Investigations of Enteric-Coated Propyl Gallate-induced Nephrotoxicity in Beagles and Human and Dog Renal Proximal Tubule Epithelial Cells

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Abstract

Permeability plays a major role in oral biotherapeutic delivery and permeation enhancers can improve the intestinal permeability of poorly absorbed active pharmaceutical ingredients such as peptides. As part of nonclinical development of an oral formulation for a glucagon-like peptide-1 (GLP-1) receptor agonist, MEDI7219, toxicology studies revealed that one of the formulation excipients, propyl gallate (PG), when administered in enteric-coated tablets, led to nephrotoxicity in beagles. While PG has been widely used in food and cosmetics as an anti-oxidant, understanding of its toxicology, metabolism and disposition has been rarely discussed. To elucidate the nephrotoxicity observed after administration of PG in an enteric coated tablet formulation, we employed dog and human renal proximal tubule epithelial cells (RPTEC). We observed greater cytotoxicity to PG in dog RPTEC compared to human cells. We also observed greater increases in response to PG treatment of glutathione in human cells compared to dog cells. Glutathione elevation is a common response to detoxify xenobiotics, especially ones that produce free radicals such as PG. Thus, we hypothesize that glutathione in human RPTECs was elevated to detoxify PG, but not in dog RPTECs, leading to greater cytotoxicity for dog RPTECs. Furthermore, to characterize disposition and metabolism of PG in both humans and dogs we developed a 10-plex, highly sensitive and robust LC-MS/MS-based quantification method of PG and its phase-I and phase-II metabolites in dog and human plasma. The methods were employed to support clinical

study (NCT03362593) and preclinical dog studies to evaluate safety, pharmacokinetics and tolerability of PG to support its use in an oral formulation for MEDI7219.

Introduction

MEDI7219 is a bis-lipidated glucagon-like peptide-1 (GLP-1) analog providing enhanced circulation time by reversible binding to albumin. It was being developed as an oral formulation to improve glycemic control and body weight loss in patients with Type 2 diabetes [1, 2]. As most currently available GLP-1 receptor agonists are administered via subcutaneous (SC) injection, MEDI7219 may provide a better option for patients by increasing compliance and ease-of-use. The formulated MEDI7219 tablets require an enteric coating to protect the peptide from degradation in the stomach, thereby helping to ensure that the peptide and absorption enhancers reach the small intestine in proximity to each other for optimal absorption. Oral administration of hydrophilic macromolecules has been challenging primarily due to low intestinal epithelial permeability. Challenges usually come from passive or carrier-mediated transcellular permeation across phospholipid bilayers, as well as restricted paracellular transport via tight junctions. Propyl gallate (PG) has been used as a permeation enhancer excipient in several clinical trials[3-5]. To increase oral bioavailability, the MEDI7219 peptide was formulated with a novel combination of excipients, with the primary permeation enhancers including PG [6].

PG is the propyl ester of gallic acid and is a Generally Regarded As Safe (GRAS) compound used in foods, cosmetics and hair products at low levels[7]. PG protects oils and fats in products from oxidation and has been added to foods containing oils and fats since 1948 [8]. It is estimated that propyl gallate is used in over 150 cosmetic products at a maximum concentration of 0.1% [9]. Free radicals of PG can inhibit the activity of enzymatic hydrolysis of adenosine triphosphate (ATP) [10]. PG may also inhibit hepatic microsomal hydrolase and demethylase activities [11]. As a food and cosmetic antioxidant, despite the presumed low toxicity, PG has been investigated to assess safety and various toxicological properties [12-14]. The European Food Safety Authority has accepted a daily intake of 0.5 mg/kg body weight for this compound, primarily

because of a gap in toxicity data more so than because of a specific safety concern. For a 60 kg individual, the proposed oral GLP-1 formulation would contain approximately 1.3-fold more PG than stipulated in these recommendations. Furthermore, the amount of PG planned for the MEDI7219 formulation is approximately 25-fold higher than in currently approved products in the United States [CDERs inactive ingredient database][24]. Early investigation regarding the toxicity profile of PG can be traced back to the 1980s and 1990s with focus on hepatic injury [11-13]. These reports indicate that PG impairs mitochondrial function thus inducing cytotoxicity of hepatocytes. Renal injury and potential species difference from PG exposure have not been reported. In advance of the first-in-human clinical study of MEDI7219 (NCT03362593), the toxicity profile of enteric-coated MEDI7219 tablets containing the intended excipients, including PG, was evaluated in dogs to supplement the literature-based safety information. Toxicology studies using the complete formulated, enteric coated tablets in dogs showed signs of renal toxicity. As this finding was observed in the presence or absence of the GLP-1 peptide, it was suspected that one or more of the excipients included in the formulation was responsible for the toxicity. To further elucidate this, a series of tablets that included different combinations of the various excipients were tested in dogs. This was followed by a second study investigating the requirement of an enteric-coated tablet to induce PG-mediated nephrotoxicity. Only enteric-coated tablets containing PG induced nephrotoxicity. The combined results of these studies indicated that PG, when formulated in enteric-coated tables, was likely inducing the renal toxicity, either directly or indirectly.

