# Initial Formal Toxicity Evaluation of APC-2, a Novel Fluorescent Tracer Agent for Real-Time Measurement of Glomerular Filtration Rate in Preparation for a first-in-man clinical trial

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## ABSTRACT

The fluorescent tracer agent 2,5-bis[N-(1-carboxy-2-hydroxy)]carbamoyl-3,6-diaminopyrazine, designated APC-2, has been developed with properties and attributes necessary for use as a direct measure of glomerular filtration rate (GFR). Comparison to known standard exogenous GFR agents in animal models has demonstrated an excellent correlation. A clinical trial to demonstrate this same correlation in humans is in preparation. A battery of formal toxicity tests necessary for regulatory clearance to proceed with a clinical trial has been recently completed on this new fluorescent tracer agent. These include single dose toxicity studies in rats and dogs to determine overall toxicity and toxicokinetics of the compound. Blood compatibility, mutation assay, chromosomal aberration assay, and several other assays were also completed. Toxicity assessments were based on mortality, clinical signs, body weight, food consumption and anatomical pathology. Blood samples were collected to assess pharmacokinetic parameters including half-life, area under the curve, and clearance. Urine samples were collected to assess distribution. Doses of up to 200-300 times the estimated human dose were administered. No test-article related effects were noted on body weight, food consumption, ophthalmic observations and no abnormal pathology was seen in either macroscopic or microscopic evaluations of any organs or tissues. All animals survived to scheduled sacrifice. Transient discoloration of skin and urine was noted at the higher dose levels in both species as expected from a highly fluorescent compound and was not considered pathological. Thus initial toxicology studies of this new fluorescent tracer agent APC-2 have resulted in no demonstrable pathological test article concerns.

- **Keywords**: GFR, renal function, pyrazine, fluorescence, optical monitoring, renal clearance, single-dose toxicity, preclinical evaluation

## 1. INTRODUCTION

Measurement of glomerular filtration rate (GFR) is widely accepted as the most reliable measure of renal function.[1] As a result there is a growing medical need for determining accurate real-time GFR for minimizing the risk of kidney injury due to acute and chronic conditions. The optimum measure of GFR is by the use of exogenous tracer agents. However all current methods are not amenable to portable bedside use, and are therefore used mainly for research purposes. The current clinical standard, measurement of serum creatinine and its use in any of the estimated GFR equations, is a time-delayed measurement. An insult to the kidney would be noted only after 24 to 48 to 72 hours. In addition, factors not related to renal function affect serum creatinine, so the measurement itself is often inaccurate as well.

To overcome the deficiencies of the research GFR tracer agents and the current clinical standard, we have synthesized APC-2, a fluorescent tracer that has exhibited characteristics essential for accurate real-time measurement of GFR.[2, 3] In rodents, this compound is freely filtered by the kidneys, is not secreted by the renal tubules, nor has demonstrated any significant metabolism *in vivo*. In dogs, APC-2 has demonstrated similar clearance curves when compared to known exogenous GFR tracer agents such as iohexol and iothalamate, suggesting that APC-2 will provide a similar GFR value and accurate status of overall kidney function in humans.

The aim of the following studies was to investigate, through a formal battery of safety and toxicity studies *(in vitro* and *in vivo*), overall toxicity and toxicokinetics of APC-2 in both rodents and dogs necessary to proceed to early human clinical evaluation

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Reporters, Markers, Dyes, Nanoparticles, and Molecular Probes for Biomedical Applications VI,
edited by Samuel Achilefu, Ramesh Raghavachari, Proc. of SPIE Vol. 8956, 89560I · © 2014
SPIE · CCC code: 1605-7422/14/$18 · doi: 10.1117/12.2039881

of this compound. The results of these studies confirm that APC-2 is a safe compound and warrants further evaluation in an early human use clinical trial. Overall the *in vitro* assays indicated no toxicity relating to CYP-450, cloned hERG channels, bacterial reverse mutation or chromosomal alteration assays. Additionally, the compound was totally compatible with human blood and plasma samples. *In vivo*, the compound exhibited no potential toxicity in a series of CNS, respiratory or cardiovascular studies in either rodents or dogs. The *in vivo* results suggest that the NOEL in rats is greater than 1200µmol/kg and in dogs greater than 200µmol/kg.

## 2. MATERIALS AND METHODS

### 2.1. Fluorescent Tracer Agent.

APC-2 is a fluorescent compound belonging to the general class of compounds known as pyrazines. The chemical structure is shown in Figure 1. It's chemical name is 2,5-bis[N-(1-carboxy-2-hydroxy)]carbamoyl-3,6-diaminopyrazine. APC-2 has a molecular weight of 372.3, with light absorption and emission maxima at 445 nm and 560 nm, respectively.[2] APC-2 has no structural relationship to other molecules that are known to be carcinogenic or raise other toxicity safety issues.

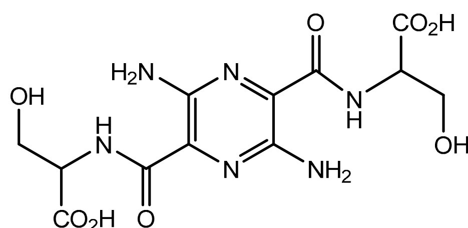


**Figure 1.** Structure of 2,5-bis[N-(1-carboxy-2-hydroxy)]carbamoyl-3,6-diaminopyrazine (APC-2)

The safety of pyrazines has been demonstrated by their use as medicinal products such as amiloride, a potassium-sparing diuretic on the market for over 30 years. Amiloride hydrochloride (marketed as Midamor) was approved prior to 1982. Natural pyrazines are found in common foods such as beef, bell peppers, cocoa butter, coffee beans, green peas, and potatoes.

### 2.2. Assays (In vitro and in vivo).

#### 2.2.1. Hemolytic Potential and Blood Compatibility in Human Blood and Plasma.

Blood (approximately 20 mL) was collected from a fasted human into heparinized tubes. Plasma was harvested from a portion of the collected blood. Whole blood and plasma for hemolytic potential testing and plasma for plasma compatibility testing were collected on the day of testing and held at room temperature until used. Hemolytic Potential Testing Test tubes were set up using test article preparations in vehicle at concentrations of 25, 50, and 100 mM; vehicle; and human whole blood, human plasma, and saponin (1%). Positive control tube included human blood incubated with 1% saponin and negative control samples included individual tubes of human plasma incubated with the three concentrations of test article.

