the same injection rates. Baseline Doppler and video intensity signals were recorded prior to each injection of AF0150.

Echocardiography and Doppler signal analysis: Fundamental (2.5 MHz) and second harmonic (5 MHz) ultrasound images of the left ventricular cavity and myocardium (septum, inferior, lateral and anterior wall) were obtained using a and were analyzed using a videodensitometry software system. Doppler signals, expressed as RMS values, were analyzed to evaluate the peak and persistence of contrast enhancement. RMS values were sampled at a rate of one per second for 15 seconds prior to and through 5 minutes after the onset of AF0150 infusion.

Results

Doppler Signal Enhancement: both bolus and infusion injections of AF0150 induced a dose-dependent increase in Doppler signal. The peak signals were reached 20-40 seconds after the bolus injection or the 1-minute infusion.

Fundamental Imaging (Continuous) (Figure 1): all 3 doses of AF0150 administrated as a bolus or infusion resulted in increase in video intensity of the left ventricular myocardium at 15 seconds after AF0150 injection. The signal enhancement had no significant dose-dependence.

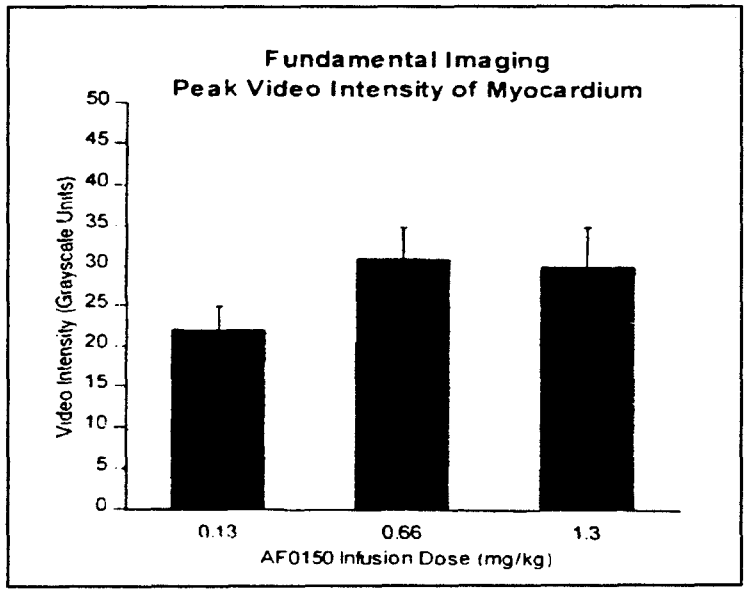

Figure 1. Peak video intensity of the LV myocardium after infusion of AF0150 (continuous fundamental ultrasound imaging). Videointensity values are background-subtracted and averaged from septum, lateral, and anterior wall regions of the LV myocardium. Data are Mean ± SEM (n=6).

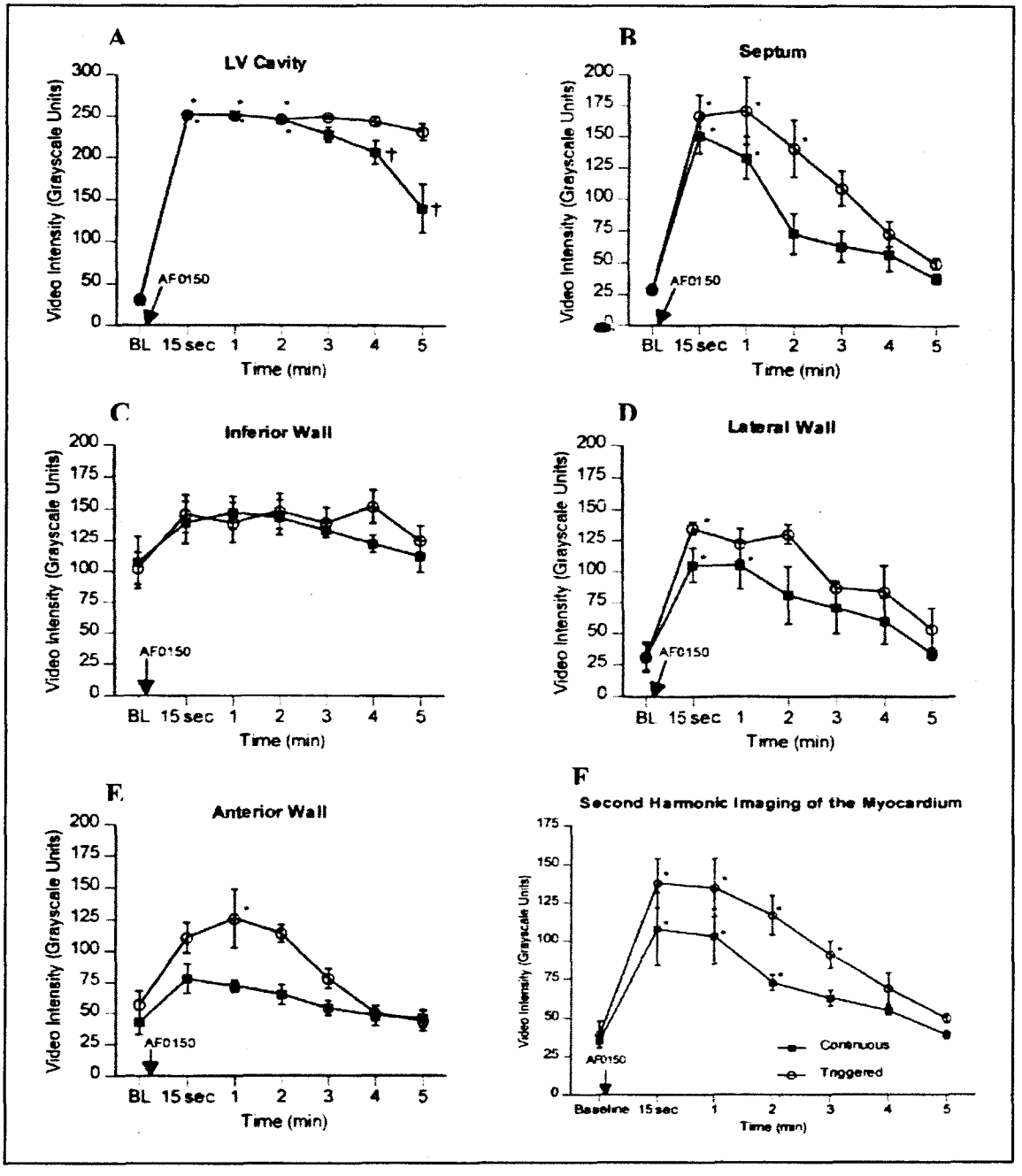

Figure 2. Videointensity of the LV cavity and myocardium with continuous or triggered second harmonic imaging after IV infusion of AF0150 at 0.66 mg/kg. *Panel A*, LV cavity; *Panel B*, Septum; *Panel C*, inferior wall; *Panel D*, Lateral wall; *Panel E*, Anterior wall and *Panel F*, summarized and averaged from the septum, lateral and anterior walls of the left ventricular myocardium. Data are Mean ± SEM (n=3). * $p<0.05$, difference from baseline in the respective groups. † $p<0.05$ difference from the triggered group at the indicated time point.

Second Harmonic Imaging (Triggered and Continuous) (Figure 2): both continuous and triggered application of ultrasound power significantly increased peak video intensity values 15 seconds post-AF0150 injection and contrast enhancement persisted for 3 to 4 minutes. There were no statistically significant differences in video intensity values of the LV myocardium between continuous and triggered ultrasounds, although the triggered harmonic imaging tended to result in better and prolonged myocardial opacification. Regional heterogeneity (in 4 regions of the left myocardial wall) in myocardial opacification was shown with both imaging modalities.

Discussion and Comments:

1. IV injection of AF0150 (either bolus or infusion) results in enhancement in fundamental and second harmonic ultrasound imaging of left ventricular cavity and myocardium. Triggered ultrasound settings showed better enhancement than continuous ultrasound due to probably ultrasound power-induced microbubble destruction.
2. Myocardial imaging enhancement at 0.66 mg/kg was slightly greater than at 0.13 mg/kg but no further improvement at 1.3 mg/kg. Therefore, to obtain better AF0150 dose-response of echocardiography, the lower dose groups need to be designed.
3. The study did not indicate whether LV cavity imaging enhancement was dose-dependence or not.
4. There was regional heterogeneity in myocardial imaging, and different signal kinetics, which is important for the clinical interpretation of ultrasound images.

Report Number: EB-95-25

Dose-response characteristics of AF0150 in dogs with normal myocardial perfusion: hemodynamic effects and echocardiographic imaging. Pilot study of AF0150 efficacy with abnormal myocardial perfusion.

Report Location:	Vol.009, p123-135
Report date:	May 24, 1999
Study Facility:	[redacted]
In-life phase:	October 1995
GLP Compliance:	No
AF0150 Lot number:	ZZ15036

Specific Aim

To determine the optimal dose and the efficacy of AF0150 for the determination of myocardial imaging in a dog with normal and abnormal (occlusion and reperfusion of coronary artery) myocardial perfusion. Also, to assess the effects on hemodynamics of bolus injections of AF0150 at various doses.

Methods

Animal preparation: Six mongrel dogs were anesthetized with phenobarbital, intubated and ventilated with room air. The left femoral artery was cannulated for the monitoring of systemic artery pressure and heart rate. A *Swan-Ganz* catheter was placed via the jugular vein for monitoring of pulmonary artery pressure and thermodilution determination of cardiac output. A Doppler flow wire was inserted in the right carotid artery to identify the coronary vessel to be occluded, and the right femoral vein was cannulated for the administration of AF0150. All animals received heparin (4000 to 5000 units) to prevent blood clotting.

Occlusion and Reperfusion: In 3 of the 6 animals studied, a pulmonary transluminal catheter for angioplasty (PTCA) was placed via a coronary guide catheter into either the left anterior descending or circumflex vessels (LAD or LCX, respectively) under fluoroscopic guidance. Echocardiographic image data were collected continuously, from multiple views, throughout the occlusion and reperfusion portion of the study. Ultrasound imaging was performed at the powers and applications described in Table 1 (random order). All 3 animals received 3 bolus injections of AF0150 (predetermined optimal dose of 0.3 or 0.6 mg/kg) as follows: prior to occlusion, during occlusion, and during reperfusion. The duration of the occlusion and reperfusion periods varied slightly, as the primary objective of this portion of the study was to observe the AF0150 image during periods of "flow" and "no flow." At the completion of imaging, the PTCA balloon was again inflated and gentian violet (1 ml) was injected into the lumen. All animals were sacrificed and the hearts were removed, perfused with a formalin solution, and subsequently transected into representative short axis planes and photographed. A pathological examination of the hearts was conducted to confirm the areas of occlusion and perfusion defect.

AF0150 Administration: AF0150 (200 mg fill per vial) was reconstituted in 10 ml SWFI to a final concentration of 20 mg/ml. Each dog received bolus injections of AF0150 at doses of 0.03, 0.09, 0.3, and 0.6 mg/kg, with 4 to 14 injections/dose (with exception of one dog which received only 2 injection at 0.03 mg/kg) and approximately 5 minutes apart from low to high doses. Total 20 to 40 separate AF0150 injections for all doses were given to each animal.

Observations: Transthoracic fundamental (2-D; 2.5 MHz) and second harmonic (5.0 MHz) ultrasound imaging of the heart was performed at closed-chest using [redacted] and [redacted] ultrasound machines. Machine settings were adjusted to maximal dynamic range and linear processing algorithms, as shown in Table 1. After initial adjustment of machine settings for optimal visualization, gain settings were maintained constant throughout the study (dose optimization and occlusion and reperfusion protocols).

Hemodynamic parameters were continuously monitored throughout the study. Specifically just prior to and 1 minute after AF0150 injection, recordings were made for systolic and diastolic systemic and pulmonary artery pressures and heart rate. Cardiac output was determined at baseline (prior to any AF0150 injection) and periodically between changes in AF0150 dose level. Data were converted to the mean arterial blood pressure by adding one-third of the difference between the systolic and diastolic pressure values to the diastolic pressure value.

