Preclinical Acute Toxicity Studies and Rodent-Based Dosimetry Estimates of the Novel Sigma-1 Receptor Radiotracer [^{18}F]FPS.

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MATERIALS AND METHODS

ACUTE TOXICITY STUDIES OF FPS

Animals. For all animals, cage size and animal care conformed to the Guidelines for the Care and Use of Laboratory Animals, 7th edition, and the U.S. Department of Agriculture through the Animal Welfare Act (Public Law 99-198).

Rats: The 24 male and 24 female rats (Charles River Laboratories, Raleigh, NC) used in this study were 7-8 weeks old when they arrived at Southern Research Institute and were 8-9 weeks of age on the day of dosing. Upon arrival, the rats were placed in quarantine and visually inspected for general health within 3 days of receipt. Each rat was allowed access to feed (Certified Rodent Diet #5002; PMI Feeds, Inc.; St. Louis, MO) and tap water (Birmingham public water supply) *ad libitum* during the quarantine and study periods. The rats were individually housed in solid-bottom, polycarbonate, shoebox cages on stainless steel racks during the quarantine and study periods in an animal room that was continuously monitored for temperature and humidity.

Rabbits: The 24 male and 24 female New Zealand white rabbits (Myrtle’s Rabbitry, Inc. (Thompson Station, TN)) used in this study were 11-12 weeks old and were 13-14 weeks of age on the first day of dosing. Upon arrival, the rabbits were placed in quarantine and were visually inspected for general health. The rabbits were fed PMI Rabbit Breeder CS 5LH3 feed (PMI Feeds, Inc. St. Louis, Mo, 125-150 g/day). Water (Birmingham municipal supply) was provided *ad libitum* during the quarantine and study periods via an automatic water system. The rabbits were individually housed in stainless steel cages on stainless steel racks in an animal room that was monitored for temperature and humidity.

Beagle dogs: The seven beagle dogs used in this study were selected from among 36 purebred adults (Marshall Farms USA, Inc., North Rose, NY). The dogs were approximately 16 months of age at the time of study. Individual animal identification was by ear tattoo. Upon arrival, all dogs were placed in quarantine and each was given a physical, including an examination of all external surfaces, organs, and orifices and a check for external parasites. Body weight and body (rectal) temperature were recorded. Each dog was allowed access to Certified Canine Diet #5007 (PMI Feeds, Inc.; St. Louis, MO) for approximately 2 hours each day during the quarantine and study periods. The dogs were housed individually in stainless steel cages during the quarantine and study periods in an animal room that was monitored for temperature and humidity.

Test Article. The test article, 1-(3-fluoropropyl)-4-[(4-cyanophenoxy)methyl]piperidine hydrochloride, was formulated in sterile saline (Phoenix Pharmaceutical, Inc.; St. Joseph, MO). Each dose formulations was prepared as a solution of test article in saline. The formulations were filter sterilized (0.45 μm nylon filters), stored at room temperature in glass bottles, and protected from light until used. The formulations were used for dosing within 8 hours after preparation and were considered stable for the use period. Immediately after the preparation of the FPS formulations, three 5 mL samples were collected from each formulation and stored at –70 $^{\circ}$C. One sample was taken from the top of the formulation, one from the middle, and one from the bottom. These samples were subjected to concentration and homogeneity analysis. Stability studies were performed by HPLC analysis.

Dose Administration Paradigms. Single dose, acute toxicity studies were conducted in male and female rats and rabbits. As a starting point, an initial mass dose limit in humans of 10 μg per intravenous administration was set (note: this was later changed to 2.8 μg max per dose after the toxicity data were obtained) and this was used to define test doses used in the toxicity studies. The procedures used to determine the FPS dose to be administered to each species requires that all doses are converted from units of mg/kg (body weight) to units of body surface area expressed in mg/m^2. Basing the dose on body surface allows for the determination of an equivalent dose to another species [7]. The equation used is:

$$\text{Dose (mg/kg)} \times F = \text{Dose (mg/m}^2\text{)}$$

Where F is a constant based on the species of animal being tested [7]. The doses used in these studies, as expressed in both mg/kg and mg/m^2, as well as species F values can be found in Table 1.