Moreover, PG has been rarely discussed as an excipient for oral delivery of biotherapeutics and detailed investigations of PG bioanalysis, metabolism, and safety considerations have been very limited to date. In order to better dissect the mechanism of PG-induced nephrotoxicity we conducted in vitro studies in human and dogs RPTECs. RPTECs have been used to evaluate the potential for renal toxicity, discovery of new biomarkers, and elucidation of mechanisms of nephrotoxicity in vitro [15-17]. In order to better characterize pharmacokinetics and metabolism of PG, we developed a high sensitivity multiplexed LC-MS/MS method to quantitatively measure PG and its nine phase-I and phase-II metabolites in dog and human plasma samples, providing

further understanding of PG metabolism in both species. Analytical methods such as gas and liquid chromatography have traditionally been the primary methods used to determine concentrations of PG as an antioxidant additive in foods [18-20]. The use of LC-MS/MS for measurement of PG has not been commonly employed and has been limited to either in food application or utilizing PG as an internal standard of a pharmacologically active constituent in rat plasma [21, 22].

Materials and Methods

Reagents and Materials

Enteric-coated tablets containing various excipients were manufactured by MedImmune (Gaithersburg, MD) as described previously[1].Tablets were be placed into size 12 gel capsules to determine dose level. Gallic acid (GA) was purchased from Sigma (St. Louis, MO). PG was purchased from USP (Rockville, MD). 4-O-methyl gallic acid (4OMGA) was purchased from VWR Scientific (Allison Park, PA) and Extrasynthese (Lyon, France). Propyl gallate-3-glucoronide (PG-3-Gluc), propyl gallate-4-glucoronide (PG-4-Gluc), gallic acid-3-glucoronide (GA-3-Gluc), gallic acid-4-glucoronide (GA-4-Gluc), propyl gallate-glutathione (PG-GSH), gallic acid-glutathione (GA-GSH), and 4-O-methyl gallic acid-3-glucoronide (4OMGA-3-Gluc) were purchased from WuXi AppTec (Shanghai, China). Gallic acid-$^{13}C_5$ and 4-O-methyl gallic acid-^{13}C, d_3 were purchased from Key Organics (Camelford, UK). Propyl gallate-d_5 was purchased from Chemtos (Austin, TX). CellTiter-Glo® luminescent cell viability assay kit and GSH/GSSG-Glo™ assay were from Promega (Madison, WI). Human renal proximal tubule epithelial cells (RPTEC) and medium were from BioIVT (Westbury, NY). Beagle Dog RPTECs and medium were purchased from BioIVT (Westbury, NY) and Innoprot (Biscay, Spain), respectively.

Acetonitrile (HPLC grade), methanol (HPLC grade), and formic acid (LC-MS grade) were purchased from Sigma-Aldrich (St. Louis, MO). 100% filtered water was purchased from EMD Millipore (Burlington, MA). Dimethyl sulfoxide (ACS grade, ≥ 99.9%) was purchased from

Honeywell (Charlotte, NC). Ammonium bicarbonate, Baker analyzed reagent (21.30–21.73%) was purchased from J.T. Baker (Phillipsburg, NJ). Dipotassium EDTA (K_2EDTA) human plasma and dog plasma were purchased from commercial sources, such as Bioreclamation (Westbury, NY) and Biological Specialty Corporation (Colmar, PA).

Cell culture

Frozen RPTECs vials were removed from liquid nitrogen storage and placed in a 37 °C water bath until the contents were completely thawed. The cells were gently resuspended and dispensed into a cell culture flask at a seeding density of ~7500 cells/cm^2. The flask was placed in a 37°C, 5% CO_2 incubator. Cell cultures were given fresh medium the following morning and then every other day until the cell culture was approximately 90% confluent. When the cell culture was 90% confluent, medium was removed and the cells were rinsed with warmed PBS. Cells were harvested and split into new flasks based on a cell density of 7500 cells/cm^2. Cells were incubated at 37°C for 3 days, with fresh medium added each day. Cells were harvested and diluted with growth medium to a density of 200,000 cells/mL. 100 µL of cell suspension per well (20,000 cells/well) was added to a clear-bottom 96-well microplate for luminescence assays. The plated cells were incubated in the 37°C incubator for 3 days.

Compound Treatment of RPTEC

Each test compound (PG, GA, 4OMPG and 4OMGA) was initially reconstituted using DMSO at a concentration of 250 mg/mL. Serial dilutions of each compound were performed to reach concentrations of 4000 µM, 2000 µM, 1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.3 µM using warmed fresh supplemented medium with 0.4 % (v/v) DMSO. 100 µL of media containing the compound solution was added to the cells. Final concentrations of the compounds were 2000 µM, 1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.3 µM, 15.7 µM. AZ13599185 (tubulysin, 100 or 500 ng/mL) or cisplatin (30 µg/mL) and buthionine sulfoximine (BSO, 200 µM) were used as positive controls for CTG and GSH assays, respectively. 0.2 % (v/v) DMSO in medium was used as the control for both assays. Three replicates for each compound at each concentration were

prepared. 6 replicates of positive or negative controls were prepared. Cells were incubated at 37°C for approximately 48 hours.

