1. AF0150 Acute Toxicity Study Procedure in Dipyridamole-pretreated Rabbits

Group	Treatment	Number of Animals M/F	I.V. Persantine®[a] Dosage (µg/kg/min)	AF0150[b] Dosage (mg/kg)
1	Saline[c]-Saline[d]	5/5	-	-
2	Saline[c]-AF0150	5/5	-	20
3	I.V. Persantine®-Saline[d]	5/5	142	-
4	I.V. Persantine®-AF0150	5/5	142	2
5	I.V. Persantine®-AF0150	5/5	142	20

[a] Infused first over 4 minutes for a total dose of 568 µg/kg
[b] Injected immediately after I.V. Persantine® (or saline) by slow IV push
[c] Saline volume/rate same as I.V. Persantine® volume/rate
[d] Saline volume/rate same as AF0150 volume/rate used for Group 5

Results

Clinical signs: all animals survived to the scheduled termination (14 days). No treatment-related toxic signs and body weight changes were observed. One of 5 males given Persantine followed by a bolus injection of 2 mg/kg AF0150 showed mucoid stool for 2 days (on days 12-13), but not at any other time intervals and animals.

Hematology and Blood Chemistry: there were no significant changes in blood chemistry and hematology parameters in any of the animals.

Pathology: There were no treatment-related changes upon macroscopic examination at necropsy. Microscopic examination of heart (atrial and ventricular epicardium, myocardium and endocardium) was not remarkable. No other organs/tissues were microscopically examined.

Discussion and Comments

The results showed that IV bolus injection of AF0150 (2 and 20 mg/kg) to the dipyridamole-pretreated rabbits had no significant toxicity. However, dipyridamole treatment alone did not induce any clinical signs, and CV stress responses (such as heart rates and other hemodynamic parameters) were not monitored before, during and after dipyridamole infusion. All these suggest that the animal model was not appropriate to address effects of AF150 under the cardiovascular stress.

Report Number: IMUS-020-TOX
Acute Intravenous Toxicity Study of AF0150 in Rabbits Preinfused with Dobutrex (Dobutamine HCl)

Report Location:	Vol.021, p001-257
Report date:	June 17, 1996
Study Facility:	[redacted]
In-life phase:	October 21-November 8, 1996
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010
AF0150 Dosage (HMD):	2 and 20 mg/kg (5.2-52 fold PCD)

Specific Aim

To evaluate single dose acute toxicity of AF0150 in rabbits pre-treated with Dobutrex (Dobutamine HCl)

Methods

Animal Preparation: Albino rabbits (30 each sex), Hra:(NZW)SPF strain, were obtained from [redacted] and were 4.5-5 months old with body weight of 2.5-3.2 kg (males) and 2.7-3.2 kg (females) at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for 20-24 days before initiation of treatment, and 25 animals of each sex meeting health and body weight requirements were randomly assigned to 5 groups (5/sex/group), as seen in Table 1. All animals were catheterized in a lateral ear vein for Dobutrex infusion followed by bolus injection of saline or AF0150.

Dobutrex Infusion and AF0150 Administration: Dobutrex solution (0.25 mg/ml in saline) or saline was infused through a catheter in the ear vein for a total dose of 380 ug/kg (10, 20, 30 ug/kg/min for 3 min each and 40 ug/kg/min for 5 min). Following the infusion, bolus injection of saline or AF0150 (2 or 20 mg/kg) was given through the same line. treatment assignments for each group were shown in Table 1.

Table 1. AF0150 Acute Toxicity Study Procedure in Dobutrex-pretreated Rabbits

Group	Treatment	Number of Animals M/F	Dobutrex®[a] Dosage (μg/kg/min)	AF0150[b] Dosage (mg/kg)
1	Saline[c]-Saline[d]	5/5	-	-
2	Saline[c]-AF0150	5/5	-	20
3	Dobutrex®-Saline[d]	5/5	[a]	-
4	Dobutrex®-AF0150	5/5	[a]	2
5	Dobutrex®-AF0150	5/5	[a]	20

[a] Infused first at 10 μg/kg/min for 3 minutes, then 20 μg/kg/min for 3 minutes, then 30 μg/kg/min for 3 minutes, and finally 40 μg/kg/min for 5 minutes for a total dose of 380 μg/kg

[b] Injected immediately after Dobutrex® (or saline) by slow IV push

[c] Saline volume/rate same as Dobutrex® volume/rate

[d] Saline volume/rate same as AF0150 volume/rate used for Group 5

Observation Parameters: clinical signs and mortality were observed twice a day, pre-dosing and up to 4 hours post doing. Body weights were recorded pre-dosing and weekly thereafter. Blood samples were taken from an ear artery or vein at 24 hours and 14 days (necropsy) post dosing for hematology and blood chemistry analyses. Macroscopic examination was performed at necropsy and selected organs/tissues were preserved. Only heart was microscopically examined and all other tissues were saved for possible future examination.

Results

Clinical signs: all animals survived to the scheduled termination (14 days). No treatment-related toxic signs and body weight changes were observed.