Dose Procedure. On Day 1, each animal received a single intravenous bolus dose of FPS formulation or sterile saline. Doses were based on the body weights recorded on Day 1 prior to dosing (and converted to mg/m^2 surface area), and amounts of 100, 1000 and 10,000 times the maximum proposed amount of 10 μg to be administered to a 70 kg person were used for the three test dose groups. Formulations were administered intravenously as this is the intended route of administration in humans. Specifically, the FPS formulations used for dosing rats were prepared at concentrations of 0.0176, 0.176, and 1.76 mg/mL (Table 2). The FPS formulations used for dosing rabbits were prepared at concentrations of 0.022, 0.22, and 2.2 mg/mL (Table 3). All formulations were stored at room temperature in glass bottles and protected from light until used. The formulations were used for dosing within 8 hours after preparation.

Observation Protocol. All animals (rats and rabbits) in this study were observed once daily during quarantine and twice daily during the study for signs of mortality, moribundity, injury, and availability of food and water. Detailed clinical observations were recorded once daily throughout the study; the examination included but was not limited to observations of the general condition, skin, fur, eyes, ears, nose, oral cavity, thorax, abdomen, external genitalia, limbs, and feet. Individual body weights were measured and recorded for each animal during the week prior to study initiation (for randomization), on Day 1 prior to dosing, and on Days 3, 8, and 15. Quantitative weekly food consumption was measured and recorded individually for all study animals throughout the study, beginning during Week 1. Both eyes of each animal were examined by indirect ophthalmoscopy during Week –1 and on Days 2 and 12. Ophthalmologic examinations were performed by a board-certified veterinary ophthalmologist.

Clinical Pathology. Blood samples were obtained from the jugular vein of each surviving rabbit on Days 3 and 15; blood samples were collected into tubes containing EDTA (hematology samples), sodium citrate (coagulation samples), or no anticoagulant (clinical chemistry samples). Blood samples were used for the determinations of the parameters outlined in Table 4.

Gross Necropsy. On Day 3, following collection of body weights and clinical pathology blood samples, half the surviving animals in each dose-sex group were sacrificed and given a complete postmortem examination. All remaining animals were sacrificed on Day 15 or 16 and given a complete postmortem examination, which included a thorough inspection of all external surfaces, organs, and orifices. The cranial, thoracic, abdominal, and pelvic cavities were opened, and the tissues/organs within each cavity were inspected. All of the organs and tissues listed in Table 5 were examined *in situ*, weighed, dissected free of fat and other contiguous tissues, and fixed in 10% neutral buffered formalin.

Histologic Processing. All tissues examined from all animals in all treatment groups were processed for histopathological examination. The fixed tissues were trimmed, processed, and microtomed. The tissue sections were mounted on glass slides, stained with hematoxylin and eosin, and coverslipped for histopathologic examination. Histologic processing was performed at HistoTechniques, Ltd. (Powell, OH). All slides and tissues were returned to Southern Research Institute, where they were fully evaluated by a board-certified veterinary pathologist.

Statistical Analyses. Group means and standard deviations were calculated when appropriate for body weights, food consumption, clinical pathology parameters, and absolute and relative organ weight data. Evaluation of body weights, clinical pathology parameters, and food consumption data for the differences between groups utilized ANOVA and Dunnett's Test for multiple comparisons. Mean organ weights and organ weight ratios for each treated group were compared to those of the control group by a two-tailed Student's t-test for each sex. In all cases, the lower limit for statistical significance was defined as $p < 0.05$.

TOXICITY STUDIES IN BEAGLE DOGS.

