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Uncommon Toxicologic Profile at Toxic Doses of CPI-613 (an Agent Selectively Alters Tumor Energy Metabolism) in Rats and Minipigs Reflects Novel Mechanism

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Abstract: Problem statement: CPI-613 is a novel anticancer agent and the toxicological profile has not been assessed. Accordingly, the objective of these studies was to thoroughly evaluate the Toxicokinetic (TK) effects of CPI-613 in rats and to compare the toxicological profile of rats to minipigs to determine if there were differences between the two animal species. **Approach:** These studies involved assessments of multiple toxicological parameters (including clinical signs as well as full panel blood work, necropsy and histology) at multiple times in animals treated with the threshold dose (i.e., a dose that did not induce significant effects), toxic dose (i.e., a dose that induced significant toxic effects) and dose that approximated the LD_{10} dose (i.e., a dose that induced death in approximately 10% of the animals). CPI-613 was given intravenously 2x weekly for 3 weeks, a dosing schedule that is used in clinical trials of CPI-613. CPI-613-treated animals were compared to absolute control treatment as well as vehicle treatment. Furthermore, the correlations among toxic effects of CPI-613, plasma concentrations and Pharmacokinetics (PK) of CPI-613 were evaluated to establish the TK effects of CPI-613. **Results:** The results demonstrated the uncommon toxicological profile at toxic doses of CPI-613, which were related to induction of inflammation as the primary and the only toxicological effects in both animal species. The induction of inflammation was consistent with the dose-related increases in plasma CPI-613. Other changes such as elevations in reticulocytes and Gamma-Glutamyl Transferase (GGT) were also observed, but they might be secondary to the inflammatory effects of CPI-613. The toxic doses of CPI-613 were ~60x the anti-tumor dose levels observed in mouse tumor xenograft models, suggesting a wide safety margin of CPI-613. **Conclusion:** CPI-613 had an uncommon toxicological profile (induction of inflammation) in both animal species, which reflects its novel mechanism of action.

Key words: Gamma-Glutamyl Transferase (GGT), toxicological profile, animal species, oxidative phosphorylation, anti-cancer agents, tumor mitochondria, metabolic syndrome, systematic evaluation, hypoxic microenvironments, necrosis and autophagia, clinical pathology

INTRODUCTION

Mitochondria of tumor cells are different from that of normal cells due to re-organization of the metabolic machinery causing tumor mitochondria to generate large amounts of biosynthetic precursors to allow tumor cells to thrive in hypo-vascularized, hypoxic microenvironments (Baggetto, 1992; Kim and Dang, 2006; Ravindran *et al*., 1996; Sakkrom *et al*., 2010). The alterations of tumor mitochondria include changes in mitochondrial membrane lipid contents, shifting reliance on glycolysis from oxidative phosphorylation as the primary sources of deriving ATP and changes in mitochondrial enzymes such as Pyruvate Dehydrogenase (PDC) and α-Ketoglutarate Dehydrogenase (KDH) (Kroemer and Pouyssegur, 2008).

CPI-613 is a novel anti-cancer agent with mechanism of action that does not belong to any existing pharmacological class of anti-cancer agents currently used in the clinics and CPI-613 is referred to

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as an Altered Energy Metabolism-Directed (AEMD) compound. Although structurally similar to lipoate, CPI-613 has activities that are distinctively different from lipoate. Specifically, CPI-613 selectively targets the altered form of mitochondrial energy metabolism found in tumor cells, causing changes in mitochondrial enzyme activities and redox status which lead to apoptosis, necrosis and autophagia of tumor cells and yet not affecting the mitochondrial energy metabolism of normal cells (Baggetto, 1992; Kroemer and Pouyssegur, 2008; Holmuhamedov *et al*., 2002; Sakkrom *et al*., 2010). These activities of CPI-613 are due to its involvement in the catalytic and regulatory functions of the tumor or altered form of PDC and KDH found in tumor cells (Kroemer and Pouyssegur, 2008).

Consistent with the proposed novel mechanism is the fact that CPI-613 has been shown to have anti-tumor activity in cell culture and animal tumor models against diverse cancers independent of multiple drug resistance. The novelty in the presumed mechanism of action for CPI-613 is further supported by the results from *ex vivo* studies demonstrating that CPI-613 is effective against various types of tumor cells excised from patients that displayed varying levels of resistance to different anti-cancer drugs currently used in the clinics. The fact that the anti-tumor activities of CPI-613 are independent of the degree of tumor cell resistance to the drugs currently used in the clinic support the notion that none of the clinically used drugs have a mechanism of action similar to that of CPI-613. The significance of CPI-613 having a novel mechanism of action that is not shared by any existing pharmacological class of anti-cancer agents currently used in the clinics is that CPI-613 is effective not only against naive tumors that have never been treated with any anti-cancer agents, but also effective against tumors that are resistant to anti-cancer agents currently used in the clinics. This is an important aspect because tumors frequently develop resistance to anti-cancer agents, limiting treatment options. The availability of such a novel anti-cancer agent would provide a new tool to treat cancer. Furthermore, CPI-613 activity is independent of cell cycle phase and signal transduction pathways. This provides CPI-613 ubiquitous efficacy against diverse cancer types, even in the presence of multiple drug resistance.

Because of the novel mechanism of action and effectiveness against a variety of tumor types, CPI-613 is undergoing clinical development as an anti-cancer agent. Early clinical results regarding CPI-613 being an anti-tumor therapy are encouraging. In spite of the attractive anti-tumor effects, the Toxicokinetic (TK) effects of CPI-613 have not been thoroughly and systemically investigated. Accordingly, the objective of the studies reported here was to perform a thorough and systematic evaluation of the TK CPI-613 in rats. The objective of this study was also to investigate the toxicological effects of CPI-613 in minipigs, for determining if there were differences in the toxicological profile of CPI-613 between these two animal species. These studies involved frequent assessments of multiple toxicological parameters including clinical signs, full panel blood work, full panel necropsy and full panel histology in animals treated with the threshold dose (i.e., a dose that did not induce significant effects), toxic dose (i.e., a dose that induced significant toxic effects) and dose that approximated the LD_{10} dose (i.e., a dose that induced death in approximately 10% of the animals). CPI-613 was given intravenously (IV) 2x weekly for 3 weeks, a dosing schedule that is used in clinical trials of CPI-613. The results from CPI-613-treated animals were compared to absolute control treatment as well as vehicle treatment. Furthermore, the correlations among toxic effects of CPI-613, plasma concentrations and Pharmacokinetics (PK) of CPI-613 were evaluated to establish the TK effects of CPI-613. These studies demonstrated the uncommon toxicological profile at toxic doses of CPI-613, which were related to induction of inflammation as the primary and possibly the only toxicological effects in both animal species. The induction of inflammation was consistent with the dose-related increases in plasma CPI-613. Other changes such as elevations in reticulocytes and Gamma-Glutamyl Transferase (GGT) were also observed, but they might be secondary to the inflammatory effects of CPI-613. The results from these studies further indicated that the toxic doses of CPI-613 were ~60x the anti-tumor dose levels observed in mouse tumor xenograft models, suggesting a wide safety margin of CPI-613.

MATERIALS AND METHODS

Drugs and vehicles: CPI-613 Injection (Cornerstone Pharmaceuticals, Inc.) was provided in 10-mL injectable formulation at a concentrated form of 50 mg/mL. In the morning of each day of dosing, CPI-613 Injection was diluted to specific concentrations with diluent (5% Dextrose in Water [D5W]) prior to IV administration. Samples of the dosing solutions of CPI-613 used in CPI-613-treated animals were collected and stored until analysis for the concentration was performed to confirm the amount of CPI-613 in the dosing solution. Vehicle (Cornerstone Pharmaceuticals, Inc.) had the same composition as CPI-613 Injection, except with the absence of CPI-613. Vehicle was also diluted with D5W in the same manner as the highest dose of CPI-613 prior to IV administration.

Table 1: Study design and treatment groups for studies using minipigs or rats.

Experiments	Group	Test Article[a]	Dose of CPI-613/Injection[b] mg kg^{-1}	Dose of CPI-613/Injection[b] mg m^{-2}	# Animals Male	# Animals Females	Day of Euthanasia[d]
A	1	D5W	0[c]	0	24	24	50% on Day 21/22 and the other
(Assessment of	2	Vehicle	0[c]	0	24	24	50% on Day 32/33
Dose-Related	3		25[c]	150	12	12	Day 21/22[e]
Toxicity, PK and	4	CPI-613	30[c]	180	24	24	50% on Day 21/22 and the other
Recovery in Rats)	5		35[c]	210	24	24	50% on Day 32/33
B	1	D5W	0	0	4	4	Day 21/22
(Assessment of	2	Vehicle	0	0	4	4	Day 21/22
Dose-Related	3		45	1575	4	4	Day 21/22
Toxicity in	4	CPI-613	50	1750	4	4	Day 21/22
Minipigs)	5		55	1925	4	4	Day 21/22

D5W = 5% dextrose in water; PK = pharmacokinetic; [a]: The test and control articles were administered intravenously twice weekly for three consecutive weeks, starting on day 1; [b]: Conversion of the dose from mg/kg to mg/m^2 was according to Freireich's conversation (Freireich *et al*., 1966); [c]: Spare rats were available and used to replace rats that died or were euthanized during the study, so that the sample size for each treatment group remained to be 12 males and 12 females at the end of the study; [d]: Upon euthanasia, gross necropsy, organ weights and histopathology were performed; [e]: There is no recovery group for Group 3 in rats because the dose of CPI-613 in this group was expected to induce minimum or no effects according to preliminary experiments. Therefore, there was hardly any recovery information that could be obtained from this group.

Animals: Adult laboratory-bred albino rats (Sprague-Dawley Harlan Indianapolis, Indiana; ~250 g body weight, both sexes) and Hanford miniature swine (Sinclair Research Center, Inc., Auxvasse, Missouri; 22-70 kg for males and 16-58 kg for females, at least 4-months old) that were free from common domestic swine diseases (e.g., leptospirosis, brucellosis, pseudorabies, transmissible gastroenteritis, porcine reproductive respiratory syndrome, toxoplasmosis) (Nunoya *et al*., 2007; Swindle, 2007) were used. Rats were individually housed in stainless steel cages whereas minipigs were housed in separate chain-link runs with vinyl-coated elevated grating. Food (Certified Rodent Chow and Pig Diet, respectively, PMI Feeds Inc.®, Richmond, IN) was available *ad libitum* for rats and provided using a pre-measured scoop for minipigs. Filtered tap water was available *ad libitum* for both species. The food and water were routinely analyzed to confirm a lack of unacceptable levels of contaminants. These analyses included detection of heavy metals, pesticides, aflatoxins and other environmental pollutants for food and heavy metals, pesticides and microbological contents for water. The rooms were well ventilated (>10 air changes per hour, with 100% non-recirculated fresh air), temperature controlled at 16-27°C for minipigs and 18-26°C for rats and relative humidity maintained at 30-70% for both species. Each room had a 12-h light/dark photoperiod. Prior to study initiation, rats were acclimated for at least 6 days whereas minipigs were acclimated to laboratory conditions for at least 25 days. Minipigs were further acclimated to sling restraint for 2 days. Handling and care of the animals were according to the USDA Animal Welfare Act (9 CFR 1 and 2) and the Guidelines for Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996).

Experimental design: These studies included two major experiments in rats and minipigs and the designs of these experiments were as outlined in Table 1. Briefly, the animals were randomly assigned to different groups by a computer generated weight-ordered distribution such that group mean body weights generally did not exceed ±10% of the overall mean weight for each sex. Diluent (i.e., D5W) was administered to the absolute control groups, vehicle was administered to control groups and various doses of CPI-613 were administered to CPI-613 treated groups, given 2x weekly (with at least 2 days between dosing) for three consecutive weeks.

Test and control articles were administered as IV bolus using asceptic technique via a venous access port which had been surgically implanted into the jugular vein in minipigs and the femoral vein in rats for IV administration. The venous access ports remained with the animals throughout the study. In minipigs, the dose volume for all animals was 5 mL kg^{-1} and the concentrations of CPI-613 were 9-10 and 11 mg^{-1} mL for the 45-50 and 55 mg kg^{-1} dose levels, respectively. In rats, the dose volume for all animals was 10 mL kg^{-1} and the concentrations of CPI-613 were 2.5-3.0 and 3.5 mg^{-1} mL for the 25-30 and 35 mg kg^{-1} dose levels, respectively.

Potential toxicological effects of CPI-613 were evaluated by monitoring the following parameters: clinical signs, body weight, clinical pathology (chemistry and hematology), ECG (minipigs only), gross necropsy, organ weights and histopathology at various times as shown in Table 2.

Plasma CPI-613 concentrations were also assessed in rats at time points as shown in Table 2. Significant changes in toxicological parameters were correlated with plasma CPI-613 concentrations and PK to assess the TK effects of CPI-613 in rats.