Cell Titer Glo and Glutathione Assays

Cell media were removed and cells were washed twice with 200 µL of PBS buffer per well. CTG and GSH assay reagents were added to the wells and the assays were performed following the protocols of the Promega assay kits. Briefly, to perform the CTG assay, 100 µL of CTG solution was added into each well. The plate was gently shaken and incubated in the dark for 30 min and luminescence was measured using a Perkin Elmer VICTOR X3 plate reader. To perform the GSH assay, luciferin-NT substrate and glutathione S-transferase were mixed into the GSH-GLo solution. 100 µL of the mixed solution was added into each well. The plate was gently shaken and incubated in the dark for at least 30 min. 100 µL of Luciferin Detection Reagent was added per well. The plate was gently shaken and incubated in the dark for at least 15 min. Luminescence was measured using a Perkin Elmer VICTOR X3 plate reader.

Preparation of plasma samples

Dog blood was collected via jugular vein in potassium (K_2) EDTA tubes and processed to plasma. Samples were centrifuged at approximately 3000 rpm for 5 minutes at approximately 4°C within 30 minutes of collection. Plasma samples were acidified with 1% (v/v) formic acid (FA) to avoid degradation of PG and metabolites in plasma within 20 minutes of the start of centrifugation and then stored frozen (≤-60 °C). Acidified plasma was extracted using internal standard-spiked solution (100 mM ammonium bicarbonate : acetonitrile = 1:4). Samples were then centrifuged to collect supernatant, followed by drying to completion. The dried samples were reconstituted with 200 uL of 0.1% (v/v) formic acid in water. Samples were vortexed and centrifuged before injection. In order to assess potential impact of hemolysis on qualification of PG and its metabolites, hemolyzed plasma was prepared by spiking fully hemolyzed whole blood at indicated percentage into plasma.

LC-MS/MS analysis

Separations were conducted on a reversed-phase column (Phenomenex, Kinetex F5, 2.6µm 100 Å, 100 x 4.6 mm) at a flow rate of 0.7 mL/min at 40°C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol. The samples were analyzed on a SCIEX 6500+ QTRAP under negative ESI mode. The total run time was 5.5 min. Eight calibration standards and three quality control (LQC, MQC and HQC) samples were used for quantification. Further details of the LC-MS/MS method are described in Supplemental Materials and Methods.

Data analysis

The targeted quantitation of PG and phase-I/II metabolites was using peak area ratio of analytes and internal standards in MultiQuant™ software (SCIEX).

For cell-based assays, data was normalized to the negative control. Cell viability and GSH level were expressed as:

$$Cell\ viability\ or\ GSH\ level = \frac{Luminescence\ (each\ well)}{Luminescence\ (Average\ of\ DMSO\ negative\ control)}$$

Animal Studies:

The in vivo dog study was conducted at the LabCorp facility (Madison, Wisconsin) in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. Female beagles 6-7 months of age and weighing 6.6-9.8 kg at initiation of dosing were used. Beagles were housed in stainless steel cages on Tenderfoot® flooring and were socially housed, unless individually housed during acclimation, for study related procedures, for behavior or health reasons, due to the number of animals available, or for individual assessment of food consumption. Starting on Day 2 of the dosing phase, animals were separated prior to dosing and were commingled approximately 0.5 hours post the last animal dosed. Certified Canine Diet #5007 (PMI Nutrition International Certified LabDiet®) was provided for 4 to 5 hours each day. Feed was offered approximately 3 h following completion of dosing on days of dosing or at approximately the same time as the expected end of dosing (+/-2 hours) on days without dosing. Water was provided ad libitum. Dogs received enteric coated tablets consisting of different combinations of the clinical formulation excipients with or without PG once daily for 27 days. For groups receiving tablets with PG, the daily dose of PG was 200 mg/kg/day. Scheduled necropsies of all surviving animals were conducted on day 28 of the dosing phase. Samples for PG toxicokinetic were collected on days 1, 14, 21 and 25 of the dosing-phase. On days 1, 14, and 21 samples were collected: pre-dose and approximately 1, 2, 3, 4, 6, 8, 12, and 24 h post-dose. Days 25: pre-dose and approximately 1, 2, 3, 4, 6, 8, 10, and 24 h post-dose. Urine for acute renal injury biomarkers (NGAL) was collected at least twice during the predose phase, on Days 8, 15, and 22 of the dosing phase, and on the day of scheduled sacrifice. Urine was collected via cystocentesis at necropsy from animals sacrificed at an unscheduled interval.

Results and Discussion

Toxicology studies of propyl gallate in MEDI7219

The toxicity profile of different excipient combinations was evaluated upon daily administration of enteric coated tablets via oral capsules to female beagle dogs for 27 days. Dogs were assigned across seven groups (3/group) and tablets containing different excipient combinations were

administered by oral gavage once daily to determine which excipient(s) is responsible for causing the renal toxicity that had been observed in repeat-dose toxicology studies conducted with MEDI7219 enteric coated tablets (**Table 1a**). Results of this investigative 4-week study of orally administered enteric coated excipient tablets in beagles revealed PG as the possible cause of renal toxicity (**Table 1b**). With different combinations of excipients including PG administered orally, nephrotoxicity was only observed in dogs dosed with formulations comprising PG. In a follow-up study, 200 mg/kg/day PG alone administered in standard gelatin capsules, which yielded similar PG systemic exposures as the enteric coated tablets, did not result in nephrotoxicity (data not shown).