Table 1. Ultrasound machines and setting

Settings	[redacted]	[redacted]
Imaging	Fundamental (2.5 MHz) and second harmonic (2.5/5.0 MHz)	Fundamental (3.5 MHz)
Power	12.5% power: -9dB 100% power: 0dB	~1% of maximal power
Mode	Continuous and triggered	Continuous and triggered

Results

Hemodynamics: hemodynamic baseline (prior to AF0150 injection) were in a normal physiological range for the anesthetized dogs: systemic artery pressure = 116±6 mmHg; pulmonary artery pressure = 11±1 mmHg; heart rate = 137±6 bpm; and cardiac output = 4.4±0.4 L/min. There were no notable changes in mean systemic artery pressure, mean pulmonary artery pressure, or heart rate associated with injection of AF0150 at any of the doses tested (0.03, 0.09, 0.3, 0.6 mg/kg) and sustained application of ultrasound power. Hemodynamic changes after AF0105 injections are summarized in Table 2. Cardiac output was measured periodically and did not change appreciably during the course of the study (3.9±0.7 L/min at end of study).

Echocardiography: AF0150 induced dose-dependent increase in video intensity of the myocardium but dose-independent increase in video intensity of LV cavity. The optimal dose for myocardial imaging was 0.3-0.6 mg/kg but could not be determined for LV cavity imaging (because of a lack of dose-response). Triggered application of ultrasound power provided better myocardial imaging contrast than continuous ultrasound. AF0150 injection (0.3 or 0.6 mg/kg) improved identification of areas of nonperfused myocardium (wall motion abnormality) which were difficult to determine with noncontrast ultrasound. The nonperfused myocardial region observed on the ultrasound imaging was correlated with pathology finding (infarct area).

Table II. Hemodynamic Changes with AF0150 Doses

Hemodynamic Parameter * **Δ Change**	**AF0150 Dose** (mg/kg) **0.03**	**0.09**	**0.3**	**0.6**
Number of Injections (total for 6 dogs)	36	46	52	39
Mean Arterial Pressure (mm Hg)	0.8 ± 0.8	0.4 ± 1.0	1.5 ± 0.9	2.0 ± 0.7
Mean Pulmonary Pressure (mm Hg)	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.2
Heart Rate (bpm)	0.1 ± 0.5	0.0 ± 0.3	0.0 ± 0.3	−1.0 ± 0.3

* For all of the hemodynamic parameters, baseline (immediately before injection) values were subtracted from the 1-minute post-injection values.

Discussion and Comments

1. Results suggest that in dogs AF0150 at doses of 0.03-0.6 mg/kg with closed-chest heart imaging enhanced ultrasound cardiac imaging with a dose-dependence for myocardium but without a dose-dependence for LV cavity. AF0150 at 0.3 and 0.6 mg/kg also allowed myocardial perfusion determination.

2. No significant hemodynamic changes were noted after AF0150 administration. However, only 1-minute post-dose data were provided, as seen in Table II. Hemodynamic observations at other time points post-dosing (till the end of the studies) were missing, particularly in the occlusion/reperfusion model (with or without AF0150 injections). The lack of hemodynamic changes following coronary artery occlusion made this model less valuable for hemodynamic evaluation.

Report Number: RMI-97-01
Doppler Signal Analysis: A customized Fortran Program

Report Location:	Vol.009, p136-139
Report date:	Jan 29, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	Jan 1997
GLP Compliance:	No
AF0150 Lot number:	N/A

Specific Aim

To design Fortran program [redacted] for Doppler signal enhancement and signal persistence analysis.

Methods/Results

Doppler signal, in mV expressed as the Root Mean Square (RMS) of the signal (RMS of 8000 data points over 200 sec), were recorded from carotid artery blood flow [*reviewer comments: data source (animal, AF0150 administration, date, etc) was not specified*]. RMS values were sampled at a rate of one per second using an [redacted]. A custom data acquisition application [redacted] was created to control data sampling from the oscilloscope.

Output from the above process is reformatted using a customized Fortran program [redacted]

1. Convert Doppler data from RMS values to mV values
2. Calculate time relative to AF0150 injection
3. Output the data in a column-delimited format with the following column headings: Sample Number; Time post-injection (seconds); and Doppler Signal (mV)

The RMS ratio is calculated using the following formula:

$$\text{RMS ratio} = (RMS_t - RMS_{bl})/RMS_{bli}$$

Where RMS_t: RMS value at time t (t=0-500 seconds postinjection)
RMS_{bl}: preinjection baseline for each specific injection
RMS_{bli}: initial baseline prior to any injections.

Doppler signals (in mV) are averaged over the following "Block Intervals" from zero: 20-40 sec; 60-80 sec; 100-120 sec; 130-150 sec; and a signal value is reported for the each time interval.

Discussion and comments

The sponsor did not indicate if this program applied to Doppler signal analysis in both pre-clinical and clinical studies. Also, statistician needs to be consulted for adequacy of the program and the calculation.

Report Number: RMI-97-02
Doppler Power Resistance Analysis: A customized Fortran Program

Report Location: Vol.009, p140-144
Report date: Jan 29, 1999
Study Facility: Alliance Pharmaceutical Corp
In-life phase: May 10 1997
GLP Compliance: No
AF0150 Lot number: N/A
Specific Aim

To design a Fortran program [redacted] for analysis of the Doppler signal's resistance to ultrasound power (calculation of the signal decrease ratio).

Methods/Results

Doppler signal, in mV expressed as the Root Mean Square (RMS) of the signal (RMS of 8000 data points over 200 msec), were recorded from carotid artery blood flow [*reviewer comments: data source (animal, AF0150 administration, date, etc) was not specified*]. RMS values were sampled at a rate of one per second using an [redacted] A custom data acquisition application [redacted] was created to control data sampling from the oscilloscope.

Output from the above process is reformatted using a customized Fortran program [redacted]

1. Convert Doppler data from RMS values to mV values
2. Calculate time relative to AF0150 injection

3. Output the data in a column-delimited format with the following column headings: Sample Number; Time post-injection (seconds); and Doppler Signal (mV)
4. Calculate linear fits to pre-ultrasound burst and ultrasound burst segments
5. Calculate monoexponential fit to the post-ultrasound burst segment

From these linear fits, signal values are extrapolated to various time intervals. For example, in the case of a ultrasound power burst that is applied 60 seconds after the injection of AF0150, for a duration of 60 seconds, Doppler signal values are extrapolated to the 60 second time point. From the extrapolated value, a formula is used to calculate the signal decrease ratio (i.e., the signal drop, S, as a percentage of the total signal):

$$S_{60} = (S_{60pre} - S_{60burst})$$

$$\text{Signal Decrease Ratio} = S_{60}/S_{60pre}$$

Discussion and comments

The "extrapolated" is not appropriate or misused for this scenario because there was nothing extrapolated from the raw data. Rather were obtained the time values from the X-axis (time, seconds). The linear fitting was just helpful for better reading, not extrapolation. The sponsor did not clarify if this program applied to Doppler signal analysis in both pre-clinical and clinical studies. Also, statistician needs to be consulted for adequate of the program and the calculation.

Report Number: PSM-97-04

Effects of AF0150 Fill Size and Constituted Concentration on Echocardiographic Imaging in a Swine Model: A Qualitative Assessment

Report Location:	Vol.009, p145-146
Report date:	April 15, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	July, 1997
GLP Compliance:	No
AF0150 Lot number:	UA16063 and UA16019

Specific Aim

To assess the effects of AF0150 fill size (100 vs. 200 mg) and reconstituted concentration (10 or 20 mg/ml) on the quality of contrast enhanced echocardiographic images in a swine model.

Methods

Two swine (17.4 to 18.5 kg) were anesthetized followed by cannulation of the jugular vein for administration of AF0150 and cannulation of the left carotid artery for monitoring hemodynamics. Each animal received the 3 solutions at the 3 different infusion rates (Table I) for

a total of 9 injections per animal. The infusion rates were chosen such that the concentration of AF0150 administered was the same for all 3 solutions at each rate. Echocardiography was performed as described in the report #EB-98-15. Two reviewers blinded to the study protocol evaluated the myocardial images. Both reviewers ranked each solution at each rate on a scale of 0 to 5 (poor to good), and the scores from both reviewers were then averaged (Table II).

Table I. AF0150 Preparation and Infusion Rates

AF0150 Solution	Constituted Concentration	Infusion Rate (ml/kg/hr)		
		High	Medium	Low
100 mg in 10 ml HNS	10 mg/mL	24	21.0	16
100 mg in 5 mL WFI	20 mg/mL	12	10.5	8
200 mg in 10 mL WFI	20 mg/mL	12	10.5	8

• HNS = half normal saline and WFI = water for injection

Table II. Qualitative Scores of AF0150 Solutions Based on Echocardiograhic Opacification

AF0150 Solution	Averaged Scores by Infusion Rate		
	High	Medium	Low
100 mg in 10 mL HNS	4.125	4.25	1.75
100 mg in 5 mL WFI	2.5	2.00	0.50
200 mg in 10 mL WFI	3.0	3.00	2.25

Results

Based on the qualitative scores of cardiac images (the higher, the better the imaging), as seen in Table II, the 100 mg fill size of AF0150 (reconstituted concentration of 10 mg/ml) seems to provide slightly better myocardial opacification from this pilot study in swine.

Discussion and Comments:

The sample size was too small to perform statistic analysis and thus it is hard to conclude which fill size and concentration is better. Variations of final data (scores), as shown in Table II, from individual animals, experimental procedure and image reading should be considered for comparison.

Report Number: EB-97-16

Effects of AF0150 Fill Size and Constituted Concentration on Doppler Signal Enhancement, Doppler Signal Persistence, and Ultrasound Power Resistance in a Rabbit Model

Report Location: Vol.009, p147-158
Report date: April 23, 1999
Study Facility: Alliance Pharmaceutical Corp
In-life phase: July, 1997
GLP Compliance: No

AF0150 Lot number: UA16063, UA16019, and ZY16042A

Specific Aim

To assess the effects of AF0150 fill size and reconstituted concentration on Doppler signal enhancement, Doppler signal persistence, and ultrasound power resistance in rabbit model.

Methods

Animal preparation: Four rabbits of either sex (3.1 to 3.5 kg) were anesthetized followed by cannulation of the jugular vein for IV administration of AF0150. A 10 MHz pulsed Doppler flow cuff transducer was placed around the carotid artery and held in place with a suture. A 3.5 MHz probe was positioned over the heart at the mid-papillary level (closed chest) at a depth setting of approximately 6 cm for application of ultrasound bursts to the heart-blood.

AF0150 preparation and administration: AF0150, 100mg or 200 mg fill vials, was reconstituted with either water for injection (WFI) or half-normal saline solution (HNS) in order to obtain an isotonic solution at a concentration of 10 or 20 mg/ml. Each animal received a 1 mg/kg IV bolus injection of three AF0150 solutions, as indicated in Table I.

Table I. AF0150 Solutions

Lot #	Fill Size (Dry Powder Weight)	Diluent for Constitution	Constituted Concentration	Dose Administered
ZY16042A (Historical Standard)	100 mg	10 mL HNS	10 mg/mL	1 mg/kg
UA16063	100 mg	10 mL HNS	10 mg/mL	1 mg/kg
UA16063	100 mg	5 mL WFI	20 mg/mL	1 mg/kg
UA16019	200 mg	10 mL WFI	20 mg/mL	1 mg/kg

• HNS = half normal saline and WFI = water for injection

Doppler analysis: Doppler signals of carotid artery blood flow were recorded in response to AF0150 bolus injection with and without a burst of ultrasound power (60 seconds at –6dB) to the heart. Signal enhancement, signal persistence and signal decrease ratio (ultrasound power resistance) were calculated using a customized Fortran program.

Results

Doppler signal enhancement and persistence: Mean signal enhancement and persistence data (from 4 animals at each solution) are shown in Table AI. There was no difference in Doppler signal enhancement (measured at 60 seconds post AF0150 injection) and Doppler signal persistence (measured at 240 seconds post AF0150 injection) regardless of fill size (100 vs. 200 mg) or reconstituted concentration (10 vs. 20 mg/ml).

Ultrasound power resistance: The application of continuous fundamental ultrasound power over the heart produced a decrease in the Doppler signal of the carotid artery blood flow. There was

no difference in resistance to ultrasound power burst regardless of fill size (100 vs. 200 mg) or reconstituted concentration (10 vs. 20 mg/ml) (Table AII).

