Pathology: macroscopic and microscopic examinations were performed on animals from the scheduled sacrifice and unscheduled death.

Unscheduled Death Rats (Table 3): there were unscheduled deaths in 2/10 rats in each of Groups 1 (saline) and 3 (16 mg/kg AF0150).

Table 3. Unscheduled Death and Pathology

Group #	Treatment	Numbers of Death	Death Day	Pathology
1	Saline	1M, 1F	2, 4	Arterial thrombosis at catheter carotid artery without identification of renal and brain infarction.
3	AF0150 (16mg/kg)	2M	3, 7	Arterial thrombosis at catheter carotid artery with renal and brain infraction

Scheduled Sacrifice (Day 8): macroscopic and microscopic examination were performed on all animals that survived to the scheduled termination day and their results are presented in Tables 4 and 5, respectively.

1. *Administration Site:* Either thromboarteritis or thrombosis was observed in the catheter carotid artery of all animals.
2. *Brain:* 2 males in Group 1 (saline) had locally extensive, unilateral regions of malacia consistent with infarcts involving the basal ganglia and cerebral cortex. One female in Group 3 (16 mg/kg AF0150) had an arterial thrombus in a meningeal artery of the longitudinal fissure.
3. *Kidneys:* pale macroscopic discoloration of kidneys was noted in many treated animals, histopathologically corresponding to ischemic and infact lesions. Renal arterial thrombosis was apparent in several AF0150-treated animals (Group 3).
4. *Testes:* unilateral pale discoloration of testes was observed in one of Group 1 (saline) and two of Groups 2 (4 mg/kg AF0150) male rats. Microscopic examination on the one in Group 1 revealed moderate coagulative necrosis consistent with infarction. No histopathology data for the two animals in Group 2 were provided.
5. *Adrenal Glands:* A dark right adrenal gland was found in one Group 3 female rat, which correlated with medullary infarction (necrosis with hemorrhage, fibrin, and hemosiderin-laden macrophages in the medulla).
6. *Lungs:* one Group 3 female rat had multifocal dark discoloration in the lungs, associated with mild alveolar hemorrhage and a few hemosiderin-laden macrophages, minimal granulocytic perivascular inflammation.
7. *Heart:* a focal dark discoloration of the heart was apparent in one of Group 2 males rats without microscopic examination.

Table 4. Macroscopic Examination on Scheduled Sacrifice Animals

Group Number	1	2	3
Tissue/Lesion Number Examined M/F	**4/4**	**5/5**	**3/5**
Administration site, right carotid artery			
-thickened, pale	3/-	1/2	3/2
Adrenal Glands			
-discoloration, dark, right			-/1
Kidney			
-discoloration		1/-	1/-
-discoloration, mottled		1/-	2/-
-discoloration, pale	1/4	3/4	3/3
-adhesion			1/-
Heart			
-discoloration, dark		1/-	
Testis			
-discoloration, pale, unilateral	1/-	2/-	
-small	1/-		
Epididymides, left caudate			
-cyst, yellow		1/-	
Lung			
-discoloration, dark			-/1
Brain, left cerebral hemisphere			
-discoloration, pale	1/-		

Table 5. Microscopic Examination on Scheduled Sacrifice Animals

Sex	M	M	M	M	M	F	F	F	F	F	M	M	M	M	M	F	F	F	F	F
Group Number			1					1					3					3		
Study Day of Euthanasia	8	8	3	8	8	4	8	8	8	8	8	8	7	8	4	8	8	8	8	8
ANIMAL NUMBER	11	12	13	14	15	16	17	18	19	20	6	7	8	9	10	26	27	28	29	30
Brain	-	+	-	+	+	-	-	+	+	-	-	-	-	-	+	-	+	-	-	+
Malacia, unilateral		4		2											3					
Gliosis		3		2											1					
Ischemic neurone		1		1	1										1					
Thromboembolus, arterial																	1			
Pigment, choroid plexus, lateral ventricle								1	1								1			1
Kidneys	NE	+	NE	NE	NE	NE	+	+	+	+	+	+	+	+	+	+	NE	+	NE	+
Infarct(s)		3						(1			4	3	4	3	3	2				
Tubulointerstitial alterations		2					2	(1	(2	(2	(2	(3		3	2	3		(3		(1
Arterial thrombosis											(1	3	(1		(2	(1				
Capsular fibroplasia												(3								
Administration site	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Thromboarteritis	4			4	4						4	4		3			4		4	
Thrombosis		3	3			2	2	3	2	2			3		1	2		3		2
Suture-associated granuloma(s)	1										2									
Testes	+	+	NE	NE	NE						NE	NE	NE	NE	NE					
Infarct, unilateral		(3																		
Degeneration	(3																			
Thymus	NE	NE	+	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	+	NE	NE	NE	NE	NE
Lymphorrhexis, cortical			2												3					
Peracute hemorrhage, multifocal			3												2					
Adrenal Glands	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	+	NE	NE	NE	NE
Infarct, unilateral																(4				
Lungs	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	+	NE	NE	NE	NE
Alveolar hemorrhage, multifocal																2				
Pigment, alveolar macrophages																1				
Perivascular inflammation, granulocytic																1				
Perivascular edema																1				
Liver	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	+	NE	NE	NE	NE	NE
Vascular/sinusoidal congestion															3					

Subjective grading of microscopic findings according to the following scale.
1=minimal, 2=mild, 3=moderate, 4=marked, NE=Tissue not examined -=no microscopic finding, +=microscopic finding, (=unilateral finding

Discussion and Comments

Intra-arterial injection of AF0150 (4 or 16 mg/kg, single dosing) or saline through the catheterized carotid artery in rats resulted in multifocal infarction and ischemic lesions in the kidneys, brain, testes and other organs in both control and AF0150-treated rats. Neurological signs from clinical observations were also consistent with the brain ischemic pathology.

Thromboarteritis or thrombosis in the catheterized carotid artery was shown in all animals, which might explain, in part, embolization and thus multiorgan infarction. However, the AF0150-treated animals tended to have a slightly higher incidence of kidney infarction (Tables 4 and 5). Also, brain infarction was identified in the two unscheduled deaths in the AF0150 (16 mg/kg) group but not in the two unscheduled deaths in the control group. Therefore, AF0150 is not recommended in patients with a right-to-left shunt.

The study procedure was not appropriate because the artery catheterization introduced thromboarteritis and thrombosis in all animals, which may mask the potential embolic effects of the microbubbles. A direct intra-cardiac injection may have been better to avoid these artifacts. A positive control, such as injection of solid microspheres, should also have been included in order to validate the experimental system.

Report Number: IMUS-043-TOX
Effects of AF0150 in the Irwin Test in Rats

Report Location:	Vol.025, p001-038
Report date:	September 9, 1999
Study Facility:	[redacted]
In-life phase:	February 5, 1999
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010 (200 mg/vial)
AF0150 Dosage (HDM):	4, 40, 100 mg/kg (up to 130-fold PCD)

Specific Aim

To assess the gross behavioral and physiological effects of IV AF0150 injection using the "Primary Observation Test" (Irwin Test).

Methods

Animal Preparation: Sprague-Dawley rats (32 males) were obtained from [redacted] 5-7 weeks old and 189-227 g weight at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation for 8 days, the animals were randomly assigned into 5 groups (6/group), as seen in Table 1.

Table 1. Study Design of Irwin Test in Rats

Group	*Treatment*	*Dose*	*Concentration*	*Dose volume*
D	Sterile saline	-	0.9% w/v	5 mL/kg
C	AF0150	4 mg/kg	2 mg/mL	2 mL/kg
A	AF0150	40 mg/kg	20 mg/mL	2 mL/kg
B	AF0150	100 mg/kg	20 mg/mL	5 mL/kg
E	Chlorpromazine hydrochloride	2 mg/kg	0.4 mg/mL	5 mL/kg

AF0150 Preparation and Administration: AF0150 (200 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 20 mg/kg and was used within 30 minutes. To make 2 mg/ml AF0150, the reconstituted AF0150 (20 mg/ml) was diluted with 0.9% saline (1:10). Chlorpromazine hydrochloride (0.4 mg/ml dissolved in 0.9% saline) was used as a reference substance. Each animal received a single IV injection of saline, AF0150 or Chlorpromazine (as shown in Table 1) via a tail vein. The tested solutions were encoded so that the observer was not aware of the identity of the treatment groups.

Observation Parameters: the parameters described in the Irwin Test (Table 2) were systematically evaluated for each animal at 15, 45, 90 and 150 minutes after dosing. Changes in the parameters were arbitrarily scored from 0 to 8. Factors normally present in animals were scored as 4, potentiation or depression of these factors were indicated as higher or lower, respectively. Factors normally absent in animals were scored as 0.

Results/Conclusion

IV injection of AF0150 up to 100 mg/kg (130-fold PCD) did not lead to significant gross behavioral or physiological changes during the 150-minute post dosing observation period. Some slight changes, including increased alertness, locomotor activity, restlessness and passivity, cage dispersion, vocalization, increased/decreased pupil size, were noted in both control and AF0150-treated rats. The positive control animals treated with chlorpromazine showed moderate to severe effects on behavior and physiology consistent with its known pharmacological activities.

