

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

^{1,2}King C. Lee, ³Claudia Maturo, ¹Robert Shorr and ¹Robert Rodriguez

¹Department of Regulatory and Clinical Affairs, Cornerstone Pharmaceuticals, Inc.,
1 Duncan Drive, Cranbury, NJ 08512 USA

²Department of Graduate Nursing Program, Faculty of Nursing, Quinnipiac University,
275 Mount Carmel Avenue, Hamden, CT 06518 USA

³Department of Program Management, Cornerstone Pharmaceuticals, Inc.,
1 Duncan Drive, Cranbury, NJ 08512 USA

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 LD₁₀ 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

Corresponding Author: King Lee, Cornerstone Pharmaceuticals, Inc., 1 Duncan Drive, Cranbury, NJ 08512, USA
Tel: 1-609-409-6037 Fax: 1-631-444-6895

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 LD₁₀ 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		# Animals		Day of Euthanasia ^d
			mg kg ⁻¹	mg m ⁻²	Male	Females	
A (Assessment of Dose-Related Toxicity, PK and Recovery in Rats)	1	D5W	0 ^c	0	24	24	50% on Day 21/22 and the other
	2	Vehicle	0 ^c	0	24	24	50% on Day 32/33
	3		25 ^c	150	12	12	Day 21/22 ^c
	4	CPI-613	30 ^c	180	24	24	50% on Day 21/22 and the other
	5		35 ^c	210	24	24	50% on Day 32/33
B (Assessment of Dose-Related Toxicity in Minipigs)	1	D5W	0	0	4	4	Day 21/22
	2	Vehicle	0	0	4	4	Day 21/22
	3		45	1575	4	4	Day 21/22
	4	CPI-613	50	1750	4	4	Day 21/22
	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/m² was according to Freireich's conversion (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 microbiological 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 aseptic 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 4 th 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 1 st , 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.)
Pharmacokinetic Parameters	Gross necropsy, organ weights and histopathology (Table 3)	Day 22 (for Experiments A and B) or Day 33 (in recovery animals in Experiment A).
	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 K₃-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		Organ Weights	Histology	
Serum Chemistry	Hematology			
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 and glandular)	-seminal vesicle
		-liver	-urinary bladder	-testis
-potassium	-platelet	-lung		-uterus
-chloride	-neutrophil	-ovary	Cardiovascular:	-vagina
	-lymphocyte	-spleen	-aorta	
	-monocyte	-testis	-heart	Gland:
Lipids:	-eosinophil			-adrenal gland
-cholesterol	-basophil			-harderian gland
-triglycerides				-mammary gland
Enzymes:	Others:		Nervous:	-pituitary gland
-alanine aminotransferase	-hemoglobin concentration		-brain (brain stem, cerebellum, cerebrum)	-prostate gland
-aspartate aminotransferase	-mean corpuscular volume		-epididymis	-salivary gland (mandibular)
-alkaline phosphatase	-red blood cell morphology		-nerve (optic, sciatic)	-thyroid gland/parathyroid gland
-gamma-glutamyltransferase	-others such as abnormal blood cells if present		-spinal cord (cervical, lumbar, thoracic)	-thymus
-lactate dehydrogenase	-hematocrit ^a			
	-mean corpuscular hemoglobin ^a		Gastrointestinal:	Musculoskeletal
Plasma Proteins:	-mean corpuscular hemoglobin concentration ^a		-tongue	-femur bone
-protein			-esophagus	-sternum
-albumin			-intestine (cecum, colon, rectum, duodenum, ileum, jejunum [with Peyer's patch])	-sternum bone marrow
-globulin ^a			-liver	-skeletal muscle
-albumin/globulin ratio ^a			-pancreas	
Others:				Others:
-glucose				-skin (abdominal)
-total bilirubin			Respiratory:	-eye
-urea nitrogen			-lung	-administration site (region of catheter insertion at femoral vein [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
Run Time:	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: Methanol: Acetonitrile: Formic Acid: Bleach (40:30:30:0.1:0.1, 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 ($p \leq 0.05$) 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 $p \leq 0.05$, then a non-parametric distribution was assumed and a Kruskal-Wallis test for independent groups was performed. A significant Kruskal-Wallis Test result ($p \leq 0.05$) 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 ($p \leq 0.05$) 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