Each mixture was incubated for 40 to 45 minutes at approximately 37°C. After incubation, the tubes were centrifuged, and the supernatant was harvested. The amount of hemoglobin in the supernatant plasma of each tube was measured spectrophotometrically on a Roche chemistry analyzer. The concentration of hemoglobin present in the supernatant plasma of the test article and vehicle mixtures was compared with the respective negative control. Hemolysis was present (recorded as a positive test result) if the concentration of hemoglobin was greater than or equal to 500 mg/dL more than the negative control. Absence of hemolysis, relative to the negative control, was recorded as a negative test result.

Plasma Compatibility Testing using the test article preparations was conducted in vehicle at concentrations of 25, 50, and 100 mM, and vehicle alone. Following the incubation period the contents of each tube were examined macroscopically. Changes in color or clarity, relative to the homologous plasma sample, and the presence of flocculation, precipitation, or coagulation were recorded.

### 2.2.2. Bacterial Reverse Mutation Assay

The tester strains used were the Salmonella histidine auxotrophs TA98, TA100, TA1535, and TA1537 (Ames et al. 1975) and the E. coli tryptophan auxotroph WP2uvrA (Green and Muriel 1976). These procedures were used in both the dose range-finding assay and the mutagenicity assays. Each plate was labeled with a code that identified the test article, test phase, tester strain, activation condition and dose level. The S9 mix and dilutions of the test article were prepared immediately prior to their use. Treatments were performed by adding 100 µL tester strain and 200 µL of test or vehicle control article to 2.5 mL of molten selective top agar (maintained at 45 ± 2°C). After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 mL minimal bottom agar in a 15 x 100 mm petri dish. After the overlay solidified, the plates were inverted and incubated for 52 ± 4 hours at 37 ± 2°C. Cultures were treated in the presence of S9 in an identical manner, except using 2.0 mL undiluted molten selective top agar and adding 500 µL S9 mix. Positive controls were administered using a 50-µL dose volume. Plates which were not evaluated immediately following the incubation period were held at >0 to 10°C until such time that colony counting and bacterial background lawn evaluation could take place.

A test article is considered to have produced a positive response if it induces a dose- dependent increase in revertant frequency that is ≥2.0-fold vehicle control values for tester strains TA98, TA100, and WP2uvrA, or ≥3.0-fold vehicle control values for tester strains TA1535 and TA1537. In addition, any response should be reproducible. A test article is considered to have produced a negative response if no dose-dependent, ≥2.0-fold or ≥3.0-fold increases are observed in tester strains TA98, TA100, and WP2uvrA, or TA1535 and TA1537, respectively.

### 2.2.3. Chromosomal Alteration Assay in Cultured Human Peripheral Blood Lymphocytes

Human venous blood from healthy, adult donors (nonsmokers without a history of radiotherapy, chemotherapy, or drug usage, and lacking current viral infections) was drawn into sterile, heparinized "vacutainers". Whole blood cultures were initiated in 15 mL centrifuge tubes by adding ~0.6 mL of fresh heparinized blood into a sufficient volume of culture medium so that the final volume was 10 mL in the assay without metabolic activation after the addition of the test article in its chosen vehicle or was 10 mL in the assay with metabolic activation after the addition of the test article in its chosen vehicle and the S9 activation mix. In the chromosomal aberration assays, duplicate cultures were used at each test article concentration, for vehicle controls, and for the positive controls.

For the assay without metabolic activation, 2 days after culture initiation, cells were incubated at 37 ± 2°C with the test article at predetermined concentrations, vehicle control and positive controls for 3 hours. For the assay with metabolic activation, 2 days after culture initiation, cultures were incubated at 37 ± 2°C for 3 hours in the presence of the test article at predetermined concentrations, vehicle control and positive controls, and the S9 activation mix. The cultures were then washed with phosphate-buffered saline, refed with complete RPMI 1640 medium and incubated for the rest of the culture period up to the time of harvest with 0.1 µg/mL Colcemid® present during the last 2 ± 0.5 hours of incubation. The cultures were then harvested (~22 hours after initiation of treatment). The cultures were centrifuged, the supernatant discarded, and the cells were swollen with 75 mM KCl hypotonic solution. The cultures were then fixed with absolute methanol: glacial acetic acid (3:1, v/v) fixative. Slides were prepared by dropping the harvested cultures on glass slides and air-dried. The slides were stained with 5% Giemsa solution, air-dried, and mounted permanently for the analysis of mitotic index and chromosomal aberrations.

For the assay without metabolic activation, 2 days after culture initiation, cells were incubated at 37 ± 2°C with the test article at predetermined concentrations, vehicle and positive controls for ~22 hours with 0.1 µg/mL Colcemid® added for the last 2 ± 0.5 hours of incubation. The cultures were then harvested. For the assay with metabolic activation, 2 days after culture initiation, cultures were incubated at 37 ± 2°C for 3 hours in the presence of the test article at predetermined concentrations, vehicle and positive controls, and the S9 activation mix. The cultures were then washed with phosphate-buffered saline. The cells were refed with complete RPMI 1640 medium and incubated for the rest of the culture period up to the time of harvest with 0.1 µg/mL Colcemid® present during the last 2 ± 0.5 hours of incubation. The cultures were then harvested ~22 hours after initiation of treatment and slides were prepared for the initial aberrations assay.

### 2.2.4 CYP-450 Enzyme Series

The CYP-450 Enzyme Series was designed to detect activation or inhibition of specific enzyme systems and to assess receptor binding of a number of common physiological and pharmacological receptor types including several CNS targets recommended by the EMEA to evaluate drug dependence potential of APC-2. Included in this lead profile screen were tested against 73 biochemical markers composed of 5 enzyme systems and 68 receptor assays. The methods employed in this study have been adapted from the scientific literature to maximize reliability and reproducibility. Where presented $IC_{50}$ values were determined by a non-linear, least squares regression analysis using MathIQ ™. Inhibition

constants (Ki) values were calculated using the methods of Cheng and Prusoff using the observed $IC_{50}$ of the test article, the concentration of the radioligand employed in the assay and the historical values for the $K_D$ of the ligand. Significant responses required >50% inhibition or stimulation for biochemical assays noted in the assays listed.

### 2.2.5. Effects on Cloned hERG Channels

HEK293 cells were stably transfected with hERG cDNA. Stable transfectants have been selected by coexpression with the G418 resistance gene incorporated into the expression plasmid. Selection pressure was maintained by including G418 in the culture medium. Cells were cultured in Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate and 500 µg/mL G418. All experiments were performed at near-physiological temperature (33 to 35 ºC). Each cell acted as its own control.