Comments

Only male rats were included in this study. However, the target patients will be of both sexes. Therefore, the behavioral effects in female animals should also have been evaluated. The observation time period (150 minutes) may not be long enough to elicit behavioral effects, particularly since animal handling (such as injection procedure) may transiently change behavioral and physiological activities at early time points.

Table 2. Irwin Screen Observations

Project Code: **Date:**

Route of administration: **Time (min) post-dose of observation:**

Observation		Score for normal animal	Test treatment and animal ID																											
			A						B						C						D						E			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Lethality																																
Dispersion in cage		0																														
Locomotor activity		4																														
Restlessness		0																														
Apathy		0																														
Writhing		0																														
Fighting		0																														
Stereotyped behaviour	Type																															
	Score	0																														
Tremor		0																														
Twitches		0																														
Convulsions	Type																															
	Score	0																														
Exophthalmos		0																														
Respiration	Type																															
	Score	0																														
Alertness		4																														
Startle response		4																														
Loss of righting reflex		0																														
Abnormal body carriage	Type																															
	Score	0																														
Abnormal gait	Type																															
	Score	0																														
Straub tail		0																														
Piloerection		0																														
Pupil diameter		4																														
Touch response		4																														
Fearfulness		4																														
Pinna reflex		0																														
Corneal reflex		0																														
Catalepsy		0																														
Passivity		0																														
Aggressiveness		0																														
Body tone		4																														
Grip strength		4																														
Cutaneous blood flow		4																														
Cyanosis		0																														
Ptosis		0																														
Lacrimation		0																														
Salivation		0																														
Pain response		4																														
Hypothermia		0																														
Paralysis		0																														
Grooming		4																														
Diarrhoea		0																														
Vocalisation		0																														
Increased urination		0																														
NAD		(✓)																														
Number of observations observed (not Group score)																																

Miscellaneous observations:

ABBREVIATIONS

NAD – No abnormalities detected

Time of observation: (for individual group times refer to TIMEPLAN)

Observation performed by:

Report Number: IMUS-044-TOX
Effects of AF0150 on Renal Function in Saline-Loaded Rats

Report Location:	Vol.025, p038-080
Report date:	September 7, 1999
Study Facility:	[redacted]
In-life phase:	February 18-19, 1999
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010 (200 mg/vial)
AF0150 Dosage (HDM):	4, 40, 100 mg/kg (5-130 fold PCD)

Specific Aim

To assess the effects of AF0150 on renal functions

Methods

Animal Preparation: Sprague-Dawley rats (56 males) were obtained from [redacted] 7-9 weeks old and 193-256 g weight at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation for 10 days, 40 animals were randomly assigned into 5 groups (8/group), as seen in Table 1.

AF0150 Preparation and Administration: AF0150 (200 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 20 mg/kg and was used within 30 minutes. To make 2 mg/ml AF0150, the reconstituted AF0150 (20 mg/ml) was diluted with 0.9% saline (1:10). Furosemide (10 mg/ml) was used as a reference substance. Each animal received a single IV injection of saline, AF0150 or furosemide (as shown in Table 1) via the tail vein. The tested solutions were encoded (A-E) so that the observer was not aware of the identity of the treatment groups.

Table 1. Study Design of Renal Function

Group	*Treatment*	*Dose*	*Concentration*	*Dose volume*
A	Sterile saline	-	0.9% w/v	5 mL/kg
E	AF0150	4 mg/kg	2 mg/mL	2 mL/kg
C	AF0150	40 mg/kg	20 mg/mL	2 mL/kg
D	AF0150	100 mg/kg	20 mg/mL	5 mL/kg
B	Furosemide	5 mg/kg	1 mg/mL	5 mL/kg

Observation Parameters: after withholding water for approximately 2 hours, rats received 25 ml/kg saline by oral gavage. Thirty minutes following saline loading, the animals received a single IV injection of either AF0150, reference substance (furosemide) or saline, as presented in Table 1, and were individually placed in metabolic cages designed for the collection of urine. Urine samples were collected at 3, 6 and 24 h post dose. During the first 6 h in the cages the rats had no access to water or food. After the 6 h urine sample collection, both food and water were offered to the rats *ad libitum.* The urine samples were analyzed for Na+, K+, Cl-, pH, and volume.

Results

Urine Volume (Table 2): IV injection of AF0150 at all three doses significantly decreased urinary output during first 3 hours, by approximately 50%, as compared with control animals. No significant difference in the urine volumes was observed at 3-6 and 6-24 hours between AF0150 treatment and saline control. Positive control, furosemide, induced significantly urinary output in the first 3 hours but decreased urinary output at 3-6 and 6-24 hours.

Table 2. Effects of AF0150 on Urine Electrolyte and Volume Levels 0-3 hours Post Dosing

Group	Treatment	Sodium Excreted (mmol/kg)	Potassium Excreted (mmol/kg)	Chloride Excreted (mmol/kg)
A	Vehicle (5 mL/kg i.v.)	2.23 ± 0.24	1.64 ± 0.15	3.07 ± 0.23
E	AF0150 (4 mg/kg i.v.)	1.50 ± 0.23†(7)	1.35 ± 0.13 (7)	2.03 ± 0.22††(7)
C	AF0150 (40 mg/kg i.v.)	1.25 ± 0.17††	0.97 ± 0.12††	1.66 ± 0.20††
D	AF0150 (100 mg/kg i.v.)	1.48 ± 0.08†	1.21 ± 0.06†	2.11 ± 0.07††
B	Furosemide (5 mg/kg i.v.)	4.82 ± 0.15***	1.66 ± 0.11	5.99 ± 0.43***

Group	Treatment	Urine Volume (mL)	pH
A	Vehicle (5 mL/kg i.v.)	3.03 ± 0.13	8.26 ± 0.22
E	AF0150 (4 mg/kg i.v.)	1.68 ± 0.29††	7.16 ± 0.39†(7)
C	AF0150 (40 mg/kg i.v.)	1.42 ± 0.20††	7.31 ± 0.31
D	AF0150 (100 mg/kg i.v.)	1.80 ± 0.17††	7.51 ± 0.25
B	Furosemide (5 mg/kg i.v.)	8.56 ± 0.35###	8.18 ± 0.17

The vehicle for AF0150 was sterile water for injection.

n = 8 rats per group, unless otherwise indicated in parentheses.

Data are expressed as mean ± s.e. mean.

*** $P < 0.001$, compared to vehicle (unpaired, 2-tailed Students' t-test).
$P < 0.001$, compared to vehicle (Mann-Whitney U-test).
†$P < 0.05$, ††$P < 0.01$, compared to vehicle (one-way analysis of variance and Dunnett's t-test).

Urinary pH (Table 2): AF0150 at all three doses decreased urinary pH during the first 3 hours and the period between 3-6 hours but did not affect urinary pH between 6-24 hours. Furosemide did not induce significant changes in urinary pH at any of time points.

Sodium Excretion (Table 2 and Figure 1): AF0150 at all three doses significantly decreased urinary Na2+ excretion (total) during the first 3 hours, but not at 3-6 and 6-24 hours, as compared with control group. AF0150 did not significantly change concentration of Na+ in urine during the 0-3 hours period (Figure 1) and other time periods. Furosemide increased at the first 3 hours but decreased urinary Na+ excretion at 3-6 and 6-24 hours.

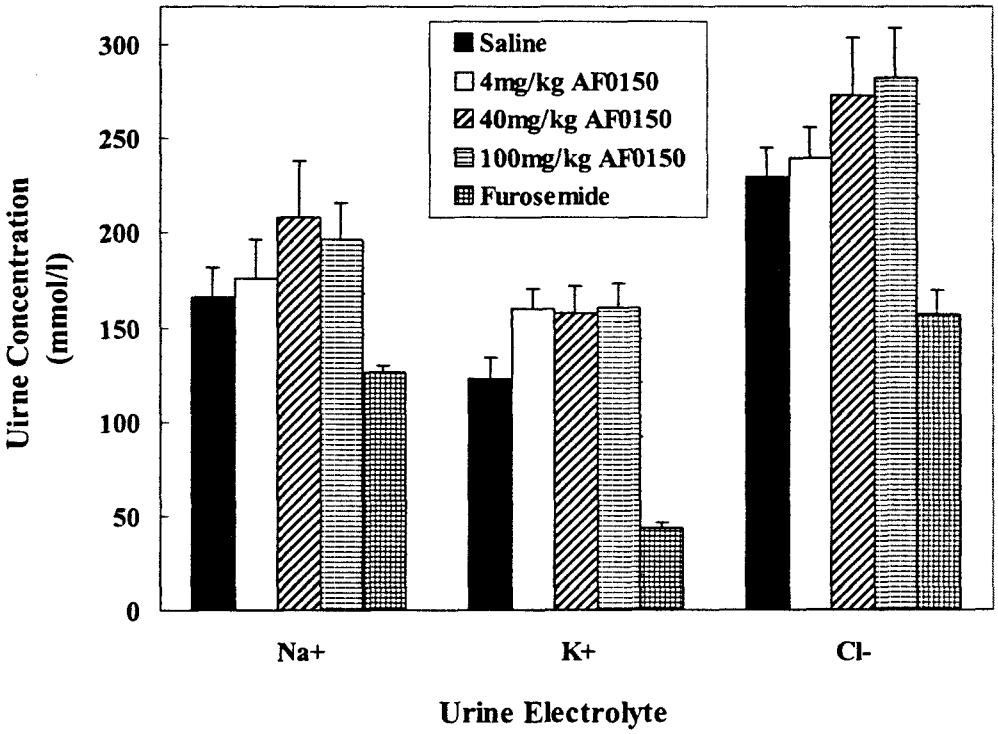

Figure 1. Concentrations of Na+, K+ and Cl- in urine collected during the 0-3 hours periods. Rats received 25 ml/kg saline by oral gavage, and 30 minutes later, AF0150 (at 4, 40 or 100 mg/kg), furosemide or saline were intravenously injected. Urine samples were individually collected in metabolic cages for analysis of Na+, K+, Cl-, pH, and volume.