Single intravenous administrations of FPS (2640 □g/kg, 132 □g/kg, or 13.2 □g/kg) were made by bolus injection into a cephalic vein of beagle dogs. Using a body surface conversion to extrapolate doses between dogs and humans, the high dose in this study (2640 □g/kg) was 10,000

times the originally anticipated maximum dose for use in humans (10 mg/70 kg). Seven dogs were arbitrarily assigned to one of 3 treatment groups. Each dog was given a single iv dose on its respective Day 1. Dose group assignments are outlined in Table 6.

Observation Protocol. Dogs were observed twice daily for signs of mortality or moribundity. When possible, all dogs were removed from their cages and examined closely for clinical signs of toxicity once pre-dose, immediately post-dose, and at 0.5, 1, 2, and 4 hours post-dose. When the dog was removed from the cage at each of these time points, a 10-lead ECG recording was obtained, respiration rate was measured and the body (rectal) temperature was recorded. A board-certified veterinary cardiologist evaluated the ECG tracings and provided a report of his findings.

FORMULATION TESTING.
All FPS formulations used for the toxicity studies were analyzed by reversed-phase HPLC methods [Alltech Prodigy C-18 analytical column (4.6 x 250 mm, 10 µm particle size); 0.1 M ammonium acetate and acetonitrile (72/25 v/v); flow rate: 2.0 ml/min; Waters 986 PDA detector]. All samples were analyzed by injection of 10 µl aliquots using Hamilton 25 µl HPLC syringes. Data were acquired and analyzed with a Waters Millennium Data Acquisition Workstation and a PC computer.

Generation of Mass versus UV Response Equation. Five different standard solutions of FPS (1 - 100 µg/ml) in saline were repared and analyzed by HPLC (injection volume = 10 µl, n = 5). The area under the curve corresponding to the FPS peak (retention time = 9.7-10.0 min) was determined and plotted against the amount of mass injected, and a line was generated (Microsoft Excel). This equation was used to determine the concentration of FPS in samples of formulations used in the acute toxicity studies.

Formulation Concentration Testing: Samples of each formulation were shipped on dry ice from Southern Research Institute and analyzed at Columbia University. Several calculated dilutions were made of each sample in sterile saline using calibrated hand-held pipetters. Samples from the vials marked “middle”, “top” and “bottom” were analyzed (n = 3). The area under the curve corresponding to FPS was determined. The dilution factors were taken into account during the final concentration calculations.

FPS saline solution stability. Two test solutions of FPS at a concentration of 10 µg/ml were prepared and stored at room temperature. Each was analyzed by HPLC at approximately 2 hours, 24 hours and 72 hours after preparation (n = 3 per analysis).

Toxicity Studies:

Studies in Rats. Transient convulsions occurred in all animals in the high dose group (8.8 mg/kg) immediately after administration; the convulsions lasted for less than 1 minute, and the rats appeared normal thereafter for the duration of the study. No drug-related clinical signs of toxicity were noted for rats in the 0, 0.088, or 0.88 mg/kg dose groups during the 15-day observation period following drug administration. No drug-related changes were noted in other parameters that were examined during this 15-day study, including body weight, food consumption, eye changes, clinical pathology parameters, gross necropsy findings, absolute and relative organ weights, or microscopic lesions. With the exception of the transient post-dose convulsions seen in the rats in the 8.8 mg/kg dose group, no evidence of CNS or cardiovascular toxicity was noted in rats. The no-observable-effect-level was 0.88 mg/kg.

Studies in Rabbits. Single iv doses of FPS (4.4 mg/kg) produced mortality in 2/6 male rabbits within 2-3 hours after drug administration and tremors/seizures and/or ataxia were also noted after dosing in animals in this dose group. These effects were transient and all surviving rabbits appeared normal the following day. FPS did not produce effects on body weight gain, food consumption, hematology, clinical chemistry, or coagulation parameters. No drug-related changes were noted in the eyes of FPS-treated rabbits, and no drug-related lesions were noted during gross or microscopic examination of these animals. The maximum tolerated dose was greater than 0.44 mg/kg, but less than 4.4 mg/kg. The no-observable-effect-level was 0.044 mg/kg.