Table AI Doppler Signal Data

Lot #	Fill Size (Dry Powder Weight)	Diluent for Constitution	Doppler Signal (mV) at 60 Seconds	Doppler Signal (mV) at 240 seconds
ZY16042A (Historical Standard)	100 mg	10 mL HNS	852.13 ± 61.61	295.24 ± 51.69
UA16063	100 mg	10 mL HNS	691.71 ± 49.73	208.23 ± 23.73
UA16063	100 mg	5 mL WFI	671.85 ± 51.89	200.17 ± 26.14
UA16019	100 mg	10 mL WFI	820.31 ± 42.62	234.91 ± 35.98

- HNS = half normal saline and WFI = water for injection
- Data are means ± SEM; (n=4).

Table AII Doppler Signal Decrease Ratio Data

Lot #	Fill Size (Dry Powder Weight)	Diluent for Constitution	Signal Decrease Ratio
ZY16042A (Historical Standard)	100 mg	10 mL HNS	0.542 ± 0.199
UA16063	100 mg	10 mL HNS	0.693 ± 0.237
UA16063	100 mg	5 mL WFI	0.614 ± 0.225
UA16019	100 mg	10 mL WFI	0.596 ± 0.199

- HNS = half normal saline and WFI = water for injection
- Data are means ± SEM; (n=4)

Discussion and Comments

This study suggests that there is no significant difference in Doppler signal enhancement, Doppler signal persistence, or signal resistance to ultrasound power burst regardless of fill size (100 vs. 200 mg) or reconstituted concentration (10 vs. 20 mg/ml).

Report Number: EB-98-14

Effect of Water-for-Constitution Gas Tension and Temperature on Constituted AF0150

Report Location: Vol.009, p159-171
Report date: April 8, 1999
Study Facility: Alliance Pharmaceutical Corp
In-life phase: April 15-Sepember 24, 1998
GLP Compliance: No
AF0150 Lot number: ZY17041 and ZY18020

Specific Aim

To determine the effects of the SWFI pretreatment with various gas tensions and temperatures on the Doppler signal of AF0150 in rabbits.

Methods

Animal Preparation: Six New Zealand White rabbits (3.1 to 3.5 kg) of either sex were anesthetized; the jugular vein was cannulated for AF0150 administration with a ⁀ tube catheter for the high dose (1.0 mg/kg; n = 3) study and a *Saf-T-Intima™* injection safety system with Y adapter for the low dose (0.2 mg/kg; n = 3) study. A 10 MHz pulsed Doppler flow cuff transducer was placed around the carotid artery and held in place with a suture.

AF0150 Preparation and Administration: AF0150 (200 mg fill) was reconstituted with 10 ml pre-treated SWFI (as described below) to a final concentration of 20 mg/ml. The high (lot ZY1704) and low (lot ZY 18020) dose studies were performed from April 15 to 17, 1998 and September 23 and 24, 1998, respectively.

SWFI pretreatment with temperatures and pressures: The SWFI (100 ml in 500-ml bottle) was equilibrated at 5 different pressures (460, 610, 760, 910, and 1060 mmHg) and 3 different temperatures (15°C, room temperature, and 30°C), as described in Table I. Each bottle was sealed and spiked with a stopcock, and left to equilibrate to the respective conditions for not less than 12 hours. The conditioned WFI was then used to constitute the AF0150. AF0150 vials were equilibrated to the same temperatures as the SWFI and maintained at that temperature until the sample was drawn from the vial. Animals received either 1.0 or 0.2 mg/kg bolus injection (3 rabbits each dose group) of the reconstituted AF0150 followed by a saline flush.

Table I. Temperatures and Pressures Used to Prepare Sterile Water for Injection Samples

	Temperatures		
	15°C	Room Temperature (19.5°C - 21.1°C)	30°C
Pressures* (mmHg)	460	460	460
	610	610	610
	760	760	760
	910	910	910
	1060	1060	1060

*460 mm Hg = -300 mm Hg from ambient pressure;
610 mm Hg = -150 mm Hg from ambient pressure;
760 mm Hg = standard ambient pressure;
910 mm Hg = +150 mm Hg from ambient pressure;
1060 mm Hg = +300 mm Hg from ambient pressure.

Doppler Signal Analysis: Doppler signals of carotid artery blood flow were collected for 150 seconds following administration of AF0150 reconstituted with the pretreated SWFI (Table I). Each consecutive injection of AF0150 was performed once signal returned to baseline. Data were processed and analyzed with a customized program.

Results

There were no market differences in the Doppler signal generated by AF0150 reconstituted in the SWFI pretreated (equilibrated) with a combination of various temperatures ([redacted]) and gas tension [redacted]) at either 1 mg/kg or 0.2 mg/kg of AF0150.

Discussion and Comments

The studies suggest that the temperature and gas tension of the SWFI used for AF0150 reconstitution have no significant effects on Doppler signal of carotid artery blood flow in rabbits. However, it is not clear how to transfer the tension-conditioned SWFI to AF0150 vials without losing tension and keep the same gas tension in the reconstituted AF0150 solution as in the SWFI. The report only mentions that the AF0150 vials were kept in the tested temperature, but does not indicate whether the AF0150 solution was also kept in the tested tension. Also, the signal for myocardial imaging was not evaluated.

Report Number: EB-98-17
Effect of Time after Constitution of AF0150 and Time after Vial Inversion of Constituted AF0150 on Doppler Signal in a Rabbit Model

Report Location:	Vol.009, p172-183
Report date:	April 7, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	February – March, 1998
GLP Compliance:	No
AF0150 Lot number:	UA16064

Specific Aim

To determine effects of the sitting time AF0150 reconstitution and the vial inversion of AF0150 solution on Doppler signals of carotid artery blood flow in rabbits.

Methods

Animal Preparation: Eight New Zealand White rabbits (3.1 to 3.5 kg) were anesthetized; the jugular vein was cannulated for AF0150 administration; and a 10 MHz pulsed Doppler flow cuff transducer was placed around the carotid artery.

AF0150 Preparation and Administration: AF0150, 100mg fill vial, was reconstituted in 10 ml 0.45% w/v sodium chloride (Half Normal Saline, HNS) to a final concentration of 10 mg/ml. For post-reconstitution time study, the AF0150 solution in vials was allowed to sit for 0, 30, 60, 90, 120 minutes. Before AF0150 was withdrawn from the vials for injection at each time point, the vials were agitated and inverted (for at least 15 seconds). For post-inversion time study, AF0150

solution was allowed to sit for at least 1 minute and then inverted. At 15, 30, 60 and 120 second after inversion, AF0150 solution was drawn for injection. Four rabbits were assigned to each study, and each animal received a total of 5 injections at the dose of 1 mg/kg AF0150. Each consecutive injection of AF0150 was performed after the Doppler signal returned to baseline.

Doppler Signal Analysis: Doppler signals of carotid artery blood flow were monitored before and after AF0150 administration. Data were analyzed with a customized Fortran program.

Results

Effects of post-reconstitution time on Doppler signals: The peak Doppler signals were not different between 0 and 30 minutes post-reconstitution of AF0150, but decreased by 15% at 60 minute postconstitution.

Effect of Postinversion time of reconstituted AF1050 on the Doppler Signal: There were no notable differences in the peak Doppler signal at 15, 30 and 60 seconds postinversion. However, the signals decreased by 26% at 120 second post-inversion.

Discussion and Comments

This study demonstrates that 60 minutes or longer post-reconstitution and 2 minutes or longer post-inversion, AF0150-generated peak Doppler signal decreased, suggesting that proper reconstitution of AF0150 is important for optimal imaging. Therefore, the reconstituted AF0150 solution should be used within 30 minutes after reconstitution and within 1 minutes following vial inversion. This time limitation should be applied to all preclinical and clinical protocols. The sponsor provided appropriate directions for reconstitution and administration of AF0150 (100 mg vial) in the appendix of this study report.

In addition to setting the time of post-reconstitution and post-inversion, it should also be tested if shaking strength and duration influence AF0105-generated Doppler signals because the shaking may determine the number and size of microbubbles.

Report Number: RE-99-47
Contribution of Two AF0150 Microbubble Size Populations to the Doppler Signal in a Rabbit Model

Report Location:	Vol.009, p184-205
Report date:	September 22, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	Not specified
GLP Compliance:	No
AF0150 Lot number:	ZZ17061 and ZY18020

Specific Aim

To assess the relative contribution of large microbubbles (>3 um) versus small microbubbles (<3 um) of AF0150 to affect Doppler signal of carotid artery blood flow.

Methods

Animal Preparation: Five New Zealand White rabbits (3.3 to 3.8 kg) were anesthetized. The jugular vein was cannulated for AF0150 sample administration. A 10 MHz pulsed Doppler flow cuff transducer was placed around the carotid artery for recording Doppler signals of the blood flow.

AF0150 Preparation: AF0150 (200-mg vial) was reconstituted with 10 ml of SWFI to a final concentration of 20 mg/ml. The large (> 3um) and small (<3 um) microbubble populations were separated from the reconstituted AF0150 with "creaming" methods ---- larger microbubbles float faster than smaller microbubbles to the top of an undisturbed suspension. The separation yielded a nearly pure population of small microbubbles (<3 gm) in the lower 9-ml portion (Lower sample, 9 ml) of a 10 ml suspension (3 parts of reconstituted AF0150 to 7 parts of sterile saline) and creamed for 30 minutes in a 20-mL *Norm-Ject* syringe. The upper 1-ml portion of the same 10-ml suspension was diluted with 8 ml of sterile (Upper sample, 9 ml). Unseparated, saline-diluted AF0150 (1 ml of reconstituted AF0150 and 2 ml sterile saline) from the same vial was used as Control sample. Bubble-size distribution (count and diameter) was measured with liquid particles counting system.

Doppler signal analysis: the Doppler signals of carotid artery blood flow were recorded after administration of Lower sample(small microbubbles), Upper sample (large microbubbles) and Control sample (unseparated microbubbles). The dosage were not specified for Lower Upper samples. Since microbubble populations were not pure, as seen in Results, the Doppler signals contributed by small and large microbubbles were theoretically resolved and predicted with a mathematical approach.

Results

AF0150 microbubble size distribution: two peaks of microbubble populations were found in reconstituted AF0150 (Control sample): one is at 1-1.5 um and another at 4-5 um in diameter. After separation, 97-99% microbubbles in the Lower sample were <3 um in diameter, and 19-24% microbubbles in the Upper sample were >3 um and the remaining 76-81% were <3 um.

Effects on the Doppler Signals: Small microbubbles (<3 um) are responsible for the initial intense response, but provided less than 50% of the signal beyond 90 seconds. Larger microbubbles (>3 um) provided a constant lower intensity signal that was responsible for the majority of the signal beyond 90 seconds.

Discussion and Comments

The study suggests that different sizes of AF0150 microbubbles contribute to the Doppler signal at different times after injection. However, this is based on correlation of *in vitro* microbubble size and *in vivo* Doppler signal of carotid artery blood flow. Correlation of microbubble size in blood with Doppler signals would be much more valuable for evaluating the effects of microbubble size on the Doppler signal response.

It is unclear if the sponsor performed the same dilution (thus the same final concentration of AF0150) to different AF0150 preparations (Upper sample, Lower sample and Control sample). The sponsor may not have injected the same total numbers of microbubbles from the Upper, the Lower and the control samples.

Report Number: RE-99-46
Effect of Ultrasound Exposure on the Size Distribution of AF0150 Microbubbles Under *in vitro* Simulated Physiological Conditions

Report Location:	Vol.009, p213-222
Report date:	September 14, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	March 12, 1999
GLP Compliance:	No
AF0150 Lot number:	ZY18032

Specific Aim

To measure the effect of clinically relevant ultrasound exposure on the size distribution of AF0150 microbubbles under *in vitro* simulated vascular system.

Methods

In vitro Circulation Model An *in vitro* vascular system model was used to simulate the key physical features of an artery or heart chambers. A 6% bovine albumin solution (in saline) simulated blood and was circulated by means of an peristaltic pump through tubing at 37°C. AF0150 (12.6 mg) was injected into 225 ml of the 6% albumin solution (circulating concentration of 0.056 mg/ml) and allowed to circulate for 90 seconds before ultrasound power (freeze, -16.2, -9.8, -4.1 and 0.0 dB, respectively) were applied to the solution in the circulating tube. 0.5 ml of the solution was taken at 0, 2, 4 and 6 minutes post-ultrasound exposure for measurement of microbubble sizes and counts [redacted] liquid particle counting system).