Table 2: Parameters monitored during the studies in Experiments A and B in rats and minipigs, respectively

	Parameters	Timing of evaluation
Toxicological Parameters	Clinical Signs	Pretest, once daily and frequently if a change was noted. On days of dosing, clinical observation was performed for several hours post-dose.
	Mortality/Moribundity	Evaluated twice daily (a.m. and p.m.)
	Body Weight	Pretest, on Study Day 1, predose on days of dosing, weekly during the recovery period, prior to terminal fasting and prior to necropsy.
	Food Consumption	Assessed weekly.
	Clinical Pathology (chemistry & hematology) (see Table 3)	Pre-dose on Day 1 (i.e., baseline), prior to the 4th dose; Study Day 22 (prior to necropsy, if took place); and Study Day 33 (prior to necropsy, for the recovery animals in Experiment A only).
	CK Isoenzymes and Troponin I	At 0.5 (minipigs only), 1 (minipigs only), 2 (minipigs only), 4, 8, 16 and 24 h after the 1st, 4th and 6th dose. Also prior to euthanasia on Study Day 22 in Experiments A and B and on Study Day 33 in recovery animals in Experiment A.
	ECG	60-sec recording obtained pre-dose and every 30 min during the first 3 h after the 1st, 4th and 6th dose, as well as prior to euthanasia on Study Day 22. (Note: ECG was recorded in minipigs of Experiment B only, not in rats of Experiment A.)
	Gross necropsy, organ weights and histopathology (Table 3)	Day 22 (for Experiments A and B) or Day 33 (in recovery animals in Experiment A).
Pharmacokinetic Parameters	Plasma CPI-613 levels	At 0.5, 1, 2, 4, 8, 16 and 24 h associated with the 1st, 2nd, 4th and 6th doses of CPI-613 in rats.[a] (Note: These samples were collected in rats of Experiment A only, not in minipigs of Experiment B.)

CK = Creatine Kinase; [a]: In rats, the total amount of blood that can be collected from each animal is limited due to the small body size of rats. Accordingly, each rat provided blood samples for only 2-4 time points out of the 7 time points listed, resulting in a sample size of 4 male rats and 4 female rats per time point even though there were 12 male rats and 12 female rats in each group

Assays and measurements: Clinical pathology was evaluated by performing serum chemistry and hematology assays. These assays were performed on samples collected from all animals of all treatment groups, as listed in Table 1. Blood samples (~1.5 mL from rats and ~2.5 mL from minipigs) were collected by puncture of the orbital sinus or by cardiac puncture in rats and from the venous access port or via a venous puncture in minipigs, after food was withheld for at least eight hours. The chemistry assay was performed using the automated Olympus AU840e analyzer on serum (~0.5 mL) isolated from blood samples, whereas the hematology assay was performed using the automated ADVIA™ 120 analyzer on EDTA-containing blood (~0.5 mL in rats and ~2 mL in minipigs). The parameters for serum chemistry and hematology that were assessed are listed in Table 3.

In minipigs, ElectroCardiogram (ECG) was also assessed. ECG was performed with the minipigs placed in slings and ECG tracings (60-sec intervals which varied slightly in some animals due to behavior) were obtained using 6 triangular leads [e.g., dorsal, axial, ventral, aVR-dorsal, aVL-axial and aVF-ventral leads] at a chart speed of 50 mm/sec from all animals. Measurements were taken from the axial lead.

Plasma CPI-613 concentrations were assayed as described below. Blood samples (~1 mL) for evaluation of plasma CPI-613 levels were collected into tubes containing K_3-EDTA. The blood samples were centrifuged and the plasma was extracted. The plasma samples were stored at-70°C until assay. In the analysis of the concentration of CPI-613, samples were prepared using solid phase extraction to obtain the total amount (free and bound) of CPI-613, followed by analysis via a Liquid Chromatography (LC)/Mass Spectrometry (MS) MS^{-1} method using negative turbo ionspray ionization and operating the instrument in the Multiple-Reaction-Monitoring (MRM) mode. The parameters and instruments for the LC^{-1} MS^{-1} MS^{-1} assay are listed in Table 4. Using D14 isotope of CPI-613 as the internal standard, the plasma assay for two concentration ranges (1.00-100 ng mL^{-1} and 50.0-5000 ng mL^{-1}) were validated for sufficient linearity, specificity, reproducibility, accuracy, precision, stability at room and refrigeration temperature.

Gross necropsy, organ weights and histopathology evaluations were performed. For these evaluations, animals were humanely euthanized (via carbon dioxide asphyxiation in rats and via anesthesia with Telazol® followed by an overdose of sodium pentobarbital and then exsanguination in minipigs). Full panel necropsy was performed, which included examination of the carcass and musculoskeletal system, all external surfaces and orifices, cranial cavity and external surface of the brain, thoracic, abdominal and pelvic cavities with their associated organs and tissues.

For organ weight determination, the weights of the following organs were determined: adrenal gland, brain, heart, kidney, liver, lung, ovary, spleen, testis and thymus. Paired organs were weighed together. Organ weights were not recorded for animals that died or were euthanized prior to scheduled termination.

Table 3: Parameters for clinical pathology, organ weights and histology assessment in Experiments A and B in rats and minipigs, respectively

Clinical Pathology				
Serum Chemistry	Hematology	Organ Weights	Histology	
Electrolytes/Ions:	Cell Counts:	-adrenal	Renal and urinary:	Reproductive:
-calcium	-red blood cell	-brain	-kidney	-ovary
-phosphorus	-white blood cell	-heart	-spleen	-cervix
-sodium	-reticulocyte	-kidney	-stomach (nonglandular	-seminal vesicle
		-liver	and glandular)	-testis
-potassium	-platelet	-lung	-urinary bladder	-uterus
-chloride	-neutrophil	-ovary		-vagina
	-lymphocyte	-spleen	Cardiovascular:	
Lipids:	-monocyte	-testis	-aorta	Gland:
-cholesterol	-eosinophil		-heart	-adrenal gland
-triglycerides	-basophil			-harderian gland
Enzymes:	Others:		Nervous:	-mammary gland
-alanine aminotransferase	-hemoglobin concentration		-brain (brain stem,	-pituitary gland
-aspartate	-mean corpuscular volume		cerebellum, cerebrum)	-prostate gland
aminotransferase	-red blood cell morphology		-epididymis	-salivary gland (mandibular)
-alkaline phosphatase	-others such as abnormal		-nerve (optic, sciatic)	-thyroid gland/parathyroid gland
-gamma-	blood cells if presence		-spinal cord (cervical,	-thymus
glutamyltransferase	-hematocrit[a]		lumbar, thoracic)	
-lactate dehydrogenase	-mean corpuscular			Musculoskeletal
	hemoglobin[a]		Gastrointestinal:	-femur bone
Plasma Proteins:	-mean corpuscular		-tongue	-sternum
-protein	hemoglobin concentration[a]		-esophagus	-sternum bone marrow
-albumin			-intestine (cecum, colon,	-skeletal muscle
-globulin[a]			rectum, duodenum, ileum,	
-albumin/globulin ratio[a]			jejunum [with Peyer's patch])	Others:
			-liver	-skin (abdominal)
Others:			-pancreas	-eye
-glucose				-administration site (region of
-total bilirubin			Respiratory:	catheter insertion at femoral vein
-urea nitrogen			-lung	[rats] and jugular vein [minipigs])
-creatinine			-trachea	-lymph node (mandibular,
				mesenteric)
				-gross lesions/masses

[a]: Calculated values

Table 4: Parameters and instruments for Liquid Chromatography (LC) and Mass Spectrometry (MS) of the plasma CPI-613 assay

HPLC	
Autosampler:	CTC Analytics Autosampler
Pump:	Perkin Elmer Series 200 Micro LC Pumps
Column Oven:	NA
Column:	Luna Phenyl-Hexyl Column; 2.0 x 50mm, 5 µm (phenomenex)
Pre-column Frit:	0.5 µm stainless-steel Precolumn Frit (Upchurch Scientific)
Total Flow Rate:	0.500 mL min^{-1}
RunTime:	2.5 minutes
Injection Volume:	5.00 µL
Pre-cleans:	0
Post-cleans:	3 of Needle Wash 1,3 of Needle Wash 2
Valve cleans:	3 of Needle Wash 1,3 of Needle Wash 2
Mobile Phase A:	0.1 % Formic Acid in Water
Mobile Phase B:	0.1 % Formic Acid in Acetonitrile
Needle Rinse 1:	Acetone: MethanoI: Acetonitrile: Forrnic Acid: Bleach (40:30:30:0.1:0.1, v:v:v:v:v)
Needle Rinse 2:	DMSO:ACN (60:40, v:v)
MS	
Mass Spectrometer:	Applied Biosystems API 4000
Interface:	Turbo-Ionspray, negative-ion mode
Scan Mode:	MRM
Source Temp:	450°C
Nominal Transitions:	m/z 387 → ~ 123 or 130, dwell time = 300 msec

For full panel histopathology, the following organs and tissues were examined *in situ*, dissected free and fixed in 10% neutral buffered formalin (except for the testes which were fixed in Modified Davidson's fixative and the eyes with optic nerve which were fixed in Davidson's fixative): adrenal gland, administration site (region of catheter insertion at femoral vein (rats) or jugular vein (minipigs)), aorta, bone (femur), bone (sternum), bone marrow (sternum), brain (brain stem, cerebellum, cerebrum), cervix, epididymis, esophagus,

eye, harderian gland, heart, intestine (cecum, colon, rectum, duodenum, ileum, jejunum (with Peyer's patch)), kidney, liver, lung, lymph node (mandibular, mesenteric), mammary gland, nerve (optic, sciatic), ovary, pancreas, pituitary gland, prostate gland, salivary gland (mandibular), seminal vesicle, skeletal muscle, skin (abdominal), spinal cord (cervical, lumbar, thoracic), spleen, stomach (nonglandular, glandular), testis, thymus, thyroid gland/parathyroid gland, tongue, trachea, urinary bladder, uterus, vagina and gross lesions/masses. Histopathology was performed on all tissues from all animals and all gross lesions. Slides were stained with hematoxylin and eosin and were microscopically evaluated.

Assessment and statistical analysis: The PK profile of each group was characterized by non-compartmental analysis of plasma concentrations of CPI-613 using validated computer software (WinNonlin, Version 3.2, Pharsight Corp., Mountain View, California, USA). The area under the "CPI-613 plasma concentrations vs time" curve (AUC) was calculated using the linear trapezoidal method with linear interpolation. Since the "CPI-613 plasma concentrations vs time" curve revealed that the elimination phase followed a 2-compartment model with biphasic appearance (i.e., an initial distribution phase followed by a terminal elimination phase), onset of the terminal elimination phase was identified by the appearance of the inflection point between the initial distribution phase and the terminal elimination phase of the TK profile. The coefficient of determination of the line fitted to the terminal elimination phase was calculated. TK parameters describing the systemic exposure of the test article in the test system was estimated from observed plasma concentration values, the dosing regimen, the AUC and the terminal elimination phase rate constant for each group.

Statistical analysis was performed on body weights, body weight changes, food consumption, hematology, clinical chemistry parameters and organ weights. To determine the appropriate statistical test, each data set was subjected to a statistical decision tree. A minimum of three animals per sex per group per interval was required for statistical analysis. Treated groups of the same sex of all treatment groups were compared at all common time-points. The Bartlett's Test was used to compare overall variance among all groups. If the Bartlett's Test had a probability of $P>0.05$, then a parametric distribution was assumed and a one-way Analysis Of Variance (ANOVA) was performed. A significant ANOVA result ($p\leq0.05$) was followed by multiple comparisons using a *post-hoc* Dunnett's test to identify differences between absolute control and each treatment group. A non-significant ANOVA result ($p>0.05$) indicated that no significant differences exist between the groups for the parameter under consideration. If the Bartlett's Test had a probability of $p\leq0.05$, then a non-parametric distribution was assumed and a Kruskal-Wallis test for independent groups was performed. A significant Kruskal-Wallis Test result ($p\leq0.05$) was followed by multiple comparisons using a *post-hoc* Dunn's procedure to reveal differences between control(s) and each treatment group. A non-significant Kruskal-Wallis Test ($p>0.05$) indicated that no significant differences exist between the groups for the parameter under consideration. For all tests and all quantitative parameters, a 95% confidence level ($p\leq0.05$) was the standard criterion for statistical significance.

RESULTS

There were no differences between males and females in their responses to CPI-613 treatment in both rats and minipigs in both experiments, with exceptions that are described below. Whenever there was a lack of differences between the two sexes, results from males and females were combined when evaluating the effects induced by CPI-613 or vehicle.

Analytical results of dosing solutions as a means to check the actual dose: In both experiments, analysis of the dosing solutions confirmed that the concentrations were as expected. Therefore, these results confirmed that the doses given to rats were as expected.

Mortality results: Experiment A in rats. The number of rats from Experiment A survived the course of the study in different treatment groups are shown in Fig. 1 and rats that died or euthanized moribund are individually listed in Table 5. Specifically, all rats in the D5W, vehicle and 25 mg kg^{-1} groups survived during the course of the study. Conversely, not all rats in the 30-35-mg kg^{-1} groups survived. Two of 48 rats (4.2%) in the 30-mg kg^{-1} group were found dead on Days 1 or 2 of the study. Autopsy and histology findings showed that the cause of death in one rat was due to significant systemic inflammation and the cause of death in the other rat could not be determined. Four of 48 rats (8.3%) in the 35-mg kg^{-1} group developed significant moribundity, which occurred on Day 2 in one rat, Day 8 in two rats and Day 20 in the remaining rat. These four rats were euthanized for humane reasons. The cause of moribundity in three of these four rats was due to significant systemic inflammation according to autopsy and histology findings, whereas the cause of moribundity in the remaining rat could not be determined. Therefore, these results showed that dose that induced ~10% mortality (LD_{10}) is approximately 35 mg kg^{-1} in rats.