Studies in Beagle Dogs. This study determined the acute toxicity induced by single iv bolus doses of FPS in male and female beagle dogs. Measured parameters included heart rate and EKG, blood pressure, body temperature (rectal probe), and any signs of physical or behavioral changes. Doses of 2.64 mg/kg (2640 μg/kg) in dogs induced unacceptable clinical signs of toxicity, particularly overt aggressiveness and EKG changes. The aggressiveness lasted approximately two hours, after which time the animals could be handled normally. A lower dose of 132 μg/kg also induced the unacceptable aggressive behavior, but no changes in dog's EKG that were of concern. A dose of 0.013 mg/kg FPS produced no observable effects in dog. Therefore, the results indicate a maximum safe dose in dogs of greater than 0.013 mg/kg but less than 0.13 mg/kg.

Formulation Testing. Dose solutions used in the toxicity studies were returned to Columbia University for analysis (HPLC). The analyst was blinded to the identity of the samples. For each dose solution, samples from the top, middle and bottom were analyzed in triplicate, and results from these samples were compared to assess homogeneity. No significant differences were detected between the top, middle and bottom from sample vials for each concentration tested, indicating that the solutions were homogeneous in nature. No extraneous peaks were noted in any of the samples. No FPS was detected in the control solutions. All solutions containing FPS were found to be within 20% of the expected concentrations.

Finally, sterile solutions of FPS in saline stored at room temperature were examined by HPLC for a period of three days. This analysis revealed that no significant degradation of FPS occurred during storage at room temperature for 72 hours.

DISCUSSION

Extended acute toxicity studies in rats and rabbits, and limited acute toxicity studies in beagle dogs suggest at least a 175-fold safety margin in humans at a mass dose limit of 2.8 µg per intravenous injection. This estimate is based on the measured no observable effect doses in these species. These data are presented in Table 9. It is worthwhile to note that post-mortem histopathology studies in rats and rabbits did not reveal any FPS related abnormalities. No changes in food consumption, organ or whole body weights, blood chemistry or other outcome measures were noted. The most notable observed effect in both species were transient seizures noted at the highest doses.

These studies also clearly demonstrated that dogs are more sensitive to FPS induced toxicity than either rats or rabbits. The main effects included overt aggressiveness and EKG changes. The aggressiveness lasted approximately two hours, after which time the animals appeared normal, and no abnormalities were detected in the EKG traces. As described in the Methods section, the test dose paradigm was initially based on a maximum proposed injected mass dose of 10 µg per administration to a 70 kg human. Since the dose (in mg/m^2) that failed to produce any signs of toxicity in dogs, the most sensitive laboratory animal species tested, was only 50 times this value, the maximum injected mass limit per dose to humans was subsequently adjusted to 2.8 µg. This adjustment this would allow for at least a 175-fold margin of safety based on the dog studies without compromising the quality of the human PET imaging studies that are planned. This margin of safety should be acceptable to allow the evaluation of this new PET tracer in human volunteers. During these initial human imaging studies, [^{18}F]FPS would be administered twice to some participants with a minimum of 14 days between doses. During the human imaging studies, it is expected that a dose of between 1 and 5 mCi of [^{18}F]FPS would be administered intravenously during each PET scan, with a specific activity greater than or equal to 300 mCi/µmol at time of injection.

LEGENDS

Table 1. Dose Conversions (in mg/kg and mg/m^2) for all Species and Dose Groups Used in The Evaluation of [^{18}F]FPS.

Note: FPS doses are based upon the originally anticipated maximum human dose of 10 μg per 70 kg subject. Species F values were obtained from Reference 7.

Table 2. Summary of Doses for Rats.

Note: [a]These animals each received single iv doses of sterile saline (4.4 ml/kg).

Table 3. Summary of Doses for Rabbits

Note: [a]These animals each received single iv doses of sterile saline (5.0 ml/kg).