The positive control was applied to two cells (n = 2). The performance of the test system was considered acceptable since application of the positive control elicited a response within ± 2 standard deviations from the historic average response. Two concentrations were selected to evaluate the concentration-response relationship based on the outcome of the initial concentration range determination. Each concentration was tested in at least three cells (n ≥ 3). Vehicle control solution was applied to three cells (n = 3). Duration of application was approximately 3.8 minutes, which was longer than the longest test article application in the study.

APC-2 at 10 µM was evaluated in five cells (n = 5). A mean inhibitory effect on hERG potassium current amplitude of 6.0% was observed. Based on this result, and the established stability of APC-2 in HB-PBS, an additional nominal concentration (300 µM) was selected to evaluate the concentration-response relationship.

One test article concentration was applied to each cell (n ≥ 3). Peak current was measured during the test ramp. A steady state was maintained for at least 20 s before applying test article or positive control. Peak current was measured until a new steady state was achieved.

### 2.2.6. Single Dose Expanded Intravenous Bolus Toxicity and Toxicokinetic Study in Rats

All dose formulations were mixed once on the day of dosing according to the mixing procedure. The test article was supplied as a sterile solution at the concentration (111.66 mM) used for dosing the high-dose group. The test article was diluted with vehicle control article using aseptic procedures to achieve the low- and mid-dose concentrations. Prepared doses were stored at room temperature and protected from light (in amber glass vials wrapped in foil). Doses were administered by bolus intravenous injection in the tail vein (over at least 30 seconds) as a single dose on Day 1 of the dosing phase at a dose volume of 10.75 mL/kg. Actual dose volumes were based on the body weight from Day 1 of the dosing phase. Male and female Hsd:Sprague Dawley®™SD®™ rats were received from Harlan Laboratories, Inc., Indianapolis, Indiana.

All animals were checked twice daily (a.m. and p.m.) for mortality, abnormalities, and signs of pain or distress. Cageside observations were done for all toxicity animals immediately postdose and approximately 5, 15, 30, 45, and 60 minutes postdose and for selected toxicity animals approximately 120 minutes postdose. On nondosing days (except for days of detailed observations), daily cageside observations were made for each toxicity animal. Detailed observations were done for all animals once during the predose phase and for toxicity animals before dosing on Day 1 and weekly thereafter and on the day of scheduled sacrifice.

### 2.2.7. Single Dose CNS Safety Pharmacology Study in Rats

A total of 80 animals (10/sex/group) were assigned to groups as shown in the following table. The animals were not fasted overnight prior to dosing. The animals were evaluated for activity measurements, and a functional observational battery test was performed as described below.

| Dose Group | Number of Animals (M/F) | Test Article | Dose Level (µmole/kg) | Dose Level (mg/kg) | Dose Conc. (mg/mL) |
|---|---|---|---|---|---|
| 1 | 10/10 | Vehicle | 0 | 0 | 0 |
| 2 | 10/10 | APC-2 | 180 | 66.96 | 6.2 |
| 3 | 10/10 | APC-2 | 600 | 223.2 | 20.8 |
| 4 | 10/10 | APC-2 | 1200 | 446.4 | 41.5 |

**Table 1**. CNS Study Parameters

The test article was administered once, as a slow bolus intravenous tail vein injection, at a dose volume of 10.75 mL/kg body weight. Control animals received the vehicle only at the same dose volume. The actual volume/weight to be administered to each animal was calculated and adjusted based on the Day 1 body weight of each animal.

Animals were observed for viability at least once in the morning and once in the afternoon, at least 4 hours apart, throughout the study. The neurobehavioral assessment included a Functional Observational Battery (FOB) and a motor activity test. The FOB and motor activity test were performed on all animals once predose and at three time points postdose (between 1 minute to 45 minutes, between 3 to 4 hours, and between 23.5 to 24.5 hours). The anticipated $T_{max}$ is 5 minutes and the $T_{1/2}$ is less than 0.5 hours postdose based on previously conducted clearance studies in this species.. Animals were randomized prior to dose administration for the purpose of conducting "blinded" FOB testing throughout the study.

This battery is comprised of 4 sets of observations. The battery includes home cage observations, handling observations, open-field observations and handling/specific testing of the animal.

### 2.2.8. Single Dose Respiratory Safety Pharmacology Study in Rats

A total of 16 male rats were assigned to groups as shown in the following table. The test article was administered once, as a slow bolus intravenous tail vein injection at a dose volume of 10.75 mL/kg body weight. Control animals received the vehicle at the same dose volume.

**Table 2**. Respiratory Study Parameters

| Dose Group | Number of Animals (M) | Test Article | Dose Level (µmole/kg) | Dose Level (mg/kg) | Dose Conc. (mg/mL) |
|---|---|---|---|---|---|
| 1 | 4 | Vehicle | 0 | 0 | 0 |
| 2 | 4 | APC-2 | 180 | 66.96 | 6.2 |
| 3 | 4 | APC-2 | 600 | 223.2 | 20.8 |
| 4 | 4 | APC-2 | 1200 | 446.4 | 41.5 |

The animals were placed in a whole body plethysmograph for a minimum of 1 hour prior to dose administration to collect baseline respiratory data. The animals were removed from the chamber briefly and the dose was administered intravenously as a bolus injection via the tail vein. After dose administration, animals were placed back into the plethysmograph chamber, and respiratory data was collected for 2 hours on the day of dosing and for 1 hour at approximately the 24-hour postdose time point. Body weights were recorded on the day of dosing, for dose calculations. Dosing was staggered over two to four days by dosing an equal number of animals from each group (1 or 2 per group per day). The following parameters were recorded and reported: respiratory rate (breath/minute, tidal volume (TV, mL/breath), and minute volume (MV, mL/minute).

Four 15-minute predose intervals were collected and the last two intervals averaged together for a single baseline mean value per rat. Postdose data was summarized as means of 5-minute intervals for the first 30 minutes and of 15-minute intervals for 30-minute to 2-hour time period.

### 2.2.9. Single Dose Expanded Intravenous Bolus Toxicity and Toxicokinetic Study in Beagle Dogs

Male and female dogs were housed individually in stainless steel cages for at least 6 hours each day and offered Certified Canine Diet #2027C (Harlan Laboratories, Inc.), and water ad libitum unless fasted for study procedures. Animals were checked twice daily (a.m. and p.m.) for mortality, abnormalities, and signs of pain or distress. Daily cage side observations were done during the predose and dosing phases, except on days detailed observations were conducted. Detailed observations were done twice during the predose phase; before dosing on Day 1 and weekly thereafter; on the day of scheduled sacrifice (only those animals sacrificed that day). Abnormal findings or an indication of normal was recorded.