Results

Sustained ultrasound exposure within 6 minutes changed little in microbubble size (median diameter range of 5 to 8 um) in 6% albumin solution with a slight shift to smaller sizes except at the highest power setting (MI 1.7). At 1.7 MI, bubble size drifted from 4.9 to 6.1 um. Microbubble counts (both small and larger bubbles) decreased with increasing time and power levels of ultrasound exposure, as seen in the Table 1. The time-course of MI setting and % decrease in total bubble counts per min is shown in Table 2.

Table 1. Bubble Counts and Median Diameter Data from the [redacted] System in Circulating Albumin Solution

MI Setting	Time (min)	Total Bubble Counts/mL (> 10 µm diameter)	Total Bubble Counts/mL	Median Diameter (µm)	Total Bubble Counts/mL (3-10 µm diameter)
1.7 (Albumin Control)	0	1.2×10^3	7.32×10^5	14.1	6.86×10^3
	2	2.9×10^3	7.36×10^5	17.3	6.19×10^3
	4	2.5×10^3	7.63×10^5	19.6	5.66×10^3
	6	2.2×10^3	6.31×10^5	19.4	5.55×10^3
0.0	0	1.2×10^4	3.96×10^6	6.3	1.02×10^6
	2	5.0×10^3	3.21×10^6	5.9	6.73×10^5
	4	3.5×10^3	3.76×10^6	5.5	9.32×10^5
	6	2.8×10^3	3.79×10^6	5.9	1.07×10^6
0.2	0	2.4×10^4	4.52×10^6	7.1	9.86×10^5
	2	2.5×10^3	3.55×10^6	5.7	7.79×10^5
	4	1.9×10^4	3.43×10^6	7.0	9.66×10^5
	6	3.6×10^3	3.41×10^6	5.8	1.00×10^6
0.5	0	1.2×10^4	3.63×10^6	6.5	6.71×10^5
	2	1.1×10^4	3.75×10^6	6.5	1.06×10^6
	4	4.9×10^3	3.29×10^6	6.3	7.92×10^5
	6	3.7×10^3	2.05×10^6	6.3	6.53×10^5
1.0	0	1.8×10^4	4.10×10^6	7.7	9.98×10^5
	2	9.4×10^3	3.65×10^6	6.5	1.01×10^6
	4	2.2×10^3	2.04×10^6	5.7	5.50×10^5
	6	1.7×10^3	1.27×10^6	6.0	2.59×10^5
1.7	0	1.2×10^3	3.80×10^5	4.9	6.64×10^5
	2	3.8×10^2	3.14×10^6	5.5	7.36×10^5
	4	3.3×10^3	1.51×10^6	6.2	2.78×10^5
	6	1.5×10^3	9.34×10^5	6.1	1.18×10^5

Table 2. Effect of Ultrasound Exposure on Microbubble Destruction (% decrease in total bubble counts/ml) *In Vitro*

Time (min)	Ultrasound Exposure Mechanical Index (MI)				
	0	**0.2**	**0.5**	**1.0**	**1.7**
0	0.0	0.0	0.0	0.0	0.0
2	18.9	21.5	-3.3	11.0	17.4
4	5.1	23.9	9.4	50.2	60.3
6	4.3	24.6	43.5	69.0	75.4

Discussion and Comments

1. Application of clinically relevant ultrasound power resulted in dissolution of AF0150 microbubble in 6% albumin-saline solution with a slight shift of microbubble size to smaller except at the highest ultrasound power (1.7 MI). The 1.7 MI exposure increased the bubble size from 4.9 to 6.1 within 6 minutes, which may be caused for concern.
2. In this simulating vascular system, 6% albumin in the circulating saline solution may not be equivalent to the albumin level in human blood. Also, this solution can not simulate human blood, since other blood components, particularly lipids and cells, are likely to influence the behavior of microbubbles. Human blood or plasma could have been better to test the sponsor's Specific Aim.
3. It is unclear if the numbers shown in Table II represent variations from repeat determinations. A great variation, particularly at low counts, is usually seen with liquid particle counting systems. Flow cytometry may be a better system to count and measure bubble size, and may be suitable for monitoring microbubble size and diameter in an *in vivo* study (in animals and human subjects).

Report Number: EB-97-04
Effect of Ultrasonic Power on Contrast Microbubble Dynamics and Signal Persistence

Report Location: Vol.009, p223-236
Report date: January 2, 1999
Study Facility: Alliance Pharmaceutical Corp
In-life phase: Not specified
GLP Compliance: No
AF0150 Lot number: ZY16042A

Specific Aim

To determine effects of ultrasound setting (power levels, continuous or triggered) on AF0150-induced Doppler signals of carotid artery blood flow in rabbits.

Methods

Animal Preparation: Thirteen New Zealand White rabbits (3.1 to 3.5 kg body weight) of either sex were anesthetized with a ketamine/xylazine/acepromazine cocktail. Animals were instrumented for monitoring of heart rate (ECG) and the jugular vein was cannulated for AF0150 administration. A 10 MHz pulsed Doppler flow cuff transducer was placed around the carotid artery for monitoring the Doppler signals of carotid artery blood flow, and a 3.5 MHz ultrasound probe was positioned over the heart (closed chest) at a depth setting of approximately 6 cm for application of ultrasound power.

AF0150 Preparation and administration: AF0150 was reconstituted in 10 ml of 0.45% saline to final concentration of 10 mg/ml. All animals received a bolus dose of 1 mg/kg AF0150 into the jugular catheter over 1 to 2 sec (followed by a 0.2 to 0.4 ml saline flush) before a baseline Doppler signal was recorded. The Doppler flow signal was measured without and with a 60-sec burst of ultrasonic power (UP) applied to the heart at 60 sec or 120 seconds after the AF0150 injection. Each animal received a control (no UP) and several continuous or triggered UP bursts with 3 to 4 min between injections. The UP levels were 0, -3, -6, -9, -12 dB, corresponding to Spatial Peak Pulse Average Intensities (I-SPPA) of 102, 51, 26, 13, and 6 W/cm2. Animals were divided into two groups: *Group A*: receiving Continuous Ultrasound Power at Various Levels (n = 7); and *Group B*: receiving Continuous *vs* Triggered Ultrasound Power (n = 6).

Doppler Signal Analysis: Doppler signals, expressed as RMS values, of carotid artery blood flow were sampled at a rate of one per second before and after AF0150 injection with or without application of ultrasound powers (various levels and continuous or triggered UP) to the heart. Data were processed with the customized programs for a power-susceptibility and signal-persistence analysis.

Results

Effects of UP levels on the Doppler Signals: The Doppler signals of carotid artery blood flow decreased during application of ultrasound power to the heart with power level-dependence. The continuous UP decreased the Doppler signals more significantly than the triggered UP. The Doppler signals returned to control levels following the release of the UP burst in the lower UP levels (lower than 0 dB).

Effects on the Doppler Signal Persistence: application of UP to the heart decreased the Doppler signal persistence by 13 seconds as compared to the control (no UP). There was no difference between the continuous and triggered UP.

Discussion and Comments

This study suggests that application of ultrasound power, particularly at continuous setting, for 60 seconds significantly destroys microbubbles and thus decreased the Doppler signal in a power-dependent manner.

Correlation of UP-induced change in the Doppler signals with microbubble count and size in the blood would be better for testing the specific aim of this study.

The duration of UP application to the heart may also be as important as the UP levels to affect the dynamic of AF0150 microbubbles and thus efficacy.

Report Number: EB-97-20-Amended
Effect of Transmission Frequency and Ultrasound Power on AF0150-Enhanced Doppler Signal in Rabbits

Report Location:	Vol.009, p237-247
Report date:	March 2, 1999 (supersedes report of February 3, 1999)
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	Not specified
GLP Compliance:	No
AF0150 Lot number:	UA16064

Specific Aim

To assess the effects of various ultrasound transmission (probe) frequencies and power levels on AF0150-induced Doppler signal of carotid artery blood flow in the rabbit.

Methods

Animal Preparation: Six New Zealand White rabbits (3.1 to 3.5 kg) of either sex were anesthetized with a ketamine/xylazine/acepromazine cocktail and the neck and chest were shaved and scrubbed. The jugular vein was cannulated for the administration of AF0150. A 10 MHz pulsed Doppler flow cuff transducer was placed around the carotid artery and held in place with a suture. Ultrasound probes (2.5, 3.5 and 4.0 MHz) were positioned over the heart (closed chest) at a depth setting of approximately 6 cm for application of ultrasound bursts.

AF0150 Preparation and Administration: Group A (3 rabbits), the Doppler flow signal was measured for approximately 5 minutes during a 60-second application of ultrasonic power (UP) burst to the heart at 1 minute post AF0150 bolus injection (1 mg/kg). Each animal received a control (no UP) and UP bursts with 3 different probes at frequencies of 2.5, 3.5 and 4.0 MHz and powers of 0, -3, -6 dB (corresponding to the I-SPPA of 102, 51 and 26 W/cm2, respectively). Group B (3 rabbits), the same protocol was used as described in Group A except that the animals were exposed to the UP burst 2 minutes post AF0150 bolus injection.

Doppler Signal Analysis: the Doppler signals of carotid artery blood flow was monitored and processed as described in the previous studies. The signal decrease ratio: $(S_{60pre}-S_{60burst})/S_{60pre}$, as described in *Report Number: RMI-97-01*, was calculated after application of UP burst.

Results

The Doppler signals of AF0150 microbubbles in carotid artery blood flow decreased with increasing ultrasound power levels (0, -3, and –6 dB) at all tested probe frequencies (2.5, 3.5 and 4 MHz) at both 1 and 2 minutes after AF0150 injection. At a given UP level, application of the higher ultrasound transmission frequency resulted in a lower decrease in the Signal Decrease Ratio.

Discussion and Comments

Both ultrasound power and transmission frequency are important to determine the behavior of AF0150 microbubbles in blood flow. However, information from the control group (AF0150 injection without UP application to the heart) was missing in Figure 2 (pp009-242) and table AI (pp009-247), which did not allow comparison with UP-treated data.

The sponsor's explanation for "When ultrasound power level is held constant with AF0150, the Doppler signal increases in response to increasing ultrasound probe frequencies"(pp009-244) is not appropriate because application of UP to the heart, whatever the power level and/or frequency, the Doppler signals recorded from carotid artery blood flow always decreased due to UP-induced destruction of microbubbles. A proper explanation for this phenomena should be that the higher ultrasound frequencies results in lower decrease in the Doppler signal.

SEM (Standard Error of Mean) is misused for data analysis in this and all other study reports in this submission. It should be SD (Standard Deviation) which represents a variation of the samples. In statistics, SEM shows the representative of a sample to its population. The values of SEM are always less than SD, which may be misleading when drawing conclusions.

Report Number: EB-98-18
Videointensity Analysis of the Left Ventricular Cavity of Swine using the [redacted] Ultrasound Imaging System

Report Location:	Vol.009, p248-259
Report date:	May 7, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	September – October, 1998
GLP Compliance:	No
AF0150 Lot number:	UA16063

Specific Aim

To determine enhancement effects of AF0150 microbubbles on left ventricular cavity imaging in swine, including analysis of the duration of image attenuation and the videointensity of the LV cavity (peak and persistence), using the [redacted] Ultrasound Imaging system.

Methods

Animal Preparation: Two swine (19.6 and 22.7 kg body weight) were anesthetized and intubated (mechanically ventilated with room air). The heart rate was monitored with ECG, and a carotid artery was cannulated to monitor arterial pressure during anesthesia. The jugular vein was cannulated for AF0150 administration. A broad band 2.5 to 3.5 MHz transducer [redacted] was positioned over the heart (closed chest) to yield a short axis view at the midpapillary muscle level, fixed in place by a mechanical arm at a depth setting of approximately 7 cm for echocardiography.

AF0150 Preparation and Administration: AFO150 (100 mg fill) was reconstituted in 10 ml of 0.45% saline to a final concentration of 10 mg/ml. Each animal received 0.125 and 0.25 mg/kg AF0150 by bolus IV injection followed by a saline flush (1 ml over 30 sec). A total of 17 AF0150 doses were administrated to each animal.