Hematology and Blood Chemistry: there were no significant changes in blood chemistry and hematology parameters in all animals.

Pathology: One of 5 males given Dobutrex and AF0150 (20 mg/ml) showed a mild focal red in jejunum at necropsy which was corresponding to hemorrhage on microscopic examination. One of 5 males in saline-saline control group had a mild focal red in liver at necropsy but with normal microscopic observation. These changes were considered spontaneous. Macroscopic and microscopic examinations of heart (atrial and ventricular epicardium, myocardium and endocardium) were not remarkable. No other organs/tissues were microscopically examined.

Discussion and Comments

The results showed that IV bolus injection of AF0150 (2 and 20 mg/kg) to the dobutrex-pretreated rabbits had no significant toxicity. However, dobutrex treatment alone did not induce any pharmacological effects, suggesting that the animal model was not appropriate to address effects of AF150 under the cardiovascular stress.

Report Number: IMUS-036-TOX
Acute Intravenous Toxicity Study of AF0150 in Rabbits Preinfused with Arbutamine Hydrochloride (GenESA)

Report Location:	Vol.022, p001-181
Report date:	June 17, 1998
Study Facility:	[redacted]
In-life phase:	March 16-31, 1998
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010
AF0150 Dosage (HMD):	2 and 20 mg/kg (5.2-52 fold PCD)

Specific Aim

To evaluate the single dose acute toxicity of AF0150 in rabbits pre-treated with Arbutamine Hydrochloride

Methods

Animal Preparation: Albino rabbits (30 each sex), Hra:(NZW)SPF strain, were obtained from [redacted] and were approximately 4 months old with body weight of 2.4-3.0 kg (males) and 2.3-2.9 kg (females) at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for 12 days before initiation of treatment, and 25 animals of each sex meeting health and body weight requirements were randomly assigned to 5 groups (5/sex/group), as seen in Table 1. All animals were catheterized in a marginal ear vein for arbutamine infusion followed by bolus injection of saline or AF0150.

Arbutamine Infusion and AF0150 Administration: Arbutamine solution (400 ng/ml in saline) or saline was infused through a catheter in the ear vein for a total dose of 500 ng/kg (within 5 min). Following the infusion, bolus injection of saline or AF0150 (2 or 20 mg/kg) was given through the same line. Treatment assignments for each group were shown in Table 1.

Observation Parameters: clinical signs and mortality were observed twice a day, pre-dosing and up to 4 hours post doing. Body weights were recorded pre-dosing and weekly thereafter. Blood samples (2-5 ml) were taken from an ear artery or vein at 24 hours and 14 days (necropsy) post

dosing for hematology and blood chemistry analyses. Macroscopic examination was performed at necropsy and selected organs/tissues were preserved. Only heart was microscopically examined and all other tissues were saved for possible future examination.

Table 1. AF0150 Acute Toxicity Study Procedure in Arbutamine-pretreated Rabbits

Group Assignment					
		Number of Animals		Arbutamine HCl (GenESA®)[a]	AF0150[b]
Group	Treatment	M	F	(ng/kg/min)	(mg/kg)
1	Saline[c]-Saline[d]	5	5	-	-
2	Saline[c]-AF0150	5	5	-	20
3	Arbutamine HCl - Saline[d]	5	5	100	-
4	Arbutamine HCl - AF0150	5	5	100	2
5	Arbutamine HCl - AF0150	5	5	100	20

[a]Infused first over approximately 5 minutes for a total dose of 500 ng/kg.
[b]Injected immediately after Arbutamine HCl (or saline) by slow IV push
[c]Saline volume/rate same as Arbutamine HCl volume/rate
[d]Saline volume/rate same as AF0150 volume/rate used for Group 5

Results

Clinical signs: all animals survived to the scheduled termination (14 days). No treatment-related toxic signs and body weight changes were observed.

Hematology and Blood Chemistry: segmented neutrophils slightly decreased in animals treated with Saline plus 20 mg/kg AF0150 (Group 2) and Arbutamine with 2 mg/kg AF0150 (Group 4) on Days 1 (females) and 15 (males). All treated female animals had higher lymphocytes than the control group on Day 15, which was considered incidental because of variation in the control animals. There were no significant changes in blood chemistry in any of the animals.

Pathology: there was an increased incidence of red distribution of the lungs in some male rabbits in groups 2-5 at necropsy, which was microscopically correlated with lung congestion and/or hemorrhage. One of 5 males treated with Saline/AF0150 (Group 2) showed trace mononuclear cell infiltration in heart ([*specific sites were not provided*]), which was considered incidental.

Discussion and Comments

The results showed that IV bolus injection of AF0150 (2 and 20 mg/kg) to the Arbutamine-pretreated rabbits had no significant toxicity. Lung hemorrhage/congestion in some treated male rabbits may be considered incidental because it was also observed in AF0150-untreated animals (group 3) without AF0150 dose-dependency. However, arbutamine treatment alone did not induce any pharmacological effects, suggesting that the animal model was not appropriate to address effects of AF150 under the cardiovascular stress.