Detailed observations were also made for each animal immediately postdose and approximately 5, 15, 30, 45, and 60 minutes postdose and detailed observations continued (for those animals that previously had abnormal skin color or other abnormal observations potentially attributed to the test article) at approximately 2 and 3 hours postdose. Abnormal findings or an indication of normal was recorded. Unscheduled observations were recorded. Body Weights Recorded twice during the predose phase; prior to dosing on the first day of the dosing phase; and on Days 3, 8, and 15 of the dosing phase

On Day 15 of the dosing phase, all surviving animals were weighed, anesthetized with sodium pentobarbital, exsanguinated, and necropsied. The necropsy included an examination of the external features of the carcass; external body orifices; the abdominal, thoracic, and cranial cavities; organs; and tissues.

### 2.2.10. Single Dose Cardiovascular Safety Pharmacology Study in Dogs

Each dog randomly receiving the Vehicle (0μmol/kg), Low (60μmol/kg), Middle (200μmol/kg) and High (600μmol/kg of the test article. Doses were administered as a slow bolus injection (<5min) in the cephalic vein and a 3-day wash-out duration occurred between sequential dose administrations. The animals were fasted overnight prior to each dose administration, and fed approximately 2 hours postdose. The dose levels and the intervals monitored for the study were selected based on available toxicity and toxicokinetic data in this species.

The test article or vehicle was administered once per day with a 3-day washout between doses. Dosing of the animals occurred at approximately the same time on each dosing day. Each animal received each dose level over the course of the study according to a Latin Square Design. All animals were observed once each morning and afternoon throughout the study for morbidity and mortality. On the days of dose administration, physical observations were conducted at approximately 2 and 3 hours post-dose (at the time of feeding and removal of food) and all room entry/exit times recorded. On non-dosing days, physical observations were conducted once daily. The dogs for this study were previously surgically implanted with Data Sciences International (DSI) transmitters (TL11M2-D70-PCT). The transmitter was used to record ECG, arterial blood pressures, and body temperature. Animals were unrestrained within their home cage during collection of the selected cardiovascular parameters. Arterial blood pressure, ECG, and body temperature were recorded by telemetry for at least 12 hours prior to the first dosing event to verify function of the radiotelemetry unit. On dosing days, data was collected from a minimum 30-minute predose period and used to generate a single baseline mean value for each parameter. Data was collected continuously for a minimum of 24 hours postdose and during this time data was recorded as mean values of 60-second time bins. Further data summarization was generated as 5-minute means for the period 0 to 30 minutes postdose, and as 15-minute means for the period 30 minutes to 24 hours postdose. Times of any entry into the room during radiotelemetry monitoring were documented.

The following parameters were analyzed: systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), heart rate (HR) and body temperature (Tb). ECG parameters evaluated included: PR Interval, QRS Interval, QT Interval, QTcV (Van de Water's corrected QT) RR interval. One-minute tracings of the ECGs were obtained at predose, 15 min, 30 min, 1 hour, 4 hours, and at 24 hours based on Sponsor reported values of $T_{1/2}$ = 0.75 hour for the test article. ECG traces were evaluated and reported by a board-certified veterinary cardiologist.

## 3. RESULTS

### 3.1 Hemolytic Potential and Blood Compatibility in Human Blood and Plasma

The results for human blood mixed with the 25, 50, or 100 mM test article concentrations, or vehicle, or 1% Saponin are listed in Table 3. The levels of hemoglobin in all the tubes were markedly lower (<14 mg/dL) than the positive control tube containing 1% Saponin (5655 mg/dL). Negative control samples prepared with equal volumes of human plasma mixed with the 25, 50, and 100 mM APC-2 concentrations, to account for the colored nature of APC-2, also resulted in markedly low level of hemoglobin (<19 mg/dL). No changes were observed macroscopically compared with homologous plasma or with vehicle. Results also showed that no hemolysis occurred when APC-2 was mixed with human whole blood. These data demonstrate that APC-2 is compatible with human whole blood and plasma.

**Table 3.** Hemolytic Potential and Blood Compatibility Test Results

| Mixture | [Hb] (mg/dL) | Result |
|---|---|---|
| Human Blood plus: | | |
| 25 mM APC-2 | 14 | - |
| 50 mM APC-2 | 10 | - |
| 100 mM APC-2 | 5 | - |
| Vehicle | 5 | - |
| 1% Saponin | 5655 | + |
| Human Plasma plus: | | |
| 25 mM APC-2 | 19 | - |
| 50 mM APC-2 | 4 | - |
| 100 mM APC-2 | 1 | - |

(+) Result = >500 mg/dL [Hb]

### 3.2 Bacterial Reverse Mutation Assay

APC-2 was evaluated in the dose range-finding assay in tester strains TA100 and WP2uvrA. Ten doses of test article, ranging from 6.67 to 5000 µg/plate, were evaluated with and without S9. No cytotoxicity was observed with either tester strain in the presence or absence of S9 as evidenced by no dose-related decreases in revertant frequency and normal bacterial background lawns.

Based upon the results of the dose range-finding assay, APC-2 was evaluated in the initial mutagenicity assay, in all five tester strains, at doses of 313, 625, 1250, 2500, and 5000 µg/plate in the presence and absence of S9 mix. All doses of the test article, as well as the concurrent positive and vehicle controls, were evaluated in triplicate plates. No positive increases in the mean number of revertants/plate were observed with any of the tester strains in the presence or absence of S9 mix. The data shown in Table 4 is with S9 present.

These results indicate APC-2 was negative in the Bacterial Reverse Mutation Assay.