Experiments	Group	Dose (mg kg ⁻¹)	Number of animals		Euthanized moribund or death						
			Male	Female	Animal	Sex	Study Day	Male (%)	Female (%)	Both sexes (%)	
Experiment A in rats	1	0 (D5W)	24	24	0.00	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

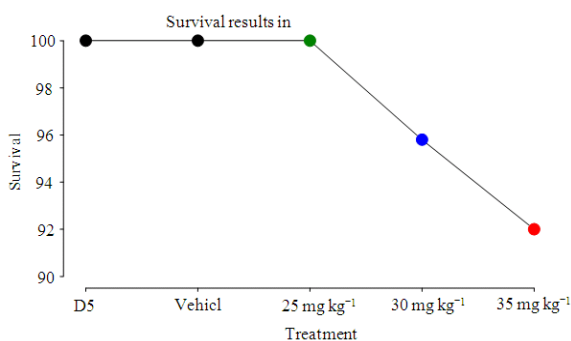


Fig. 1: Incidence of survival in different treatment groups of rats treated with 5% Dextrose in Water (D5W), vehicle, or CPI-613 at 25, 30 or 35 mg kg⁻¹, given 2x weekly for three consecutive weeks from Experiment A. Survival rats included those that did not die during the study, as well as those that 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

Experiments	Sex	Group	Values	Study days							
				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	24
		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	--		

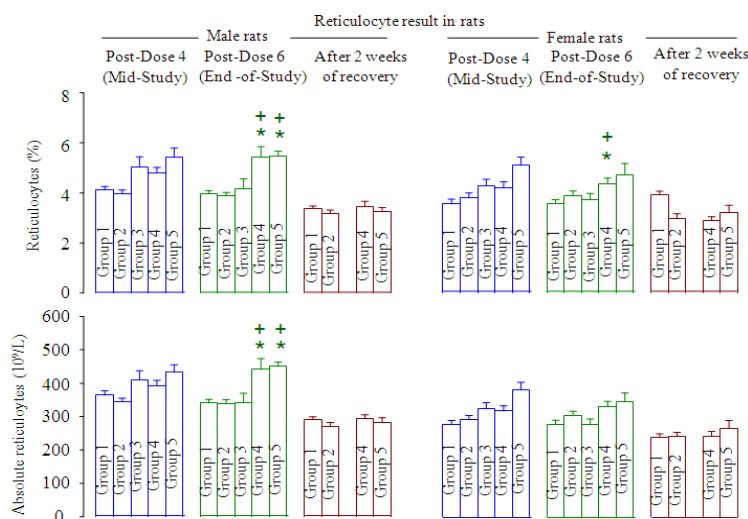


Fig. 2: Reticulocytes (% and absolute) in male and female rats treated with 5% Dextrose in Water (D5W) in Group 1, vehicle in Group 2 and CPI-613 at 25, 30 or 35 mg kg⁻¹ in Groups 3, 4 and 5, respectively, given 2x weekly for three consecutive weeks from Experiment A. There were 24 males and 24 females 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≤0.05 level

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

Sex	Group	Values	Food consumption				
			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