Potassium Excretion (Table 2 and Figure 1): AF0150 at the doses of 40 and 100 mg/kg but not 4 mg/kg decreased urinary K+ excretion (total) during the first 3 hours without effect at 3-6 and 6-24 hours. AF0150 did not significantly change concentration of K+ in urine during the 2-3 hours period (Figure 1) and other time periods. Furosemide had no effects on K+ excretion during 24-hour observation period.

Chloride Excretion (Table 2 and Figure 1): AF0150 at all three doses decreased urinary Cl- excretion (total) during the first 3 hours but had no effect at 3-6 and 6-24 hours. AF0150 did not

significantly change concentration of Cl- in urine during the 2-3 hours period (Figure 1) and other time periods. Furosemide increased Cl- excretion during the first 3 hours and decreased urinary Cl- excretion at 3-6 and 6-24 hours.

Discussion and Comments

1. IV injection of AF0150 at 4, 40 and 100 mg/kg (5-130 fold PCD) significantly decreased urinary volume, pH, and urinary Na+, K+ and Cl- excretion during the first 3 hours post dosing (Table 3). AF0150-induced urinary parameter changes had no dose-dependency. This suggests that the **NOAEL** must be below 4 mg/kg (HED: <0.65 mg/kg; HDM: <5-fold PCD). There were no significant changes in urine concentration of Na+, K+, and Cl- in the AF015-treated rats. This suggests that glomerular filtration may be a target site of AF0150. AF0150 must be used with caution in patients with decreased renal function.

Table 3. Effects of AF0150 IV Injection on Urinary Excretion at the First 3 Hours in Rats

Treatment	Volume	pH	Na+	K+	Cl-
AF0150 (4 mg/kg	↓	↓	↓	-	↓
AF0150 (40 mg/kg)	↓	↓	↓	↓	↓
AF0150 (100 mg/kg)	↓	↓	↓	↓	↓
Furosemide (5 mg/kg)	↑	-	↑	-	↑

2. More information should be provided regarding the rationale to use the saline-loaded rat to test renal function.

3. Only male rats were tested in this study, which is inadequate for safety assessment in application of AF0150 in female patients in the clinical setting.

4. Individual animal data on page074-076 were not labeled clearly. It is assumed that they were from three observation periods, 0-3 hours on page 074, 3-6 hours on page 075 and 6-24 hours on page 076.

Report Number: IMUS-045-TOX
Effects of AF0150 on the Gastrointestinal Transit of a Charcoal Meal in Rats

Report Location:	Vol.025, p081-116
Report date:	September 7, 1999
Study Facility:	[redacted]
In-life phase:	February 12, 1999
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010 (200 mg/vial)
AF0150 Dosage (HDM):	4, 40, 100 mg/kg (5-130 fold PCD)

Specific Aim

To assess the effects of AF0150 on gastrointestinal functions

Methods

Animal Preparation: Sprague-Dawley rats (44 males) were obtained from [redacted], 5-7 weeks old and 190-216g weight at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation for 10 days, 40 animals were randomly assigned into 5 groups (8/group), as seen in Table 1.

Table 1. Study Design of GI Transit

Group	*Treatment*	*Dose*	*Concentration*	*Dose volume*
B	Sterile saline	-	0.9% w/v	5 mL/kg
E	AF0150	4 mg/kg	2 mg/mL	2 mL/kg
C	AF0150	40 mg/kg	20 mg/mL	2 mL/kg
D	AF0150	100 mg/kg	20 mg/mL	5 mL/kg
A	Morphine hydrochloride	10 mg/kg	2 mg/mL	5 mL/kg

AF0150 Preparation and Administration: AF0150 (200 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 20 mg/kg and was used within 30 minutes. To make 2 mg/ml AF0150, the reconstituted AF0150 (20 mg/ml) was diluted with 0.9% saline (1:10). Morphine hydrochloride (2 mg/ml in saline) was used as a reference substance. Each animal received a single IV injection of saline, AF0150 or morphine (as shown in Table 1) via a tail vein. The tested solutions were encoded (A-E) so that the observer was not aware of the identity of the treatment groups.

Charcoal Meal Preparation: the charcoal meal was prepared by mixing charcoal, flour and reverse osmosis water in the ratio of 1:3:6.

Procedure and Administration: After being withheld from food overnight, the animals received a single IV injection of either AF0150, saline, or morphine. Five minutes after dosing, each animal was given 1 ml of a charcoal meal suspension by oral gavage. The animals were sacrificed at 30 min post charcoal meal. The intestine length and the distance of the charcoal meal traveling along the intestine from the pyloric sphincter were measured. The stomach was also weighed to estimate the stomach contents and thus the degree of gastric emptying.

Results

Gastrointestinal Transit (Table 2): IV injection of AF0150 at all three doses had no significant effects on the charcoal traveling distance along the intestine, when expressed as % of total intestine length, as compared with saline-treated animals. In morphine-treated rats, the charcoal did not enter the intestine from the stomach, suggesting significant inhibition on GI transit.

Table 2. Effects of AF0150 and Morphine on Gastrointestinal Transit and Gastric Emptying in Rats

Group	Treatment	% Distance Travelled	Weight of Stomach plus Contents (g)
B	Vehicle (5 ml/kg i.v.)	53.2 ± 1.4	3.7 ± 0.6 (6)
E	AF0150 (4 mg/kg i.v)	53.9 ± 3.1	2.8 ± 0.1 (7)
C	AF0150 (40 mg/kg i.v)	57.1 ± 1.2	2.9 ± 0.1
D	AF0150 (100 mg/kg i.v)	52.1 ± 2.2	3.2 ± 0.2
A	Morphine HCl (10 mg/kg i.v)	0 ± 0	3.6 ± 0.3

The vehicle for AF0150 was sterile water for injection.

n = 8 rats per treatment group (except where indicated in parentheses).

Data are expressed as mean ± s.e. mean.

Gastric Emptying (Table 2): AF0150 at low doses tended to increase gastric emptying based on changes in stomach weights as compared with the saline control group. However, morphine-treated rats had no significant change in the stomach weight, even though the charcoal was still present in the stomach. This suggests that changes in the stomach weight may not provide an appropriate estimate of gastric emptying.

Discussion and Comments

IV administration of AF0150 appeared not to affect gastrointestinal transit in rats given a charcoal meal. However, it is hard to conclude an effect on stomach emptying since there was no difference in stomach weight between AF0150, negative control (saline), and positive control (morphine) groups. A charcoal-untreated control group was not included. On the other hand, the sponsor did not provide an appropriate rationale for using charcoal meal method to evaluate GI function.

Report Number: IMUS-046-TOX
Effects of AF0150 on Respiration Rate and Body Temperature

Report Location:	Vol.025, p117-156
Report date:	September 7, 1999
Study Facility:	[redacted]
In-life phase:	March 11, 1999
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA16010 (200 mg/vial)
AF0150 Dosage (HDM):	4, 40, 100 mg/kg (5-130 fold PCD)

Specific Aim

To asses effects of AF0150 on respiration and body temperature in conscious rats.

Animal Preparation: Sprague-Dawley rats (90 males) were obtained from [redacted], 6-7 weeks old and 206-243g weight at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. After acclimation for 7-9 days, 40 animals were randomly assigned into 5 groups (8/group), as seen in Table 1.

Table 1. Study Design of Effects of AF0150 on Respiration and Temperature in Rats

Group	*Treatment*	*Dose*	*Concentration*	*Dose volume*
F	Sterile saline	-	0.9% w/v	5 mL/kg
I	AF0150	4 mg/kg	2 mg/mL	2 mL/kg
H	AF0150	40 mg/kg	20 mg/mL	2 mL/kg
J	AF0150	100 mg/kg	20 mg/mL	5 mL/kg
K	Chlorpromazine hydrochloride	10 mg/kg	2 mg/mL	5 mL/kg

AF0150 Preparation and Administration: AF0150 (200 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 20 mg/kg and was used within 30 minutes. To make 2 mg/ml AF0150, the reconstituted AF0150 (20 mg/ml) was diluted with 0.9% saline (1:10). Chlorpromazine hydrochloride (2 mg/ml in saline) was used as a reference substance. Each animal received a single IV injection of saline, AF0150 or chlorpromazine (as shown in Table 1) via the tail vein. The tested solutions were encoded (F, H-K) so that the observer was not aware of the identity of the treatment groups.