Report Number: AC-00-08
Comparison of AF0150 Bubble Size and Distribution at Constitution and at 30 and 60 minutes Post-Constitution

Report Location:	Supplemental submission (Fax of April 6, 2000)
Report date:	February 28, 2000
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	Not specified
GLP Compliance:	No
AF0150 Lot number:	UA18041, UA18042, UA18047, ZZ19017, ZZ19018, ZZ19019
AF0150 Dosage (HMD):	

Specific Aim

To determine microbubble size distribution of AF0150 at 30 and 60 minutes post reconstituting

Methods

AF0150 Preparations: QA-audited release and stability data from 6 lots of AF0150 were compiled from the database which contains all data before December 22, 1999 for all clinical-quality lots of AF0150. Counting and sizing of the reconstituted AF0150 microbubbles were performed according to [redacted] "Determination of Bubble Counts and Size Distribution in Constituted AF0150" [*not provided with this submission*].

Observation Parameters: Total counts/ml, volume median diameter (VMD), counts/ml with bubble size >10 um, and 3-10 um were measured at 0 (T=0, right after reconstitution), 30 (T=30) and 60 (T=60) minutes post reconstitution. The following data were used:

1. Release data for AF0150 lots ZZI9017, ZZ18018, and ZZ19019. Two samples from each lot were tested at time 0, 30, 60 min (T=0, 30, 60) post-constitution.

2. Stability data at 25°C after 3 months for lots ZZ19017, ZZ19018 and ZZ19019. Two vials from each lot (one upright and one inverted) were tested at T=0, 30, and 60 minutes.

3. Stability data at 25°C after 9 and 12 months for lots UA18041, UA18042 and UA18047. For the 9-month evaluation, only inverted vials were tested. Both upright and inverted vials were evaluated at the 12-month time point at T=0, 30, and 60 minutes.

Data analysis: Mean, SD, relative SD (RSD) and range were calculated for each analytical test and time point. Student's t-test and ANOVA were used to analyze the differences. A variation factor (percent change relative to the results at the reconstitution time) was calculated by the following formula:

$$\text{Variation Factor (\%)} = \frac{\text{Test result (T)} - \text{Test Result (T=0)}}{\text{Test Result (T=0)}} \times 100$$

Where T=30 or 60 minutes post reconstitution, and *Test Result* is bubble counts/ml (total, >10um, or 3-10 um) or volume median diameter .

Results and Discussion

Total Bubble Counts (Table 1): there was no difference in total bubble counts/ml between reconstitution time and 30 min post reconstitution, but the bubble count was statistically decreased at 60 min post reconstitution.

Table 1. Change in Total Microbubble Counts Post Constitution

Time Post Constitution (min)	Sample Numbers	Counts/ml ($x10^8$)	Range ($x10^8$)	Variation Factor
0	21	9.79±1.17	[redacted]	0
30	21	9.12±1.09	[redacted]	-6.9%
60	21	8.58±1.02	[redacted]	-11.7%*

* $p<0.05$ with Student's t-test

Volume Median Diameter (Table 2): There were no significant differences in volume median diameter between at time 0 and at 30 or 60 minute post AF0150 reconstitution.

Table 2. Change in Volume Median Diameter Post Constitution

Time Post Constitution (min)	Sample Numbers	Diameter (um)	Range ($x10^8$)	Variation Factor
0	21	5.98±.541	[redacted]	0
30	21	5.88±.627	[redacted]	-1.4%
60	21	6.00±.530	[redacted]	-0.6%

Counts of >10um Bubbles (Table 3): The counts/ml of larger than 10 um microbubbles had no significant changes at 30 and 60 minutes post reconstitution as compared to the results at the time of reconstitution. However, the exact bubble sizes and number above 10 um were not specified.

Table 3. Change in Counts of >10 um Microbubbles Post Constitution

Time Post Constitution (min)	Sample Numbers	Counts/ml ($x10^6$)	Range ($x10^6$)	Variation Factor
0	21	1.51±0.607	[redacted]	0
30	21	1.36±0.806	[redacted]	-10.7%
60	21	1.50±0.739	[redacted]	-0.4%

Counts of 3-10 um Bubbles (Table 4): the mean counts/ml of 3-10 um microbubbles slightly decreased by 3.9% and 4.4% at 30 and 60 minute post reconstitution, respectively, as compared to the results at right after reconstitution. The decreases were not statistically significant.

Table 4. Change in Counts of 3-10 um Microbubbles Post Constitution

Time Post Constitution (min)	Sample Numbers	Counts/ml ($x10^8$)	Range ($x10^8$)	Variation Factor
0	21	1.81±0.158	[redacted]	0
30	21	1.73±0.250	[redacted]	-3.9%
60	21	1.73±0.250	[redacted]	-4.4%

Discussion and Comments

It appeared that the count and size of AF0150 microbubbles did not significantly change at 30 and 60 minute post reconstitution, as compared to the results right after reconstitution. However, total counts/ml at 60 minutes post reconstitution were statistically decreased (Table 5).