Table 5: Incidence of euthanized moribund and death in rats and minipigs from Experiments A and B, respectively

			Number of animals		Euthanized moribund or death					
Experiments	Group	Dose (mg kg^{-1})	Male	Female	Animal	Sex	Study Day	Male (%)	Female (%)	Both sexes (%)
Experiment A in rats	1	0 (D5W)	24	24	.	.	.	.	0.00	0.00
	2	0 (Vehicle)	24	24	.	.	.	0.00	0.00	0.00
	3	25	12	12	.	.	.	0.00	0.00	0.00
	4	30	24	24	1	Female	1	0.00%	8.30%	4.10%
					1	Female	2			
	5	35	24	24	1	Female	2	12.50%	4.20%	8.30%
					1	Male	8			
					1	Male	8			
					1	Male	20			
Experiment B in minipigs	1	0 (D5W)	4	4	.	.	.	0.00	0.00	0.00
	2	0 (Vehicle)	4	4	.	.	.	0.00	0.00	0.00
	3	45	4	4	.	.	.	0.00	0.00	0.00
	4	50	4	4	.	.	.	0.00	0.00	0.00
	5	55	4	4	.	.	.	0.00	0.00	0.00

D5W = 5% dextrose in water

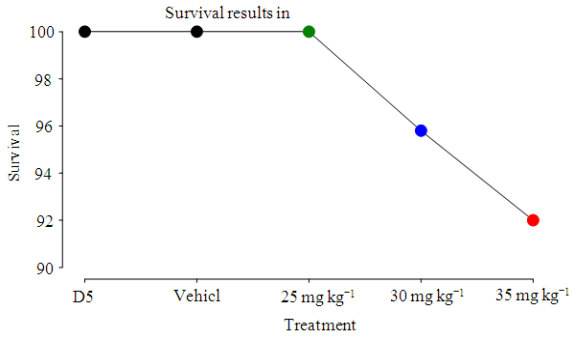

Fig. 1: Incidence of survival in different treatment groups of rats treated with 5% Dextrose in Water (D5W), vehicle, or CPI-613 at 25, 30 or 35 mg kg^{-1}, given 2x weekly for three consecutive weeks from Experiment A. Survival rats included those that did not die during the study, as well as those that did not develop significant moribund. There were 24 males and 24 females in all treatment groups, except for the 25 mg kg^{-1} group which had 12 males and 12 females. See Table 1 for specific treatment groups

Experiment B in minipigs. None of the minipigs from any of the five treatment groups died or euthanized moribund during the course of the study (Table 5). Therefore, the lethal dose for CPI-613 was >55 mg kg^{-1} in minipigs.

Clinical observations: Experiment A in rats. The incidence and duration of clinical observations are listed in Table 6. In males, the following drug-related clinical signs were observed after administration of 30 and 35 mg kg^{-1} of CPI-613: distended abdomen, hunched posture, impaired limb function, decreased activity, abnormal stool, decreased defecation, material in pan/bedding, necrosis at the site of administration, extended penis, abnormal testes and material around the nose. In females, the following drug-related clinical signs were observed after administration of 35 mg kg^{-1} of CPI-613: impaired limb function, necrosis at the site of administration and skin discoloration.

Experiment B in minipigs. The only apparent drug-related clinical sign observed was vomiting that was transient and reversible and usually occurred on the days after administration of CPI-613 of all doses and the severity was dose-related (Table 6).

Body weight and food consumption: Experiment A in rats. There were no adverse effects on body weight (Table 7), body weight changes, or food consumption (Table 8) in rats treated with different doses of CPI-613 or vehicle, when compared to D5W-treated animals.

Experiment B in minipigs: There were no adverse effects on body weight (Table 7) or body weight changes in animals treated with various doses of CPI-613 or vehicle, when compared to D5W-treated animals. There was rare occasional low food consumption in minipigs treated with CPI-613 at 45 and 55 mg kg^{-1}, which did not occur in other treatment groups (Table 9).

Clinical Pathology (chemistry and hematology): The clinical pathology data from males and females were analyzed separately due to significant differences between males and females in both species.

Table 6: CPI-613 related clinical observation during the studies from experiment A and B in rats and minipigs, respectively

Types	Incidence	Group 1	Group 2	Group 3	Group 4	Group 5
Experiment A in rats-males						
Distended Abdomen	# of Observations	.	.	.	.	1
	# of Animals	.	.	.	.	1
	Duration (Days from-to)	.	.	.	.	8-8
Hunched Posture	# of Observations	.	.	.	.	2
	# of Animals	.	.	.	.	2
	Duration (Days from-to)	.	.	.	.	8-8
Impaired Limb Function	# of Observations	.	.	.	3	6
	# of Animals	.	.	.	1	2
	Duration (Days from-to)	.	.	.	14-16	22-18
Activity Decreased	# of Observations	.	.	.	.	1
	# of Animals	.	.	.	.	1
	Duration (Days from-to)	.	.	.	.	8-8
Abnormal Stool	# of Observations	.	.	.	9	14
	# of Animals	.	.	.	4	2
	Duration (Days from-to)	.	.	.	11-24	11-23
Defecation Decreased	# of Observations	.	.	.	.	5
	# of Animals	.	.	.	.	1
	Duration (Days from-to)	.	.	.	.	7-3
Material in Pan/Bedding	# of Observations	2	.	.	1	5
	# of Animals	1	.	.	1	5
	Duration (Days from-to)	11 12	.	.	4-4	1-8
Necrosis at Injection Site	# of Observations	.	.	.	.	2
	# of Animals	.	.	.	.	2
	Duration (Days from-to)	.	.	.	.	19-22
Abnormal Testes	# of Observations	.	.	1	23	.
	# of Animals	.	.	1	3	.
	Duration (Days from-to)	.	.	22-22	16-29	.
Penis Extended	# of Observations	.	.	.	3	5
	# of Animals	.	.	.	1	1
	Duration (Days from-to)	.	.	.	22-24	3-7
Material Around Nose	# of Observations	11	17	14	23	25
	# of Animals	3	6	4	3	5
	Duration (Days from-to)	15-22	12-27	12-22	11-33	3-26
Experiment A in rats-females						
Impaired Limb Function	# of Observations	.	.	.	9	14
	# of Animals	.	.	.	4	4
	Duration (Days from-to)	.	.	.	1-15	1-22
Necrosis at site of administration	# of Observations	.	.	.	.	14
	# of Animals	.	.	.	.	1
	Duration (Days from-to)	.	.	.	.	20-33
Skin Discolored	# of Observations	.	.	.	.	9
	# of Animals	.	.	.	.	3
	Duration (Days from-to)	.	.	.	.	20-33
Experiment B in Minipigs-Males						
Vomitus	# of Observations	.	.	.	3	8
	# of Animals	.	.	.	1	4
	Duration (Days from-to)	.	.	.	1-15	1-19
Vomitus, Discolor	# of Observations	.	.	.	2	4
	# of Animals	.	.	.	1	2
	Duration (Days from-to)	.	.	.	1-4	1-19
Experiment B in Minipigs-Females						
Vomitus	# of Observations	.	.	3	3	3
	# of Animals	.	.	2	2	2
	Duration (Days from-to)	.	.	4-15	1-5	4-19
Vomitus, Discolor	# of Observations	.	.	3	1	3
	# of Animals	.	.	2	1	2
	Duration (Days from-to)	.	.	4-15	4	4-15

Experiment A in rats: There were no significant changes in any of the clinical pathology parameters in male or female rats based on statistical analyses and comparison to historical control data, with the exception of reticulocytes and GGT. For reticulocytes, CPI-613 at 30-35 mg kg^{-1} increased both the % and absolute values after the 6th or last dose, when compared to concurrent values from D5W and vehicle treatments, in male rats (Fig. 2).

Table 7: Body weights during the studies in rats (gm) and minipigs (kg) from Experiments A and B, respectively

				Study days							
Experiments	Sex	Group	Values	Day 1	Day 4-5	Day 8	Day 11-12	Day 15	Day 18-19	Day 21-22	Day 32
Experiment A in Rats	Male	1	Mean	358	366	377	358	389	396	388	417
			SD	16	16	19	21	20	20	28	20
			N	24	24	24	24	24	24	24	12
		2	Mean	356	368	378	359	389	401	386	425
			SD	16	15	14	13	13	14	16	13
			N	24	24	24	24	24	24	24	12
		3	Mean	346	355	367	342	378	388	375	--
			SD	17	15	17	16	21	21	24	--
			N	12	12	12	12	12	12	12	--
		4	Mean	363	369	375	355	381	391	377	418
			SD	19	22	22	20	23	22	20	18
			N	24	24	24	24	24	24	24	12
		5	Mean	364	368	371	356	386	396	370	431
			SD	22	24	30	27	29	29	34	23
			N	24	24	24	22	22	22	21	11
	Female	1	Mean	249	251	256	252	263	268	259	282
			SD	16	11	13	14	15	16	13	14
			N	24	24	24	24	24	24	24	12
		2	Mean	251	252	255	255	266	271	266	284
			SD	14	14	14	12	14	14	14	17
			N	24	24	24	24	24	24	24	12
		3	Mean	248	250	254	240	262	263	262	--
			SD	13	12	11	11	10	12	11	--
			N	12	12	12	12	12	12	12	--
		4	Mean	255	255	258	255	267	271	264	283
			SD	15	13	13	14	13	15	16	11
			N	24	24	24	24	24	24	24	12
		5	Mean	247	251	254	255	265	270	268	280
			SD	15	15	15	13	17	17	19	16
			N	24	24	24	24	24	24	24	12
Experiment B in minipigs	Male	1	Mean	25	26	27	28	28	29	30	--
			SD	3	3	2	3	3	4	4	--
			N	4	4	4	4	4	4	4	--
		2	Mean	24	25	26	26	27	28	29	--
			SD	1	2	2	2	1	1	1	--
			N	4	4	4	4	4	4	4	--
		3	Mean	26	28	29	29	30	30	33	--
			SD	2	2	2	2	1	2	2	--
			N	4	4	4	4	4	4	4	--
		4	Mean	25	26	26	27	26	27	28	--
			SD	1	2	2	2	2	2	1	--
			N	4	4	4	4	4	4	4	--
		5	Mean	26	27	27	28	28	28	29	--
			SD	3	3	3	4	4	5	4	--
			N	4	4	4	4	4	4	4	--
	Female	1	Mean	21	22	23	23	24	24	25	--
			SD	3	3	3	3	3	4	3	--
			N	4	4	4	4	4	4	4	--
		2	Mean	22	22	23	23	24	25	25	--
			SD	2	2	2	2	2	3	2	--
			N	4	4	4	4	4	4	4	--
		3	Mean	21	22	23	23	24	24	26	--
			SD	4	4	4	4	5	4	5	--
			N	4	4	4	4	4	4	4	--
		4	Mean	21	22	23	24	24	25	26	--
			SD	3	3	3	3	3	4	3	--
			N	4	4	4	4	4	4	4	--
		5	Mean	23	24	24	25	26	27	27	--
			SD	3	4	4	3	3	4	4	--
			N	4	4	4	4	4	4	4	--

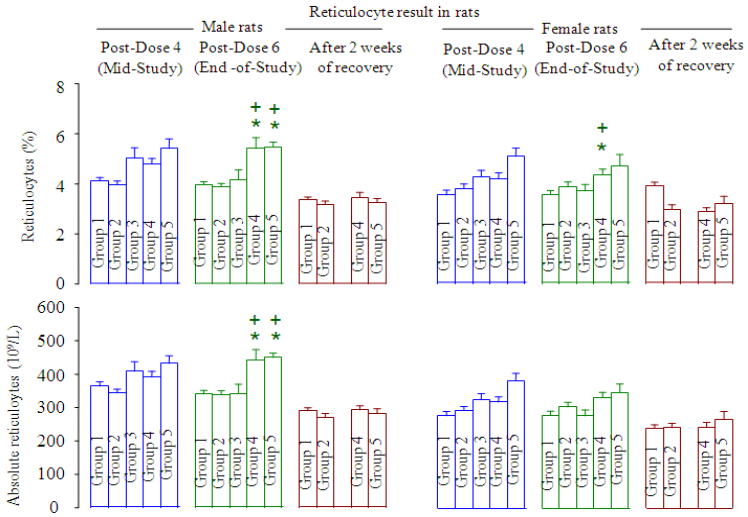

Fig. 2: Reticulocytes (% and absolute) in male and female rats treated with 5% Dextrose in Water (D5W) in Group 1, vehicle in Group 2 and CPI-613 at 25, 30 or 35 mg kg^{-1} in Groups 3, 4 and 5, respectively, given 2x weekly for three consecutive weeks from Experiment A. There were 24 males and 24 females in all treatment groups, except for the 25-mg/kg group which had 12 males and 12 females. In all treatment groups other than the 25 mg kg^{-1} groups, one-half of the animals were euthanized on day 22, whereas the other half of the animals were euthanized on day 33. For the 25 mg kg^{-1} group, all animals were euthanized on day 22. See Table 1 for specific treatment groups. Results are presented as mean ± Standard Error of the Mean (SEM). + = significantly higher than the D5W group and * = significantly higher than the vehicle group, at $p \leq 0.05$ level

Table 8: Food consumption (g/day) during the studies in rats from Experiment A.