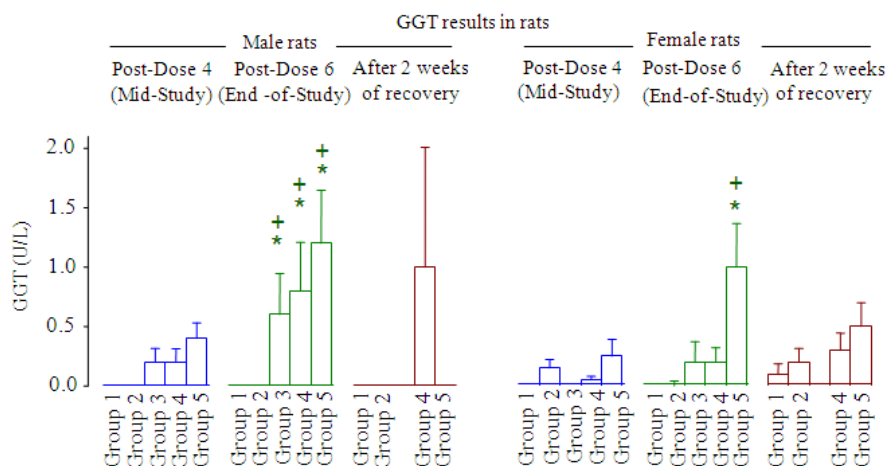


Fig. 3: Gamma-Glutamyltransferase (GGT) in male and female rats treated with 5% Dextrose in Water (D5W) in Group 1, vehicle in Group 2 and CPI-613 at 25, 30 or 35 mg kg⁻¹ in Groups 3, 4 and 5, respectively, given 2x weekly for three consecutive weeks from Experiment A. There were 24 males and 24 females 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<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
	Low ^a	Days	6, 17
		# of Observations	.	.	3	.	1
Females	Scant ^a	# of Animals	.	.	2	.	1
		Days Observed	.	.	2, 20, 21	.	18
		# of Observations
	Low ^a	# of Animals
		Days Observed
		# of Observations	.	.	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 histopathological 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 (12 were examined)	429	EYE: Discoloration, pale, left, TGL-small
			435	THYMUS: Discoloration, mottled, TGL-small
			439	SKIN-back: Crust, red, TGL-5×10 mm, above port site
	F	Day 33 (12 were examined)	441	EYE-cornea: Crust, dark, left, TGL-small
			455	SKIN-forefoot: Thick, left, TGL-small
			426	SKIN-back: Crust, red TGL: 3×4 mm, at port site
2	M	Day 22 (12 were examined)	432	SKIN-back: Crust, dark TGL: 5×11 mm
			440	LIVER-medial lobe: Nodule, tan TGL-7×7 mm
			468	SKIN-subcutaneous: Nodule, back, dark TGL-15×15 mm, over port site, consistent with a hematoma
			473	EPIDIDYMIS: Missing, left.
			491	TESTIS: Small, left, TGL-small
			491	SKIN-subcutaneous: Nodule, inguinal, mottled, TGL-12×12mm, left side, near the administration site
	F	Day 33 (12 were examined)	495	SKIN-back: Crust, brown, TGL-10×10 mm, over port site
			513	LYMPH NODE-mandibular: Discoloration, dark, right, TGL-small
			517	LIVER-medial lobe: Adhesion was to diaphragm, TGL-small
			472	SKIN-back: Crust, brown TGL-small, on port site
			478	UTERUS: Enlarged, bilateral TGL
			484	SKIN-subcutaneous: Nodule, back, green TGL: 10x20mm, at port site
3	M	Day 22 (12 were examined)	486	LYMPH NODE-iliac: Enlarged, bilateral TGL-small
			496	SKIN-subcutaneous: Nodule, inguinal, green TGL-15x10mm, near administration site, left
			502	KIDNEY-capsule: Focus, white, left, multiple TGL-3x1mm, LYMPH NODE-mandibular: Discoloration, mottled, left TGL-small
			506	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
	F	Day 22 (12 were examined)	514	SKIN-inguinal: Crust, brown, left TGL-3×3 mm, located near staples
			519	SKIN-inguinal: Nodule, subcutaneous, tan, TGL-10×15 mm, under staples
			537	THYMUS: Focus, dark, multiple, TGL-1×2 mm
			539	ADMINISTRATION SITE: Nodule, tan, TGL-10×15 mm
			541	EPIDIDYMIS: Mass, tan, left, TGL-15×25 mm, adhered to abdominal wall
			520	LYMPH NODE-mediastinal: Enlarged, red, TGL-small
4	M	Day 22 (12 were examined)	526	TESTIS: Adhesion to scrotum, left TGL-small
			530	SKIN-back: Crust, red, TGL-5×12 mm, over port
			532	THYMUS: Discoloration, mottled TGL
			536	SKIN-inguinal: Nodule, subcutaneous, tan TGL-12×15 mm, near administration site, left
			547	SKELETAL MUSCLE-back: Thick, right TGL-small, psoas major muscle
			547	SKIN-subcutaneous: Fluid, pink: fluid around port site
4	M	Day 22 (12 were examined)	551	SKIN-subcutaneous: Nodule, hindlimb, dark, left TGL-3×3 mm, near administration site
			553	LIVER-lateral lobe: Adhesion, to lumbar musculature, right, TGL-small
			557	SKIN-inguinal: Nodule, subcutaneous, green, TGL-13×14 mm, near administration site, left
			559	LYMPH NODE-iliac: Mass, left, TGL-20×20 mm
			561	SKIN-inguinal: Nodule, subcutaneous, tan, TGL-20×20 mm, left, near administration site