Microscopic evaluation of kidneys from animals administered PG via enteric tablets showed renal nephropathy consisting of tubular degeneration and regeneration and occasionally focal interstitial infiltrates of mononuclear cells or brown-yellow pigment in tubular epithelial cells. In addition, dilation of glomeruli was observed in some affected tubules. Additionally, these animals also had an increase in urine neutrophil gelatinase-associated lipocalin (NGAL) levels, one of the 6 renal injury biomarkers recommended by the FDA for clinical monitoring. Time-dependent increases in NGAL levels were observed in dogs receiving excipients with PG, shown in **Figure 1**. Other excipients such as sodium chenodeoxycholate (NaCDC) did not induce NGAL levels. This biomarker correlated well with the histopathological nephropathy observed in these animals.

In vitro assessment of propyl gallate toxicity in primary renal proximal tubule epithelial cells

The metabolism pathway for PG is depicted in **Figure 2** [12, 23]. The proposed mechanism of toxicity for PG and GA arises from the formation of a reactive phenoxy radical species leading to the formation of an unstable o-quinone. The o-quinone then undergoes detoxification by GSH conjugation. While both PG and GA can lead to the formation of phenoxy radical and quinone intermediates, PG is much more lipophilic than GA and is expected to cross cell membranes more readily (Log P for PG is 2.13 and for GA is 0.91) [23].

To study the mechanism of PG renal toxicity and its translatability to humans in human and dog primary renal proximal tubule epithelial cells (RPTECs), in vitro assessments of PG and its metabolites GA and 4OMGA were performed. A concentration-dependent decrease in cell viability was observed with PG for both dog and human RPTECs (**Figure 3**). The relative difference of PG in sensitivity between human (EC_{50} ~1529 µM) and dog (EC_{50} ~46.5 µM) was estimated to be ~33-fold, indicating that dogs may be more sensitive to PG-induced renal toxicity than humans in vivo. PG was more cytotoxic for dog than its metabolites, GA and 4OMGA, as well as 4-O-methyl propyl gallate (4OMPG), a synthetic analog of PG that is unable to form a reactive o-quinone. Compound structures are depicted in Supplemental Figure 1.

The hypothesis that dogs are more sensitive to PG than humans due to PG-mediated glutathione depletion was also studied. The results show that glutathione levels were induced by PG in human but not in dog cells, suggesting that GSH-mediated detoxification of the reactive quinone of PG may be less effective in dog RPTEC than human RPTEC (**Figure 4**). Recent studies indicate that while dog and human glutathione-s-transferase (GST) show substantial similarity some differences in activity towards some substrates such as isothiocyanates exist with dog GST being less active than human[24]. High concentrations of PG and GA induced a reduction of GSH levels in both dog and human cells likely due to decreased cell numbers at these high concentrations of PG and GA. High concentrations of 4OMPG also led to increases of GSH in human cells, possibly due to demethylation of 4OMPG.

Development of LC-MS/MS assay for PG and its phase-I and phase-II metabolites in dog and human plasma

LC-MS/MS analysis of PG and its metabolites in both dog and human plasma was performed to further understand the potential differences of PG disposition and its metabolism between humans and dogs. A highly sensitive 10-plex method for the quantification of PG and its phase I & II metabolites was developed. **Figure 5** shows a chromatogram of all targeted metabolites and an example of PG assay performances including PG intra/inter assay quality controls in human plasma, demonstrating appropriate precision and accuracy. The 3-plexed method of PG and its

phase I metabolites (GA and 4OMGA) was fully validated per 2018 FDA guidance [18], with LLOQ of propyl gallate at 20 pg/mL in human plasma and 40 pg/mL in dog plasma. Other major metabolites such as glutathione and glucuronide conjugates were detected at low nanogram levels, which were sufficient for quantifying the study samples. **Table 2** summarizes LLOQ of each analyte.

Unforeseen matrix interferences, that were not observed in pre-dose or placebo samples, were observed in plasma samples upon administration of PG. The quantitative measurement of PG and 4OMGA from pharmacokinetic samples was confounded by matrix interferences. To address the method limitations due to interference, extensive chromatographic optimizations such as optimizing columns, gradients, mobile phases, flow rate and column temperature were evaluated to resolve the analyte peak and to separate the analyte from matrix interference (Supplemental Figure 2). An impact of hemolysis on PG and GA quantification was also observed even at such low levels as 0.05 %. However, their respective internal standards also showed similar response as the analytes. As a result, the impact of hemolysis on propyl gallate quantification can be controlled with a stable labeled internal standard (Supplemental Figure 3).

Pharmacokinetic assessment of propyl gallate in humans using validated method

Healthy volunteers participating in the clinical study NCT03362593 were dosed orally with single or multiple ascending doses of MEDI7219 tablets**.** Healthy volunteers received enteric coated MEDI7219 tablets containing 200, 400 and 800 mg PG per tablet. The clinical plasma samples were analyzed using the validated method described above. **Figure 6** shows representative PG pharmacokinetic data. After oral administration of tablets containing PG, the T_{max} of PG was reached around 3-4 hours post-dose. PG exposure (mean $AUC_{0\text{-}t}$ and C_{max}) generally increased over the study duration with increasing dose levels.