			Food consumption				
Sex	Group	Values	Days 1-8	Days 8-15	Days 15-22	Days 22-29	Days 29-32
Male	1	Mean	29	26	26.0	27	25
		SD	3	2	1.5	1	1
		N	24	23	23.0	12	12
	2	Mean	29	27	27.0	27	26
		SD	2	2	2.5	1	2
		N	24	23[a]	23.0[a]	12	12
	3	Mean	29	27	27.0	.	.
		SD	3	3	2.0	.	.
		N	12	12	12.0	.	.
	4	Mean	28	27	27.0	29	24
		SD	2	3	4.5	4	5
		N	24	24	24.0	12	12
	5	Mean	26	27	26.0	29	26
		SD	5	3	3.0	2	2
		N	24	22[a]	21.0[a]	11[a]	11[a]
Female	1	Mean	23	22	21.0	25	20
		SD	1	2	2.0	2	2
		N	23	24	24.0	12	12
	2	Mean	22	23	21.5	25	19
		SD	4	1	2.5	2	3
		N	23	24	24.0	12	12
	3	Mean	24	23	23.0	.	.
		SD	1	1	2.0	.	.
		N	12	12	12.0	.	.
	4	Mean	24	23	23.0#	26	19
		SD	5	1	1.5	1	1
		N	23[a]	23[a]	24.0	12	12
	5	Mean	23	23	22.5	27	19
		SD	2	3	2.0	2	2
		N	22[a]	22[a]	22.0[a]	12	12

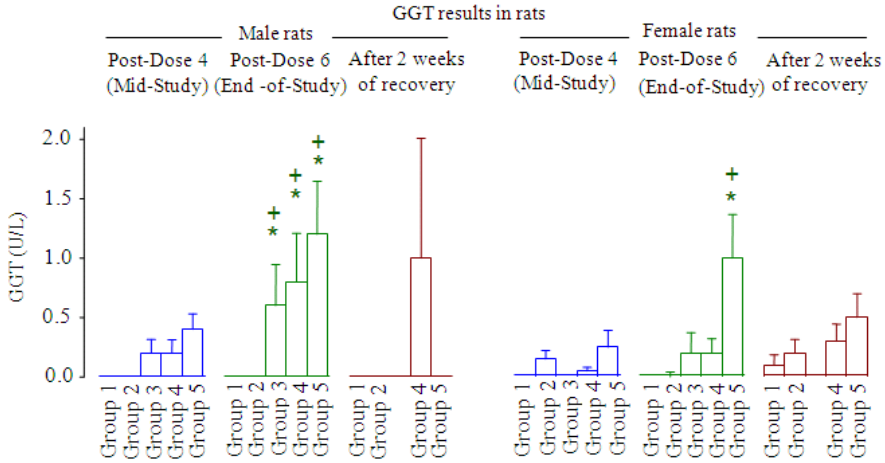

Fig. 3: Gamma-Glutamyltransferase (GGT) in male and female rats treated with 5% Dextrose in Water (D5W) in Group 1, vehicle in Group 2 and CPI-613 at 25, 30 or 35 mg kg^{-1} in Groups 3, 4 and 5, respectively, given 2x weekly for three consecutive weeks from Experiment A. There were 24 males and 24 females in all treatment groups, except for the 25 mg kg^{-1} group which had 12 males and 12 females. In all treatment groups other than the 25 mg kg^{-1} groups, one-half of the animals were euthanized on day 22, whereas the other half of the animals were euthanized on day 33. For the 25 mg kg^{-1} group, all animals were euthanized on day 22. See Table 1 for specific treatment groups. Results are presented as mean ± Standard Error of the Mean (SEM). +: Significantly higher than the D5W group and *: Significantly higher than the vehicle group, at $p \leq 0.05$ level

Table 9: Food consumption pattern during the studies in minipigs from experiment B

Sex	----------Food consumption----------		Group 1	Group 2	Group 3	Group 4	Group 5
Male	Scant[a]	# of Observations	.	.	.	.	2
		# of Animals		.	.	.	1
		Days	.	.	.	.	6, 17
	Low[a]	# of Observations	.	.	3	.	1
		# of Animals	.	.	2	.	1
		Days Observed	.	.	2, 20, 21	.	18
Females	Scant[a]	# of Observations	.	.	.	.	.
		# of Animals	.	.	.	.	.
		Days Observed	.	.	.	.	.
	Low[a]	# of Observations	.	.	1	.	.
		# of Animals	.	.	1	.	.
		Days Observed	.	.	20	.	.

[a]: Normal: No food remaining; Low: Up to half of the previous food allocation remaining; Scant: More than half of the previous food allocation remaining

CPI-613 also increased the % values of reticulocytes after the 6th dose, when compared to concurrent values from D5W and vehicle treatments, in female rats. At the end of the recovery period (i.e., two weeks after the last dose of CPI-613), the reticulocyte values in the CPI-613 treated groups were no longer higher than the D5W or vehicle groups, suggesting that the CPI-613-induced increases in reticulocytes were reversible upon termination of CPI-613 treatment.

For GGT, CPI-613 at all three doses increased GGT values when compared to D5W and vehicle after the 6th doses (i.e., during mid-study and end-of-study assessments, respectively) and the magnitude of increases were in a dose-related manner in male rats (Fig. 3). In female rats, CPI-613 at the highest doses increased GGT values when compared to D5W and vehicle after the 6th dose. The mean GGT values in the 35-mg kg^{-1} group were lower two weeks after the study when compared to those at the end of the study in both male and female rats, suggesting that the increases in GGT values might be reversible. Interestingly, vehicle also slightly increased GGT values, when compared to D5W, after the 4th dose and after the 2-week recovery period in female rats. Therefore, vehicle might be partially responsible for the increased GGT induced by CPI-613.

Experiment B in minipigs: There were no toxicologically or biologically significant changes in hematology in males or females during the study. CPI-613 at all doses tested did not cause toxicologically or biologically significant changes in any clinical chemistry parameters during the study.

ECG: ECG was assessed in minipigs in Experiment B. There were no changes of any biological or toxicological significance associated with D5W, vehicle or different doses of CPI-613. Specifically, there were no toxicologically significant effects on heart rate, RR interval, PR interval, QRS duration, QT interval, or QTc. No treatment-related arrhythmias were detected.

Gross pathology: Experiment A in rats. Gross pathology findings are listed in Table 10. Nodules, masses, or crusts at the injection site, inguinal skin and subcutaneous tissues near the port, as well as histological evidence of inflammation, were observed in CPI-613 treated rats and the severity and frequency were dose-related. Gross lesions tended to be more frequent in males than female rats. The gross inflammatory process at the injection site extended to the reproductive organs (prostate, epididymis, seminal vesicle or testis) of Group 5 male rats (treated with 35 mg kg^{-1} of CPI-613) and a single Group 3 male rat (treated with 25 mg kg^{-1} CPI-613). Many of these findings were consistent with the clinical observation of the current study.

In Recovery rats (rats that were assessed after a 2-week recovery period), there was a significant reduction in the incidence of gross findings of skin crust or subcutaneous nodules at or near the injection site or port in mid-and high-dose male rats and high-dose female rats and a pronounced reduction in findings of nodules and masses of male reproductive organs of high-dose males. These results suggested that the gross pathological findings were reversible upon termination of treatment with CPI-613.

Experiment B in minipigs. CPI-613-related gross lesions were observed and they included nodules, masses, crusts or fluid at the administration site and subcutaneous tissue near the port (Table 11). These gross findings most often correlated with ulcerative and/or chronic-active inflammation. The incidences were not dose-dependent.

Organ weights: Experiments A and B in rats and minipigs, respectively. In rats, there were no statistically significant differences in absolute organ weight values or changes in organ weights from baseline, with the exception of the higher spleen weights observed at the end of the treatment period in CPI-613 treated rats when compared with D5W rats (0.833±0.022 g Vs. 1.192±0.103 g). This may be associated with the hematopoietic cell proliferation of the spleen possibly secondary to the inflammatory effects of CPI-613. In minipigs, there were no abnormal effects of CPI-613 on organ weights.

Histopathology: Experiment A in rats. The results from histopathology performed at the end of the study on day 22 as well as those performed at the end of the recovery period on day 33 are shown in Table 12. The histopathology results showed a dose-dependent exaggeration in incidence and severity of granulomatous or acute, chronic or chronic active inflammation at the administration site (inguinal vein), skin and subcutaneous tissue near the port and inguinal skin and subcutaneous tissue near the inguinal vein were observed. Lymphoid hyperplasia in drainage lymph nodes (iliac and mediastinal), hematopoietic cell proliferation of the spleen and sternal bone marrow hyperplasia were considered secondary to inflammation initiated at the administration site or port, rather than primary effects of CPI-613 due to a lack of other effects such as a lack of increase in blood cell counts.

According to histopathology performed after a 2-week recovery period, there was a reduction in the incidence and severity of moderate or marked inflammation at or adjacent to the administration site or port observed in mid-and high-dose males and females. The most notable difference was a reduction in the incidence of inflammation of the abdominal organs that was considered an extension of the inflammatory reaction initiated at the administration site, including inflammation and spermatic granuloma of the epididymis, inflammation of the seminal vesicle and testes in high-dose males. Once again, these results suggested that the histopathlogical findings were reversible.

Experiment B in minipigs: The results from histopathology performed at the end of the study on day 22 are shown in Table 13. These results showed that thromboses (unilateral or bilateral) surrounding the catheter and involving the vein wall were noted in all CPI-613 groups of both sexes. A significant, chronically-active inflammation involving the vein wall and perivascular tissue was noted in several animals, but there was no relationship to the dose. Skin lesions in areas noted related to the administration site (primarily head and back) were relatively common and consisted primarily of ulcerative inflammation and/or hyperkeratosis. There was no dose relationship. Chronically-active inflammation and an abscess were noted in the lung of one Group 5 male (treated with 55 mg kg^{-1} of CPI-613), but it is believed that the inflammation was closely related to inflammation at the administration site. Marked bone marrow hyperplasia was also noted in this animal. Moderate lymphoid hyperplasia of the Bronchiole-Associated Lymphoid Tissue (BALT) was noted in two Group 5 females (both treated with 55 mg kg^{-1} of CPI-613), but not in any other animals.

Table 10: Findings from full gross necropsy in rats from Experiment A. Full panel necropsy included examination of the carcass and musculoskeletal system, all external surfaces and orifices, cranial cavity and external surface of the brain, thoracic, abdominal, and pelvic cavities with their associated organs and tissues

Group	Sex	Day Performed	Rats	Observations
1	M	Day 22	429	EYE: Discoloration, pale, left, TGL-small
		(12 were examined)	435	THYMUS: Discoloration, mottled, TGL-small
			439	SKIN-back: Crust, red, TGL-5×10 mm, above port site
			441	EYE-cornea: Crust, dark, left, TGL-small
		Day 33	455	SKIN-forefoot: Thick, left, TGL-small
		(12 were examined)		
	F	Day 22	426	SKIN-back: Crust, red TGL: 3×4 mm, at port site
		(12 were examined)	432	SKIN-back: Crust, dark TGL: 5×11 mm
			440	LIVER-medial lobe: Nodule, tan TGL-7×7 mm
		Day 33	468	SKIN-subcutaneous: Nodule, back, dark TGL-15×15 mm,
		(12 were examined)		over port site, consistent with a hematoma
2	M	Day 22	473	EPIDIDYMIS: Missing, left.
		(12 were examined)		TESTIS: Small, left, TGL-small
			491	SKIN-subcutaneous: Nodule, inguinal, mottled, TGL-
				12×12mm, left side, near the administration site
		Day 33	495	SKIN-back: Crust, brown, TGL-10×10 mm, over port site
		(12 were examined)	513	LYMPH NODE-mandibular: Discoloration, dark, right, TGL-small
			517	LIVER-medial lobe: Adhesion was to diaphragm, TGL-small
	F	Day 22	472	SKIN-back: Crust, brown TGL-small, on port site
		(12 were examined)	478	UTERUS: Enlarged, bilateral TGL
			484	SKIN-subcutaneous: Nodule, back, green TGL: 10x20mm, at port site
			486	LYMPH NODE-iliac: Enlarged, bilateral TGL-small
		Day 33	496	SKIN-subcutaneous: Nodule, inguinal, green TGL-15x10mm, near
		(12 were examined)		administration site, left
			502	KIDNEY-capsule: Focus, white, left, multiple TGL-3x1mm,
				LYMPH NODE-mandibular: Discoloration, mottled, left TGL-small
				SKIN-back: Crust, brown TGL-7×9 mm, over port site
			506	SKIN-back: Laceration TGL-3x6mm, suture material present on one
				side of lesion
			508	SKIN-subcutaneous: Nodule, inguinal, green TGL-5×7 mm, near
				administration site, left
			514	SKIN-inguinal: Crust, brown, left TGL-3×3 mm, located near staples
3	M	Day 22	519	SKIN-inguinal: Nodule, subcutaneous, tan, TGL-10×15 mm,
		(12 were examined)		under staples
			537	THYMUS: Focus, dark, multiple, TGL-1×2 mm
			539	ADMINISTRATION SITE: Nodule, tan, TGL-10×15 mm
				EPIDIDYMIS: Mass, tan, left, TGL-15×25 mm, adhered to abdominal wall
				LYMPH NODE-mediastinal: Enlarged, red, TGL-small
				TESTIS: Adhesion to scrotum, left TGL-small
			541	SKIN-back: Crust, red, TGL-5×12 mm, over port
	F	Day 22	520	THYMUS: Discoloration, mottled TGL
		(12 were examined)	526	SKIN-inguinal: Nodule, subcutaneous, tan TGL-12×15 mm, near
				administration site, left
			530	SKELETAL MUSCLE-back: Thick, right TGL-small, psoas major muscle
			532	SKIN-subcutaneous: Fluid, pink: fluid around port site
			536	SKIN-subcutaneous: Nodule, hindlimb, dark, left TGL-3×3 mm, near
				administration site
4	M	Day 22	547	LIVER-lateral lobe: Adhesion, to lumbar musculature, right, TGL-small
		(12 were examined)		SKIN-inguinal: Nodule, subcutaneous, green, TGL-13×14 mm, near
				administration site, left
			551	LYMPH NODE-iliac: Mass, left, TGL-20×20 mm
			553	SKIN-inguinal: Nodule, subcutaneous, tan, TGL-20×20 mm, left, near
				administration site
			557	SKIN-inguinal: Nodule, subcutaneous, tan, TGL-12×22 mm, left, near
				administration site
			559	SKELETAL MUSCLE-hindlimb: Nodule, left, TGL-10×15 mm
			561	LYMPH NODE-iliac: Discoloration, dark, left, TGL-small
				LYMPH NODE-renal: Discoloration, dark, left, TGL-small
			563	LYMPH NODE-iliac: Enlarged, left, TGL-small
				SKIN-inguinal: Nodule, subcutaneous, green, TGL-10×15 mm, left, near
				administration site
			565	LYMPH NODE-mediastinal: Enlarged, TGL-small
				SKIN-inguinal: Mass, subcutaneous, tan, TGL-30×15mm, left side, near
				administration site