Echocardiography: Echocardiographic imaging was performed using fundamental ultrasound transmission, with both continuous and intermittent mode. Fundamental intermittent imaging was performed with ECG dual triggering on the T and R waves for every heartbeat. Mechanical index (MI, corresponding to ultrasound power levels) was increased from 0.1 to 1.4 (0.250 mg/kg AF0150) or 1.7 (0.125 mg/kg AF0150). The baseline ultrasound images were collected before each AF0150 injection.

Videointensity Analysis: Images were recorded on 1.25 cm VHS videotape followed by videodensitometric quantitation for the LV cavity using [redacted] Baseline videointensity values (pre-injection) were subtracted from the peak contrast intensity determined at approximately 5 seconds after the period of attenuation, and the videointensity at 90 seconds post-AF0150. The 90-second time point represents the persistence of the videointensity signal. The duration of attenuation was measured from the time that the contrast agent reached the LV cavity until the image "shadow" completely subsided. Videointensity data from each of 2 swine are presented individually in graph and table without statistical analysis.

Results

Imaging Attenuation (see Table 1 in the follow study EB-98-19): Attenuation of the ultrasound signal, visible in images as "shadowing," is indicative that a high concentration of microbubbles is present in the field of imaging. The duration of image attenuation of the LV cavity was reduced at high MI (high ultrasound power) and at the low AF0150 dose (0.125 mg/kg).

Peak Videointensity: LV cavity peak videointensity increased slightly with increasing MI, from 0.1 to approximately 0.8, remaining at a similar level at MI above 1.0, and decreased at MI 1.4-1.7. There were no notable differences in peak videointensity between low (0.125 mg/kg) and high (0.250 mg/kg) dose AF0150.

Persistence of Videointensity Signal: Persistence of videointensity, measured at 90 seconds post-AF0150 injection, decreased with increasing MI setting. At the MI 0.4, the signal was

approximately 70% (0.125 mg/kg AF0150) and 45% (0.25 mg/kg AF01050) of the peak videointensity. This decrease in videointensity was more marked with a low dose (0.125 mg/kg) of AF0150.

Discussion and Comments

This study demonstrated the effects of ultrasound power levels and modes on the intensity and persistence of echocardiographic imaging induced by AF0150 microbubbles. The results may provide some information for optimization of AF0150 application in clinical setting. However, the sample size (total 2 animals) used in the study was too small to draw a firm conclusion. Increasing animal numbers in each dose group is recommended and appropriate statistical analyses need to be followed.

Report Number: EB-98-19
Videointensity Analysis of the Left Ventricular Cavity of Swine using the [redacted] Ultrasound Imaging System

Report Location:	Vol.009, p260-269
Report date:	May 7, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	July 1998
GLP Compliance:	No
AF0150 Lot number:	UA16064

The same experimental protocol and conditions were used in this study as presented in the *Study EB-98-18*, except using the [redacted] Ultrasound system instead of the [redacted] Ultrasound Imaging System, and the mechanical settings were slight different from those in *EB-98-18*. Single dose of AF0150, 0.125 mg/kg, was administrated to 2 swine. The similar results were obtained, as seen in the report *#EB-98-18*. However, there were significant individual differences in attenuation duration and videointensity signals in this study. Again, more animals are needed to draw proper conclusions. Attenuation data from both studies are summarized in Table 1.

Table 1. Effects of MI on Attenuation of LV Cavity Imaging
(Summarized from the studies EB-98-18 and EB-98-19)

Mechanical Index	AF0150 (mg/kg)	
	0.125*	0.25#
0.1	22.3±2.8	53.0±5.7
0.2	18.3±3.1	47.0±12.7
0.3	13.3±6.2	19.0±0.0
0.4	12.0±4.5	18.5±0.7
0.6	7.3±6.6	4.5±2.1
0.8	3.8±5.2	3.0±4.2
1.0	1.8±2.9	2.5±2.1
1.4	1.0±1.7	2.5±3.5
1.7	0.0	ND

ND: no data
* Mean ± SD of 4 swine (2 from each study).
# Mean ± SD of 2 swine from the study EB-98-18.

Report Number: EB-98-20
Videointensity Analysis of the Left Ventricular Cavity of Swine using the Ultrasound Imaging System

Report Location: Vol.009, p270-282
Report date: May 7, 1999
Study Facility: Alliance Pharmaceutical Corp
In-life phase: May-June 1998
GLP Compliance: No
AF0150 Lot number: UA18009

Specific Aim

To assess the effects of the ultrasound power (or mechanical index) and the frame rates of ultrasound imaging on AF0150-induced left ventricular cavity imaging in swine.

Methods

Animal Preparation: Three swine (20.3 ± 0.3 kg) were anesthetized and intubated (mechanically ventilated with room air). Heart rate was monitored with ECG and a carotid artery was cannulated for monitoring arterial blood pressure during anesthesia. The jugular vein was cannulated for AF0150 administration. A broad band 3.2 MHz transducer was placed above the heart on the closed chest by a mechanical arm at a depth setting of approximately 7 cm for echocardiography.

AF0150 Preparation and Administration: AF0150 (100 mg fill) was reconstituted in 10 ml of 0.45% saline to a final concentration of 10 mg/ml. Each animal received 0.125 and 0.25 mg/kg

AF0150 by bolus IV injection followed by a saline flush (1 ml over 30 sec) at the different ultrasound settings. A total of 18 AF0150 injections were administrated to each animal.

Echocardiography: Echocardiographic imaging was performed using fundamental ultrasound transmission, with both continuous and intermittent mode. Fundamental intermittent imaging was performed with ECG dual triggering on the T and R waves for every heartbeat. Mechanical index (MI, corresponding to ultrasound power levels) was 0.1, 0.4 and 0.7. At each of these MI, 3 different frame rates were evaluated (18, 28, and 78 frames/sec). After baseline ultrasound images were collected at MI 0.1 and 18 frames/sec, the animal received a bolus IV injection of AF0150. All ultrasound imaging was performed in the following sequence: fundamental continuous imaging (~45 sec), fundamental intermittent imaging (12 beats, -32 to 38 sec), followed by fundamental continuous imaging until no contrast was visible in the LV cavity (-5 min).

Videointensity Analysis: Images were recorded on 1.25 cm VHS videotape followed by videodensitometric quantitation for the LV cavity using [redacted] Baseline videointensity values (pre-injection) were subtracted from the peak contrast intensity determined at approximately 5 seconds after the period of attenuation, during intermittent imaging, and the videointensity at 90 seconds post-AF0150. The 90-second time point represents the persistence of the videointensity signal. The duration of attenuation was measured from the time that the contrast agent reached the LV cavity until the image "shadow" completely subsided. Videointensity data from 3 swine are presented as Mean ± SEM but without statistical analysis.

Results

Image Attenuation: With frame rate constant, duration of attenuation was reduced at high MI and at the low dose (0.125 mg/kg) of AF0150. There was no significant change in the duration of attenuation with varying frame rates for both AF0150 doses.

Videointensity: Peak videointensity of LV cavity, measured approximately 5 seconds post-attenuation (during both continuous and intermittent imaging), decreased slightly with increasing MI (from 0.1 to 0.7) at both AF0150 doses except that the dose of 0.250 mg/kg AF0150 slightly increased the signals at MI of 0.7. The persistence of videointensity of LV cavity, measured at 90 seconds post-AF0150 injection, decreased with increasing MI setting at both AF0150 doses. Both peak videointensity and persistence of videointensity decreased with increasing frame rates without significant difference between high and low AF0150 doses.

Discussion and Comments

Increasing frame rates of ultrasound imaging with different ultrasound power levels had no effects on duration of attenuation but decreased the LV cavity videointensity (peak and persistence) at either low and high AF0150 doses (0.125 and 0.25 mg/kg). However, like previous studies (EB-98-18 and EB-98-19), small sample size (n=2-3) and lack of proper statistical analysis makes it difficult to judge the significance of the differences.

Report Number: EB-98-16
Effect of External Pressure Applied to Constituted AF0150 on Doppler Response in Rabbits

Report Location:	Vol.009, p283-292
Report date:	February 3, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	May 14, 1998
GLP Compliance:	No
AF0150 Lot number:	ZY18020

Specific Aim

To determine the effects of external pressures, applied to AF0150 microbubbles via a pressurized syringe during intravenous administration, on the efficacy (Doppler signal of carotid artery blood flow) of AF0150 in the rabbit.

Methods

Animal Preparation: One New Zealand White rabbit (4.5 kg) was anesthetized and intubated (allowed to free-breathe). Heart rate and arterial oxygen saturation were monitored with a pulse oximeter placed on the ear. The jugular vein was cannulated for AF0150 administration. A 10 MHz pulsed Doppler flow cuff transducer was placed around the carotid artery for recording the Doppler signal of blood flow.

AF0150 Preparation and Pressure Treatment: AF0150 (200 mg fill) was reconstituted with 10 ml of SWFI to a final concentration of 20 mg/ml. The AF0150 solution was treated with different pressure levels (760, 842, 925, 1008 and 1090 mmHg) in a Pressurized Syringe System for 2 minutes prior to injection into the rabbit. The pretreated AF0150 solution was then given to the animal at the dose of 1 mg/kg bolus IV injections with 3 injection at each of the 5 pressures (total of 15 injections).

Doppler Signal Analysis: Doppler signals of carotid artery blood flow was monitored and processed with a customized program as described in previous studies. Baseline Doppler signals were collected prior to each injection and each consecutive AF0150 injection was performed after the Doppler signal returned to baseline. The data were presented as Mean ± SEM from 3 repeated injection at each pressure level without statistical analysis.

Results

Increasing external pressure application to reconstituted AF0150 tended to decrease AF0150-induced Doppler signal (both peak and persistence), as compared with the ambient pressure level

(760 mmHg). The highest test pressure, 1090 mmHg, decreased the peak Doppler signal by approximately 21%.

Discussion and Comments

1. One rabbit was used in this study and the sponsor reported that the high pressure (330 mmHg above ambient pressure), but not the lower pressures, decreased the Doppler signals of carotid artery blood flow. However, the data shown in the submitted table indicate that the Doppler signals decreased with increasing external pressures. More rabbits should be included in this study in order to make a final conclusion.

2. In the clinical setting, the pressures in the syringe containing reconstituted AF0150 solution need to be evaluated and correlated to this study system. A standardization procedure may need to be developed to allow consistent pressure effects on the microbubbles, and thus imaging efficacy.

3. Information about changes in microbubble size and counts after pressure application and before injection should be included for comparison with *in vivo* Doppler data. Also, the sponsor should compare the results with those from previous study *Report #EB-98-14 (constitution of AF0150 in the temperature- and pressure-pretreated water)*

4. The effects of vascular blood pressure (hypertension) on AF0150 microbubble behavior and thus cardiac imaging need to be assessed because hypertension patients are one of the major target populations of AF0150.

Report Number: EB-98-22
Effect of Hypertension on AF0150-Enhanced Doppler Signal in Rabbits and Videointensity Levels in Swine

Report Location:	Vol.009, p293-307
Report date:	June 16, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	November-December, 1998
GLP Compliance:	No
AF0150 Lot number:	ZY18020

Specific Aim

To assess the effect of the high systemic arterial blood pressure and left ventricular pressure on AF0150-induced Doppler signals in rabbits and left ventricular cavity imaging in swine.

Methods

Part I: Rabbit Doppler Studies

Animal Preparation: Seven New Zealand White rabbits (3.5 + 0.6 kg) of either sex were anesthetized and intubated (allowed to free-breathe). The jugular vein was cannulated for the administration of AF0150 and for the infusion of phenylephrine HC1. A 10 MHz pulsed Doppler flow cuff transducer was placed around one carotid artery and held in place with a suture. A second carotid artery was cannulated for the measurement of arterial blood pressure. Heart rate and blood oxygen saturation was monitored with a pulse oximeter placed on the ear.

Doppler Signal Analysis: The Doppler signals of carotid artery blood flow was monitored and processed, as described in previous rabbit studies. The signal data were collected before (for baseline) and after bolus (10 sec) IV injections of AF0150 at 0.2 mg/kg (n=5) or 1.0 mg/kg (n=4), followed by 1 ml saline flush over 30 seconds. Doppler signals were recorded for 150 seconds following AF0150 administration. The animals were then given a continuous IV infusion of phenylephrine HCI (15-50 ug/min) to increase peripheral vascular resistance and thus lead to systemic hypertension. During the steady-state hypertensive response, Doppler signals were again measured following bolus IV injections of AF0150 at the same doses as before phenylephrine treatment.