Procedure and Observations: The rats received a single IV injection of either AF0150, chlorpromazine or saline, and were immediately placed in a small plethysmography chamber (1 rat/chamber) which was supplied with 0.5 l/min of air. The chamber was connected to a flow

transducer and computer. Approximately 15 min after dosing, a 10-second record of respiratory waveform was frozen on the screen and the number of respiratory cycles was counted. Duplicate measurements were taken from each animal to obtain a mean. Immediately after obtaining readings, the animals were removed and their core temperature measured from the rectum by a thermocouple connected to a digital thermometer.

Results

Respiration Rate (Table 2): AF0150 at all three doses had no significant effects on respiration rate as compared with saline control. Chlorpromazine, however, significantly decreased respiration rate by approximately 15%.

Table 2. Effects of AF0150 and Chlorpromazine on Respiration Rate and Core Body Temperature

Group	Treatment	Respiration Rate (breaths/min)	Body Temperature (°C)
F	Vehicle (5 ml/kg i.v.)	130.5 ± 3.8	38.2 ± 0.1
I	AF0150 (4 mg/kg i.v.)	120.8 ± 2.6	37.9 ± 0.1
H	AF0150 (40 mg/kg i.v.)	131.3 ± 4.4	37.9 ± 0.0
J	AF0150 (100 mg/kg i.v.)	130.1 ± 1.6	37.7 ± 0.1†
K	Chlorpromazine HCl (10 mg/kg i.v.)	111.0 ± 7.6*	36.0 ± 0.2***

The vehicle for AF0150 was sterile water for injection.

n = 8 rats per group.

Data are expressed as mean ± s.e. mean.

* $P < 0.05$, *** $P < 0.001$, compared to vehicle (unpaired, 2-tailed Student's t-test).
† $P < 0.05$, compared to vehicle (one-way analysis of variance and Dunnett's t-test).

Body Temperature (Table 2): AF0150 at the dose of 100 mg/kg, but not at 4 and 40 mg/kg, slightly decreased rat rectal temperature (statistically significant) as compared with the saline control group. In chlorpromazine-treated rats, the body temperature was significantly lower than that in control rats.

Discussion and Comments

IV injection AF0150 at all three doses (4, 40, 100 mg/kg) had no significant effects on respiration rate and at the high dose (100 mg/kg) slightly decreased core body temperature at 15 minutes post dosing. However, the observation time was too short (only 15 minute post AF0150 dosing) which may provide an optimal assessment of the effects of AF0150 on respiratory parameters.

Blood gas analysis could also have provided a better assessment of functional effects of Aaf0150 on the respiratory system.

Summary of Safety Pharmacology Studies

Studies regarding safety assessment of AF0150 in hemodynamic (pulmonary and cardiovascular systems), neurological, renal and gastrointestinal systems were conducted in rats, rabbits, dogs and monkey. Most of the studies were not in compliance with GLP regulations (however, this is not considered a deficiency). *Human dose multiples (fold the PCD) are calculated based on the body surface area.*

Cardiovascular Safety Assessment: Cardiovascular safety of AF10150 intravenous administration 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 examined 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 period (Report # EB-95-19). Similarly, changes were not noted in rabbits that were either pre- or co-treated with cardiac stress agents (see Table 9 in **Overall Summary**), such as adenosine, arbutamine and dobutamine (EB-98-05/07/08). However, in rabbits treated with another cardiac stress agent dipyridamole (EB-98-06), AF150 at both doses (2 and 20 mg/kg) transiently (about 20 minutes) increased dipyridamole-induced tachycardia without affecting MAP. The mechanism of this effect is still unclear.

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 a thromboxane-induced pulmonary hypertension model in rabbits (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 rabbit model (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 myocardial ischemic/infarction areas, during the 30-minute post dosing observation period.

In anesthetized dogs, heart rate, MAP and cardiac output were not different between pre and 1 minute post dosing, at AF0150 doses up to 0.6 mg/kg (2.6-fold PCD, with and without sustained application of ultrasound) (EB-95-25), or 10 minute post dosing at the AF0150 dose up to 1.6 mg/kg (6.9-fold PCD) (EB-95-27). Intravenous infusion of AF0150 at the dose of 20 mg/kg (52-fold PCD) with ultrasound application had no effects on heart rate and MAP (EB-97-13) during the 4-hour post dosing observation period.

In a conscious monkey study (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 these parameters between AF0150 and control groups during the 1-hour observation period.

Electrocardiograph (ECG) examination was performed in two GLP studies, one in anesthetized dogs and other in non-anesthetized cynomolgus monkeys. Six limb leads of ECG were recorded before and after AF0150 administration. There were no remarkable differences in most ECG parameters from both studies between AF0150 and saline controls, and between pre and post AF0150 dosing. Time-dependent increase in the uncorrected QT intervals were observed in anesthetized dogs (IMUS-035-TOX) following IV infusion of AF0150 at the dose of 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 up to a 60-minute post dosing observation period at AF0150 doses of 10, 20 and 40 mg/kg (26-40 fold PCD).

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

In a dog study (EB-95-27), AF0150 at the dose of up to 1.6 mg/kg (6.9-fold PCD) had no changes on PAP within 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 the 1-minute post dosing observation period after AF0150 dose of up to 0.6 mg/kg (2.6-fold PCD). In a rabbit study (EB-98-13), AF0150 at the dose up to 10 mg/kg (26-fold PCD) did not change the pharmacologically-induced pulmonary hypertension during the 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.

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 suggestions were forwarded to the sponsor on April 10, 2000:

1. To ensure that enough microbubbles can be observed, AF0150 dose may need to be increased to approximately 10-fold of PCD.

2. 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.

3. A positive control (such as solid microspheres) needs to be included to validate the assay system.

4. Coadministration or pretreatment of the animals with pharmacological stress agents is suggested to test microbubble behavior in the presence of vasoconstriction and vasodilation.

5. 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 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 AF105 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 tracks 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 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.

Conclusion and Comments

1. 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.

2. AF0150 had no significant effects on arterial blood pressure, heart rate, cardiac output and pulmonary arterial pressure following IV administration at 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 hemodynamic parameters in animals treated with AF0150.

3. 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.

4. 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.

5. 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).

6. 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.

7. 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 observation period post dose.

8. 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).

9. The following studies are recommended to the sponsors:

   i. Blood gas analyses in non-anesthetized animals (dogs or monkeys) following IV injection of AF0150 at doses from 5 to 200 fold PCD.

   ii. Renal effects need to be further evaluated at lower AF0150 doses in order to achieve a **NOAEL**. Animal species besides rat may be considered, such as dog or monkey.

   iii. A microcirculation study needs to be conducted to evaluate AF0150 microbubble behavior and effects on blood flow and capillary endothelial cells. The AF0150 dosage should be high (10-fold or more) to ensure that enough microbubbles are being observed. 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 hyperlipidemic animal models.

   iv. The sponsor needs 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 setting.

28. TOXICOLOGY

The selected organs/tissues for histopathology examination performed in the single and multiple dose toxicity studies are listed in Table 1 of **Toxicology Summary** on page 141. The **NOAELs** from all toxicity studies are listed in Table 11 of **Overall Summary** on page 248.

Report Number: IMUS-037-TOX
Single Dose Intravenous Toxicity Study with AF0150 in Mice

Report Location:	Vol.010, p179-225
Report date:	February 5 1999
Study Facility:	[redacted]
In-life phase:	May 28 – June 11, 1998
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ16054 (400 mg/vial)
AF0150 Dosage (HDM):	0-1600 mg/kg (up to 1037-fold of PCD)

Specific Aim

To evaluate acute toxicity of AF0150 at a single dose IV injection in mice.

Methods

Animal Preparation: Mice, Hsd:ICR (CD-1) strain, were obtained from [redacted]. The mice were 27-41 days old and weighed 20-27.2 g at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The animals were acclimated for at least 7 days before the study, and 25 mice were randomly assigned into 5 groups (5/sex/group), as seen in Table 1.

Table 1. Acute Single Dose Toxicity Study in Mice

Group	AF0150 Dose* (mg/kg)	Number of Mice**		IV Volume (ml/kg)
		Male	Female	
1 (Control)#	0	5	5	40
2	200	5	5	5
3	400	5	5	10
4	800	5	5	20
5	1,600	5	5	40

* AF0150 was reconstituted in SWFI to a final concentration of 40 mg/ml.
** All animals were terminated on one time point (day 15).
\# Received 0.9% NaCl for Injection

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. Animals received 0 to

1,600 mg/kg AF0150 through IV injection into a lateral tail vein. Dose groups are shown in the Table 1.

Observation Parameters: Clinical signs were observed pre-dosing (on the day of dosing) and at 5, 30 minutes, 1, 2, 3, 4 hours on the day 1 post-dosing, followed by once a day for 14 days. Mortality was observed twice daily (AM and PM). Body weights were recorded on days 1, 8 and 15. All animals were subjected to gross necropsy on day 15 without histopathological examination. No statistical analysis was performed for any of the data.