Table 10: Continue

		Day 33 (12 were examined)	571	SKIN-subcutaneous: Nodule, inguinal, green, TGL-7×15 mm, near administration site left
			579	KIDNEY: Mass, left, TGL-35×53 mm, filled with green fluid SEMINAL VESICLE: Nodule, tan, left, TGL-3×5 mm SPLEEN: Enlarged, TGL-small
			581	ADMINISTRATION SITE: Nodule, green, TGL-10×10 mm
			585	SPLEEN- capsule: Focus, white, TGL-15x8mm
			587	PREPUTIAL GLAND: Discoloration, green, left, TGL-small SKIN-inguinal: Crust, brown, left,-TGL-3x3mm, located near staples SKIN-subcutaneous: Nodule, inguinal, tan, TGL-8x8mm, located under staples, left
			589	SKIN-back: Crust, dark, TGL-2×2 mm, at port
	F	Day 22 (12 were examined)	544a	Tissues were autolyzed LYMPH NODE-mandibular: Discoloration, red, bilateral TGL-small
			546	LYMPH NODE-mediastinal: Enlarged TGL-small OVARY: Cyst, clear, right TGL-small
			550	SKIN-subcutaneous: Nodule, inguinal, tan TGL-5×5 mm URINARY BLADDER-Nodule: white TGL-10×0 mm, on right outer surface
			556	LYMPH NODE-mediastinal: Enlarged, TGL-small
			566	LYMPH NODE 0 iliac: Enlarged, bilateral TGL-small
			1120	ADMINISTRATION SITE: Nodule, green TGL-10×10 mm EYE: Discoloration, dark, right TGL-small
		Day 33 (12 were examined)	568	SKIN-subcutaneous: Nodule, inguinal, tan TGL-10×10 mm, near administration site, left
			574	LIVER-all lobes: Discoloration, pale TGL
			578	SKIN-inguinal: Crust, brown, left, multiple TGL-5×1 mm, near staples
			586	LYMPH NODE-iliac: Enlarged, left TGL-small LYMPH NODE-mediastinal: Enlarged, TGL-small SKIN-subcutaneous: Nodule, inguinal, tan, left TGL-10×12 mm, located under staples
			1116a	Tissues were autolyzed ADMINISTRATION SITE-subcutaneous: Thick, clear TGL-small, gelatinous
5	M	Day 22 (12 were examined)	591	PREPUTIAL GLAND: Nodule, green, left, TGL-15×18mm
			593	ADMINISTRATION SITE: Mass, TGL-30×30 mm KIDNEY: Discoloration, pale, left, TGL-small SEMINAL VESICLE: Adhesion to mass, left, TGL-small SKIN-tail: Discoloration, dark TGL-small TESTIS: Discoloration, pale, left, TGL-small
			595	HEART-ventricle: Focus, depressed, right, multiple TGL: 1×1 mm, visible on outside, surface SKIN-subcutaneous: Nodule, inguinal, dark TGL: 8×11 mm, left, near administration site
			597	ADMINISTRATION SITE: Nodule, yellow TGL-8×5 mm ADRENAL GLAND: Enlarged, bilateral TGL-small INTESTINE-rectum: Adhesion TGL-small KIDNEY: Enlarged, mottled, bilateral TGL-small KIDNEY-pelvis: Dilation, bilateral TGL-small LUNG-all lobes: Discoloration, mottled TGL-small LYMPH NODE, mediastinal: Enlarged, mottled TGL-small THYMUS: TGL-small URETER: Dilation, bilateral TGL-small URINARY BLADDER: Enlarged TGL-40×30 mm CAVITY-pelvic: Nodule, tan TGL-12×10 mm
			599b	EPIDIDYMIS: Adhesion to mass, left TGL-small KIDNEY: Focus, yellow, multiple TGL-2×2 mm KIDNEY-pelvis: Dilation, left TGL-small LYMPH NODE-mediastinal: Enlarged, bilateral TGL-small SEMINAL VESICLE: Mass, bilateral TGL-30×25mm TESTIS: Adhesion to mass, red, left TGL-small THYMUS: Small TGL-small
			601b	CAVITY-abdominal: Fluid, clear HEART-ventricle: Focus, depressed, right, multiple TGL-2×3 mm, visible on outside, surface KIDNEY: Focus, yellow, bilateral multiple TGL-3×3 mm KIDNEY-pelvis: Dilation, bilateral TGL-small LYMPH NODE 0 iliac: Enlarged, red TGL-small

Table 10: Continue

		LYMPH NODE-mediastinal: Enlarged, bilateral TGL-small
		THYMUS: Small TGL-small
		URINARY BLADDER: Enlarged TGL-small
	603	CAVITY-abdominal: Nodule, green, left TGL-23×20 mm, perirenal
	605	SKIN-back: Nodule, subcutaneous, green TGL-20×20 mm, near port
	609	EPIDIDYMIS: Thick, left TGL-small
		LYMPH NODE-inguinal: Enlarged, bilateral TGL-small
		LYMPH NODE-mesenteric: Discoloration, dark TGL-small
		SKIN-inguinal: Nodule, subcutaneous, green TGL 22×30 mm, near administration site, left
		TESTIS: Discoloration, pale, left TGL-small
Day 33 (12 were examined)	619	SKIN-subcutaneous: Nodule, inguinal, tan TGL-10×8 mm, near administration site, left
	621	LYMPH NODE-iliac: Enlarged, left TGL-small
	631	LYMPH NODE-iliac: Enlarged, bilateral TGL-small
	633b	CAVITY-abdominal: Fluid, clear: gelatinous
		SKIN-inguinal: Nodule, subcutaneous, green TGL: 15×30 mm, near administration site
		SKIN-tail: Discoloration, dark TGL-small
	635	SPLEEN-capsule: Focus, white TGL-3×4 mm
	592	LYMPH NODE-iliac: Enlarged, right TGL-small
		SKELETAL MUSCLE-hindlimb: Thick, pale, left TGL-small
		SKIN, Abdominal-subcutaneous: Mass, mottled, right TGL-7×15 mm
		SPLEEN: Enlarged, TGL-small
	594	SKIN-subcutaneous: Nodule, hindlimb, green, right TGL-20×10 mm
		SPLEEN: Enlarged TGL-small
	596	ADMINISTRATION SITE: Nodule, tan TGL-8×8 mm, multiple
		LYMPH NODE-mediastinal: Enlarged TGL-small
	606	LYMPH NODE-iliac: Enlarged, bilateral TGL-small
		SKIN-subcutaneous: Nodule, inguinal, green TGL-10×12 mm, under staples
	610b	HEART-apex: Focus, dark TGL-2×2 mm, may be due to terminal blood collection via cardiac puncture
		URINARY BLADDER: Enlarged TGL-small
	612	ADMINISTRATION SITE: Nodule, green TGL-20×10 mm
	614	SKELETAL MUSCLE-abdominal: Mass, green, left TGL-15×18 mm
	1122	SKIN-tail: Discoloration, black TGL-small, tip of tail
Day 33 (12 were examined)	616	SKIN-back: Crust, brown TGL-3×1 mm
		SKIN-back: Laceration TGL-4×5 mm
	622	SKIN-back: Ulcer, TGL-3×4 mm
		SKIN-subcutaneous: Nodule, back, green TGL-20×20 mm, over port site, consistent with a chronic abscess
	634	ADMINISTRATION SITE-subcutaneous: Nodule, tan TGL-18×15mm
		SKIN-tail: Crust, dark TGL-20×5 mm

F: Female; M: Male; TGL: Trackable Gross Lesions; [a]: Rat 544 was found dead on day 1 and Rat 1116 on day 2. Gross pathology was performed on the same day when the rats were found dead; [b]: Moribund euthanasia and gross pathology were performed on day 8 in rats 599 and 601, day 20 in rat 633, and day 2 in rat 610

Table 11: Findings from full gross necropsy in minipigs from Experiment B. Full panel necropsy included examination of the carcass and musculoskeletal system, all external surfaces and orifices, cranial cavity and external surface of the brain, thoracic, abdominal and pelvic cavities with their associated organs and tissues

Group	Sex	Day conducted	Mini-pig#	Observations
1	M	Day 22	1687	EPIDIDYMIS: Right, TGL - small
		(4 total examined)		EPIDIDYMIS: Cyst; clear; right, TGL - 22×20 mm
				TESTIS: Right, TGL - small
	F	Day 22	1686	SKIN - hindlimb: Crust, brown, right, multiple, TGL - 10×10 mm
		(4 total examined)	1688	SKIN - back: Crust, dark, left, multiple, TGL - 5×5 mm
				SKIN - subcutaneous, neck: Nodule, brown, TGL - 20×20 mm
				SKIN - subcutaneous: Accumulation, red, ventral, TGL - small, hematoma per pathologist
				THYMUS: Discoloration, red, TGL - small
2	M	Day 22	1697	SKIN - Neck: Crust, tan, TGL - 5×5 mm
		(4 total examined)	1703	ADMINISTRATION SITE: Thick, right, TGL - small
	F	Day 22	1698	LYMPH NODE - bronchial: Discoloration, mottled, TGL - small
		(4 total examined)		SKIN - back: Crust, dark, left, TGL - 5×5 mm, at port site
				SKIN - neck: Crust, dark, bilateral, TGL - 5×20 mm
			1702	SKIN - forelimb: Crust, brown, left, TGL - 5×15mm
3	M	Day 22	1713	SKIN - back: Crust, brown, TGL - 10×4mm, at port site

Table 11: Continue

		(4 total examined)		SKIN - neck: Crust, brown, bilateral, TGL - 5×10 mm
				SKIN - forelimb: Laceration, brown, bilateral, TGL - 20×2 mm
			1715	LUNG - all lobes: Failed to collapse upon opening of thoracic cavity, discoloration, mottled, TGL - small
				LYMPH NODE, MEDIASTINAL - Discoloration, dark, TGL - small
				SKIN - back: Nodule, subcutaneous, green, TGL - 7×15 mm; at port site.
				SKIN - back: Ulcer, tan, left, multiple, TGL - 4×10 mm, at port site
				THYMUS; Discoloration; dark (TGL)
			1717	STOMACH - mucosa: Focus, dark, TGL - 15×15 mm
	F	Day 22	--	No visible lesions in all animals.
		(4 total examined)		
4	M	Day 22	1725	TESTIS: Cyst, clear, unilateral, TGL - 12×20 mm
		(4 total examined)		
	F	Day 22	1722	SKIN - forelimb: Crust, brown, left, TGL - 20×30 mm
		(4 total examined)	1728	LYMPH NODE - mediastinal: Discoloration, dark, TGL - small
5	M	Day 22	1739	EAR: Crust, brown, left, TGL - 4×7 mm
		(4 total examined)		LUNG - caudal lobe: Adhesion to thoracic cavity, right, TGL - small
				LUNG - left lobe: Mass, TGL - 30×40 mm
				LUNG - all lobes: Nodule, TGL - 3×8 mm
				SKIN; back; Crust; brown; left (TGL): 10×15 mm
				THYMUS; Small (TGL)
			1741	SKIN - neck: Ulcer, yellow, left, TGL - 10×10 mm, at port site
	F	Day 22	1738	EAR: Crust, brown, bilateral, TGL - 3×4 mm
		(4 total examined)	1744	THYMUS: Discoloration, brown, TGL - small

F: Female; M: Male; TGL: Trackable Gross Lesions

Table 12: Summary on the number of rats with histopathology findings from Experiment A. Severity levels are: A = minimal; B = mild; C = moderate; D = marked. Only tissue/organs with histopathologic findings are listed