Pharmacokinetic assessment of propyl gallate and its metabolites in human and dog

The primary metabolites of PG were measured in dog and human plasma following oral administration of enteric coated MEDI7219 tablets to examine a potential mechanism of the PG-

mediated renal toxicity in dog and its possible translatability to humans. Plasma concentrations of PG, its phase-I metabolites GA and 4OMGA and phase-II glucuronide and glutathione conjugates were measured. The human and dog samples, collected through 8 hours after administration, were analyzed and the results were compared (**Figure 7**). Based on allometric scaling method the highest daily dose administered in humans was approximately 8-fold less compared with the dose level tested in dogs. However, at these doses the observed exposures to PG, GA or 4OMGA were comparable between the two species, thus suggesting that humans may have greater oral bioavailability of PG than dogs. However, as to our knowledge PG exposure data following IV administration is not available for either humans or dogs and therefore a potential species difference in PG bioavailability cannot be confirmed. The metabolism pattern between the two species was largely similar with some differences observed in exposure of PG glucuronides and GA glucuronides. PG glucuronides had higher exposure while GA-3-glucoronides showed lower exposure in humans than in dogs. Both PG-GSH and GA-GSH conjugates had very low exposure in circulation with potential minor differences between humans and dog observed.

Conclusion

Oral delivery, as a non-invasive drug administration route for peptides and biologics, has been of growing interest for the development of chronically administered medications since it can significantly improve the patient experience. Novel design of formulation is one of the means to enhance drug properties such as permeability and solubility. Propyl gallate, an excipient commonly used in food and cosmetics to avoid oxidation, has been recently found to enhance the bioavailability of a GLP-1 agonist, MEDI7219, when formulated as an enteric coated tablet. As a novel excipient (ie, used at higher levels than in approved oral medications), pharmacokinetic and toxicokinetic measurements of PG and its metabolites are important to establish exposure-based safety margins. Preclinical toxicology studies in dogs have revealed that PG delivered via enteric-coated tablets induced renal toxicity. In vitro assessments using RPTEC identified possible sensitivity difference between dogs and humans, providing further understanding of potential translatability of PG-induced dog renal toxicity to humans. Mechanistic investigations by cell-

based assays with RPTEC suggested GSH-mediated detoxification was less effective in dog cells than in human cells. A highly multiplexed, robust and sensitive LC-MS/MS-based method was developed for the quantification of PG and its nine phase I&II metabolites in both dog and human plasma. The methods were evaluated and validated comprehensively to support evaluation of PG safety and pharmacokinetics. Species differences in PG drug metabolism at molecular level were further investigated using the LC-MS/MS assay. Some exposure differences in phase-II metabolites of PG were observed in circulation between humans and dogs as was observed a difference in overall exposure, assuming allometric scaling.

Because nephrotoxicity did not occur when PG was administered in an immediate release capsule, it seems unlikely that PG is solely responsible for the renal injury observed in dogs when receiving PG via enteric coated tablets. One hypothesis is that while PG enhances the gastrointestinal permeability of the MEDI7219 peptide, it may also allow other substances to cross the gastrointestinal membrane, such as inflammatory factors and uremic toxins. Leaky gut has been implicated as a source of inflammation in chronic kidney disease in humans[25]. It is possible that enhanced gut permeability by PG in conjunction with a higher sensitivity to compounds requiring GSH-mediated detoxification may be responsible for the nephrotoxicity observed in dogs receiving PG via enteric coated tablets. Further studies are required to better understand the exact mechanism of PG-induced nephrotoxicity in dogs and the contribution of enteric coating as well as potential species differences. Additionally, non-targeted proteomics, lipidomic and metabolomics approaches can be employed to gain more comprehensive understanding of enteric-coated PG-induced dog renal toxicity.