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).

However, detailed information about bubbles larger than 10 um was not provided, although percentage of those bubbles was less than 0.2%. No stability results in terms of bubble sizes and counts were provided or discussed.

Additionally, the experimental procedure and data analysis were unclear. The protocol was referred to [redacted] "Determination of Bubble Counts and Size Distribution in Constituted AF0150". However, the document was not provided with this submission. The number of "Analytical Results ("n =21 in all original tables)" and "Tests" need to be clarified. In this review, "sample numbers" is used to replace "n" in the sponsor's tables.

APPEARS THIS WAY
ON ORIGINAL

Summary of Special Toxicology Studies

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

Immunotoxicity Studies: Two GLP *in vivo* studies were conducted in guinea pigs to test the antigenicity and delayed hypersensitivity of AF0150. The antigenicity (IMUS-021-TOX) was evaluated with active systemic anaphylaxis (ASA) and passive cutaneous anaphylaxis (PCA) methods. Animals were first induced by subcutaneous injection of 2 mg AF0150 with and without Freund's Complete Adjuvant (FCA). Two weeks following induction, blood samples were collected and meantime the animals received IV injection of 20 mg/kg AF0150 (35-fold PCD) for challenge (ASA test). Imunoactivity of serum from the blood samples were assayed with a skin test in a separate guinea pig groups (PCA test). AF0150 did not induce significant active systemic anaphylactic or passive cutaneous anaphylactic reactions.

The delayed hypersensitivity (IMUS-029-TOX) of AF0150 was tested in guinea pigs using Magnusson and Kligman maximization assay (guinea pig maximization study). The animals were subjected to intradermal injection of AF0150 (2 mg) with and without FCA for induction, followed by skin patching (with filter papers saturated with 20 mg/ml AF0150) for challenge. The dermal reactions were recorded following AF0150 skin patching, and no significant findings were observed. However, no concurrent but separate positive control group 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 levels (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 levels.

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 hear 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 dated on April 6, 2000, as requested on March 30, 2000's T-Con with the sponsor. The submission included data on AF0150 microbubble dosages expressed as bubble numbers/kg body weight for all GLP studies.

As summarized in Table 1 (from report AC-00-08), there appeared to be no significant changes on the microbubble size and corresponding counts/ml of AF0150 at 30 and 60 minute post reconstitution, as compared to results from immediately after reconstitution. However, a significant decrease in total bubble counts/ml was noted at 60 minutes post reconstitution. The percentage of bubbles larger than 10 um was less than 0.2% at all time points post reconstitution.

Table 1. 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).

Microbubble size analyses were also performed in some of the pharmacodynamics studies. In study RE-99-47 (reviewed under 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 was 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.

Effects of ultrasound power on AF0150 microbubble size was tested using a simulated *in vitro* circulation system using bovine albumin solution (RE-99-46, reviewed under General Pharmacology). Sustained ultrasound exposure did not alter microbubble size (median diameter range of 5 to 8 um) in the albumin solution with only slight shift in bubble sizes 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 ultrasound exposure time and power levels.

AF0150 Dosage Conversion of mg/kg to bubbles/kg: As per our request on March 30, 2000's T-Con, the sponsor also provided a data sheet in the April 6, 2000's fax submission, in which

AF0150 dosages in GLP studies were converted to microbubbles per kg body weight. The information in Table 2 was extracted from the data sheets. After reconstitution, the bubble count per mg of AF0150 was identical, 4.9 bubbles/mg, in all fill sizes. The sponsor converted doses in mg/kg to bubble counts/kg by simply multiplying the mg by 4.9 bubbles/mg for all studies. For example, 20 mg/kg for any animal species was $9.8x10^8$ bubbles/kg.

Table 2. 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

31. REPRODUCTIVE TOXICOLOGY

Report Number: IMUS-022-TOX
An Intravenous Study of Fertility and Early Embryonic Development to Implantation of AF0150 in Rats

Report Location:	Vol.025, p170-279; Vol.026, p001-328
Report date:	January 13, 1999
Study Facility:	[redacted]
In-life phase:	February 26 – April 11, 1997
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ16051, ZZ16052, 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 on fertility and early embryonic development in rats (Segment I).

Methods

Animal Preparation: Crl:CD(SD)BR rats (100 each sex, 45 days old) were obtained from [redacted] Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation and quarantine for 13 days (for males) and 17 days (for females, the first day of smearing for estrus), animals (namely Fo generation) meeting good health and acceptable body weight requirements were randomly assigned into 4 groups (25 rats/sex/group) and received designed treatments, as seen in Table 1.

Table 1. Fertility and Early Embryonic Development Study Design in Rats

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

Animals were dosed from pre-mating (28 days) till the sacrifice day for male rats, and from pre-mating (14 days) till the gestation Day 7 and sacrificed on gestation day 13.
* AF0150 was reconstituted in SWFI to final concentration of 40 mg/ml.