Results

Mortality and Body Weights: All animals survived till the sacrificed day. There was no difference in body weight gain between AF0150 and control groups.

Clinical Signs: 2 males and 2 females at 1,600 mg/kg group showed transient hypoactivity at 5 minutes post dosing and returned to a normal appearance at 30 minutes. Otherwise, all animals appeared clinically normal during the 14-day observation period.

Gross Pathology: at necropsy, the cecums of 4 males at 1,600 mg/kg group and 2 males at 400 mg/kg showed diffusely thickened walls, containing multiple, clear, fluid-filled cysts of variable size. No gross change was found in other animals.

Discussion and Comments

1. IV injection of AF0150 up to 1000-fold of PCD had no significant toxicity in mice except transient hypoactivity and dyspnea in a few mice at the highest dose. Also cecal inflammation was found in some mice at 1,600 mg/kg and 400 mg/kg group, but no histopathological examination was followed. The **NOAEL** was 200 mg/ml (HED: 16 mg/kg and HDM: 130-fold) for cecal inflammation, 800 mg/kg (HED: 65 mg/kg and HDM: 518-fold) for transient hypoactivity.

2. Food consumption data was missing, and clinical signs were not specified, particularly for the pulmonary system.

3. Only one termination time point (on day 15) was used without an interim termination time. Any reversible toxic injury that may have occurred at the early time point may have been missed.

4. No histopathology, clinical pathology and hematology.

Report Number: IMUS-010-TOX
Intravenous Escalating Dose Toxicity Study with AF0150 in Rats

Report Location:	Vol.010, p226-343
Report date:	January 17, 1996
Study Facility:	[redacted]
In-life phase:	July 27-August 11, 1995
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ15031 (400 mg/vial)
AF0150 Dosage (HDM):	0-1600 mg/kg (up to 2073-fold of PCD)

Specific Aim

To assess the acute toxicity of AF1050 in rats with IV injection in escalating doses

Methods

Animal Preparation: male and female rats (30 each), Crl:CD (SD) BR VAF/Plus, were obtained from ' [redacted] The animals were 41-47 day-old and weighed 170.8-197.2 g (males) and 132.6-179.5 g (females) at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The rats were acclimated for 10 days before initiation of treatment. 25 rats from each sex (after exclusion of those with body weight exceeding ±2SD) were randomly assigned into 5 groups (5/sex/group), as seen in table 1.

AF0150 Preparation and Administration: AF0150 (400 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 40 mg/ml and used within 30 minutes. Animals received 0 (saline) to 1600 mg/kg AF0150 through IV injection into a lateral tail vein. Individual doses were calculated based on the day 1 body weight. Dose groups are shown in the Table 1.

Table 1. Acute Single Dose Toxicity Study in Rats

Group	AF0150 Dose* (mg/kg)	Number of Rats**		IV Volume (ml/kg)
		Male	Female	
1(Control)#	0	5	5	40
2(Low)	200	5	5	5
3(Low-Mid)	400	5	5	10
4(Mid)	800	5	5	20
5(High)	1,600	5	5	40

* AF0150 was reconstituted in SWFI to a final concentration of 40 mg/ml.
** All animals were terminated on one time point (day 15).
# Received 0.9% NaCl for Injection

Observation Parameters: mortality and toxicity signs were observed twice daily (AM and PM) for 14 days. On the day 1, animals were examined predose and at 5 minutes, 0, 5, 1, 2, 3, 4 hours

postdose for signs of toxicity. Body weight and food consumption were recorded weekly. The necropsy for macroscopic examination was performed at the end of the study (the day 16).

Results

Clinical Signs: all animals survived till sacrificed on day 16. At approximately 5 minutes postdose, red skin (lips, nose, ears, paws, and/or tail) was observed in one male and two females at 800 mg/kg and in all animals at 1600 mg/kg. At approximately 0.5 hour postdose, red skin (lips, nose, ears, and paws) was observed in three males at 1600 mg/kg. During subsequent observation intervals treatment-related effects were not noted.

Body Weight and Food Consumption: AF0150 at all doses had no effects on body weight gains and food consumption during the 15-day observation period.

Macropathology: observation at necropsy were not remarkable, including thoracic viscera.

Discussion and Comments

IV bolus injection of AF0150 at 200-400 mg/kg (130-260 fold PCD) had no significant toxicity in rats.

However, at the doses of 800 and 1600 mg/kg, a transient reddening (about 1 hour) of the lips, nose, ears, paw and tail was noted, suggesting effects of AF0150 on the microcirculation through vasodilation. The **NOAEL** was 400 mg/kg (HED: 65 mg/kg and HDM: 518-fold). The clinical observations may be due to direct effects of AF0150 on blood vessels and/or indirect effects of an anaphylactoid pathway (immune response and release of histamine or other mediators). No further information was provided to address the mechanism.

Body weight gain at 800 mg/kg in males was lower and had greater variation than the other groups. An appropriate explanation was not provided.

Only one termination time point (on day 15) was used without interim termination times. Any reversible toxic injury that may have occurred at the early time points may have been missed.

No histopathology, clinical pathology and hematology.

Report Number: IMUS-011-TOX
Single Dose Intravenous Toxicity Study with AF0150 in Rats

Report Location:	Vol.011, p001-276; vol.012 p001-326
Report date:	January 17, 1996
Study Facility:	
In-life phase:	August 14-28, 1995

GLP Compliance: Yes (with QA Statement)
AF0150 Lot number: ZZ15031 (400 mg/vial)
AF0150 Dosage (HDM): 0-400 mg/kg (up to 518-fold of PCD)

Specific Aim

To assess acute toxicity of AF0150 with single IV injection in rats

Methods

Animal Preparation: male and female rats (90 each), Crl:CD (SD) BR VAF/Plus, were obtained from [redacted]. The animals were 38-44 day-old and weighed 136.6-176.2 g (males) and 108.8-153.4 g (females) at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The rats were acclimated for 7 days before initiation of treatment. 80 rats from each sex (after exclusion of those with body weight exceeding ±2SD) were randomly assigned into 4 groups (20/sex/group), as seen in table 1.

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. Animals received 0 (saline), 50, 200, or 400 mg/kg AF0150 through an IV injection into a lateral tail vein. Individual doses were calculated based on the day 1 body weight. Dose groups are shown in the Table 1.

Observation Parameters: mortality and toxicity signs were observed twice daily (AM and PM) for 14 days. On the day 1, animals were examined predose and at 5 minutes, 0,5, 1, 2, 3 hours postdose for toxicity signs. Body weight and food consumption were recorded weekly. Blood samples were taken from the jugular vein on Days 2 and 8 (5 rats/sex/group), on Day 4 (10 rats/sex/group), and on Day 15 (all remaining rats) for hematology and clinical chemistry analysis. The necropsy was performed from 5 rats/sex/group on Days 2 and 8 and remaining rats on Day 15, including organ weights, macroscopic and histopathological examination (the selected organs/tissues were listed in Table 1 in the Toxicology Summary on page 141).

Table 1. Expanded Acute Toxicity Study in Rats

Group	AF0150 Dose* (mg/kg)	Number of Rats**		IV Volume (ml/kg)
		Male	Female	
1(Control)#	0	20	20	10
2(Low)	50	20	20	1.25
3(Mid)	200	20	20	5
4(High)	400	20	20	10

* AF0150 was reconstituted in SWFI to final concentration of 40 mg/ml.
** Sacrificed 5 rats/sex/group on Days 2 and 8, and 10 rats/sex/group on Day 15.
# Received 0.9% NaCl for Injection

Results

Clinical Signs: no overt signs of toxicity were noted after an IV injection of AF0150 at doses of 50-400 mg/kg. On Day 4, one male rat in the control group and 3 rats (1 male and 2 female) in 50 mg/kg AF0150 group, died following blood collection. The deaths were very likely related to the blood collection procedure but not to treatment. All other animals survived to the scheduled sacrifice.

Body Weight and Food Consumption: AF0150 treatment had no effects on body weight gain and food consumption.

Hematology and Clinical Chemistry: slight decreases in platelets, neutrophils, RBC, Hb, HCT, serum total protein and albumin, and slight increases in BUN, serum creatinine and inorganic phosphorous were reported in some of AF0150-treated rats. However, the changes in these parameters were independent of AF0150 doses, sex of animals and time post dosing, suggesting these findings were probably not related to AF0150 treatment.

Organ Weight: no significant change in the absolute weights and relative weights (organ-to-body weight and organ-to-brain) was found in most organs, including lung, heart and brain. The following slight changes were seen in AF0150-treated male rats on Day 15: decrease in the absolute weights and relative weights (organ-to-brain) of right adrenal gland, left kidney at the dose of 400mg/kg and increase in the absolute weight of liver at the dose of 50 mg/kg. These changes seem not to be related to AF0150 dose, sex and/or contralateral organs and thus may not be associated with AF0150 treatment

Pathology: there were no remarkable macroscopic changes associated with AF0150 treatment at all doses and time points. However, histopathologic observation showed that AF0150 induced infiltration of vacuolated macrophages in the spleen and mesenteric lymph nodes at all dose groups in a dose-dependent and time-dependent manner. The no observed effect level (NOEL) could not be estimated since the vacuolated macrophages were found in rats given 50 mg/kg, the lowest dose group in this study.