			563	SKIN-inguinal: Nodule, subcutaneous, tan, TGL-12×22 mm, left, near administration site
			563	SKELETAL MUSCLE-hindlimb: Nodule, left, TGL-10×15 mm
			565	LYMPH NODE-iliac: Discoloration, dark, left, TGL-small
			565	LYMPH NODE-renal: Discoloration, dark, left, TGL-small
			565	LYMPH NODE-iliac: Enlarged, left, TGL-small
4	M	Day 22 (12 were examined)	563	SKIN-inguinal: Nodule, subcutaneous, green, TGL-10×15 mm, left, near administration site
			565	LYMPH NODE-mediastinal: Enlarged, TGL-small
			565	SKIN-inguinal: Mass, subcutaneous, tan, TGL-30×15mm, left side, near administration site
			565	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-15×8mm
			587	PREPUTIAL GLAND: Discoloration, green, left, TGL-small SKIN-inguinal: Crust, brown, left,-TGL-3×3mm, located near staples SKIN-subcutaneous: Nodule, inguinal, tan, TGL-8×8mm, 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 (4 total examined)	1687	EPIDIDYMIS: Right, TGL - small
				EPIDIDYMIS: Cyst; clear; right, TGL - 22×20 mm
	F	Day 22 (4 total examined)	1686	SKIN - hindlimb: Crust, brown, right, multiple, TGL - 10×10 mm
			1688	SKIN - back: Crust, dark, left, multiple, TGL - 5×5 mm
2	M	Day 22 (4 total examined)	1697	SKIN - subcutaneous, neck: Nodule, brown, TGL - 20×20 mm
				SKIN - subcutaneous: Accumulation, red, ventral, TGL - small, hematoma per pathologist
	F	Day 22 (4 total examined)		THYMUS: Discoloration, red, TGL - small
				SKIN - Neck: Crust, tan, TGL - 5×5 mm
				ADMINISTRATION SITE: Thick, right, TGL - small
3	M	Day 22	1698	LYMPH NODE - bronchial: Discoloration, mottled, TGL - small
				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
			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 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) STOMACH - mucosa: Focus, dark, TGL - 15×15 mm No visible lesions in all animals.
			1715	
	F	Day 22 (4 total examined)	--	
4	M	Day 22 (4 total examined)	1725	TESTIS: Cyst, clear, unilateral, TGL - 12×20 mm
	F	Day 22 (4 total examined)	1722	SKIN - forelimb: Crust, brown, left, TGL - 20×30 mm
			1728	LYMPH NODE - mediastinal: Discoloration, dark, TGL - small
5	M	Day 22 (4 total examined)	1739	EAR: Crust, brown, left, TGL - 4×7 mm 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 (4 total examined)	1738	EAR: Crust, brown, bilateral, TGL - 3×4 mm
			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			
Adrenal gland	Pigmentation						1A				
	Vacuolation, cortex										
Bone marrow, sternum	Vacuolation, cortex, focal	1A	1A								
	Hyperplasia			2A,1B	4A,1B,1C	3A,4B	3A	2A	2A	3A	5A,1B,1C
Bone, femur	Granuloma										
	Foreign material										
	Fibrosis, focal										
	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						
Bone, sternum	Fibrosis					1A					2B
	Accessory structure, physis, cartilage										
	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										
	Inflam, chronic, active				1D						
Cavity, abdominal	Granuloma, spermatic, unilateral					1D	--	--	--	--	--
	Granuloma, spermatogenic, unilateral						1D	--	--	--	--
Epididymis	Inflam, chronic, active										--
	Infiltration, lymphocytic	1A		1D			--	--	--	--	--
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											
	Inflam, chronic, active						1A					
Kidney	Cyst						1C					
	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				1A			
	Inflam, chronic, active											
	Inflam, chronic, active, pelvis											
	Dilation, pelvis											
	Infiltration, lymphocytic											
Liver	Hematopoiesis, increased				1B				1A	1A		3A,1B
	Hypertrophy, Kupffer cell											1B