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. 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). 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). The male rats received daily dosing from 28 days pre-mating till sacrifice day, and the female rats were dosed daily from 14 days pre-mating till gestation day 7. Individual dosages were based on the most recent body weights. All animals were treated at approximately the same time each day.

Observations: the animals were observed twice a day for mortality and moribundity, and detailed clinical signs were recorded daily. Body weights were measured twice weekly throughout the dosing period (up to day 55). The females with evidence of mating were weighed on gestation days 0, 3, 7, 10 and 13. Food consumption was recorded twice weekly or on the gestation days for body weight measurement, expressed as g/animal/day and g/kg/day.

Estrous Cycles (for Fo females): Vaginal smears (for determining the stage of estrous) were conducted daily for each Fo female for 10 days pre-dosing and throughout the 14-day pre-mating/dosing period. Once the animals were paired, smearing was continued until evidence of copulation was observed, or until termination of the mating period.

Breeding Procedures: Following a 28-day dosing period, Fo males were paired with Fo females (which had undergone a 14-day dosing) from the same treatment group. The animals were paired on a 1:1 basis within each treatment group. Each mating set was examined daily. Positive evidence of mating was confirmed by the presence of sperm in a vaginal smear or a vaginal copulatory plug. The day with evidence of mating was identified as the gestation day 0. If evidence of copulation was not detected after 10 days of pairing, a female was placed with a proven male from the same treatment group for an additional 5 days. Pre-coital intervals were calculated according to a 12-hour dark cycle counted for one paired day. Mating and fertility indices were calculated according to the equations described in Table 2.

Table 2. Calculation of Mating and Fertility Indices

$$\text{Male (Female) Mating Index (\%)} = \frac{\text{Numbers of Males (Females) with Positive Mating}}{\text{Total Numbers of Males (Females) used for Mating}} \times 100$$

$$\text{Male Fertility Index (\%)} = \frac{\text{Numbers of Males Siring at least 1 Liter}}{\text{Total Numbers of Males Used for Mating}} \times 100$$

$$\text{Female Fertility Index (\%)} = \frac{\text{Numbers of Females with Confirmed Pregnancy}}{\text{Total Numbers of Females used for Mating}} \times 100$$

Gestation Day 13 Uterine Examination: females with evidence of mating were sacrificed on gestation day 13. The number of corpora lutea on each ovary and the number/location of all embryos, early resorptions and implantation sites in the uterus were recorded. Additionally, the thoracic and abdominal cavities of females with evidence of mating were examined. Viability of

the embryos was determined with a dissecting stereomicroscope. Data were processed with the equations described in Table 3.

Table 3. Gestation Data Process

Group Mean Litter Basis

$$\text{Post-Implantation Loss/Litter} = \frac{\text{Numbers of Dead Embryos, Resorptions (Early/Late)/Group}}{\text{Numbers of Gravid Females/Group}}$$

Proportional Litter Basis

$$\text{Summation per Group (\%)} = \frac{\text{Total Post-Implantation Loss/Litter/Group (\%)}}{\text{Numbers of Litters/Group}}$$

$$\text{Total Post-Implantation Loss/Litter/Group (\%)} = \frac{\text{Numbers of Dead Embryos, Resorptions/Litter}}{\text{Numbers of Implantation Sites/Litter}} \times 100$$

Spermatogenetic Endpoint Evaluations: male rats was sacrificed and necropsied after the female necropsy, and spermatogenetic endpoints were evaluated, including testicular and epididymal sperm numbers, sperm production rate, sperm motility and sperm morphology.

Macroscopic Examination: all animals were subjected to a complete necropsy at the scheduled sacrifice (on gestation day 13). The necropsy included examination of the external surface, all orifices, the cranial cavity, the external surface of the brain and spinal cord, and the thoracic, abdominal and pelvic cavities. The absolute and relative weight (to final body weight) of testes, epididymides (total and cauda), ovaries, brain and pituitary gland were determined.

Microscopic Examination: at necropsy, the following tissues/organs were collected and preserved for possible future histopathological examination:

Coagulating gland
Ovaries and oviduct (2)
Pituitary
Prostate
Seminal vesicles (2)
Right testis with epididymis (1)[a]
and vas deferens
Uterus with vagina
All gross lesions[b]

[a] = The right testis and epididymis were fixed in Bouin's solution.
[b] = Representative sections of corresponding organs from control animals were preserved for comparison.

Results

Male Reproductive Observations

Clinical Signs, Body Weight and Food Consumption: All male rats survived to the schedule sacrifice. No AF0150-related toxic signs were noted at the daily observations. Transient changes (increase or decrease) in body weight gain were noted in some AF0150-treated animals at certain time points, but without dose-dependency. For example, body weight gains in all males on Days 27 and 48 in both control and AF0150 groups (vol 025, Table 7, p230) were much lower than those at other time points. No appropriate explanation was provided. Food consumption slightly decreased (by 5%, $p<0.05$) in the 100 and 200 mg/kg groups at the end of study (Days 51-55), and no remarkable changes were observed in other groups and at any time points.