**Table 4.** Bacterial Reverse Mutation Assay Results (with S9)

| Strain | Agent | Dose (ug/plate) | Revertants/Plate | T:V |
|---|---|---|---|---|
| TA98 | APC-2 | 5000 | 21.0 | 1.1 |
| | | 2500 | 17.3 | 0.9 |
| | | 1250 | 21.7 | 1.1 |
| | | 625 | 26.3 | 1.4 |
| | | 313 | 18.7 | 1.0 |
| | PBS | ---- | 19.3 | --- |
| TA100 | APC-2 | 5000 | 86.0 | 1.0 |
| | | 2500 | 80.0 | 0.9 |
| | | 1250 | 80.0 | 0.9 |
| | | 625 | 78.0 | 0.9 |
| | | 313 | 82.7 | 1.0 |
| | PBS | ---- | 85.7 | --- |
| TA1535 | APC-2 | 5000 | 11.0 | 1.5 |
| | | 2500 | 8.0 | 1.1 |
| | | 1250 | 10.3 | 1.4 |
| | | 625 | 7.3 | 1.0 |
| | | 313 | 9.0 | 1.2 |
| | PBS | ---- | 7.3 | --- |
| TA1537 | APC-2 | 5000 | 5.3 | 1.0 |
| | | 2500 | 4.3 | 0.8 |
| | | 1250 | 5.3 | 1.0 |
| | | 625 | 5.7 | 1.1 |
| | | 313 | 6.3 | 1.2 |
| | PBS | ---- | 5.3 | ---- |
| WP2uvrA | APC-2 | 5000 | 13.7 | 0.8 |
| | | 2500 | 11.3 | 0.7 |
| | | 1250 | 18.0 | 1.1 |
| | | 625 | 19.3 | 1.2 |
| | | 313 | 20.3 | 1.2 |
| | PBS | ---- | 16.3 | --- |
| TA98 | BP | 2.5 | 263.7 | 13.6 |
| TA100 | 2AA | 2.5 | 1121.3 | 13.1 |
| TA1535 | 2AA | 2.5 | 151.7 | 20.7 |
| TA1537 | 2AA | 2.5 | 54.7 | 10.3 |
| WP2uvrA | 2AA | 25.0 | 404.3 | 24.8 |

BP = Benzo{a}pyrene
2AA = 2-aminoanthracene

### 3.3 Chromosomal Aberration Assay in Cultured Human Peripheral Blood Lymphocytes

The initial chromosomal aberrations assay was conducted testing concentrations of 0.0678, 0.0969, 0.138, 0.198, 0.282, 0.404, 0.576, 0.824, 1.18, 1.68, 2.40, 3.43, 4.90, 7.00, and 10.0 mM without and with metabolic activation with a 3-hour treatment and all cultures were harvested ~22 hours from the initiation of treatment. Chromosomal aberrations were analyzed from the cultures treated with 4.90, 7.00, and 10.0 mM without and with metabolic. The high dose selected for analysis, 10.0 mM, is the high dose recommended for this assay by the OECD Testing Guidelines. No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed without or with metabolic activation. These data are shown in Table 5.

**Table 5.** Chromosomal Aberration Assay Results (with Metabolic Activation)

| Treatment | [uM] | % Mitotic Index A Culture | % Mitotic Index B Culture | Average Mitotic Index | % Mitotic Index Reduction |
|---|---|---|---|---|---|
| Vehicle | ---- | 10.6 | 9.4 | 10.0 | 0 |
| APC-2 | 3.43 | 10.4 | 10.7 | 10.6 | 0 |
| APC-2 | 4.90 | 10.8 | 11.6 | 11.2 | 0 |
| APC-2 | 7.00 | 10.0 | 9.6 | 9.8 | 2 |
| APC-2 | 10.0 | 9.7 | 9.5 | 9.6 | 4 |

The test article, APC-2, was negative for inducing chromosomal aberrations in cultured human lymphocytes without and with metabolic activation.

### 3.4 CYP-450 Enzyme Series

There were no significant findings (>50% stimulation or inhibition) for any of the enzyme system or receptors assayed. These results indicate that APC-2 does not cause significant activation or inhibition of the enzyme systems tested or bind to the wide cross section of receptors evaluated.

### 3.5 Effects on Cloned hERG Channels

The *in vitro* effects of APC-2 on ionic currents in voltage-clamped human embryonic kidney cells (HEK293) that stably express the human ether-à-go-go-related gene (hERG) were determined. Two concentrations of APC-2 (10 and 300 µM) were tested at near-physiological temperature. APC-2 inhibited hERG current by (Mean ± SEM) 6.0 ± 3.1% at 10 µM (n = 5) and 2.9 ± 0.7% at 300 µM (n = 3) versus 1.9 ± 0.8% (n = 3) in control. The $IC_{50}$ for the inhibitory effect of APC-2 on hERG potassium current was not calculated but was estimated to be greater than 300 µM. Under identical conditions, the positive control (60 nM terfenadine) inhibited hERG potassium current by (Mean ± SD; n = 2) 85.2 ± 5.1%. This result confirms the sensitivity of the test system to hERG inhibition.

The results are tabulated in Table 6. In Figure 2, the upper panel [Current (pA); Time (ms)] shows superimposed, records of hERG potassium currents obtained in a single cell during application of vehicle control, test article and reference substance. hERG potassium currents were evoked by the voltage protocol shown in the lower panel [Voltage (mV)].

**Table 6.** hERG Channel Assay Results

| **Compound** | **[Concentration]** | **Mean % Inhibition** | **SD** | **N** |
|---|---|---|---|---|
| | | | | |
| APC-2 | 0 uM | 1.9 | 1.5 | 3 |
| APC-2 | 10 uM | 6.0 | 6.9 | 5 |
| APC-2 | 300 uM | 2.9 | 1.2 | 3 |
| Terfenadine | 60 nM | 85.2 | 5.1 | 2 |