Table 4. Clinical Chemistry Determinations for Extended Acute Toxicity Testing in Rats and Rabbits

Table 5. Organs Weighed and Examined by Histopathology for Extended Acute Toxicity Testing in Rats and Rabbits

Table 6. Summary of Doses for Dogs

Table 7. [^{18}F]FPS Biodistribution Data Used in Human Dosimetry Estimatations. Values are Given in %ID/g as Obtained in Adult Male Wistar Rats (n = 3).

Table 8 Organ and Whole Body Extrapolated Human Dosimetry Estimation Obtained from Rat [^{18}F]FPS Biodistribution Data.

Table 9. Summary of the "No Observable Effect Levels" (NOEL) for FPS as Determined in Rats, Rabbits and Beagle Dogs.

Table 1.

Group I			
Species	dose mg/kg	F	dose mg/m^2
Rat	0.088	6	0.528
Rabbit	0.044	12	0.528
Beagle	0.0132	20	0.264
Group II			
Species	dose mg/kg	F	dose mg/m^2
Rat	0.88	6	5.28
Rabbit	0.44	12	5.28
Beagle	0.132	20	2.64
Group III			
Species	dose mg/kg	F	dose mg/m^2
Rat	8.8	6	52.8
Rabbit	4.4	12	52.8
Beagle	2.64	20	52.8

Table 2.

Group No.	Article Administered	Dose		Number of Study Animals	
		Level (mg/kg)	Volume (mL/kg)	Males	Females
1[a]	Saline	0	2	6	6
2	FPS formulation	0.044	2	6	6
3	FPS formulation	0.44	2	6	6
4	FPS formulation	4.4	2	6	6

Table 3.

Group No.	Article Administered	Dose		Number of Study Animals	
		Level (mg/kg)	Volume (mL/kg)	Males	Females
1	Saline	0	5	6	6
2	FPS formulation	0.088	5	6	6
3	FPS formulation	0.88	5	6	6
4	FPS formulation	8.8	5	6	6

Table 4.

HEMATOLOGY		
WBC	Total leukocyte count	$10^3/mm^3$
RBC	Erythrocyte count	$10^6/mm^3$
HGB	Hemoglobin	g/dL
HCT	Hematocrit	%
MCV	Mean corpuscular volume	fL
MCH	Mean corpuscular hemoglobin	pg
MCHC	Mean corpuscular hemoglobin conc.	g/dL
PLT	Platelet count	$10^3/mm^3$
RETIC	Reticulocyte count	$10^5/mm^3$
	Differential leukocyte counts	$10^3/mm^3$
	RBC morphology (recorded)	
COAGULATION		
PT	Prothrombin time	sec
APTT	Activated partial thromboplastin time	sec
CLININCAL CHEMISTRY		
BUN	Blood urea nitrogen	mg/dL
Crea	Creatinine	mg/dL
Gluc	Glucose	mg/dL
TP	Total protein	g/dL
Alb	Albumin	g/dL
Glob	Globulin	g/dL
A/G	Albumin/globulin ratio	
ALT	Serum alanine aminotransferase	U/L
AST	Serum aspartate aminotransferase	U/L
ALP	Alkaline phosphatase	U/L
TBIL	Total bilirubin	mg/dL
Chol	Cholesterol	mg/dL
CK	Creatine kinase	U/L
NA	Sodium	mEq/L
K	Potassium	mEq/L
CL	Chloride	mEq/L
Ca	Calcium	mg/dL
Phos	Phosphorus	mg/dL

Table 5.

All gross lesions including tumors & masses	Ovary [2]
Adrenal [2]	Pancreas
Bone marrow smear (femur, sternum)	Pituitary gland
Brain	Small intestine, duodenum
Epididymis [2]	Small intestine, ileum
Esophagus	Small intestine, jejunum
Heart	Spleen
Kidney [2]	Spinal cord (two levels)
Large intestine, cecum	Stomach, glandular
Large intestine, colon	Stomach, nonglandular
Liver	Testis [2]
Lung [2]	Thymus
Lymph node, mesenteric	Thyroid/parathyroid gland [2]
Mammary gland (both sexes)	Urinary bladder
Uterus	