Reproductive Performance (Table 4): There were no significant AF0150-related effects on male reproductive performance at all dose levels tested, except for a slight decrease (by 5%) in fertility index noted in the males from the 200 mg/kg group without statistical significance as compared to the control group.

Table 4. Male Reproductive Performance

Parameter	AF0150 (mg/kg/day), *n=25*			
	0	50	100	200
Mating Index (%)	96	96	96	95.8
Fertility Index (%)	92	91.7	100	87.5*
Pre-coital Interval (days, Mean ± SD)	2.3 ± 1.27	2.7 ± 1.33	2.9 ± 1.02	2.9 ± 2.45

* The decrease was not statistically significant as compare to the control (92%) with Chi-square test.

Spermatogenetic Endpoint Evaluation (Table 5): Mean epididymal sperm number in the 200 mg/kg/day group decreased by 14 % with statistical significance. The other spermatogenic parameters including mean testicular sperm numbers, sperm production rate, sperm motility and morphology were not significantly different between AF0150 (at all dose levels) and control groups.

Table 5. Testicular and Epididymal Sperm Counts

Organs	AF0150 (mg/kg/day), *n=25*			
	0	50	100	200
Left Testis (Mean ± SD)	88.3 ± 15.9	95.9 ± 16.3	98.6 ± 13.6*	83.3 ± 13.6
Left Epididymis (Mean ± SD)	514.5 ± 93.2	464.4 ± 85.7	479.8 ± 142.7	443.6 ± 73.5*

* Compared to the control (0 mg/kg/day, saline) group with Dunnett's Test, $p<0.05$

Macroscopic Examination: No remarkable AF0150-related macroscopic findings were noted at necropsy. The organ weights (absolute and relative) of brain, testis (right and left), epididymis (right and left) and pituitary were not different between the treated and control groups.

Female Reproductive Observations

Clinical signs: all female rats survived to the schedule necropsy. No AF0150-related toxic signs were noted at the daily observations.

Body Weight: During the pre-mating period, mean body weight and body weight gains at all dose levels and at most time points were not different from the control group, except that body weight gains increased in the 200 mg/kg/day during Days 13-16 and in 50 and 100 mg/kg/day groups during days 44-48 (with statistical significance). During the gestation period, AF0150 at all doses did not change body weight and body weight gain as compared with the control group.

Food Consumption: During the pre-mating period, food consumption (g/animal/day and g/kg/day) in all AF0150 dose groups at most time points were not different from the control group, except that a slight but statistically significant decrease (by 6-7%) in the 100 mg/kg/day on Days 16-20 was noted. During the gestation period, there were no differences in food consumption at all AF0150 doses and time points as compared to the control group.

Reproductive Performance: there were no significant differences in mating index, fertility index and pre-coital intervals between the AF0150-treated and the control groups. AF0150 at all doses had no effects on the regularity and duration of the estrous cycle.

Gestation Day 13 Uterine Examination: AF0150 at all doses had no effects on pre- and post-implantation losses, numbers of viable embryos, corpora lutea and implantation sites, as compared with control.

Macroscopic Examination: No remarkable findings at the scheduled and unscheduled necropsy were observed in both AF0150-treated and control groups. Some incidental findings were noted, such as dilated renal pelvis (1/24 rats at 50 mg/kg/day), dark red lung (1/24 rats at 50 mg/kg/day), hemorrhagic/thickened right parietal skull and a cyst in the left oviduct (1/24 rats at 200 mg/kg/day). AF0150 at all doses had no adverse effects on the organ weights (absolute and relative) of brain, ovaries and pituitary gland.

Discussion and Comments

The effects of AF0150 on fertility were assessed in male and female rats. AF0150, at the doses of 50, 100 and 200 mg/kg/day, was given to the animals from 2 (female) and 4 (male) weeks pre-mating till post-mating sacrifice day (male) and gestation day 7 (female). Slight decreases in male fertility (by 5%, not statistically significant) and mean epididymal sperm numbers (by 15%, $p<0.05$) were observed in the high dose groups (200 mg/kg/day), with **NOAELs** of 100 mg/kg/day (HED: 16.2 mg/kg/day and HDM: 130-fold of PCD) for both findings.

There were no significant differences in the other observations of 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) between AF0150-treated and control groups. The female necropsy on gestation day 13 showed that AF0150 had no significant effects on pre- and post-implantation losses, number of viable embryos, implantation sites and corpora lutea.

However, at the high AF0150 dose of 200 mg/kg/day, no maternal toxicity was noted, including changes on body weights, organ weights (brain, testis, ovary and pituitary) and food consumption. Minimal toxicity is generally expected in the high-dose group for appropriate dose selection.

Other comments: the term "total occurrence/no. of animal" was not defined clearly. Some AF0150-treated animals had an increased body weight, and an appropriate explanation was not provided. Since AF0150 decreased renal function (in saline-loaded rat study), it is possible that the body weight change might be related to Na+/H2O retention. However, this was not discussed by the sponsor in any study report.