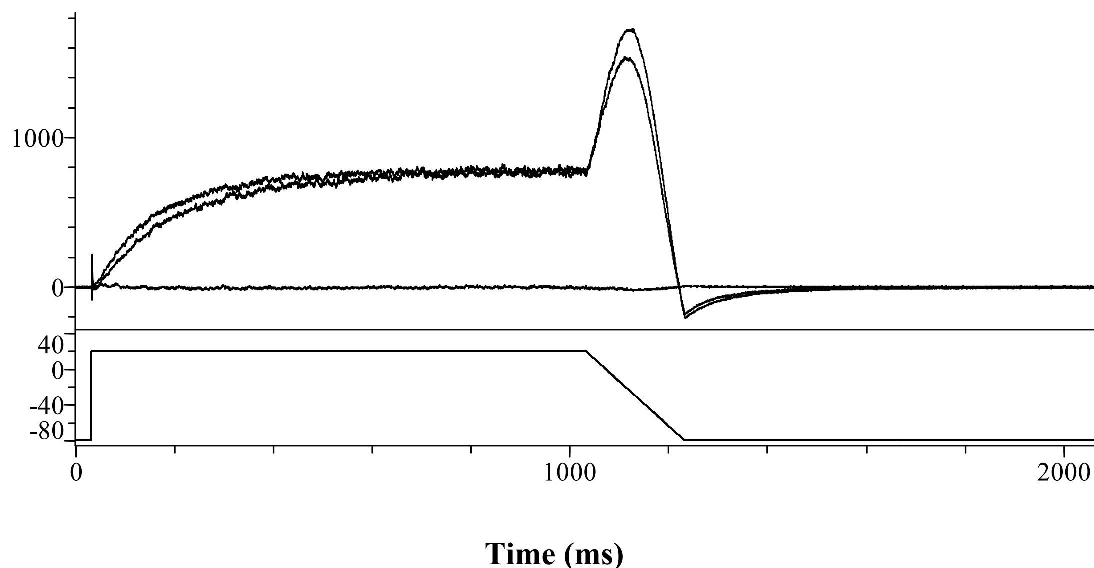


**Figure 2.** hERG Potassium Current Traces

### 3.6 Single Dose Expanded Intravenous Bolus Toxicity and Toxicokinetic Study in Rats

All animals survived to their scheduled sacrifice. APC-2 -related clinical signs were observed in treated animals at all dose levels tested. The most common clinical signs included yellow discoloration of the skin at various anatomic locations (entire body, tail, paws, ears, leg, mouth, nose, penis, periorbital area, perioral area, perineal area, scrotum, testes, and/or vagina) and/or discolored urine (yellow or orange). These were attributed to the colored nature of the test article and were not deemed adverse.

Yellow discoloration of the skin was noticed at all dose levels, although it was more widespread and appeared more rapidly in animals given 600 or 1200 µmoles/kg. Immediately following dosing, yellow discoloration of the skin was noted on the entire body, tail, paws, ears, mouth, nose, periorbital area, scrotum, and/or vagina in animals given 600 or 1200 µmoles/kg, however, in animals given 180 µmoles/kg, the skin discoloration was noted only on the tail (most animals) and/or paws (one male). Between 5 and 45 minutes postdose, yellow discoloration of the skin persisted at one or more of these anatomic locations. Additionally, yellow discoloration of the skin was observed on the leg, penis, testes, perineal area, and/or perioral area of some animals between 15 and 45 minutes postdose. Normal skin coloration returned at some locations, although yellow discoloration of the skin persisted in some animals at several other locations (ears, nose, paws, penis, perineal area, tail, and/or testes) at 60 minutes postdose. On animals given 1200 µmoles/kg that were examined at 2 hours postdose, normal skin color returned on the nose and ears, however yellow discoloration of the skin persisted in the paws, penis, perineal area, tail, and/or testes. Yellow discoloration of skin on tail and/or testes was also noted in some animals sacrificed on Day 3 of the dosing phase.

Discolored urine (yellow or orange) was observed primarily in animals given > 600 µmoles/kg and in 4 animals given 180 µmoles/kg, on Day 1 of the dosing phase. Yellow hair coat in the perineal area, midline ventral thorax, and/or penis was noted on Day 1 in some animals, however, it was observed more often in animals given 1200 µmoles/kg. All other clinical signs, including red discharge from the eye, sores, or scabs, appeared infrequently. These clinical signs were incidental findings and were not considered test article-related. No abnormal clinical signs were noted after Day 3 of the dosing phase.

No test article-related effects on body weight or body weight gain were observed during the dosing phase. No test article-related alteration in food consumption was noted. Although low mean food consumption was recorded on Days 2 and 14 of the dosing phase, it was unrelated to test article administration since it was low for treated as well as control groups and due to animals being fasted prior to clinical pathology blood collection and/or necropsy. No abnormal ophthalmic observations were noted.

The mean concentration-time profiles for males and females show that exposure to APC-2 increased with the increase in APC-2 dose level from 180 to 1200 µmoles/kg. After intravenous bolus administration, APC-2 concentrations readily declined generally in a mono-exponential manner and with concentrations below the lower limit of quantitation by 24 hours. The t1/2 values ranged from 0.392 to 0.417 hours. Values for t1/2 were generally dose independent. Values for CL ranged from 0.402 to 0.485 L/hr/kg and appeared to be dose independent.

For each sex, Groups 2 through 4 were compared with Group 1 (control) at the 5%, two-tailed probability level. Only data collected on or after the first day of treatment were analyzed statistically. None of the data collected from the toxicokinetic animals were statistically analyzed. Statistical significance is designated throughout the text of this report by the term significant.

Test article-related macroscopic findings included yellow discolored intravenous sites in three males given 180 to 600 µmoles/kg and four females given ≥180 µmoles/kg and yellow discolored scrotal skin in four males given ≥600 µmoles/kg. The yellow discoloration of the intravenous and scrotal sites was attributed to the yellow color of the test article. The remaining macroscopic findings in interim sacrifice animals were incidental or normal background findings commonly reported in animals of this species and age.

The microscopic findings in interim sacrifice animals were incidental or normal background findings commonly reported in animals of this species and age. All terminal sacrifice animals survived to their scheduled sacrifice. Significant relative organ weight changes in terminal sacrifice animals were few in number, random in nature, not dose-responsive, and not test article-related. The macroscopic and microscopic findings in terminal sacrifice animals were incidental or normal background findings commonly reported in animals of this species and age.

Values for Vss ranged from 0.192 to 0.205 L/kg and were also dose independent. Values for Vss were less than the total body water of a 0.25 kg rat but greater than the blood volume, indicating that APC-2 was moderately distributed after intravenous bolus administration APC-2.

No sex differences (> 2-fold) were observed in APC-2 Cmax and AUC0-24 values. The increases in Cmax and AUC0-24 for males and females were, in general, dose proportional. Few statistically significant or otherwise notable differences for clinical pathology test results were observed between control and treated animals. All of the differences were consistent with normal variation and considered incidental. All of the differences were characterized by one or more of the following: very small magnitude, no relationship to dose, inconsistency between sexes, and absence of correlative findings. All interim sacrifice animals survived to their scheduled sacrifice. Significant absolute and relative organ weight changes in interim sacrifice animals were few in number, random in nature, not dose-responsive, and not test article-related.