		Number of Rats with Histopathology on Day 22 in Main Study									
		Males					Females				
Location or tissue type	Histopathology findings	Grp1 n = 12	Grp2 n = 12	Grp3 n = 12	Grp4 n = 12	Grp5 n = 10	Grp1 n = 12	Grp2 n = 12	Grp3 n = 12	Grp4 n = 12	Grp5 n = 12
Administration site	Hemorrhage	1A						1B			
	Thrombosis								1B		
	Inflam, chronic	1A	1A,2B,1C	2A,1B	1A,2B	2A,1B	2A,3B	1A,2B	2A,2B	2A,2B	1B
	Inflam, chronic, active	1B,1C	4C	2C,1D	1B,3C	1B,3D	1A,2B,1C	1B,1C	2B,1C	2C	2B,3C,1D
	Foreign material	9	8	5	8	3	1	9	3	4	6
	Inflam, granulomatous	2A,7B	1A,2B,1C	1A,2B,1C	1B,4C	1A,1B,1C	1A	7B	3B	1A,3B,1C	3A,1B
	Mineralization							1A			
	Pigmentation						1A				
Adrenal gland	Vacullation, cortex										
	Vacuolation, cortex, focal	1A	1A								
Bone marrow, sternum	Hyperplasia			2A,1B	4A,1B,1C	3A,4B	3A	2A	2A	3A	5A,1B,1C
	Granuloma										
	Foreign material										
	Fibrosis, focal										
Bone, femur	Inflam, chronic	1B	1B			2B	1A				
	Inflam, chronic, periosteum			1B							
	Inflam, chronic, synosium	1A,1B				1B					
	Inflam, chronic, active		1C							1C	1D
	Inflam, chronic, active, periosteum				1C						
	Foreign material		1								
	Hyperostosis					1B					1A,1C
	Osteomalacia			1A	1B						
	Fibrosis					1A					2B
	Accessary structure, physis, cartilage										
Bone, sternum	Degeneration										
	Degeneration, cartilage	1A	1A		1B	1A	3A	3A	3A	6A	2A
	Inflam, chronic, cartilage										
	Inflam, chronic									1B	
	Inflam, chronic, active									1B	
	Hyperostosis									1B	
Cervix	Cyst										
Cavity, abdominal	Inflam, chronic, active				1D						
Epididymis	Granuloma, spermatic, unilateral					1D	--	--	--	--	--
	Granuloma, spermatogenic, unilateral					1D	--	--	--	--	--
	Inflam, chronic, active			1D			--	--	--	--	--
	Infiltration, lymphocytic	1A					--	--	--	--	--
Eye	Ulceration, cornea									1D	

Table 12: Continue

	Inflam, chronic, cornea				1A						
	Inflam, chronic, muscle										
	Inflam, chronic, muscle, unilateral										
	Mineralization, cornea										
	Inflam, chronic, active									1D	
	Inflam, chronic, active, cornea	1C									
	Phthisis Bulbi, unilateral	1D									
Harderian gland	Inflam, chronic, bilateral							1C			
	Inflam, chronic, unilateral		1B		1A	1A				1A,2B	1A
	Inflam, chronic, active									1	
	Infiltration, lymphocytic								1A		
Heart	Hemorrhage, myocardium										
	Cardiomyopathy					1A.1C	1A			1A	
	Fibrosis, focal										
	Inflam, chronic, active, valve										1D
	Bacteremia										1
Intestine, cecum	Inflam, chronic		1A		1A						
Intestine, duodenum	Inflam, chronic										
	Inflam, chronic, serosa			1B	1A						
Intestine, ileum	Inflam, chronic		1A		1A		3A	2A	2A	1A	2A
Intestine, jejunum	Inflam, chronic	1A					1A	1A		2A	1A
Intestine, rectum	Inflam, chronic, serosa					1A					
	Inflam, chronic, active					1C					
Kidney	Cyst										
	Chronic, progressive, nephropathy	1A	9A	8A,1B	1A,1B	6A	7A	5A	4A	8A	9A
	Degeneration, tubule	1A				1A			1A		
	Hyperplasia, transitional cell, epithelium					1B					
	Mineralization	1A				1A					
	Mineralization, pelvic			2A							
	Inflam, chronic, capsule				1B	1B			1A		
	Inflam, chronic, active					1C,1D					
	Inflam, chronic, active, pelvis					1A					
	Dilation, pelvis				1B	1A				1A	
	Inflitration, lymphocytic					1A					
Liver	Hematopoiesis, increased				1B	1A		1A		1A	3A.1B
	Hypertropy, Kupffer cell										1B
	Infiltration, histiocytic					1A					
	Infiltration, lymphohistiocytic	1A	1A	5A	2A	2A	5A	4A	3A	4A	3A
	Necrosis				1A						
	Necrosis, single cell									1A	
	Inflam, chronic, capsule		1A		1B						
	Thrombosis, vein										1A
	Hepatodiaphragmatic nodule						1				
	Vacuolation, hepatocyte, cytoplasm										
Lung	Foreign material	2	1			1			1	3	
	Hemorrhage					1A	1A			1A,1B	
	Hyperplasia, alveolar epithelium, focal		1C								
	Granuloma	1A	1A	2A	1A				1A	2A	
	Granuloma, vein									1B	
	Granuloma, vein, multiple					1B					
	Inflam, subacute								1A		
	Inflam, Subacute, periarterial		1A								
	Inflam, granulomatous									1A	1A
	Inflam, granulomatous, focal									1A	
	Inflam, granulomatous, periarterial	1A									
	Inflam, chronic	3A,1B		1A			1A.2B	2A,1B	1B	2A	1A
	Inflam, chronic, interstitium			1A,1C		1B					
	Inflam, chronic, arterial							1A			
	Inflam, chronic, active				1A						
	Inflam, chronic, active, artery	1A.1B									
	Histiocytosis, focal	1A									
	Metaplasia, osseous					1A					
	Hypertrophy, artery						1A.1B	1A,1B			
	Pigmentation						1A				
Lymph node, iliac	Hemorrhage				1B						
	Hyperplasia, lymphoid							1C			2C
	Hyperplasia, lymphoid, mucosal										
Lymph node, inguinal	Inflam, chronic, active				1D						
	Hyperplasia, lymphoid, bilateral					1C					
Lymph node, mediastinal	hyperplasia, lymphoid					1A				1A,1B	1A
	Hemorrhage					1A					
	Edema									1B	
Lymph node, mesenteric	Atrophy					1B					
	Hyperplasia, lymphoid		1A	1A		1A	1A				

Table 12: Continue

Lymph node, renal	Hemorrhage				1B						
Lymph node, mandibular	Hemorrhage			1B							
	Hypoplasia, lymphoid										1B
	Hyperplasia, lymphoid		1A	5A	5A	1A	2A			1B	
	Inflam, chronic										
Mesentery	Inflam, chronic, active					1C					
Skeletal muscle	Degeneration				1A						
	Necrosis										1D
	Inflam, chronic										1A
	Inflam, chronic, active				1D				1D		2D
Skeletal muscle, psoas	Inflam, chronic, active								1D		
Nerve, Optic	Necrosis									1B	
Nerve, Sciatic	Degeneration								1C		
Ovary	Hyperplasia, sertoli cell, unilateral	--	--	--	--	--		1A			
Pancreas	Atrophy, acinar cell, focal			1A,1B	1A						1B
	Inflam, chronic, active										
	Inflam, chronic, interstitium					1A					
	Inflam, acute										
	Edema					1A					
	Inflam, subacute							1A			
Pituitary gland	Cyst, pars distalis										
Prostate gland	Inflam, chronic	1A,1C	1A	1A	2A	3A,1C	--	--	--	--	--
	Inflam, chronic, active			1B		2C,1D	--	--	--	--	--
	Infiltration, lymphocytic		2A				--	--	--	--	--
	Inflam, subacute				1A		--	--	--	--	--
Preputial gland	Abscess					1D	--	--	--	--	--
Seminal vesicle	Atrophy					1B	--	--	--	--	--
	Atrophy, unilateral					1A	--	--	--	--	--
	Inflam, chronic, active					1A,1B	--	--	--	--	--
	Inflam, chronic, active, unilateral					1D	--	--	--	--	--
	Inflam, chronic, active, bilateral						--	--	--	--	--
Skin	Erosion										
	Hemorrhage		1C								
	Hyperplasia, epidermis	1B		1B			1B	1B			
	Necrosis										1D
	Ulceration	1B		1B				1B			
	Inflam, chronic				1D				1A,1C		
	Inflam, chronic, active	1B	1B	1B,1D	2D	2D	2B	1A		1C	1C,1D
	Inflam, chronic, active, subcutaneous				1C,1D			1C			
	Inflam, chronic, osseous										
	Congestion					1C					
	Thrombosis, vein										
Skin, abdominal	Inflam, chronic, subcutaneous	1A									1B
	Edema										1B
Spleen	Hematopoiesis, increased		1A	4A	2A,3B	4A,3B	1A	1A	1A	3A	6A,1B,1C
	Hyperplasia, follicle					1B	1A				1A
	Inflam, chronic, capsule										
	Angectasis										
Stomach, glandular	Dilation, glands		1A								
	Ectopia				1A						
	Edema								1A		
Testis	Atrophy, germinal epithelium		1A				--	--	--	--	--
	Hypoplasia, unilateral		1D				--	--	--	--	--
	Infarction, unilateral			1D		2D	--	--	--	--	--
	Inflam, chronic, active, unilateral			1D		1D	--	--	--	--	--
	Inflam, acute, capsule, unilateral					1C	--	--	--	--	--
Thymus	Atrophy, cortex										1A
						1D					
	Hemorrhage	8A	5A	4A	5A	4A	5A	3A	6A,1B	4A	3A
Thyroid gland	Hyperplasia, epithelial, focal						1A				
	Infiltration, lymphohistiocytic										
	Infiltration, lymphocytic										
	Extopia										
Treachea	Inflam, chronic				1A		1A				
Ureter	Dilation						1C				
	Inflam, chronic					1B					
Urinary bladder	Hyperplasia, epithelium, transitional cell					1B					
	Hyperplasia, transitional cell, epithelium	1B				1B					
	Inflam, chronic	1A				1A					
	Inflam, chronic, active					1A					
	Inflam, chronic, active, serosa									1C	
Uterus	Hemorrhage	--	--	--	--	--				1A	
	Hyperplasia, stromal	--	--	--	--	--			1A		
	Deciduoma, single	--	--	--	--	--		1C			
	Inflam, chronic, active, endometrium	--	--	--	--	--		1A			
Vagina	Inflam, chronic	--	--	--	--	--	1A				
		--	--	--	--	--				1B	
	Inflam, acute										
Cavity, Pelvic	Inflam, chronic, active					1C					

Table 12: Continue

Location or Tissue Type	Histopathology findings	Number of Rats with Histopathology on Day 33 in Recovery Study							
		Males				Females			
		Grp1 n=12	Grp2 n=12	Grp4 n=12	Grp5 n=11	Grp1 n=12	Grp2 n=12	Grp4 n=12	Grp5 n=12
Administration site	Hemorrhage								
	Thrombosis								
	Inflam, chronic	2A,1B	6A,1B	3A,5B	1C	5A,1B	3A	3A,4B	1B
	Inflam, chronic, active			1B,1D		2B,1C	3C,2D	1C	1B,2C,1D
	Foreign material	9	1		1	3	12	2	9
	Inflam, granulomatous	3A,6B	1A		3A,6B,1C	1A	1A,3B	2B	1A,5B,1C
	Mineralization							1A	
	Pigmentation								
Adrenal gland	Vacullation, cortex	1A		1A					
	Vacuolation, cortex, focal						1A		
Bone marrow, sternum	Hyperplasia			4A,1B		2A	5A	1A	3A,2B
	Granuloma								1B
	Foreign material								1
	Fibrosis, focal						1A		
Bone, femur	Inflam, chronic	1A		1B	1A,1B	1A	2B	1A	1B
	Inflam, chronic, periosteum								
	Inflam, chronic, synosium								
	Inflam, chronic, active							2B	
	Inflam, chronic, active, periosteum								
	Foreign material						1		
	Hyperostosis				1A		1B		
	Osteomalacia								
	Fibrosis								
	Accessary structure, physis, cartilage			1					
Bone, sternum	Degeneration		1B			1A			
	Degeneration, cartilage	2A,1B	2A	3A	4A	9A	8A,1B	8A	1A,2B
	Inflam, chronic, cartilage						1B		
	Inflam, chronic								
	Inflam, chronic, active								
	Hyperostosis								
Cervix	Cyst	--	--	--	--		1		
Cavity, abdominal	Inflam, chronic, active								
Epididymis	Granuloma, spermatic, unilateral								
	Granuloma, spermatogenic, unilateral								
	Inflam, chronic, active								
	Infiltration, lymphocytic								
Eye	Ulceration, cornea								
	Inflam, chronic, cornea						1A		1B
	Inflam, chronic, muscle					1B			
	Inflam, chronic, muscle, unilateral				1A				
	Mineralization, cornea					1B	1A		1A
	Inflam, chronic, active								
	Inflam, chronic, active, cornea								
	Phthisis Bulbi, unilateral								
Harderian gland	Inflam, chronic, bilateral								
	Inflam, chronic, unilateral	1B				1B	1A	2A	
	Inflam, chronic, active								
	Infiltration, lymphocytic								
Heart	Hemorrhage, myocardium							1B	
	Cardiomyopathy		2A			1A			
	Fibrosis, focal								1A
	Inflam, chronic, active, valve								
	Bacteremia								
Intestine, cecum	Inflam, chronic								
Intestine, duodenum	Inflam, chronic	1A						1A	
	Inflam, chronic, serosa								
Intestine, ileum	Inflam, chronic		3A	1A	2A	4A	3A		1A
Intestine, jejunum	Inflam, chronic	1A		1A	3A	1A	1A		1A
Intestine, rectum	Inflam, chronic, serosa								
	Inflam, chronic, active								
Kidney	Cyst	1	1			1	1	1	1
	Chronic, progressive, nephropathy	11A	11A	8A,1B	1A	5A	6A,1B	8A	6A,1B
	Degeneration, tubule					1A			
	Hyperplasia, transitional cell, epithelium								
	Mineralization				1A	2A		1A	
	Mineralization, pelvic								
	Inflam, chronic, capsule								1B