Discussion and Comments

IV injection of AF0150 at the doses of 50-400 mg/kg (65-516 fold of PCD) did not result in significant acute toxicity in rats. However, histopathologic examination showed that AF0150 induced macrophage vacuolation in the spleen and mesenteric lymph nodes at all dose groups in a dose-dependent and time-dependent manner. The fate of the vacuolated macrophages, particularly potential effects on macrophage function, needs to be further addressed. The issue was raised during pre-NDA meeting (July 1999). However, no appropriate response was provided in this NDA submission.

Unlike in mice, no cecal lesions were noted in rats treated with the same doses of AF0150 as in mice.

Report Number: IMUS-012-TOX
Single Dose Intravenous Toxicity Study with AF0150 in Dogs

Report Location:	Vol.013, p001-399
Report date:	January 30, 1996
Study Facility:	[redacted]
In-life phase:	August 7-21, 1995
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ15031
AF0150 Dosage (HDM):	0-200 mg/kg (up to 865-fold of PCD)

Specific Aim

To assess acute toxicity of AF0150 with single IV injection in dogs

Methods

Animal Preparation: male and female purebred beagles (24 each sex) were obtained from [redacted] The animals were 5.5-7 months old and weighed 6.2-9.9 kg at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The dogs were acclimated for 14 days before initiation of treatment, and randomly assigned into 4 groups (6/sex/group) (Table 1).

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. Animals received 0 (saline), 50, 100, 200 mg/kg AF0150 through IV injection into a cephalic vein at a rate of no more than 20 ml/min (to maintain the integrity of the suspended microbubbles). Individual doses were calculated based on the day 1 body weight. Dose groups are shown in the Table 1.

Table 1. Expanded Acute Toxicity Study in Dogs

Group	AF0150 Dose* (mg/kg)	Number of Dogs**		IV Volume (ml/kg)
		Male	Female	
1(Control)#	0	6	6	6
2(Low)	50	6	6	1.25
3(Mid)	100	6	6	2.5
4(High)	200	6	6	5

* AF0150 was reconstituted in SWFI to final concentration of 40 mg/ml.
** Sacrificed 2 dogs/sex/group on Days 2, 8, and 15.
# Received 0.9% NaCl for Injection

Observation Parameters: mortality and toxicity signs were observed twice (AM and PM) and once daily, respectively, for 14 days. On the day 1, animals were examined predose and at 0, 5, 1, 2, 3 and 4 hours postdose for toxicity signs. Body weights were recorded weekly and food consumption was measured daily beginning on Day -7 (predose). Blood samples were taken from

the jugular vein on Days 2, 8 and 15 (a sample on Day 4) for hematology and clinical chemistry analysis. Two dogs/sex/group on Days 2, 8, 15 were sacrificed for necropsy, organ weight measurement, and histopathology examination of most organs (the selected organs/tissues were listed in Table 1 in the Toxicology Summary on page 141).

Results

Clinical Signs: all animals survived to the scheduled sacrifice. The incidence of vomitus, excessive salivation, hypoactivity, and injected sclera increased in AF0150-treated dogs within the first 1 hour post dosing. These changes were resolved within 3 hours.

Body Weight and Food Consumption: there were no changes in body weight gains and food consumption in any of AF0150-treated dogs.

Hematology and Clinical Chemistry: slight decrease in hematocrit, PTT, RBC, serum Ca2+ and inorganic phosphorous and slight increase in PT, neutrophils, serum glucose and Na+ were observed in some animals without dose- or time-dependency.

Organ Weights: AF0150 treatment had no significant effects on absolute organ weights or relative organ weights (organ-to-body and organ-to-brain).

Pathology: Some histopathological changes in the liver were noted in dogs, particularly on day 2 (Table 2), which may be associated with AF0150 treatment. The main findings included moderate perivascular hemorrhage and congestion in the liver and at the serosal surface of the gallbladder, and pigment in Kupffer cells. Since no histopathology examination was performed on selected organs/tissues from 50 and 100 mg/kg AF0150 group, the **NOAEL** can not be determined.

Table 2. Liver Histopathological Findings (perivascular hemorrhage and/or congestion) in Dogs

Treatment	Day 2†		Day 8†		Day 15‡	
	Male	Female	Male	Female	Male	Female
Control	0/2*	0/2	2/2	1/2	2/2	2/2
200mg/kg AF0150	2/2	1/2	2/2	2/2	2/2	2/2

* two dogs/sex/group for examination.

† perivascular hemorrhage and congestion in the liver and at serosal surface of the gallbladder.

‡ congestion only in the liver.

Discussion and Comments

1. Hemorrhage in liver and gallbladder of some animals treated with AF0150 at the dose of 200 mg/kg (865-fold PCD) was observed on day 2 post dosing. Similar changes were noticed in the control animals and there were no corresponding serum AST and ALT increases in the animals. It is likely that these changes were incidental. However, the sample size was to small (only 2 dogs/sex/group) and animals in the lower dose groups (50 and 100 mg/kg) were not

examined, it is not possible to draw a conclusion and **NOAEL** from these data. Liver function needs to be closely monitored during clinical application of the agent.

2. PTT decrease, although no dose- or time-dependency, suggests that AF0150 may affect coagulation system, at least, transiently. Platelet function (bleeding time) and blood coagulation may need to be carefully monitored in clinical application.

3. Transient increase in the incidence of vomiting, excessive salivation, hypoactivity, and injected sclera was noted in AF0150-treated dogs. These changes occurred within first 1 hour post dosing and were resolved within 3 hours. No **NOAEL** was achieved for this finding.

4. No histamine or TxB2 was measured, so it is not possible to determine if the dogs showed an immune response to AF0150.

Report Number: IMUS-039-TOX
Single Dose Intravenous Toxicity Study with AF0150 in Dogs

Report Location:	Vol.014, p001-333
Report date:	January 10, 1999
Study Facility:	[redacted]
In-life phase:	June 30-July 14, 1998
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ16055 (400 mg/vial)
AF0150 Dosage (HDM):	0-400 mg/kg (up to 1731-fold of PCD)

Specific Aim

To assess acute toxicity of AF0150 with single IV injection in dogs

Methods

Animal Preparation: male and female purebred beagles (24 each sex) were obtained from [redacted] The animals were 4-5 months old and weighed 6.3-8.1 kg at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The dogs (17 each sex) were acclimated for 14 days before initiation of treatment, and randomly assigned into 4 groups (6/sex/group) (Table 1).

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. Animals received 0 (saline), 200, 400 mg/kg AF0150 by IV injection through an indwelling catheter into a cephalic vein. Individual doses were calculated based on the day 1 body weight. Dose groups are shown in the Table 1.

Table 1. Expanded Acute Toxicity Study in Dogs

Group	AF0150 Dose* (mg/kg)	Number of Dogs**		IV Volume (ml/kg)
		Male	Female	
1(Control)#	0	5	5	10
2(Low)	200	5	5	5
3(High)	400	5	5	10

* AF0150 was reconstituted in SWFI to final concentration of 40 mg/ml.
** Sacrificed 2 dogs/sex/group on Days 8 and 3 dogs/sex/group on Day 15.
# Received 0.9% NaCl for Injection

Observation Parameters: mortality and toxicity signs were observed twice (AM and PM) and once daily, respectively, for 14 days. On the day 1, animals were examined predose and at 5 and 30 minutes and 1, 2, 3 4 hours postdose for signs of toxicity. Body weights were recorded weekly and food consumption was measured daily beginning one week prior to dosing. Blood samples were taken [*sources and sites, vein or artery, were not specified*] on Days –6, 2, 4, 7, and 14 for hematology and clinical chemistry analysis. Urine samples were collected on Days –6, 7 and 14 for urinalysis. Two dogs/sex/group on Day 8 and 3 dogs/sex/group on Day 15 were necropsied, including macroscopic examination and organ weight measurement. Histopathological examination was performed in animals from the control and high dose groups (see Summary Table for selected organs).

Results

Clinical Signs: All animals survived to scheduled sacrifice. The incidence of pale gum, vomitus, excessive salivation, ocular discharge, and injected sclera increased in AF0150-treated dogs within the first 30 minutes post dosing. These reactions were no longer evident by 3 hours post dosing.

Body Weight and Food Consumption: there were no changes in body weight gain and food consumption in any of the AF0150-treated dogs.

Hematology and Clinical Chemistry: AF0150 treatment transiently induced a slight decrease in platelet counts for both sexes in the 400 mg/kg group on Day 2 only, and slightly increased serum triglycerides on Day 2 and ALT activity on Days 2 and 4 in male dogs given 400 mg/kg. All these changes appeared not to be correlated with clinical and pathological findings.

Urinalysis: Urine glucose, ketone, blood, urobilinogen and bilirubin (measured with the urine chemistry [redacted] and urine sediment were analyzed. Data from individual animals for all these parameter were provided, however, only urine volume, specific gravity (SP GR) and urine pH data were summarized. It seems that all observations were not remarkable.

Organ Weights: There were no significant changes in absolute organ weights and relative organ weights (organ-to-body and organ-to-brain) in AF0150-treated dogs.