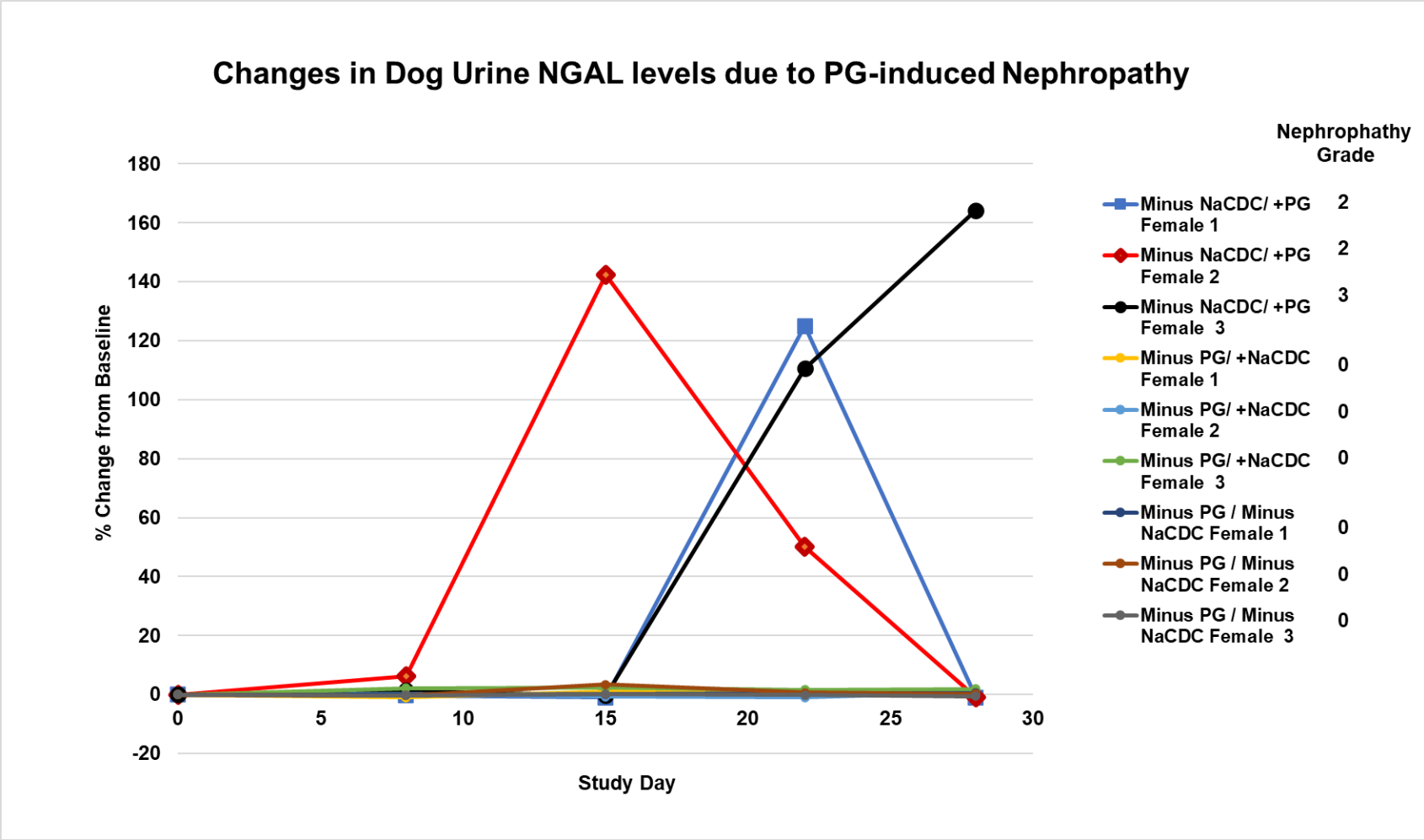

Figure 1. Changes in dog urine NGAL levels due to PG-induced nephropathy. Acute renal injury biomarker - urinary NGAL increased in all animals receiving PG but not in other groups. N = 3 dogs per group. Individual animal profiles are shown.

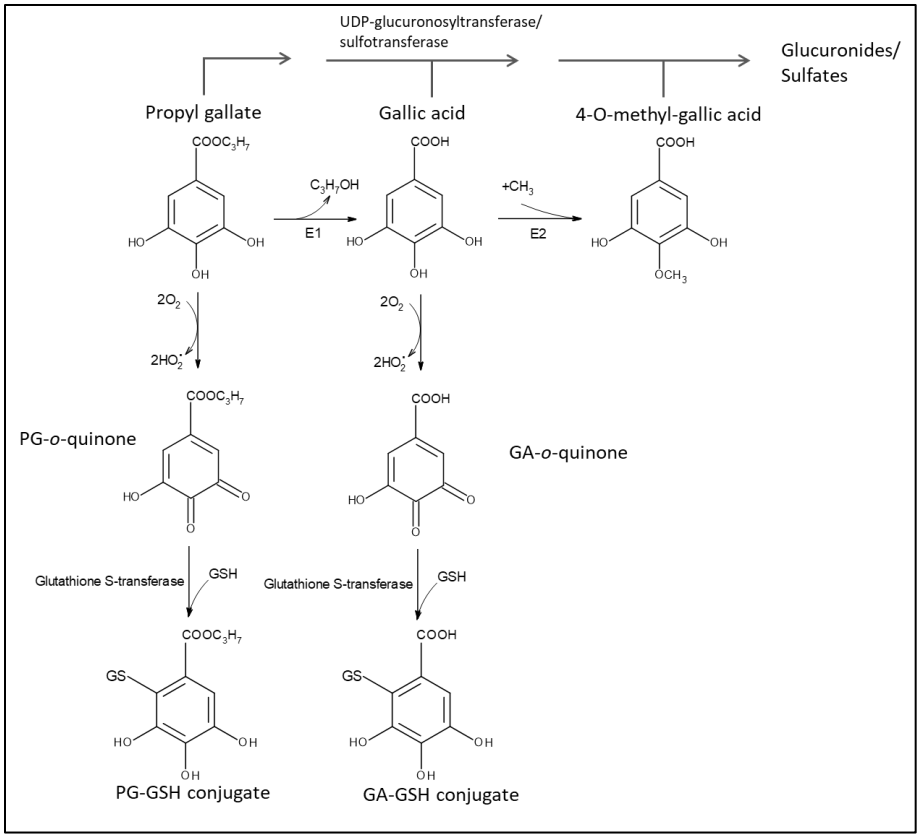

Figure 2. Overview of propyl gallate metabolism. Adapted from Nakagawa et al., 1995; Galati et al., 2006.