Table 12: Continue

	Inflam, chronic, active			1D					
	Inflam, chronic, active, pelvis								
	Dilation, pelvis			1A					
	Inflitration, lymphocytic								
Liver	Hematopoiesis, increased			1A					
	Hypertropy, Kupffer cell								
	Infiltration, histiocytic								
	Infiltration, lymphohistiocytic	1A	3A	1A		2A	4A	2A	1A
	Necrosis								
	Necrosis, single cell								
	Inflam, chronic, capsule								
	Thrombosis, vein								
	Hepatodiaphragmatic nodule		1						
	Vacuolation, hepatocyte, cytoplasm						1A		
Lung	Foreign material		2			2			
	Hemorrhage								
	Hyperplasia, alveolar epithelium, focal								
	Granuloma		1A			2A			
	Granuloma, vein								
	Granuloma, vein, multiple								
	Inflam, subacute								
	Inflam, Subacute, periarterial								
	Inflam, granulomatous								
	Inflam, granulomatous, focal								
	Inflam, granulomatous, periarterial								
	Inflam, chronic			2A		1A			
	Inflam, chronic, interstitium								
	Inflam, chronic, arterial	1A	1A						
	Inflam, chronic, active								
	Inflam, chronic, active, artery								
	Histiocytosis, focal	1A						1A	
	Metaplasia, osseous		1A						
	Hypertrophy, artery								
	Pigmentation								
Lymph node, iliac	Hemorrhage								
	Hyperplasia, lymphoid				1B			1C	
	Hyperplasia, lymphoid, mucosal				1B				
Lymph node, inguinal	Inflam, chronic, active								
	Hyperplasia, lymphoid, bilateral								
Lymph node, mediastinal	hyperplasia, lymphoid							1A	
	Hemorrhage							1B	
	Edema								
Lymph node, mesenteric	Atrophy								
	Hyperplasia, lymphoid		1A						
Lymph node, renal	Hemorrhage								
Lymph node, mandibular	Hemorrhage			1A			1A		
	Hypoplasia, lymphoid								
	Hyperplasia, lymphoid	3A	4A	2A,1B	1B	2A	1A		2A
	Inflam, chronic			1A					
Mesentery	Inflam, chronic, active								
Skeletal muscle	Degeneration								
	Necrosis								
	Inflam, chronic								
	Inflam, chronic, active								
Skeletal muscle, psoas	Inflam, chronic, active								
Nerve, Optic	Necrosis								
Nerve, Sciatic	Degeneration	1A				1B			
Ovary	Hyperplasia, sertoli cell, unilateral								
Pancreas	Atrophy, acinar cell, focal							1A	
	Inflam, chronic, active			1B					
	Inflam, chronic, interstitium								
	Inflam, acute					1C			
	Edema								
	Inflam, subacute								
Pituitary gland	Cyst, pars distalis	2			1				
Prostate gland	Inflam, chronic	1A		2A	1A,1C	--	--	--	--
	Inflam, chronic, active			1B,1C		--	--	--	--
	Infiltration, lymphocytic					--	--	--	--
	Inflam, subacute					--	--	--	--
Preputial gland	Abscess			1D		--	--	--	--
Seminal vesicle	Atrophy					--	--	--	--
	Atrophy, unilateral					--	--	--	--
	Inflam, chronic, active			1D		--	--	--	--
	Inflam, chronic, active, unilateral					--	--	--	--
	Inflam, chronic, active, bilateral				1D	--	--	--	--

Table 12: Continue

Skin	Erosion		1B	1B			1A	1B	
	Hemorrhage					1C			
	Hyperplasia, epidermis		1B	1B			2B,1C	1B	1B
	Necrosis								
	Ulceration						1D		1C,1D
	Inflam, chronic						1B		1B
	Inflam, chronic, active		1A	1B,1C,1D	1C	1C	1B,1C,2D	2D	1C,1D
	Inflam, chronic, active, subcutaneous								
	Inflam, chronic, osseous	1D							
	Congestion								
	Thrombosis, vein								1C
Skin, abdominal	Inflam, chronic, subcutaneous								
	Edema								
Spleen	Hematopoiesis, increased		1A	1B				1A	1A
	Hyperplasia, follicle								
	Inflam, chronic, capsule			1B					
	Angectasis				1B				
Stomach, glandular	Dilation, glands								
	Ectopia								
	Edema								
Testis	Atrophy, germinal epithelium					--	--	--	--
	Hypoplasia, unilateral					--	--	--	--
	Infarction, unilateral					--	--	--	--
	Inflam, chronic, active, unilateral					--	--	--	--
	Inflam, acute, capsule, unilateral					--	--	--	--
Thymus	Atrophy, cortex								
	Hemorrhage	9A	7A	5A	9A	6A	5A	4A	9A
Thyroid gland	Hyperplasia, epithelial, focal								
	Infiltration, lymphohistiocytic							1A	
	Infiltration, lymphocytic		1A		1A				
	Extopia		1A				1A	1A	
Treachea	Inflam, chronic			1A	1A	1A			
Ureter	Dilation								
	Inflam, chronic								
Urinary bladder	Hyperplasia, epithelium, transitional cell								
	Hyperplasia, transitional cell, epithelium								
	Inflam, chronic								
	Inflam, chronic, active								
	Inflam, chronic, active, serosa								
Uterus	Hemorrhage	--	--	--	--				
	Hyperplasia, stromal	--	--	--	--				
	Deciduoma, single	--	--	--	--				
	Inflam, chronic, active, endometrium	--	--	--	--				
Vagina	Inflam, chronic	--	--	--	--				
		--	--	--	--				
	Inflam, acute	--	--	--	--				1B
Cavity, Pelvic	Inflam, chronic, active								

Table 13: Summary on the number of minipigs with histopathology findings from Experiment B. Severity levels are: A = minimal; B = mild; C = moderate; D = marked. Only tissue/organs with histopathologic findings are listed. Histology was performed on day 22

		Males					Females				
Location or Tissue Type		Grp1	Grp2	Grp3	Grp4	Grp5	Grp1	Grp2	Grp3	Grp4	Grp5
	Histopathology findings	n=4	n=4	n=4	n=4	n=4	n=4	n=4	n=4	n=4	n=4
Administration site	Hemorrage						1C		1B		
	Infiltration, lymphocytic	1B	1C							1C	
	Inflammation, chronic, active		1D	2D						1B	1C
	Thrombosis	1A,1B	2C	1B		1B,1C	1B,2C	2B	1A,2B	2C	
	Thrombosis, bilateral	1B,1C	1C,1D	1C,1D	1A,1C,1D	2B	1C	2C	1C	2C	3C
Bone marrow, sternum	Hyperplasia					1D					
Brain, brain stem	Hemorrhage, multifocal	1B					2A			1A	
Brain, cerebellum	Hemorrhage, multifocal								2A		
Brain, cerebrum	Hemorrhage, multifocal	1A		1A	1A	1A	1A.1B	1B	1A	1A	
Epididymis	Atrophy, right	1C					--	--	--	--	--
	Dilation, duct, right	1C					--	--	--	--	--
	Spermatid giant cells, duct, bilateral					1A	--	--	--	--	--
			1B				--	--	--	--	--
	Spermatid giant cells, duct, unilateral		1B				--	--	--	--	--
Intestine, jejunum	Hemorrhage, mucosa			1B							
Kidney	Hyperplasia, transitional cell, bilateral					1C					
	Inflitration, lymphocytic	2A	1A	1A	1A	2A	1A	1A	1A	3A	1A
	Vacuolation, cytoplasmic,					1B					

Table 13: Continue

	transitional cell, bilateral										
Liver	Infiltration, lymphohistiocytic								1A		
Lung	Foreign material, focal				1A				1A		
	Hemorrhage, bronchus		1B								
	Hyperplasia, lymphocytic										2C
	Infiltration, lymphohistiocytic	1A	2A	2A,B	3A	2A	2A,1B	3A	3A,1B	2A	
	Inflammation, acute			2C							
	Inflammation, chronic, acute					1D					
	Inflammation, granulamatous		1A		1A				2A		
	Abscess					1D					
Lymph node, mediastinal	Erythrophagocytosis									1D	
	Hemorrhage			1B							
	Mistiocytosis			1B							
	Inflammation, acute				1B						
Lymph node, mesenteric	Histiocytosis			1B		1B					1B
Lymph node, bronchial	Hemorrhage							1B			
Lymph node, mandibular	Hemorrhage									1B	
	Histiocytosis	2B,2C		1B	1B		2B				
	Infiltration, neutrophilic								1B		
Skeletal muscle	Infiltration, histiocytic								1A		
Pancreas	Hemorrhage			1B							
Pituitary gland	Cyst, pars nervosa						1B		1A		
Salivary gland, mandibular	Infiltration, luymphocytic	2A	1A	1A	1A	1A	2A	2A			
Skin, abdominal	Inflammation, chronic								1A		
Skin, treated sites	Inflammation, chronic, focal			1B							
	Hyperkeratosis, focal			1B							
	Abscess					1D					
	Inflammation, ulcerative			1C		1D					
Skin, untreated	Inflammation, chronic		1C	1B				1B,1C			
	Inflammation, ulcerative, focal			1C		1D	1D	1D		1B	1B
	Hyperkeratosis		1B	1C		1B	2B	1B,1C		1C	1B
	Hyperkeratosis, focal						1C				
	Hemorrhage, subcutaneous						1C				
Spinal cord, cervical	Hemorrhage, multifocal								1A		
Spinal cord, thoracic	Hemorrhage, multifocal	1A	1A		1A		2A	1A		1A	
Spinal cord, lumbar	Hemorrhage, multifocal	1A									
Stomach	Hemorrhage, mucosa			1B							
Testis	Atrophy, right	1C					--	--	--	--	--
	Degeneration, seminiferous tubule, germinal epithelium				1B		--	--	--	--	--
	Spermatid giant cells, seminiferous tubule, bilateral		1B			1B	--	--	--	--	--
	Spermatid giant cells, seminiferous tubule, unilateral		1B		1B	1B	--	--	--	--	--
Thymus	Depletion										1D
	Edema						1C				
	Hemorrhage			1C			1C				
	Infiltration, neutrophilic		1B								
	Involution					1B					
	Fibrosis										1D
Thyroid gland	Hemorrhage						1C				
Tongue	Hemorrhage					1B					
Treachea	Infiltration, lymphocytic		1B								

D5W = 5% Dextrose in Water; Grp1 = Group 1 minipigs treated with D5W; Grp2 = Group 2 minipigs treated with vehicle of CPI-613; Grp3 = Group 3 minipigs treated with CPI-613 at 45 mg kg^{-1}; Grp4 = Group 4 minipigs treated with CPI-613 at 50 mg kg^{-1}; Grp5 = Group 5 minipigs treated with CPI-613 at 55 mg kg^{-1}; n = number of minipigs with histology performed on day 22

Since BALT is prominent in this species, moderate hyperplasia is not considered biologically significant. Minimal or mild multifocal hemorrhage in the brain and/or spinal cord was noted in animals from most groups (control and treatment groups included) and was most likely associated with removal of the tissue at necropsy.

Total (free plus bound) plasma concentrations of CPI-613 and toxicokinetics: The plasma CPI-613 concentrations after administrations of different doses of CPI-613 were assessed in rats in Experiment A. These concentration-time curves of CPI-613 (Fig. 4) revealed an apparent 2-compartment model with biphasic appearance, characterized by an initial distribution phase followed by a terminal elimination phase with a terminal half-life ($T_{1/2}$) of approximately 2-5 hours. The onset of the terminal phase was between 1-8 hours post dose. There were no apparent differences in these results between genders. Interestingly, there were no statistical differences among different dose groups, possibly because of the narrow dose range used in this study.