Pathology: Some animals in both control and 400 mg/kg AF0150 groups on Days 8 and 15 showed some background microscopic changes, including pituitary cysts, thyroid C-cell hyperplasia, renal tubule regeneration, chronic liver inflammation, renal pelvis mineralization, lung and kidney lymphocytic infiltrates. The incidence and severity of these findings appeared not to be significant different between control and AF0150 treatment. The morphology of the testes and ovaries from all animals (both control and AF0150-treated) was consistent with sexual immaturity.

Discussion and Comments

IV injection of AF0150 transiently and slightly decreased blood platelets at the dose of 400 mg/kg, otherwise, no significant toxicity was found at doses of 200 and 400 mg/kg (865-1731 fold of PCD) in both male and female dogs. Closely monitoring platelet counts and function during clinical trials is still recommended.

The incidence of pale gum, vomiting, excessive salivation, ocular discharge, and injected sclera transiently increased in AF0150-treated dogs. These reactions occurred within the first 30 minutes post dosing and resolved at 3 hours post dosing. No **NOAEL** was achieved. The clinical signs in this study were consistent with those reported for study IMUS-012-TOX.

Report Number: IMUS-013-TOX
28-Day Repeated Dose Intravenous Toxicity Study with AF0150 in Rats

Report Location:	Vol.015, p001-305; vol.016, p001-361
Report date:	February 2, 1996
Study Facility:	[redacted]
In-life phase:	August 29 – October 11, 1995
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ15031, ZZ15032 (400 mg/vial)
AF0150 Dosage (HDM):	0, 50, 200, 400 mg/kg/day x 29 days (0-516 fold PCD)

Specific Aim

To assess toxicity of AF0150 with 29 daily IV injection in rats followed by a 14-day recovery period.

Methods

Animal Preparation: male and female rats (95 each), Crl:CD (SD) BR VAF/Plus, were obtained from [redacted] The animals were 39-45 days old and weighed 151.7-189.1 g (males) and 149.4-199.0 g (females) at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The rats were acclimated for 8 days before initiation of treatment and, after exclusion of those with body weight exceeding ±2SD), randomly assigned into 4 groups (20/sex/group), as seen in table 1.

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. Animals received 0 (saline), 50, 200, or 400 mg/kg AF0150 through IV injection. Individual doses were calculated based on the most recent recorded body weight. Dose groups are shown in the Table 1.

Observation Parameters: toxicity signs were observed daily. Body weigh and food consumption were recorded weekly. Ophthalmic examination was performed before initiation of treatment and during week 4 of treatment. Blood and urine samples were collected on Days 18 and 44 (5 rats/sex/group) and on Days 30 or 31 (10 rats/sex/group) for hematology, blood chemistry and urinalysis. At each scheduled sacrifice (as indicated in Table 1), animals were necropsied for macroscopic examination including measurement of organ weights and histopathology.

Table 1. Repeated Dose Toxicity Study in Rats

Group	AF0150 Dose* (mg/kg/day)	IV Volume (ml/kg/day)	Number of Rats**	
			Male	Female
1(Control)#	0	10	20	20
2(Low)	50	1.25	20	20
3(Mid)	200	5	20	20
4(High)	400	10	20	20

* AF0150 was reconstituted in SWFI to final concentration of 40 mg/ml.

** 5 rats/sex/group were sacrificed on day 17; 10 rats/sex/group were sacrificed on day 29 or 30; 5 rats/sex/group were treated daily for 29 days and then sacrificed after 15-day recovery period.

Received 0.9% NaCl for Injection (10 ml/kg).

Results

Clinical Signs: all animals survived to scheduled termination. There were no remarkable toxic signs associated with AF0150 treatment.

Body Weight and Food Consumption: AF0150 treatment had no significant effects on body weight and food consumption in any of the animals.

Ophthalmology: no remarkable ophthalmic changes were observed pre- and week 4 post-AF0150 treatment.

Clinical Chemistry: on Day 30/31, the following changes were observed and were statistically significant: lower blood creatinine in male rats for all doses; lower blood total protein for male and lower globulin for males and females at the doses of 200 and 400 mg/kg/day; lower aspartate aminotransferase for males given 400 mg/kg/day and females at all dose levels; lower alanine aminotransferase for males and females given 200 or 400 mg/kg/day, and lower alkaline phosphatase for males at all dose levels. After a 15-day recovery period, alanine aminotransferase remained decreased in females given 200 and 400 mg/kg/day. On Day 18, statistically significant

decreases in blood urea nitrogen and alanine aminotransferase were shown in females given 200 and 400 mg/kg/day.

Hematology and Urinalysis: Hematology and urinalysis were not remarkable.

Organ Weight: there was no significant organ weight changes on Day 18. But on Day 30/31, liver and spleen weights significantly increased in female rats given 200 and 400 mg/kg/day and the spleen weight continued to increase at the end of 15-day recovery period in rats treated with 400 mg/kg/day. Although the weights of the liver and spleen were not significantly increased on day 30-31 in the male rats given AF0150, there appeared to be a trend toward increased spleen weight.

Histopathology:

1. Vacuolated macrophages were observed in the followed organs on Days 18 and 30/31 without significant reduction after the 14-day recovery period.

- spleen and mesenteric lymph node of animals (both sexes) given the AF0150 at all dose levels with the higher incidence and severity at the dose of 400 mg/kg/day;
- Liver Kupffer cells in some animals (both sexes) given 200 mg/kg/day and most animals given 400 mg/kg/day;
- Thymus, bronchial-associated lymphoid tissue (lung), adrenal medulla and glomeruli of some animals given 400 mg/kg/day;
- Uterus and cervix of females given 200 and 400 mg/kg/day;
- Lymph nodes in mammary gland;
- Negative vacuolation in bone marrow cells;

2. Eosinophil infiltrates in mesenteric lymph nodes and perivascular eosinophil infiltrate in the lungs increased in males and females given 200 and 400 mg/kg/day on Days 18. On Day 30/31, eosinophil infiltrate was shown only in mesenteric lymph nodes. These changes decreased after the recovery period. **NOAEL** was 50 mg/kg/day (HED: 8 mg/kg/day; HDM: 65-fold)

3. Increase in extramedullary hematopoiesis in the spleen was observed with a dose-dependence in males, and at 400 mg/kg/day in females on Day 18. Only males showed increased extramedullary hematopoiesis at 200 or 400 mg/kg/day. These change slightly increased after recovery period.

Discussion and Comments

1. AF0150 at all dose levels induced macrophage vacuolation in multiple tissues in a dose-dependent manner on Day 30 and at the end of a 15-day recovery period. The organs/tissues rich in monocytes/macrophages showed the highest incidence of vacuolated macrophages, such as in spleen and lymph nodes, but not in bone marrow. Eosinophil infiltrates in

mesenteric lymph nodes and perivascular eosinophil infiltrate in the lungs increased in males and females, and these changes decreased after the recovery period. Increase in extramedullary hematopoiesis in the spleen was observed at 200 or 400 mg/kg/day. These changes slightly increased after the recovery period. **NOAEL** for both effects was 50 mg/kg/day (HED: 8 mg/kg/day; HDM: 65-fold).

2. It is not clear if the source of vacuolation macrophages in tissues was from vacuolated circulating monocytes. if the macrophages in tissues take up the microbubbles, the sponsor did not discuss how the microbubbles cross the endothelium.
3. The nature of the vacuole in the macrophages was not described. It is unclear if the vacuole represent phagocytosed microbubbles.
4. The fate of the vacuolated macrophages and long-term effects of the vacuolation on macrophage function needs to be addressed, such as phagocytosis, cytokine production, antigen presentation; and host resistance to bacterial challenge (macrophage/monocytes-mediated defense)
5. Potential effects on atherosclerotic lesions of vascular wall were not discussed. The vacuolated macrophages may play an important role in worsening or promoting the plague to detach remote areas.
6. Most blood chemistry parameters with AF0150-related changes were associated with liver function. Although increases rather than decreases in these parameters are generally related to organ acute toxicity (increased or destroyed cell membrane permeability), chronic toxicity to the liver may decrease liver function (without significantly affecting the cell member intact) and thus lower liver enzyme markers in the blood. Increase in the liver weight could be supportive of a chronic injury process. Together with positive findings in the liver pathology from the single dose toxicity study in dogs (IMUS-012-TOX), the liver function needs to be closely monitored in clinical application of AF0150.
7. There were no cecal lesions found in rats treated with multiple doses of AF0150 from this study.

Report Number: IMUS-027-TOX
One Week Intravenous Toxicity Study with AF0150 in Dogs

Report Location:	Vol.017, p001-055
Report date:	June 13, 1996
Study Facility:	[redacted]
In-life phase:	June 15-June 22, 1995
GLP Compliance:	No
AF0150 Lot number:	SD-18APR95-A (200 mg/vial)
AF0150 Dosage (HDM):	10 mg/kg/day x 7 days (up to 43 fold PCD)

Specific Aim

To assess toxicity of AF0150 with 7 daily IV injection in dogs.