Part II: Swine Video Intensity Studies

Animal Preparation: Four swine (21.94- 1.4 kg) of either sex were anesthetized and intubated for mechanical ventilation with room air. Jugular veins were cannulated for AF0150 administration and phenylephrine HCI infusion. A carotid artery was cannulated for BP measurement. A pressure transducer
was inserted into the left ventricular cavity via other carotid artery for the measurement of left ventricular pressure. Heart rate was monitored with ECG.

Echocardiography: A broad band 3.2 MHz transducer was positioned on closed chest to yield a short axis view at the midpapillary muscle level and then fixed in place by a mechanical arm at a depth setting of approximately 7 to 8 cm. Echocardiographic imaging was performed using fundamental (continuous and intermittent) ultrasound transmission. Mechanical Index (MI) was increased from 0.2 to 1.0. A dynamic range of 70 dB, frame rate between 57 and 61 frames/sec and a gain to optimize baseline images at each MI tested were used. Fundamental intermittent imaging was performed by ECG dual triggering on the T and R waves for every heartbeat.

For normotensive controls, cardiac ultrasound imaging was recorded before (baseline) and after a bolus (10 sec) IV injection of AF0150 (0.25 mg/kg) at each of MI (0.2, 0.6 and 1.0) in the following sequence: fundamental continuous imaging (-45 sec), and fundamental intermittent imaging (15 beats, -10 sec duration).

For the hypertension test, following the control (normotensive) sequence described above, the same animals received an IV infusion of phenylephrine HC1 (15-50 ug/min) to increase systemic vascular resistance and thus mimic systemic hypertension. During the steady-state hypertensive

response, bolus injections of AF0150 were administered and ultrasound images were collected in the same sequence as described above.

Videointensity Analysis: Video image data were recorded on 1.25 cm VHS videotape and videodensitometric quantitation of the contrast intensity was performed for the left ventricular middle cavity. Baseline video intensity levels (i.e., pre AF0150 injection) were subtracted from the peak contrast intensity determined at approximately 5 seconds after the end of image attenuation.

Data Analysis: heart rate, arterial pressure, and left ventricular pressure, before and after phenylephrine infusion, were reported as Means ± SEM. The differences were analyzed using a Student's t test. Videointensity and Doppler signal were expressed as Means ± SEM. The effects of normotension versus hypertension on AF0150-induced Doppler signal and videointensity were analyzed using two-way ANOVA. $p < 0.05$ was defined as statistically significant difference.

Results

Effects of Hypertension on AF0150-induced Doppler Signal in Rabbits (Figure 1): 7 rabbits received a continuous IV infusion of phenylephrine and systolic arterial pressure increased by 138% (from 60 mmHg to 130 mmHg). Phenylephrine slightly increase heart rate but without a statistical significance. The hypertensive status was maintained at a steady-state level throughout the duration of the AF0150 injection and Doppler signal monitoring. The phenylephrine-induced hypertension had no effect on Doppler signal at either 0.2 mg/kg (5 rabbits) or 1.0 mg/kg (4 rabbits) AF0150 (2 of 7 rabbits received both doses) as compared to controls (normotensive state).

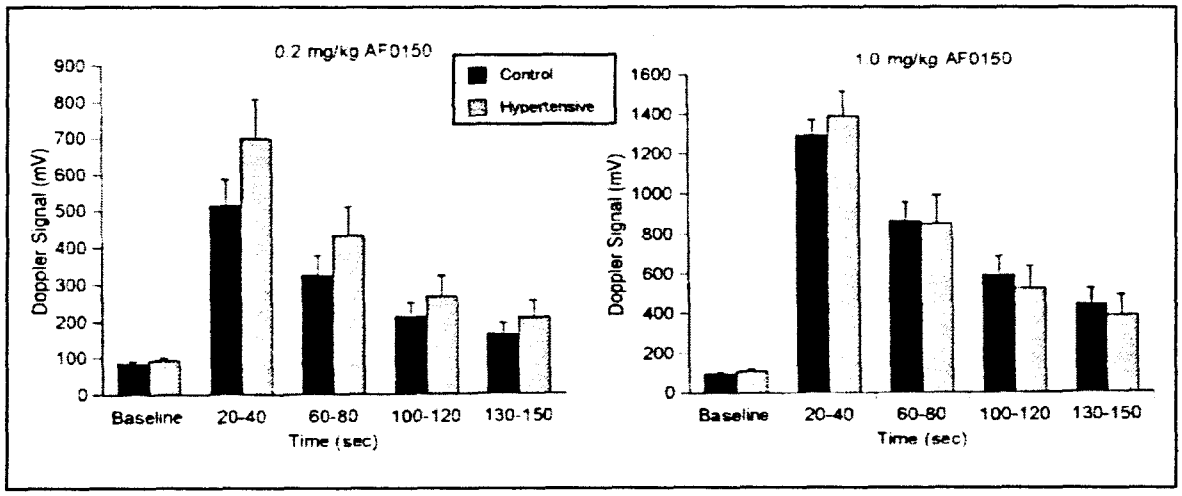

Figure 1. Effects of phenylephrine-induced hypertension on the Doppler signals of carotid artery blood flow in rabbits following IV injection of bolus AF0150 (0.2 mg/kg and 1.0 mg/kg).

Effects of Hypertension on AF0150-induced Cardiac Imaging in Swine (Figure 2): 4 swine were given a continuous IV infusion of phenylephrine, systolic arterial pressure increased by

approximately 42% and left ventricular pressure increased by approximately 44% without significant effects on heart rate. While the hypertension was maintained at a steady-state level, injection of a bolus AF0150 (0.25 mg/kg) had no significant effects on images of the left ventricular middle cavity region and AF0150-enhanced videointensity levels throughout the range of MI (0.2-1.0), as compared to controls (normotensive state).

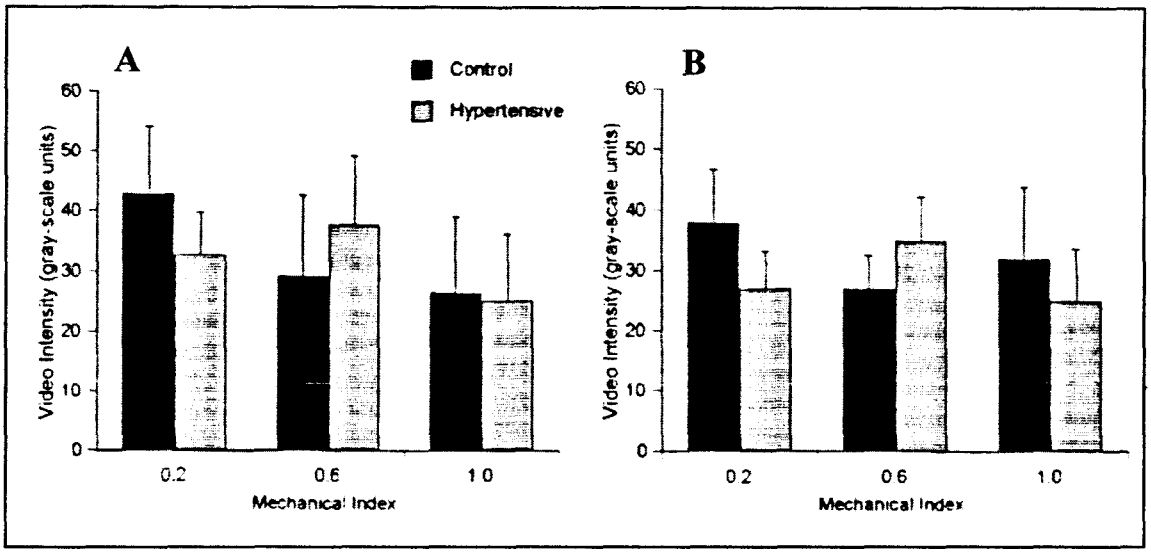

Figure 2. Effects of Phenylephrine-induced hypertension on videointensity of the left ventricular middle cavity imaging in swine following IV injection of bolus AF0150 (0.25 mg/kg). Images were recorded during fundamental continuous (***Panel A***) and intermittent (***Panel B***) ultrasound imaging followed by videodensitometric quantitation analysis. Data are mean ± SEM of 4 swine.

Discussion and Comments

1. This study showed that AF0150-enhanced Doppler signal of carotid artery blood flow in rabbits and left ventricular cavity imaging is not affected by high arterial blood pressure and high left ventricle pressure induced by phenylephrine. The results suggest that AF0150 microbubbles, at doses of 0.2 or 1.0 mg/kg, remain stable in circulation under hypertensive conditions and are likely effective for use in patients with hypertension.

2. Phenylephrine (15-50 ug/min IV infusion to both swine and rabbits) increased the blood pressure to a lower degree in swine (44%) than in rabbits (138%), probably due to dose difference (based on either body surface area or body weight). Therefore, it would have been more useful to use higher doses of phenylephrine or another alpha1 adrenergic receptor agonist, to induce BP increase equivalent to human hypertension.

3. Hypertension, or phenylephrine pretreatment, increased the Doppler signals of carotid blood flow in rabbits and increased videointensity of left ventricular cavity at MI of 0.6 following AF0150 injection. Appropriate explanation for these phenomena was not provided.

Report Number: EB-98-15
Effect of High Inspired Oxygen Concentrations on the Efficacy of AF0150 in Swine

Report Location:	Vol.009, p308-322
Report date:	March 22, 1999
Study Facility:	Alliance Pharmaceutical Corp
In-life phase:	January-March, 1998
GLP Compliance:	No
AF0150 Lot number:	UA16064

Specific Aim

To assess the effects of high O2 inspiration on AF0150-induced Doppler flow signal and echocardiographic image in swine.

Methods

Animal Preparation: Four swine of either sex (23.0±1.2kg) were anesthetized and intubated for mechanical ventilation. O2 concentrations in the inspired air were measured with an in-line O2 sensor placed in the ventilator gas line. Heart rate was monitored with ECG. A jugular vein was cannulated for AF0150 administration. Carotid arteries were cannulated to monitor hemodynamics and to collect blood samples. A 10 MHz pulsed Doppler flow cuff transducer was placed around a carotid artery for the measurement of carotid artery blood flow. A 3.2 MHz ultrasound probe was positioned over the heart at a depth setting of approximately 6 cm for closed chest ultrasound cardiac imaging.
AF0150 Administration: AF0150 (100 mg fill) was reconstituted with 10 ml of 0.45% sodium chloride to a final concentration of 10 mg/ml. Each animal received a bolus injection of AF0150 at 0.125 mg/kg and 0.25 mg/kg.

Doppler Signal and Cardiac Imaging Analysis: Baseline Doppler signal of carotid artery blood flow and cardiac ultrasound images were recorded in all animals at room air (21% O2) before AF0150 administration. Inhaled O2 concentration was then increased in a stepwise from 21% to 65%. The animal was allowed to equilibrate for 30 mines at each O2 level prior to blood gas analysis and AF0150 administration. Doppler signal and videointensity (of left ventricular cavity) data were analyzed at various time points post-AF0150 injection. Doppler signal was measured at the signal peak (20-40 seconds post-AF0150) and persistence (60-80 seconds post-AF0150) during simultaneous ultrasound power application (echocardiography) throughout the study.

Data Analysis: Data are expressed as mean ± SEM of 4 tested animals. One-way ANOVA was used for the effects of high O2 inspiration on Doppler signal and videointensity levels, and two-way ANOVA for the effects of different doses of AF0150 on videointensity levels or Doppler signal response at various inspired O2 levels. Significance level was defined at $p < 0.05$.

Results

Arterial Blood O2 Tension (Figure 1) and Hemodynamics: After 30 minutes of equilibration at each inspired O2 concentration, PaO2 levels increased with increasing O2 concentration in the inspired air from 21% to 65%. There was no significant change in the arterial carbon dioxide (PaCO2) and arterial pH values. Heart rate and mean arterial pressure were not notably affected by the different O2 concentrations (21% to 65%).