### 3.7 Single Dose CNS Safety Pharmacology Study in Rats

A neurobehavioral assessment using a Functional Observational Battery (FOB) and a motor activity test were performed on all animals pretest and postdose at 1 minute to 45 minutes, 3 to 4 hours, and 23.5 to 24.5 hours. There were no test article-related changes in FOB or motor activity parameters. Table 7 summarizes the 1-45 minute data. Remaining data were unremarkable and are not tabulated.

| Table 7. Mean Motor Activity: 1-45 Minutes Post Dose (Males and Females) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Group (Males)** | **Mean SD** | **DT (cm)** | **RT (sec)** | **ST (sec)** | **AT (sec)** | **BSM** | **HC** | **AC** | **V1C** | **V1B** |
| Group 1 0 mg/kg | Mean SD | 1272.1 521.6 | 432.4 121.8 | 281.0 62.9 | 186.6 63.7 | 146.0 32.7 | 1409.5 493.0 | 829.3 363.8 | 121.0 90.9 | 23.1 13.8 |
| Group 2 180uM/kg | Mean SD | 1508.6 505.4 | 354.7 85.8 | 331.2 35.7 | 214.1 59.8 | 161.4 20.9 | 1743.5 375.4 | 1050.9 313.0 | 165.6 90.5 | 29.7 16.7 |
| Group 3 600uM/kg | Mean SD | 1361.8 653.9 | 422.0 139.5 | 284.8 64.4 | 193.2 81.2 | 143.1 29.6 | 1568.7 635.1 | 949.5 467.1 | 150.2 106.0 | 33.1 22.6 |
| Group 4 1200uM/kg | Mean SD | 864.3 486.7 | 478.6 120.5 | 290.3 71.5 | 131.1 66.2 | 134.1 29.3 | 1165.2 434.5 | 579.0 312.2 | 121.0 90.9 | 23.1 13.8 |
| | | | | | | | | | | |
| **Group (Females)** | **Mean SD** | **DT (cm)** | **RT (sec)** | **ST (sec)** | **AT (sec)** | **BSM** | **HC** | **AC** | **V1C** | **V1B** |
| Group 1 0 mg/kg | Mean SD | 1891.9 718.5 | 348.8 107.4 | 313.5 53.1 | 237.7 81.0 | 154.3 25.8 | 2262.3 566.2 | 1477.2 494.0 | 254.2 107.6 | 44.1 16.3 |
| Group 2 180uM/kg | Mean SD | 1801.2 699.1 | 393.8 104.9 | 290.7 53.5 | 215.5 74.7 | 146.1 18.8 | 2021.3 637.4 | 1333.0 535.9 | 247.4 143.0 | 44.3 21.0 |
| Group 3 600uM/kg | Mean SD | 2050.0 985.4 | 354.1 113.8 | 303.2 50.1 | 242.7 84.8 | 152.2 24.1 | 2339.0 995.0 | 1590.4 841.8 | 254.0 144.4 | 44.5 20.0 |
| Group 4 1200uM/kg | Mean SD | 1616.3 685.0 | 414.1 99.1 | 282.7 45.7 | 203.2 64.6 | 143.2 22.5 | 1826.5 666.4 | 1187.5 525.5 | 183.1 99.1 | 34.4 15.6 |

**Key to Motor Activity Data**

| Column | Description |
|---|---|
| DT | Distance Traveled |
| RT | Resting Time |
| ST | Stereotypic Time |
| AT | Ambulatory Time |
| BSM | Bursts of Stereotypic Movement |
| HC | Horizontal Counts |
| AC | Ambulatory Counts |
| V1C | Vertical Counts |
| V1B | Vertical Breaks |

### 3.8 Single Dose Respiratory Safety Pharmacology Study in Rats

Respiratory rate was significantly higher at 30 minutes postdose for rats receiving 180 or 600 µmole/kg. Tidal volume was significantly lower than control at 10 minutes postdose (1200 µmole/kg), and at 23.75 hours postdose (180, 600, 1200 µmole/kg). These findings were not considered test article-related as there were no clear correlations to dose level or trends following dose administration. Minute volume had no significant changes between dose groups.

There were no test article-related changes in the respiratory parameters measured. The NOEL (No-Observable Effect Level) on respiratory parameters in the rat was >1200 µmole/kg APC-2, the high dose of this study, when given as a single intravenous bolus injection.

### 3.9 Single Dose Expanded Intravenous Bolus Toxicity and Toxicokinetic Study in Beagle Dogs

All animals survived to their scheduled sacrifice. APC-2-related clinical signs included yellow discoloration of various anatomic locations and discolored urine (yellow or orange). These were attributed to the colored nature of the test article and were not considered adverse. Yellow discoloration of various anatomic locations was primarily seen on Day 1 of the dosing

phase in animals given 200 or 600 µmoles/kg. These locations included eyes, gums, ears, legs, and abdomen. Yellow discoloration appeared and progressed faster and lasted longer in animals given the highest dose (600 µmoles/kg). These animals exhibited yellowish discoloration of eyes and gums during dosing, followed by yellowish discoloration of all other locations during dosing or immediately postdose that lasted for up to 1 hour postdose. At 2 hours postdose, yellow discoloration appeared to fade from certain body parts and was localized to one or more sites (eyes, ears, front legs, or abdomen) except for one male and one female that still had yellow discoloration of the entire body. By 3 hours postdose, the color of all body parts was normal. In animals given 200 µmoles/kg, yellow discoloration was first noticed in the gums immediately postdose, followed by other body parts within 15 minutes of dosing. By 45 minutes postdose, yellow color started to disappear from most of these body parts and was mainly localized to the ears, gums and eyes, and by 1 hour postdose yellow color was only seen in eyes. By 2 hours postdose, all animals given 200 µmoles/kg regained normal coloration. Discolored urine (yellow or orange) was observed in all three APC-2-treated groups on Day 1 of the dosing phase. It was also observed in a few animals the next morning

Day 2 of the dosing phase: Two of these animals showed discolored urine only on Day 2 of the dosing phase, which indicated that discolored urine observed on Day 2 of the dosing phase might not have been the residual urine from the previously recorded observation on Day 1 of the dosing phase. All other signs were considered incidental given the sporadic occurrence or similar incidence in control and test article-treated animals.

No APC-2-related effects on body weight or body weight gain were observed during the dosing phase. Lower mean body weight on Days 3 of the dosing phase was considered to be due to overnight fasting for clinical pathology blood collection. No effects were noted on food consumption.

All concentration values of APC-2 in the control group were below the lower limit of quantitation.