Methods

Animal Preparation: six Beagle dogs (3 each sex) were obtained from [redacted] The animals were 8 months old with body weight of 12.1-15.9 kg at initiation of treatment. Routine procedures were followed for housing, handling, feeding and care of the animals. The dogs were acclimated for 14 days before initiation of treatment.

AF0150 Preparation and Administration: AF0150 (200 mg fill) was reconstituted with 10 ml SWFI to a final concentration of 20 mg/kg. Animals received a daily injection of 10 mg/kg AF0150 (0.5 ml/kg) by IV injection [*name of the vein was not specified*] for 7 days without control groups.

Observation Parameters: clinical signs and mortality were recorded daily and the first 30 minute after dosing on Day 1. Body weight data were collected on Days –13, -7, 0 and 7 (day of necropsy). Blood samples (3 ml each time) were taken from the jugular vein on Days –2, 0 (10 and 60 minutes after the end of dosing), 1, 2, 3, 4, 5, and 6 (before and 10, 60 minutes after dosing), and 7 for hematology (without blood chemistry). Urine samples were collected from the bladder at necropsy (Day b7) for urinalysis. Macroscopic examination was performed at necropsy together with organ weight measurement. Selected organs, as shown in summary table, were subjected to histopathological examination.

Results

Clinical Signs: All animals survived to scheduled sacrifice. On Day 0, 1, 2, and 3, one male dog (#3) showed moderate paleness of mucous membranes and staggering gait that began 11 minutes and ended 20 minutes after dosing. On day 3, one female dog (#4) exhibited increased pulse from 5 to 10 minutes after dosing.

Body Weight: body weights were not changed as compared with pretreatment data. However, it is impossible to conclude if AF0150 treatment affected body weight because of the lack of a control group.

Hematology: one male dog (#3) showed decrease in WBC and platelets consistently 10 minutes after dosing and returned to "normal" level. Similar effects were noted in another male dog (#2) but only on the first day. The other animals had no remarkable changes. Slight decreases in RBC, Hb and hematocrit were noted and attributed to the drawing of blood.

Urinalysis: unremarkable. However, there was no control and no normal values for dogs were provided.

Organ Weights: no meaning information could be elucidated from the organ weight data because of lack of control or pretreatment data.

Pathology: No macroscopic findings were noted. However, Toxocara canis were present in the jejunum in 5 of 6 dogs. This is harmless to adult dogs and is not considered to have affected the outcome of the study. The presence of Toxocara canis is reported to be responsible for the microscopic changes such as subacute alveolitis, alveolar hemorrhage and interstitial pneumonia seen in most of the dogs. This was attributed to larval migration caused by the Toxocara canis. Other microscopic changes included perivascular hemorrhage and accumulation of neutrophils at the injection site.

Discussion and Comments

The study was poorly designed due to lack of control groups, limited pretreatment information, and only one dose. However, based on the time-course of some parameters during the 7-day observation period, 10 mg/kg AF0150 daily IV administration to dogs for 7 days appeared not to cause significant toxic effects. The changes in WBC and platelets in one male dog (#3) corresponded to clinical signs in the same dog, which may be due to a general health problem and may or may not be related to AF0150 treatment.

Report Number: IMUS-014-TOX
28-Day Repeated Dose Intravenous Toxicity Study with AF0150 in Dogs

Report Location:	Vol.017, p056-055; Vol.018, p001-269
Report date:	January 30, 1996
Study Facility:	[redacted]
In-life phase:	September 1-October 13, 1995
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	ZZ15033, ZZ15034, ZZ15035, ZZ15038
AF0150 Dosage (HDM):	0, 25, 50, 100 mg/kg/day x 28 days (0-433-fold PCD)
Specific Aim	

To assess toxicity of AF0150 with 28 daily IV injection in dogs followed by a 14-day recovery period

Methods

Animal Preparation: male and female purebred Beagle dogs (32 each sex) were obtained from [redacted] The animals were 6 to 7.5 months old and weighed 5.9-12.2 kg at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The dogs were acclimated for 17 days before initiation of treatment, and randomly assigned into 4 groups (8/sex/group), as seen in Table 1.

AF0150 Preparation and Administration: AF0150 was reconstituted with 10 ml SWFI to a final concentration of 40 mg/kg and used within 30 minutes. Animals received 0 (saline), 25, 50, 100

mg/kg AF0150 by IV injection into a cephalic or saphenous vein at an approximate rate of no more than 20 ml/min. Dose groups are shown in the Table 1.

Table 1. Repeated Dose Acute Toxicity Study in Dogs

Group	AF0150 Dose* (mg/kg/day)	Number of Dogs**		IV Volume (ml/kg/day)
		Male	Female	
1(Control)#	0	8	8	2.5
2(Low)	25	8	8	0.625
3(Mid)	50	8	8	1.25
4(High)	100	8	8	2.5

* AF0150 was reconstituted in SWFI to final concentration of 40 mg/ml.

** Three dogs/sex/group were sacrificed on Days 14 and 28, respectively, and 2 dogs/sex/group were treated for 28 days followed by 14-day recovery period and then sacrificed.

Received 0.9% NaCl for Injection

Observation Parameters: toxicity signs were observed twice (AM and PM) and once daily (pre-dosing). Each animal was observed on Days 1, 7, 14, and 28 at 15 minutes, 1 and 4 hours post-dosing. Body weights were determined weekly before initiation of treatment, on the first day of treatment and weekly thereafter. Food consumption was recorded daily. Ophthalmic examination were performed once before the initiation and once during week 4 of treatment. Blood pressure was measured between 5 and 10 minutes post dosing on Day 1 and once during Weeks 2 and 4. Blood and urine samples were taken on Days –10, 15, 29 and 43 of treatment for hematology, blood chemistry and urinalysis. All animals were subjected to necropsy. Selected organs/tissues (as seen in summary table) were removed and weighted followed by histopathological examination.

Results

Clinical Signs: all animals survived to the scheduled termination. Possible AF0150 treatment-related effects included pale mucous membrane, uncoordinated and/or hypoactive behavior and vomitus in some animals, as seen in Table 2. The changes in the mucous membranes and behavior generally appeared within 15 minutes and resolved within 4 hours after the administration of AF0150 on Day 1. These changes were not noted on other days during the study except for a single incidence each of pale mucous membrane and hypoactivity on Day 14. Vomiting was observed sporadically throughout the study.

Body Weight and Food Consumption: AF0150 treatment had no significant effects on body weights and food consumption.

Ophthalmic Examination: unremarkable.

Table 2. Clinical Observation

Transient Toxic Signs (not seen in control)	AF0150 Dose (mg/kg/day)		
	25	50	100
Pale mucous membranes	1/6	2/16	4/16
Uncoordinated and/or Hypoactive behavior	1/16	1/16	2/16

Blood Pressure: Systolic, diastolic and mean arterial blood pressures were significantly lower in female dogs 5 min after the administration of 100 mg/kg of AF0150 on Day 1 as compared to saline controls. Mean arterial pressure was reduced by 42% to an average of 69 mmHg. Although the changes in blood pressure in male dogs did not attain statistical significance, there appeared to be a trend toward lower blood pressure after the administration of AF0150 on Day 1. AF0150 did not affect blood pressures when measured during weeks 2 and 4 of the study. Mean blood pressure significantly increased by 22% ($p<0.05$) in males given 50 mg/kg on Week 2, but not seen in any other group and other time points.

Hematology, Clinical Chemistry and Urinalysis: decreases in serum globulin (by 17% in the 25 and 50 mg/kg groups on day 29, $p<0.05$) and total protein (by 8%, $p<0.05$) were noted in female dogs treated with AF0150 without dose- or time-dependency. Serum cholesterol decreased in female on days 15 and 29 in a dose dependent manner (by 10-19%, $p<0.05$). The NOAELs could not be achieved because the decrease was seen in the lowest dose group. During recovery period, the differences appeared reversed slightly but not to control levels. Hematology and urinalysis were not remarkable.

Organ Weight: absolute (about 2-fold, $p<0.05$) and relative (about 1.5-fold, $p<0.05$) spleen weights increased in some AF0150-treated groups without dose- and time-dependency and did not correlate with microscopic findings. No changes were observed during the recovery period.

Histopathology: Microscopic examination was not remarkable. Vacuolated macrophages were not found in spleen, lymph nodes and any other tissues as noted in the rat study. There were chronic inflammatory reactions at the injection sites, such as perivascular hemorrhage and fibrosis of blood vessel walls in both control and AF0150-treated dogs. Therefore, these changes were likely attributed to mechanical trauma associated with multiple injections.

Discussion and Comments

Twenty-eight daily IV injection of AF0150 at dose up to 400-fold the planned clinical dose in dogs had a transient and reversible toxic effects characterized by pale mucous membrane, uncoordinated and hypoactive behavior and vomiting, lower serum protein and cholesterol. These effects were not dose- or time-dependent. These changes were not correlated with Histopathological examination. No vacuolated macrophages were found in any of the treated groups. Mean blood pressure decreased by 42% in female dogs 5 minutes post dosing at 100 mg/kg/day of AF0150 on Day 1. Mean blood pressure significantly increased by 22% in males

given 50 mg/kg on Week 2, but this was not seen in any other group. No cecal lesion was found in any animals.