	Infiltration, histiocytic											
	Infiltration, lymphohistiocytic	1A	1A	5A	2A	2A	5A	4A	3A	4A	3A	
	Necrosis				1A							
	Necrosis, single cell											
	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											
	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											
	Hyperplasia, lymphoid	1A		5A	5A	1A	2A			1B		1B
	Inflam, chronic											
Mesentery	Inflam, chronic, active											
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								
	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											
Stomach, glandular	Angiectasis											
	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
Thyroid gland	Hemorrhage	8A	5A	4A	5A	4A	5A	3A	6A,1B	4A		3A
	Hyperplasia, epithelial, focal						1A					
	Infiltration, lymphohistiocytic											
	Infiltration, lymphocytic											
Treachea	Extopia											
Ureter	Inflam, chronic				1A			1A				
	Dilation							1C				
Urinary bladder	Inflam, chronic					1B						
	Hyperplasia, epithelium, transitional cell					1B						
	Hyperplasia, transitional cell, epithelium	1B				1B						
	Inflam, chronic					1A						
	Inflam, chronic, active	1A				1A						
	Inflam, chronic, active, serosa											1C
Uterus	Hemorrhage	--	--	--	--	--						1A
	Hyperplasia, stromal	--	--	--	--	--						
	Deciduoma, single	--	--	--	--	--		1C		1A		
	Inflam, chronic, active, endometrium	--	--	--	--	--		1A				
Vagina	Inflam, chronic	--	--	--	--	--		1A				1B
		--	--	--	--	--						
Cavity, Pelvic	Inflam, acute											
	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	Vacuolation, 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								
	Accessory 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					
Liver	Infiltration, lymphocytic								
	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					
Lung	Vacuolation, hepatocyte, cytoplasm						1A		
	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								
Lymph node, iliac	Pigmentation								
	Hemorrhage								
	Hyperplasia, lymphoid					1B			1C
Lymph node, inguinal	Hyperplasia, lymphoid, mucosal					1B			
	Inflam, chronic, active								
Lymph node, mediastinal	Hyperplasia, lymphoid, bilateral								
	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								
	Spleen	Edema								
Hematopoiesis, increased		1A	1B					1A	1A	
Hyperplasia, follicle				1B						
Stomach, glandular	Inflam, chronic, capsule			1B						
	Angectasis				1B					
	Dilation, glands									
	Ectopia									
Testis	Edema									
	Atrophy, germinal epithelium					--	--	--	--	
	Hypoplasia, unilateral					--	--	--	--	
Thymus	Infarction, unilateral					--	--	--	--	
	Inflam, chronic, active, unilateral					--	--	--	--	
	Inflam, acute, capsule, unilateral					--	--	--	--	
	Atrophy, cortex									
Thyroid gland	Hemorrhage	9A	7A	5A	9A	6A	5A	4A	9A	
	Hyperplasia, epithelial, focal									
	Infiltration, lymphohistiocytic							1A		
	Infiltration, lymphocytic		1A		1A					
Treacha	Extopia		1A				1A	1A		
	Inflam, chronic			1A	1A	1A				
Urinary bladder	Dilation									
	Inflam, chronic									
Uterus	Hyperplasia, epithelium, transitional cell									
	Hyperplasia, transitional cell, epithelium									
	Inflam, chronic									
	Inflam, chronic, active									
Vagina	Inflam, chronic, active, serosa									
	Hemorrhage	--	--	--	--					
	Hyperplasia, stromal	--	--	--	--					
Cavity, Pelvic	Deciduoma, single	--	--	--	--					
	Inflam, chronic, active, endometrium	--	--	--	--					
Cavity, Pelvic	Inflam, chronic	--	--	--	--					
	Inflam, acute	--	--	--	--				1B	
	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