Videointensity: for LV cavity imaging (*Figure 1*), increasing the inspired O2 concentration had no effect on videointensity levels up to 55% O2. There was no notable difference in videointensity between the two AF0150 doses (0.125 and 0.250 mg/kg). At the O2 concentration of 65%, videointensity levels decreased by approximately 24% and 45% at the 0.125 mg/kg and 0.250 mg/kg doses, respectively.

For LV myocardium (septum and lateral wall) imaging (*Figure 2*), videointensity levels decreased with increasing O2 concentrations (from 21% to 65%) at both AF0150 doses (0.125 and 0.250 mg/kg). The videointensity levels were lower than those in the LV cavity. There were slight differences between two AF0150 doses. However, the variations are too large to reach statistical significant level.

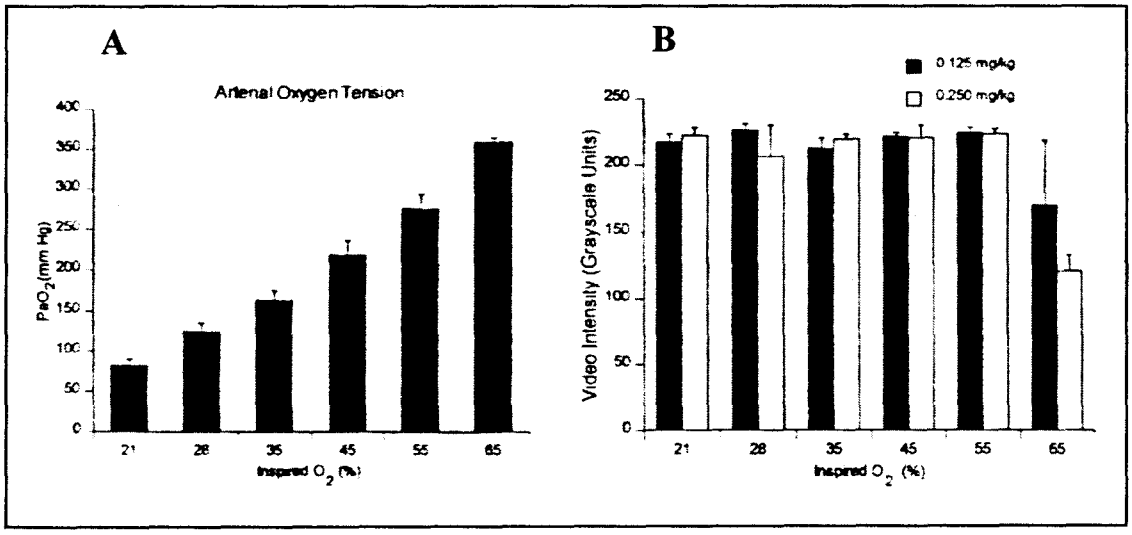

Figure 1. Effects of inspired high O2 on videointensity of left ventricular cavity imaging in swine following IV injection AF0150 at 0.125 and 0.25 mg/kg. ***Panel A,*** arterial O2 tension (PaO2) analysis after the animals inspired O2 at different concentrations prior to AF0150 injection; ***Panel B,*** videointensity of LV cavity image. Data are Mean ± SEM of 4 swine.

Doppler Signal (Figure 3): the inspired O2 concentrations higher than 45% decreased both peak and persistent Doppler signals at low dose (0.125 mg/kg) and at high dose (0.250 mg/kg) of AF0150 without statistical difference between two AF0150 dose groups. The decrease at the high dose of AF0150 was lower than at the low dose.

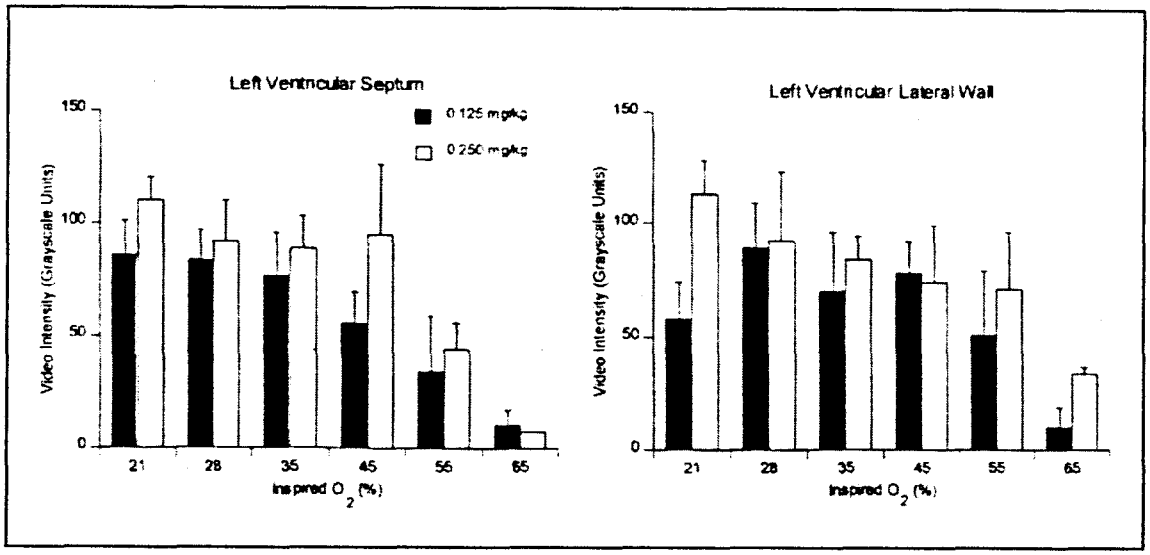

Figure 2. Effects of inspired high O2 on videointensity of left ventricular myocardium (septum and lateral wall) imaging in swine following IV injection AF0150 at 0.125 and 0.25 mg/kg. Data are Mean ± SEM of 4 swine.

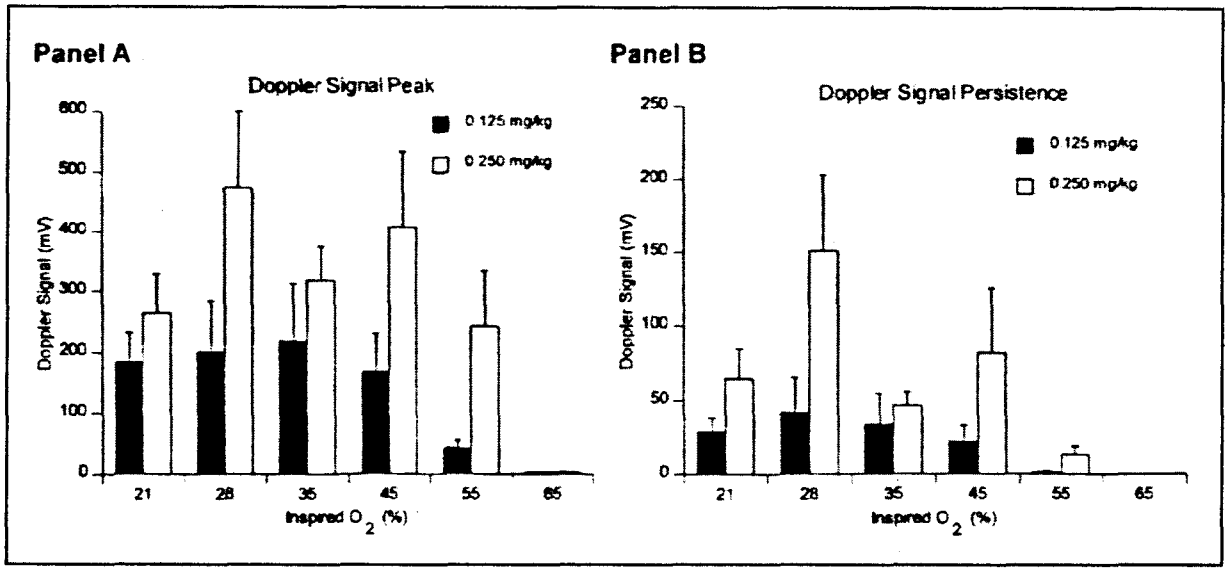

Figure 3. Effects of increasing O2 concentration in inspired air on Doppler signals (peak signal in ***Panel A***, and persistent signal in ***Panel B***) in swine following bolus IV injection of AF0150 at the doses of 0.125 and 0.25 mg/kg. Data are Mean ± SEM of 4 swine (except at 65% O2, n=2).

Discussion and Comments

The results suggest that inhalation of high O2 (more than 50%) air decreases ultrasound signals (Doppler flow signals and echocardiographic images) induced by AF0150 without significant AF0150 dose-dependence. However, all differences were not statistically significant. This is due to the high variations of most data as presented in Figures (the true variations are even higher

because of misusing SEM instead of SD). The sponsor also provided some results, as an appendix, from a pilot study in which 21-35% O2 concentrations were tested and showed that swine inhalation of 35% O2 decreased Doppler signals and videointensity of cardiac images without indication of statistical significance.

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Summary of General Pharmacology

Efficacy and efficacy-related issues of AF0150 microbubble agent were addressed by monitoring the effects on Doppler flow signal and echocardiographic imaging of left ventricular cavity. The *in vivo* and *in vitro* systems were used to test the effects of the following conditions on AF0150 microbubble behavior and ultrasound signal enhancement: dose-response, administration modes (IV bolus or infusion), reconstitution conditions, ultrasound power settings, and external pressure.

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

AF0150 Administration Modes: Studies in rabbits and swine assessed the comparative efficacy of AF0150 when administered as a bolus injection or an infusion. Dose-dependent increases in Doppler signal enhancement were observed in both administration modes. In the swine study, optimal videointensity was achieved with both fundamental and second harmonic imaging after the 1-minute infusion of 0.66 mg/kg AF0150.

AF0150 Reconstitution Conditions: the vial fill sizes (100 mg vs. 200 mg), reconstituted concentration (10 mg/kg vs. 20 mg/kg), microbubble stability (time post reconstitution and vial inversion), physical conditions (pretreated water, application of pressure to reconstituted AF0150) and microbubble sizes were tested in terms of effects on AF0150-enhanced ultrasound signals. Studies in swine and rabbits demonstrated that fill sizes and reconstituted AF0150 concentration had no significant effects on AF0150 Doppler signal and myocardium imaging enhancements. Pretreatment of SWFI with different temperatures (15° to 30°C) and gas tensions (460 to 1060 mm Hg) prior to reconstituting AF0150 had no significant effect on the Doppler signal, except at 15°C for all gas tensions and 30°C for the low gas tension (460 mm Hg), where the Doppler signal slightly decreased. Optimal efficacy in rabbits was obtained within the first 30 minutes following AF0150 reconstitution and within the first 60 seconds after vial inversion. After the reconstituted AF0150 were separated into two microbubble populations by creaming (large bubbles float to the top of the suspension), the small microbubbles (<3 um diameter, 97-99%) contributed more to the Doppler peak signal (initial intense response), and the large microbubbles (>3 um in diameter, 19-24%) contributed more to Doppler persistent signal.

Effects of Ultrasound Settings on AF0150 Microbubble: Exposure of AF0150 microbubbles to increasing ultrasound powers (mechanical index, MI) or time in an *in vitro* simulated circulation

system decreased microbubble count with a slight shift, suggesting that ultrasound exposure destroys microbubbles in a power-and time-dependent manner. Application of ultrasound burst (continuous and triggered) to the heart (closed-chest) decreased AF0150-induced Doppler signal (peak and persistence) enhancement of carotid artery blood flow in rabbits, with increased ultrasound power levels. Triggered ultrasound application caused less decrease than the continuous ultrasound. Echocardiography studies in closed-chest swine with three different ultrasound imaging equipments showed that application of increasing ultrasound power or frame rate diminished signal detection of LV cavity imaging at AF0150 doses of 0.125 mg/kg (0.9 fold the PCD based on the body surface area) and 0.250 mg/kg (1.9 fold the PCD based on the body surface area).

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

Conclusion and Comments:

1. The results from above studies showed that AF0150 microbubble agent appeared effective to enhance ultrasound signals (Doppler signal of carotid artery blood flow and left ventricle imaging) in animal models, suggesting a potential effectiveness in the clinical application.

2. Animal numbers in major studies which address the potential efficacy of AF0150 in clinical application were not high enough for statistical analyses, although results from those studies showed a similar tendency. These studies are: EB-98-18 (2 swine); EB-98-19 (2 swine); EB-98-20 (3 swine); EB-98-16 (2 rabbit).