Report Number: IMUS-023-TOX
An Intravenous Study of the Effects of AF0150 on Embryo/Fetal Development in Rats

Report Location:	Vol.027, p001-350
Report date:	September 14, 1998
Study Facility:	[redacted]
In-life phase:	November 19-December 10, 1996
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ16049 (400 mg/vial)
AF0150 Dosage (HMD at BSA):	50, 100, 200 mg/kg/day (up to 260-fold of PCD)

Specific Aim

To assess the teratogenicity of AF0150 in rats (Segment II).

Methods

Animal Preparation: Crl:CD(SD)BR female rats (125, sexually mature and virgin, 10 weeks old) were obtained from [redacted] Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation for 12 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) on a 1:1 basis 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 228-295 g). The animals were treated with AF0150 or saline starting on gestation day 6 till day 17, as shown at Table 1.

Table 1. Teratology 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. Animals were dosed from gestation days 6 to 17 and sacrificed on gestation day 20.

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 daily administered by IV injection via a lateral caudal vein (dilating with warm water, if necessary) from gestation 6-17. 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.

Maternal Observations:

Clinical Sings and Food Consumption: All animals were observed twice a day for mortality and moribundity, and detailed clinical signs were recorded daily till scheduled sacrifice day (gestation day 20). Food consumption was recorded on gestation days 0, 6-18 (daily) and 20 and reported as g/animal/day and g/kg/day for each corresponding body weight changes.

Body Weights and Gravid Uterine Weights: Maternal body weights were recorded on gestation days 0, 6-18 (daily) and 20. A 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 6-9, 9-12, 12-18, 6-18 and 0-20. The net body weights were determined by exclusion of the uterus and content weights on gestation day 20 (scheduled laparohysterectomy) from body weight on Day 20. Net body weight changes were calculated by exclusion of the uterus and content weights from the day 0-20 body weight change.

Gestation Day 20 Laparohysterectomy: All maternal animals were sacrificed on gestation day 20 followed by necropsy including the thoracic, abdominal, pelvic cavities and their contents. The number of corpora lutea in each ovary was recorded. The trimmed uterus was weighed and opened. The number and location of all fetuses, early and late resorptions, and the total number of implantation sites were collected. Data were processed according to equations described in Table 2.

Table 2. Gestation Data Process

Group Mean Litter Basis

$$\text{Post-Implantation Loss/Litter} = \frac{\text{Numbers of Dead Fetuses, Resorptions (Early/Late)/Group}}{\text{Numbers of Gravid Females/Group}}$$

Proportional Litter Basis

$$\text{Summation per Group (\%)} = \frac{\text{Total Post-Implantation Loss/Litter/Group (\%)}}{\text{Numbers of Litters/Group}}$$

$$\text{Total Post-Implantation Loss/Litter/Group (\%)} = \frac{\text{Numbers of Dead Fetuses, Resorptions/Litter}}{\text{Numbers of Implantation Sites/Litter}} \times 100$$

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 in each group allocated to soft tissue or skeletal examinations was not specified*].

Results

Clinical Signs and Food Consumption: all animals survived to the scheduled necropsy on gestation day 20. AF0150 did not result in significant toxic effects at any time points or any dose. Food consumption slightly increased in the 50 mg/kg/day AF0150 group during gestation days 6-18 (statistically significant as compared to the control group). No food consumption change was noted in other dose groups.

Body Weights and Gravid Uterine Weights: mean net body weight gain increased in the 50 mg/kg/day AF0150 group (statistically significant as compared to the control group). AF0150 at all other dose groups had no significant effects on mean body weight, body weight gain, gravid uterine weight, net body weight and net body weight gain.

Maternal Macroscopic Examination: At the scheduled necropsy (gestation day 20), one rat in 200 mg/kg/day group had clear fluid contents in the uterus, one rat in each of the 50 and 100 mg/kg/day 50 mg/kg/day groups had dilation of one or both renal pelves. One rat in the 50 mg/kg/day group had an accessory spleen. One rat in the control group had uterine hypoplasia

(the right horn was present as a filamentous segment). No other internal findings were noted at any AF0150 dose level.

Gestation Day 20 Laparohysterectomy: There were no significant differences in post-implantation loss, viable litter size, fetal body weights, fetal sex ratios, the numbers of corpora lutea and implantation sites between AF0150 at all dose levels and control groups. [*Placenta examination was not indicated*].

Fetal Morphology (Table 3): Malformation findings in the litters and fetuses available for morphological evaluation were summarized in Table 3.

External Malformations and Variations: One fetus each in the control, 50 and 100 mg/kg/day groups had external malformations. The findings included right anophthalmia and left microphthalmia, maxillary and mandibular micrognathia, aglossia and astomia (one fetus in the control); anasarca and artophthalmia (one fetus in the 50 mg/kg/day group); localized fetal edema (head, neck and thorax), a malrotated left hind limb and a cleft palate (one fetus in the 100 mg/kg/day group); astomia, mandibular agnathia, anophthalmia (bilateral) and cebocephaly with a proboscis-like nose (one fetus in the 200 mg/kg/day group). No external developmental variations were noted at any AF10150 dose level.