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

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

Dose	Day 1 1 st Dose			Day 4 2 nd Dose			Day 11 4 th Dose			Day 18 6 th Dose		
	(mg kg ⁻¹)			(mg kg ⁻¹)			(mg kg ⁻¹)			(mg kg ⁻¹)		
	25	30	35	25	30	35	25	30	35	25	30	35
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 × 10 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-∞} (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-∞} /AUC _{0-∞} (%)	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-∞} = 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

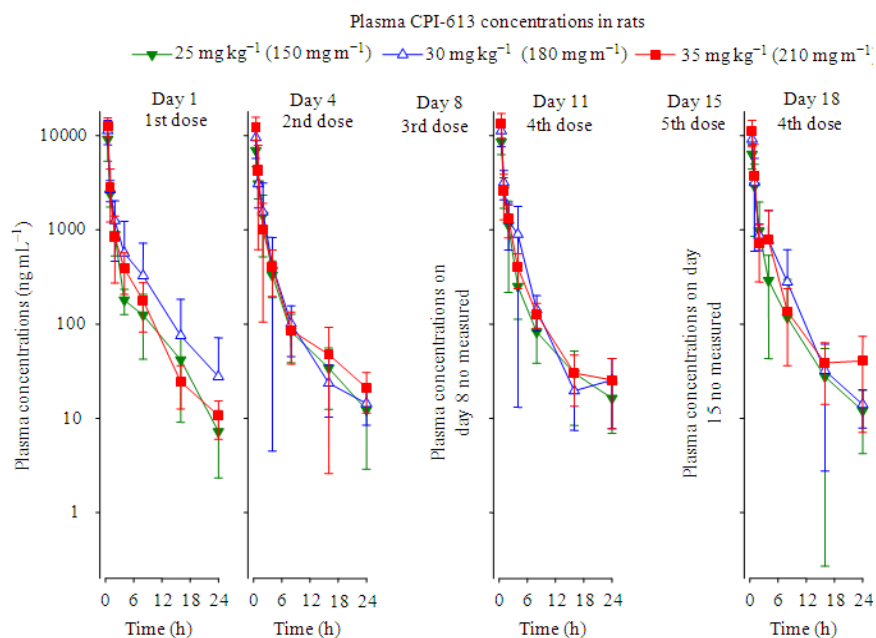


Fig. 4: Plasma concentrations of CPI-613 in rats treated with 25, 30 or 35 mg kg⁻¹ of CPI-613, given 2x weekly for three consecutive weeks from Experiment A. There were 4 rats per sex at each time point of each dose level. Results are presented as mean ± standard of deviation

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 (C_{max}) and AUC from dosing time to the final observation at 24 h (AUC_{0-24hr}) were proportional to dose levels (Fig. 5).

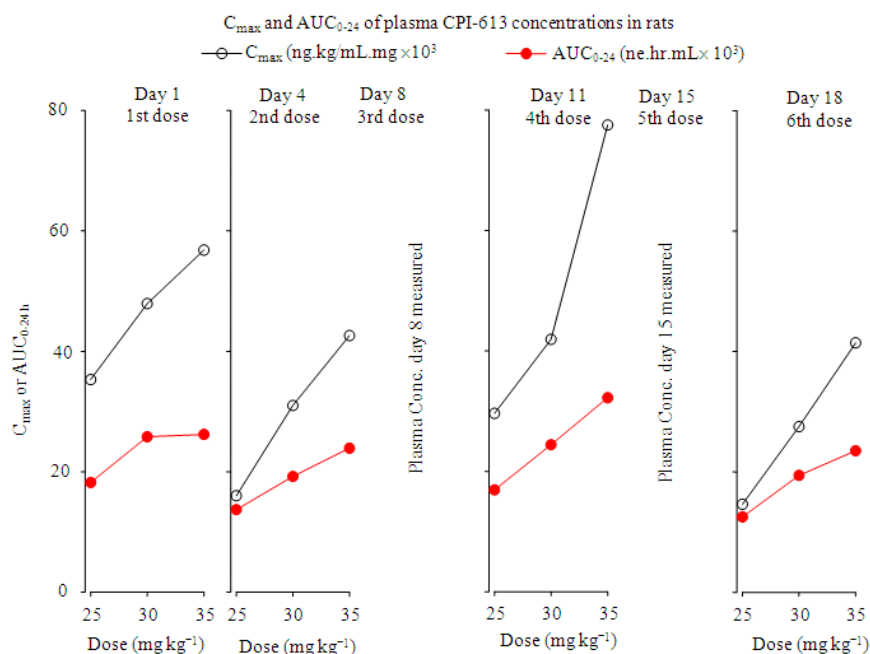


Fig. 5: Area under the curve from dosing time to the final observation at 24 h (AUC_{0-24h}) and maximum observed concentration extrapolated to time at 0 h (C_{max}) in rats treated with intravenous CPI-613 at 25, 30 or 35 mg/kg, given 2x weekly for three consecutive weeks from Experiment A. These pharmacokinetic 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 ($\sim 55 \text{ mg kg}^{-1}$ or $\sim 1925 \text{ mg}^{-1} \text{ m}^2$) being significantly higher than those of rats ($30\text{-}35 \text{ mg kg}^{-1}$ or $180\text{-}210 \text{ mg}^{-1} \text{ m}^2$).

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 $\sim 60\text{x}$ 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.