3. Measurement of Doppler signals of carotid artery blood flow was used in most studies to optimize condition of AF0150 application. This was different from the clinical indication, where left ventricular cavity imaging will be conducted. A proper explanation for equivalence of the two imaging end point was not provided.

4. SEM (Standard Error of Mean) instead of SD (Standard Deviation) was used for data process and analysis in all study reports. SD, but not SEM, should be used for demonstration of variation between samples.

27. PHARMACOLKINETICS AND TOXICOKINETICS

Report Number: IMUS-041-TOX
Elimination of Perfluorohexane from AF0150 in Expired Air in Rats with Pharmacokinetic Evaluation of Perfluorohexane in Blood

Report Location:	Vol.032, p080-179
Report date:	June 28, 1999
Study Facility:	[redacted]
In-life phase:	December 14, 1998 - February 27, 1999
GLP Compliance:	Yes (with QA Statement)
AF0150 Lot number:	UA18027 (400 mg/vial)
AF0150 Dosage (HDM):	20 (26-fold of PCD)

Specific Aim

To determine pharmacokinetics of AF0150 (intravenous injection with a single bolus) by quantifying perfluorohexane (PFH) in expired air in rats

Methods

Animal Preparation: Rats (both sexes), Crl:CD (SD)IGS BR, were obtained from [redacted] The animals were 9-11 weeks old with body weights of 269-315 g for males, and 10-17 weeks old with body weights of 345-376 g for females at initiation of treatment. Standard procedures were followed for housing, handling, feeding and care of the animals. The rats were acclimated for at least 1 week before initiation of treatment. Seven and 5 rats each sex were used for expired air PFH and blood PFH analyses, respectively.

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 20 mg/kg AF0150 through IV injection into a pre-inserted indwelling catheter in the tail vein. A single bolus injection was given to each animal. For the expired air evaluation, rats were sealed within a glass tube with the catheter exteriorized through a rubber seal. Dose was calculated based on pre-test body weight.

Observations: Mortality and clinical signs were observed twice daily. Expired air and blood samples were collected after AF0150 administration as follows, and PFH in these samples was analyzed with [redacted]

Expired Air PFH Analysis: Expired air samples were collected from each of 7 rats/sex. The samples were drawn from the glass tube (where each individual animal was retained) into the sample tubes (2/sex, Figure 1) or the gas bags (5/sex, Figure 2). Airflow was 300 ml/minute (based on a pilot study results) and went past the animal body and snout. With the gas bag sampling, 100% of the expired air was collected for 3 hours into 80-liter Tedlar bag. Samples

after the 3-hour time point were directly collected onto adsorbent cartridges containing [redacted] PFH in the expired air samples was measured by [redacted]. For the tube sampling, expired air samples were collected with the adsorbent trapping procedure in real time: for the first 5 minutes (1-minute interval), the second 5 minutes (2.5-minutes intervals), next 50 minutes (12.5-minute interval) up to 180 minutes (100% expired air) post-dosing. The animals were then returned to the chamber for additional sampling at 8, 24 and 48 hours after dosing.

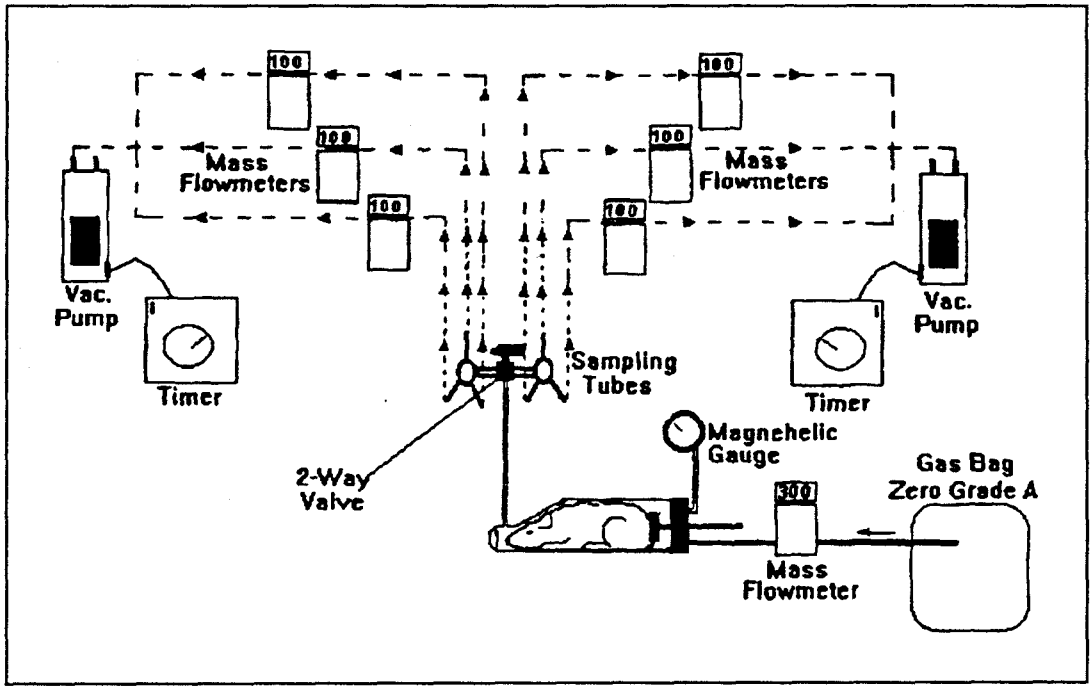

Figure 1. Expired Air Sample Collection (***Tube Sampling***). At the first 3 hours post dosing, 100% expired air was collected in real time in the tube containing trapping adsorbent [redacted] Thereafter, additional samples were collected at 8, 24 and 48 hours.

Blood PFH Analysis: Eleven serial blood samples (0.25ml/sample) were taken from each of 5 rats/sex via a pre-inserted femoral vein catheter up to 24 hours post doing. Collection time-points were 0 (pre-dosing), immediately (within 1 minute), 2, 5, 15, 30, 45 minutes; 1, 4, 8 and 24 hours after dosing.

Dose Verification: Aliquots (0.25 ml) of reconstituted AF0150 were taken from each vial at immediately pre-dosing and post-dosing for each animal. PFH levels in the samples were analyzed using [redacted] The mean value of these two determinations was used for calculation of total AF0150 dose (PFH, ug) and PFH recovery.

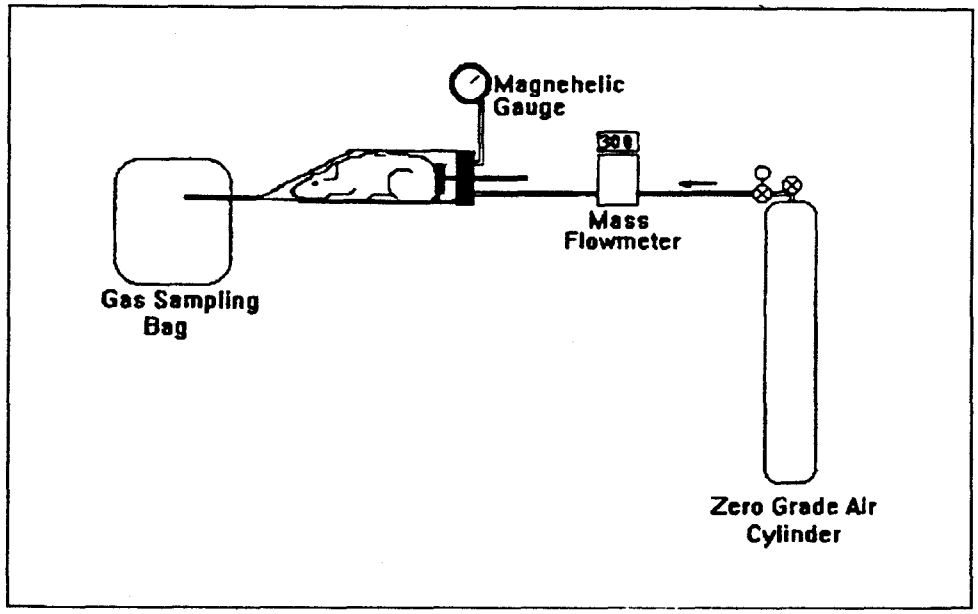

Figure 2. Expired Air Sample Collection (***Gas Bag Sampling***). At the first 3 hours post dosing, 100% of the expired air was collected into 80-liter Tedlar bag with a total 54 liters. Additional samples were collected at 6-11 and 24-28 hours.

Results

PFH Analysis in the Injected Dose: The mean PFH concentration from pre- and post-dose measurements of 13 vials of reconstituted AF0150 was 80-162 ug/ml and the exact dose to the animals ranged from 18 to 45 ug PFH per animal in Expired Air Study group. In Blood PFH Study group, PFH concentration in reconstituted AF0150 (11 vials) was 104.7-137.5 ug/ml and the exact animal dose was 33-44 ug PFH.

PFH Analysis in Expired Air: PFH recoveries with gas bag sampling was approximately 94% within the first 3 hours, 98% at 6-11 hours and 103% at 24-28 hours after dosing, as summarized in Table 1. One animal from each sex was excluded from the recovery calculation because only half of air volume was left in the sampling bags before arrival at the lab for analysis. The injected PFH in this animal was 25.9 ug and the PFH recoveries were more than 150% at all time points. With the tube sampling, the PFH recoveries were 78% at the first 3 hours, 87% at 6-10 hours and 87% at 24-28 hours post dosing, as summarized in Table 2.

The excretion kinetics of PFH from the expired air fitted a two-compartment model within the first 3 hours, as seen in Figure 3. The initial rapid elimination phase was from plasma, followed by a slow elimination phase contained distribution phase, which was most likely from adipose tissues. The half-life values for the initial elimination phase and slow elimination phase were not provided.

Table 1. PFH Recovery from Expired Air after IV Injection of AF0150 in Rats
(Gas Bag Sampling)

Animals	Injection Dose PFH (ug)	PHF Recovery Post Dosing					
		3 Hours		6-11 Hours		24-28 Hours	
		PFH (ug)	%	PFH (ug)	%	PFH (ug)	%
Male (n=4)*	40.5±4.6	39.9±6.6	99.8±21.9	40.9±6.4	102.5±22	42.9±6.9	103.3±22.8
Female (n=5)*	28.3±6.9	24.9±7.1	87.9±13.9	26.6±7.3	93.9±12.8	32.4±3.3	103.3±17.3
Total (MF)	34.4±8.5	32.4±10.2	93.8±18.2	33.8±9.9	98.2±17.3	37.6±7.5	103.3±18.1

* One animal was excluded from the data process because the collected air volumes were suspected.

Table 2. PFH Recovery from Expired Air after IV Injection of AF0150 in Rats
(Tube Sampling)

Animals	Injection Dose PFH (ug)	PHF Recovery Post Dosing							
		3 Hours		6-10 Hours		24-28 Hours		48-59 Hours	
		PFH (ug)	%	PFH (ug)	%	PFH (ug)	%	PFH (ug)	%
Male (n=2)	35.8 ±0.9	24.7 ±0.8	69.2 ±3.8	26.5 ±1.1	74.1 ±4.7	29.0 ±0.2	81.2 ±2.6	30.7 ±1.6	85.9 ±6.5
Female (n=2)	30.9 ±0.4	27.0 ±0.04	87.2 ±1.1	28.5 ±0.5	92.3 ±0.5	30.4 ±	99.3 ±	31.4 ±0.5	101.5 ±0.5
Total (MF)	33.3 ±2.8	25.9 ±1.4	78.2 ±1.4	27.5 ±1.4	83.2 ±10.8	29.7 ±0.8	87.2 ±10.6	31.0 ±1.0	93.7 ±9.7

PFH Analysis in Blood: Blood PFH level decreased by 78±20.1% during the first 2 minutes following IV injection of AF0150 (20 mg/kg). PFH was not detectable in blood by 24 hours post dosing. The terminal half-life of PFH was 87.8 minutes for males and 88.8 minutes for females based on combined data from all male and female rats (but not from individual half-life calculations). The elimination kinetics fitted to a two-compartment model, as seen in Figure 4, which is consistent with the PFH excretion kinetic profile from expired air.

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