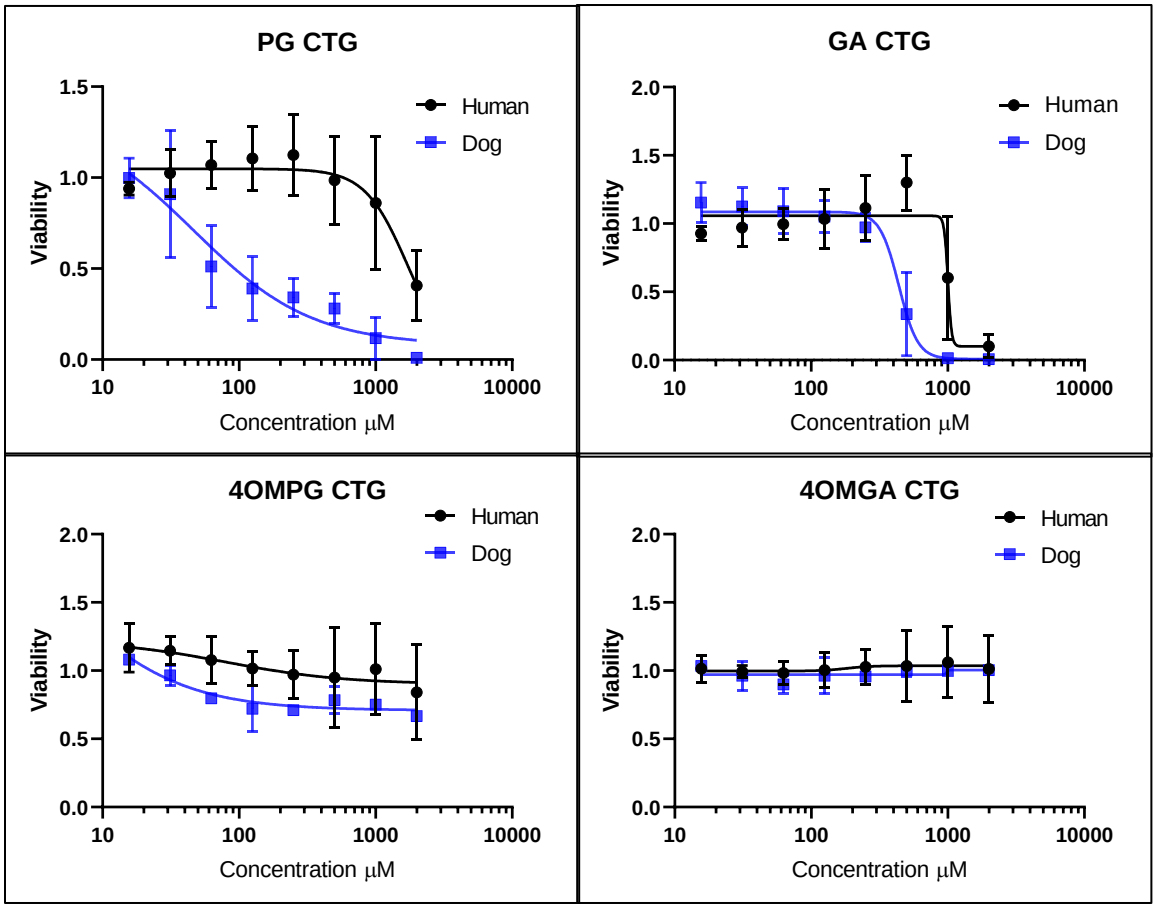

Figure 3. Cytotoxicity estimations in dog and human RPTECs after 48 h exposure to PG, GA, 4-O-methy-propyl gallate (4OMPG), and 4-O-methyl gallic acid (4OMGA). All human RPTEC determinations were from 5 independent experiments (2 different donors). For dog RPTECs PG and GA determinations were from 5 independent experiments, for 4OMPG and 4OMGA determinations were from 3 independent experiments (2 different donors). All experiments had 3 replicates per experiment. 4OMPG is a methylated form of GA and was used as a control compound.