Table 3. Fetal Morphological Examination in Rats

	AF0150 (mg/kg/day)			
	0	**50**	**100**	**200**
Number of Litter	23	22	24	22
Number of Fetus	340	326	352	313
Total Malformation (Fetuses/Litters)	4/3	3/2	1/1	0/0
External	1/1	1/1	1/1	0/0
Soft Tissue	1/1	0/0	0/0	0/0
Skeletal	3/2	2/2	0/0	0/0

Visceral Malformations and Variations: No visceral malformations and developmental variations were observed in any group except that one fetus in the control group had situs inversus.

Skeletal Malformations and Variations: One fetus in the control group had a rib anomaly (fusion of rib nos. 9 and 10). Two fetuses each in the control and 50 mg/kg/day groups had vertebral anomalies with or without associated rib anomalies. These findings included absent or extra arches, ribs and centra; fused ribs and arches; arches and centra that were smaller than normal; malpositioned centra; fused, absent or malpositioned costal cartilage; misshapen, unossified or malaligned sternebrae; and the presence of only six cervical vertebrae. No other skeletal malformations were noted at any AF0150 dose level. Some skeletal developmental variations were noted in both control and AF0150 groups, including ossification of cervical centrum (number 1), unossified sternebrae (number 5 and/or 6), 14th rudimentary ribs and bent ribs.

Discussion and Comments

In this teratology study, pregnant rats received AF0150 at the doses of 50, 100 and 200 mg/kg/day by IV injection from gestation day 6 to 17 and were sacrificed on gestation day 20. There were no significant differences in fetal development, viability, body weights, sex ratio, corpora lutea number, implantation sites, postimplantation loss and morphology (external, visceral and skeletal malformations/variations) between AF0150-treated and control groups.

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.

Report Number: IMUS-024-TOX
An Intravenous Study of the Effects of AF0150 on Embryo/Fetal Development in Rabbits

Report Location:	Vol.031, p001-337
Report date:	September 14, 1998
Study Facility:	[redacted]
In-life phase:	December 1-31, 1996
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ16049, ZZ16050, ZZ16051 (400 mg/vial)
AF0150 Dosage (HMD at BSA):	50, 100, 200 mg/kg/day (up to 518-fold of PCD)

Specific Aim

To assess the teratogenicity of AF0150 in rabbits (Segment II)

Methods

Animal Preparation: New Zealand white female rabbits (98, sexually mature and virgin, 5 months old with body weight of 2525-3787 g) were obtained from [redacted] Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation for 26 days, animals meeting good health and acceptable body weight requirements (6 month old, 3000-4500 g) were randomly assigned to 4 groups, 22/group for insemination and treatment (Table 1).

Insemination: Semen was individually collected from 11 resident male rabbits of the same strain and supplier as the females. Semen with greater than 50% motility was diluted with saline to a final concentration of more than 3 million motility sperms/ml. The diluted semen from one male was used to inseminate two females in each group. A 0.25-0.5 ml of the diluted semen was deposited into the anterior vagina of each female with a glass insemination pipette, immediately

followed by IV injection of HCG (100 USP units) to ensure ovulation. The insemination day was designated as gestation day 0.

Table 1. Teratology Study Design for AF0150 IV Injection in Rabbits

Group	AF0150 Dose* (mg/kg/day)	Number of Female Rabbits	IV Volume (ml/kg/day)
1 (Control)	(Saline)	22	5.0
2 (AF0150)	50	22	1.25
3 (AF0150)	100	22	2.5
4 (AF0150)	200	22	5.0

* AF0150 was reconstituted in SWFI to final concentration of 40 mg/ml. Animals were dosed from the gestation day 7 to day 20, and sacrificed on gestation day 29.

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 marginal ear vein (dilated with warm water if necessary), from gestation days 7 through 20. The AF0150 dosages were 50, 100 and 200 mg/kg/day for the assigned groups, and 5 ml/kg/day of saline for the control group (Table 1) based on the most recent body weights. All animals were treated at approximately the same time each day.

Maternal 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 (gestation days 0-29).

Food consumption: Daily food consumption was recorded from gestation days 0-29 and reported as g/animal/day and g/kg/day for each corresponding body weight changes.

Body Weights and Gravid Uterine Weights: Maternal body weights were recorded on gestation days 0, 7-21 (daily), 24 and 29. A 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 7-10, 10-13, 13-21, 21-29 and 0-29. The net body weights were determined by exclusion of the uterus and content weights on gestation day 29 (scheduled laparohysterectomy) from body weight on Day 29. Net body weight changes were calculated by exclusion of the uterus and content weights from the day 0-29 body weight change.

Gestation Day 20 Laparohysterectomy: All animals were sacrificed on gestation day 29 followed by necropsy including the thoracic, abdominal, pelvic cavities and their contents. The number of corpora lutea in each ovary was recorded. The trimmed uterus was weighed and opened. The