ACKNOWLEDGEMENT

Researchers gratefully acknowledge the contributions of Ms. Kathleen O'Donnell (Cornerstone Pharmaceuticals, Inc.) for her contributions in reviewing and editing of this manuscript.

REFERENCES

- Baggetto, L.G., 1992. Deviant energetic metabolism of glycolytic cancer cells. *Biochimie*, 74: 959-974.
DOI: 10.1016/0300-9084(92)90016-8

- Barret, J. and K.L. Blanc, 2009. Cancer Chemotherapy and Immune Regulation. *Am. J. Immunol.*, 5: 8-16. DOI: 10.3844/ajisp.2009.8.16
- Freireich, E.J., E.A. Gehan, D.P. Rall, L.H. Schmidt and H.E. Skipper, 1966. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother Rep.*, 50: 219-244. PMID: 4957125
- Holmuhamedov, E., L. Lewis, M. Bienengraeber, M. Holmuhamedova and A. Jahangir *et al.*, 2002. Suppression of human tumor cell proliferation through mitochondrial targeting. *FASEB J.*, 16: 1010-1016. DOI: 10.1096/fj.01-0996com
- Kim, J.W. and C.V. Dang, 2006. Cancer's molecular sweet tooth and the Warburg effect. *Cancer Res.*, 66: 8927-8930. DOI: 10.1158/0008-5472.CAN-06-1501
- Krob, H.A., A.B. Fleischer, Jr., R. D'Agostino, Jr., C.L. Haverstock and S. Feldman, 2004. Prevalence and relevance of contact dermatitis allergens: A meta-analysis of 15 years of published T.R.U.E. test data. *J. Am. Acad. Dermatol.*, 51: 349-353. PMID: 15337975
- Kroemer, G. and J. Pouyssegur, 2008. Tumor cell metabolism: Cancer's Achilles' heel. *Cancer Cell*, 13: 472-482. DOI: 10.1016/j.ccr.2008.05.005
- Nunoya, T., K. Shibuya, T. Saitoh, H. Yazawa and K. Nakamura *et al.*, 2007. Use of miniature pig for biomedical research, with reference to toxicologic studies. *J. Toxicol. Pathol.*, 20: 125-132. DOI: 10.1293/tox.20.125
- Nystrom, P.O., 1998. The systemic inflammatory response syndrome: Definitions and aetiology. *J. Antimicrob. Chemother.*, 41: 1-7. DOI: 10.1093/jac/41.suppl_1.1
- Ravindran, S., G.A. Radke, J.R. Guest and T.E. Roche, 1996. Lipoyl domain-based mechanism for the integrated feedback control of the pyruvate dehydrogenase complex by enhancement of pyruvate dehydrogenase kinase activity. *J. Biol. Chem.*, 271: 653-662. DOI: 10.1074/jbc.271.2.653
- Retter, A.S., R. Shorr, R. Rodriguez, K. Hoffman and F. Volterra *et al.*, 2010. Phase I trial of CPI-613, a lipoic acid analog, and gemcitabine in patients with advanced solid tumors. ASCO, Chicago, IL. http://www.asco.org/ASCOv2/Meetings/Abstracts?&vmview=abst_detail_view&confID=74&abstractID=43689
- Sakkrom, P., W. Pompimon, P. Meepowpan, N. Nuntasana and C. Loetchutinat, 2010. The effect of *phyllanthus taxodiifolius* beille extracts and its triterpenoids studying on cellular energetic stage of cancer cells. *Am. J. of Pharmacology and Toxicology*, 5: 139-144. DOI: 10.3844/ajptsp.2010.139.144
- Shaw, D.W., L.F. Eichenfield, T. Shainhouse and H.I. Maibach, 2004. Allergic contact dermatitis from tacrolimus. *J. Am. Acad. Dermatol.*, 50: 962-965. DOI: 10.1016/j.jaad.2003.09.013
- Swindle, M.M., 2007. *Swine in the Laboratory: Surgery, Anesthesia, Imaging and Experimental Techniques*. 2nd Edn., CRC Press, Boca Raton, FL., ISBN-10: 0849392780, pp: 471.
- Yamada, J., H. Tomiyama, M. Yambe, Y. Koji and K. Motobe *et al.*, 2006. Elevated serum levels of alanine aminotransferase and gamma glutamyltransferase are markers of inflammation and oxidative stress independent of the metabolic syndrome. *Atherosclerosis*, 189: 198-205. DOI: 10.1016/j.atherosclerosis.2005.11.036