The mean concentration-time profiles for males and females show that exposure to APC-2 increased with the increase in dose level from 60 to 600 µmoles/kg. After intravenous bolus administration, APC-2 concentrations readily declined generally in a bi-exponential manner and with almost all concentrations below the lower limit of quantitation by 24 hours. The mean t1/2 values ranged from 0.693 to 0.767 hours. Values for t1/2 were generally dose independent. Values for mean CL ranged from 0.222 to 0.247 L/hr/kg and appeared dose independent. Values for mean Vss ranged from 0.121 to 0.152 L/kg and also appeared dose independent. Values for mean Vss were less than the total body water of a 10 kg dog but greater than the blood volume, indicating that APC-2 distributed throughout the extracellular space (0.276 L/kg) after intravenous administration.

No marked sex differences were observed in mean APC-2 Cmax and $AUC_{0\text{-}24}$ values, as the differences were less than two-fold. The increases in mean Cmax and $AUC_{0\text{-}24}$ for males and females were generally dose proportional.

Clinical Pathology Findings: Very few notable differences for clinical pathology test results were observed between control and treated animals, and all were consistent with normal variation and considered incidental. All differences were characterized by one or more of the following: very small magnitude, no relationship to dose, similarity to a difference present before initiation of dosing, and absence of correlative findings.

No unscheduled deaths occurred. Significant changes in body and organ weights for the dosing phase interim and final sacrifices were not found. Microscopic findings related to the test article were not found. A variety of observations were recorded but were considered incidental for this age, sex, and species of animal.

### 3.10 Single Dose Cardiovascular Safety Pharmacology Study in Dogs

No animals died or were deemed moribund during the study. Test article-related clinical observations were limited to one dog following the 600 µmoles/kg dose that became limp immediately after administration of APC-2. The animal recovered within 20 minutes and did not exhibit any further test article-related observations.
Blood Pressure parameters were significantly lower from 5 to 20 minutes (systolic pressure) and from 5 to 25 minutes (diastolic and mean pressure) postdose for the high dose group, 600 µmoles/kg APC-2. These group mean changes in the high dose were the result of one dog that had a marked drop in blood pressures during the 20- minute postdose period that subsequently recovered to normal values following this period.

Heart Rate and ECG intervals had some significant findings in the 5- to 30-minute postdose period but these were not considered test article-related findings since primary changes in HR during this period were due to excitation related to dose administration techniques and changes in the ECG parameters were considered related to heart rate change. Body

temperatures were not affected by administration of APC-2. This study evaluated the potential effects of a single intravenous bolus administration of 0, 60, 200, or 600 µmoles/kg APC-2 on the cardiovascular system in the dog. The measurements provided an evaluation of potential effects on blood pressure, heart rate, and electrocardiogram as well as body temperature. There was one incidence of markedly low blood pressure immediately postdose in one of four dogs receiving 600 µmoles/kg APC-2 with associated clinical signs of listlessness. This decrease in blood pressure was considered adverse; however, the effect was of short duration (resolution within 25 minutes of dose completion) and produced no long-term adverse effects. There were no other test article-related effects within the physiological parameters measured. The NOEL (No-Observable-Effect Level) was determined to be 200 µmoles/kg APC-2 (74.4 mg/kg).

## 4. Discussion and Conclusions

The novel fluorescent tracer, APC-2, designed and synthesized to be an optical agent for determining real-time GFR in humans was evaluated is a series of *in vitro* and *in vivo* studies to assess potential toxicity of the agent. *In vitro* assays included: Hemolytic Potential and Blood Compatibility in Human Blood and Plasma, Bacterial Reverse Mutation Assay, Chromosomal Alteration Assay in Cultured Human Peripheral Blood Lymphocytes, CYP-450 Enzyme Series and Effects on Cloned hERG Channels. *In vivo* assays included: Single Dose Expanded Intravenous Bolus Toxicity and Toxicokinetic Study in Rats, Single Dose CNS Safety Pharmacology Study in Rats, Single Dose Respiratory Safety Pharmacology Study in Rats, Single Dose Expanded Intravenous Bolus Toxicity and Toxicokinetic Study in Beagle Dogs, Single Dose Expanded Intravenous Bolus Toxicity and Toxicokinetic Study in Beagle Dogs and Single Dose Cardiovascular Safety Pharmacology Study in Dogs. The accumulated results indicate that the toxicity profile should be conducive to start a first-in-human clinical trial.

**Table 8.** Nonclinical Studies and Results

| Study Title | Doses or concentrations | Number per Group | Results |
|---|---|---|---|
| Single Dose Expanded IV Bolus Toxicity and Toxicokinetic Study in Rats | 180, 600, 1200 µmol/kg | Tox: 10M, 10F<br>TK: 9M, 9F | *NOAEL 1200 µmol/kg |
| Single Dose Expanded IV Bolus Toxicity and Toxicokinetic Study in Beagles | 60, 200, 600 µmol/kg | 4M, 4F | *NOAEL 600 µmol/kg |
| Hemolytic Potential and Blood Compatibility in Human Blood and Plasma | 12.5, 25, 50 mM | NA | Negative |
| Bacterial Reverse Mutation Assay with Confirmation | 5 mg / plate | NA | Negative |
| Chromosomal Aberration Assay in Cultured Human Peripheral Blood Lymphocytes | 10mM and lower | NA | Negative |
| CYP-450 enzyme screen | NA | NA | Negative |
| Effect of APC-2 on Cloned hERG Potassium Channels Expressed in Human Embryonic Kidney Cells | 10 and 300 µM | NA | Negligible hERG inhibition |
| Single Intravenous Dose CNS Safety Pharmacology Study in Rats | 0, 180, 600, or 1200 µmole/kg | 10M, 10F | NOEL >1200 µmole/kg |
| Single Intravenous Dose Respiratory Function Safety Pharmacology Study in Rats (Respiratory Study in Conscious Rats) | 0, 180, 600, 1200 µmole/kg | 4M | NOEL >1200 µmole/kg |
| Single Intravenous Dose Cardiovascular Safety Pharmacology Study in Female Beagle Dogs (Cardiovascular Safety Assessment Study in Male Beagles) | 0, 60, 200, 600 µmole/kg | 4F | NOEL 200 µmole/kg (74.4 mg/kg) |
| Cardiovascular Evaluation; Single Intravenous Dose Cardiovascular Safety Pharmacology Study in Female Beagle Dogs | 0, 60, 200, 600 µmole/kg | 4F | No toxicologic effects on cardiac rhythm or ECG morphology |

* Eventual limit is higher than reported number, this was the highest concentration employed; could not administer higher concentration due to volume consideration.

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