Table 14: Pharmacokinetics (PKs) in rats from Experiment A. PK results presented in this table are combined results from males and females, due to a lack of apparent difference in these values between the two sexes

		Day 1 1st Dose			Day 4 2nd Dose			Day 11 4th Dose			Day 18 6th Dose		
	(mg kg^{-1})	25	30	35	25	30	35	25	30	35	25	30	35
Dose	(mg m^{-2})	150	180	210	150	180	210	150	180	210	150	180	210
C_{max} (ng/mL ×10^3)		35.30	47.90	56.50	16.00	31.00	42.60	29.60	41.90	77.50	14.60	27.50	41.40
C_{max}/Dose (ng.kg/mL.mg)		1411.00	1597.00	1614.00	640.00	1033.00	1218.00	1182.00	1395.00	2214.00	586.00	916.00	1183.00
AUC_{0-24hr} (ng.h/mL x10^3)		18.20	25.80	26.20	13.70	19.20	23.90	16.90	24.30	32.30	12.50	19.40	23.50
$AUC_{0-\infty}$ (ng.h/mL ×10^3)		18.20	26.00	26.30	13.80	19.30	17.20	17.10	24.50	37.80	12.60	19.50	20.60
$AUC_{24-\infty}/AUC_{0-\infty}$ (%)		0.23	0.70	0.22	0.55	0.46	0.49	1.17	0.48	0.48	0.74	0.40	1.10
AUC_{0-24hr}/Dose (ng.kg/mL.mg)		727.00	862.00	750.00	549.00	641.00	682.00	676.00	812.00	922.00	501.00	648.00	671.00
K_{el} (/h)		0.18	0.15	0.19	0.17	0.17	0.17	0.12	0.22	0.18	0.16	0.20	0.11
Terminal $T_{1/2}$ (h)		4.07	4.78	3.74	4.16	4.05	4.06	6.71	3.18	3.84	4.93	3.66	6.47
Vd (mL/kg ×10^3)		8.30	8.00	7.20	11.00	9.70	11.90	15.50	5.60	5.10	14.20	8.20	15.90
Cl (mL h^{-1} kg^{-1})		1502.00	1154.00	1335.00	1825.00	1648.00	2031.00	1513.00	1227.00	926.00	1984.00	1545.00	1703.00

$AUC_{0-24\ h}$ = Area Under the Curve (AUC) from dosing time to the final observation at 24 h; $AUC_{0-\infty}$ = area under the curve from the dosing time extrapolated to infinity; Cl = total body clearance; C_{max} = maximum observed concentration extrapolated to time 0 min; K_{el} = terminal elimination phase rate constant; Terminal $T_{1/2}$ = plasma half-life derived from the second or terminal phase of elimination; Vd = apparent volume of distribution

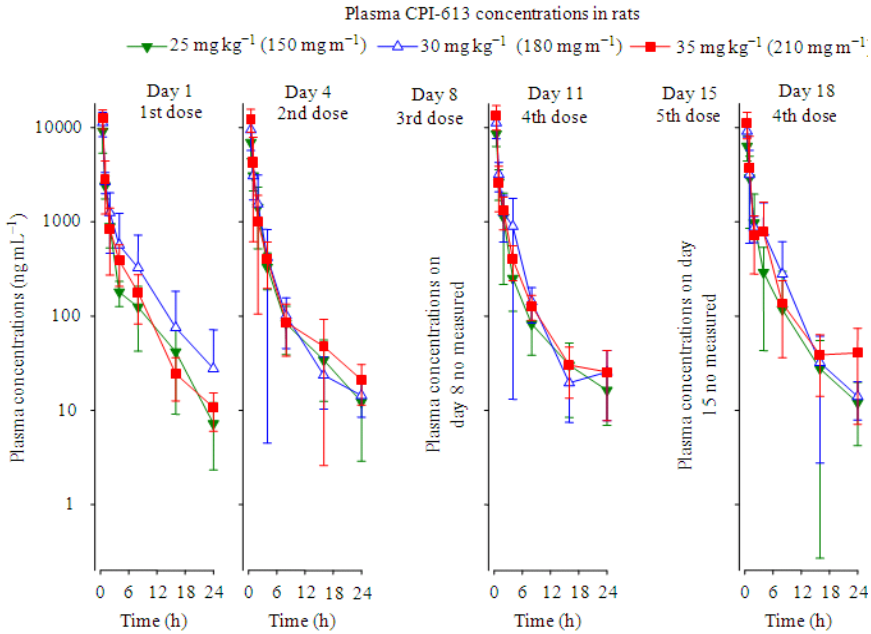

Fig. 4: Plasma concentrations of CPI-613 in rats treated with 25, 30 or 35 mg kg^{-1} of CPI-613, given 2x weekly for three consecutive weeks from Experiment A. There were 4 rats per sex at each time point of each dose level. Results are presented as mean ± standard of deviation

The PK results, derived from group mean values of plasma CPI-613 concentrations, are shown in Table 14. Significant amounts of CPI-613 were detected 24 h after each of the 6 administrations of CPI-613. Despite detectable concentrations of CPI-613 at 24 h post dose, there was no evidence of accumulation of CPI-613 associated with the "twice weekly for 3 weeks" dosing regimen. The lack of drug accumulation and a terminal $T_{1/2}$ of 2-5 h indicated that a single administration is representative of the steady state kinetics at the dose levels tested with this dosing regimen.

The apparent volume of distribution (Vd) and total body clearance (Cl) of CPI-613 were estimated beyond physiological meaning, with considerable variability in the volume parameter. These results suggested that the distribution was thorough and clearance was rapid.

The values of the maximum observed concentration extrapolated to time 0 (Cmax) and AUC from dosing time to the final observation at 24 h (AUC_{0-24hr}) were proportional to dose levels (Fig. 5).

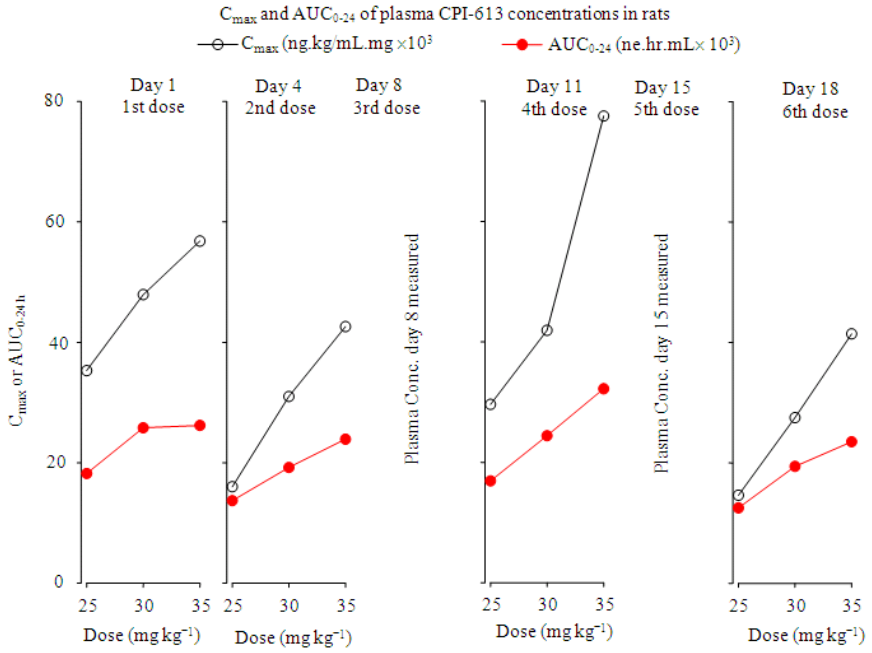

Fig. 5: Area under the curve from dosing time to the final observation at 24 h ($AUC_{0\text{-}24\ h}$) and maximum observed concentration extrapolated to time at 0 h (C_{max}) in rats treated with intravenous CPI-613 at 25, 30 or 35 mg/kg, given 2x weekly for three consecutive weeks from Experiment A. These pharmacokinetic parameters were derived from the mean values of 4 rats per sex at each time point at each dose level

DISCUSSION

Study objectives and rationale: The objective of these studies was to systematically investigate the dose-related TK effects of CPI-613 in rats. The objective of these studies was also to investigate the toxicological profile of CPI-613 in minipigs, to determine if there were differences in the toxicological profile and sensitivity of CPI-613 between these two animal species. CPI-613 was given IV twice weekly for three consecutive weeks in both animal species. The "twice weekly for three weeks" dosing schedule was the intended clinical dosing schedule and results from these studies may provide insight to possible adverse events in study subjects of clinical trials. The investigation of these doses helped to determine the safety of CPI-613 and to reveal the potential toxicity associated with CPI-613. Also, these toxic doses of CPI-613 were ~60x the anti-tumor dose levels observed in mouse tumor xenograft models, suggesting a wide safety margin of CPI-613.

The results from these studies revealed the uncommon toxicological profile at toxic doses of CPI-613, which were related to induction of inflammation as the primary and possibly the only toxicological effects, in both rats and minipigs. The severity of the inflammatory effect was consistent with the dose-related increases in plasma CPI-613. Other changes such as elevations in reticulocytes and GGT) were also observed, but they might be secondary to the inflammatory effects of CPI-613.

Assessment of dose-related toxicity, recovery and PK of CPI-613 in rats: In the rat studies, the criteria for choosing the three doses, which were based on results from previous preliminary studies, were that the lowest dose would induce minimum effects (i.e., the threshold dose), the mid-dose would induce significant toxic effects and the highest dose would approximate the LD_{10}. Based on these criteria for selecting the doses in these studies, the dose range turned out to be relatively narrow and was 25-35 mg kg^{-1} for rats. These studies were also for evaluation of reversibility of the toxic effects of CPI-613, by comparing the intensity of toxic effects of CPI-613 immediately after treatment with CPI-613 vs. those two weeks after the last dose of CPI-613. The results showed that all toxic effects of CPI-613 were reversible.

The major toxic effect of CPI-613 in rats was inflammation. Inflammation occurred beyond the injection site and the severity of inflammation-related symptoms was mostly related to the dose levels. As a

matter of fact, the cause of death or moribund condition after treatment with CPI-613 revealed significant systemic inflammation. Inflammation being the primary toxicity at toxicological/lethal doses of CPI-613 was supported by necropsy and histological assessments. Systemic inflammation, which was nicely reviewed by (Nystrom, 1998), is similar to sepsis, except that it was not of microbial etiology. Regulation of the immune system by chemotherapeutic agents has previously been reported (Barret and Blanc, 2009).

The exact mechanism for the inflammatory effects of CPI-613 is unknown. A possible explanation may be related to non-specific chemical effects of CPI-613, since this compound has some detergent-like structural properties. High local concentrations of detergents (which occur at the site of injection) can induce local inflammation-like effects (Krob *et al.*, 2004; Shaw *et al*., 2004). Another possible explanation is that, although selectively effective against tumor cells, high concentration (as occurs at the site of injection) can induce adverse effects or death in normal cells, leading to inflammation. Regardless of the mechanism of action for inflammatory effects of CPI-613, the severity and frequency can be attenuated or even eliminated by lowering the concentrations of injectate, slowing the rate of infusion of CPI-613, or both. This is consistent with our experience to date in animal safety studies as well as ongoing clinical trials (Retter *et al*., 2010). An approach to eliminate local inflammation at the site of injection of a peripheral vein is to infuse CPI-613 via a central venous catheter. This approach was implemented once we learned that CPI-613 still induces local reaction even with dilution of the injectate and slow rate of infusion of CPI-613, which is currently the route of administration in ongoing clinical trials of CPI-613.

In rats, increases in reticulocytes and GGT were observed after treatment with high doses of CPI-613. The rise in reticulocytes may be associated with hematopoietic cell proliferation of the spleen and sternal bone marrow hyperplasia, which are considered secondary to inflammation initiated at the administration site or port rather than primary effects of CPI-613. The indirect nature of CPI-613 effects on the rise of reticulocytes is further supported by a lack of effects on other blood cell counts. For the rise in GGT in high doses of CPI-613, although it could reflect injury of the kidney and liver, it was not associated with histopathology findings or other parameters of kidney or liver functions. Therefore, the elevation was unlikely related to toxicity of the kidney or liver. Rather, it may be related to systemic inflammation, as reported by (Yamada *et al*., 2006).

PK was assessed concurrently with toxicological evaluation. The TK profile of CPI-613 was characterized by an apparent 2-compartment model with biphasic appearance. Systemic exposure to CPI-613 was proportional to dose level in rats and there was no evidence of test article accumulation or sex differences in CPI-613 exposure.

Assessment of dose-related toxicity of CPI-613 in minipigs: The toxicological effects at toxic doses of CPI-613 were also assessed in minipigs. The nature of the toxicological effects (such as inflammation at the site of injections and symptoms of systemic inflammation) were similar to rats. However, minipigs were less sensitive to the toxicological effects of CPI-613 than rats, as reflected by the toxic dose of CPI-613 in minipigs (~55 mg kg^{-1} or ~1925 mg^{-1} m^2) being significantly higher than those of rats (30-35 mg kg^{-1} or 180-210 mg^{-1} m^2).

CONCLUSION

These studies demonstrated the uncommon toxicological profile at toxic doses of CPI-613, which were related to induction of inflammation as the primary and possibly the only toxicological effects, in both rats and minipigs. The severity of the inflammatory effects correlated with the dose-related increases in plasma CPI-613. Other changes such as elevations in reticulocytes and Gamma-Glutamyl Transferase (GGT) were also observed, but they might be secondary to the inflammatory effects of CPI-613. This uncommon toxicological profile of CPI-613 reflects its novel mechanism of action.

The TK profile of CPI-613 was characterized by an apparent 2-compartment model with biphasic appearance. Systemic exposure to CPI-613 was proportional to dose level, with no evidence of test article accumulation or sex differences in CPI-613 exposure.

Additionally, the toxic doses of CPI-613 observed in these study were ~60x the anti-tumor dose levels observed in mouse tumor xenograft models, suggesting a wide safety margin of CPI-613. Therefore, the dose of CPI-613 to be used in the clinic is expected to be significantly below the sub-lethal dose and the risk of CPI-613 inducing significant toxicity in the clinic is unlikely.

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