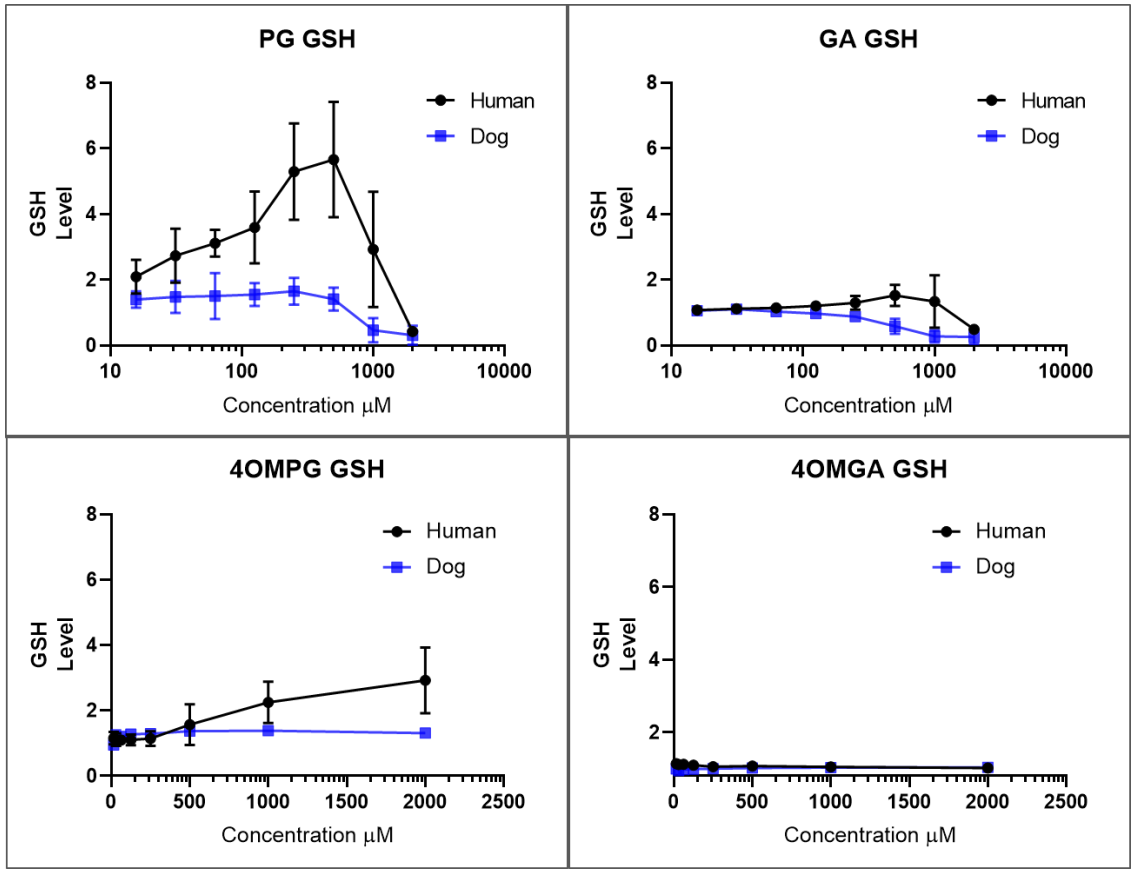

Figure 4. Relative GSH levels in dog and human RPTECs after ~48 h exposure to PG, GA, 4OMPG, and 4OMGA. All human RPTEC determinations were from 5 independent experiments (2 different donors). For dog RPTECs PG and GA determinations were from 5 independent experiments (2 different donors), for 4OMPG and 4OMGA determinations were from respectively 2 and 3 independent experiments (1 donor). All experiments had 3 replicates per experiment. PG induces glutathione (GSH) levels in human

cells, but not in dog cells. All data (luminescence (CPS)) was normalized to DMSO-treated negative control (luminescence (CPS)) for each donor for each experiment.

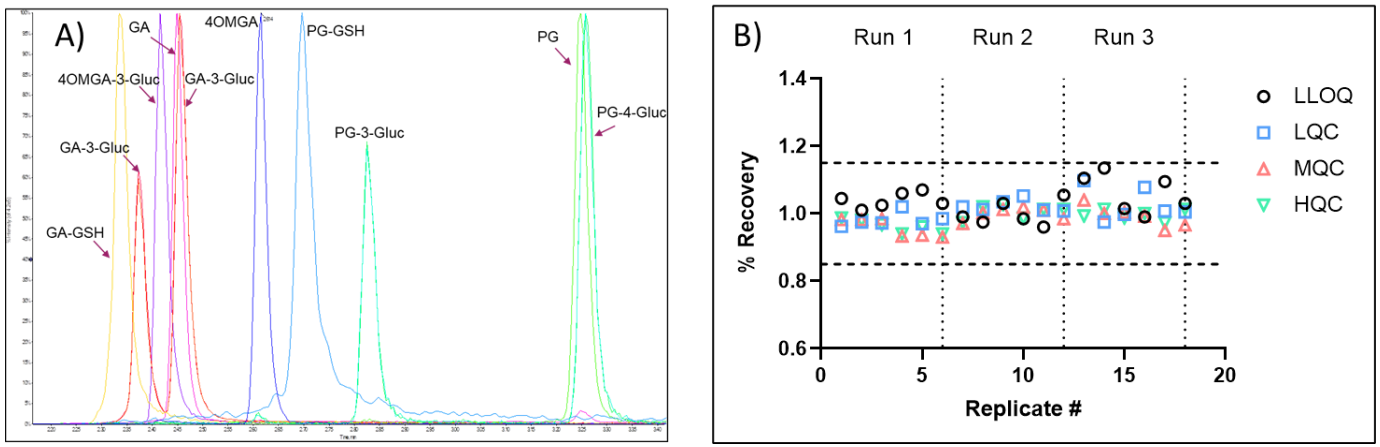

Figure 5. A). A representative chromatogram of PG and its nine phase-I and phase-II metabolites standards in 0.1% formic acid in water. B) PG intra/inter assay quality controls in human plasma demonstrating acceptable assay precision and accuracy. GA and 4OMGA had similar performances.

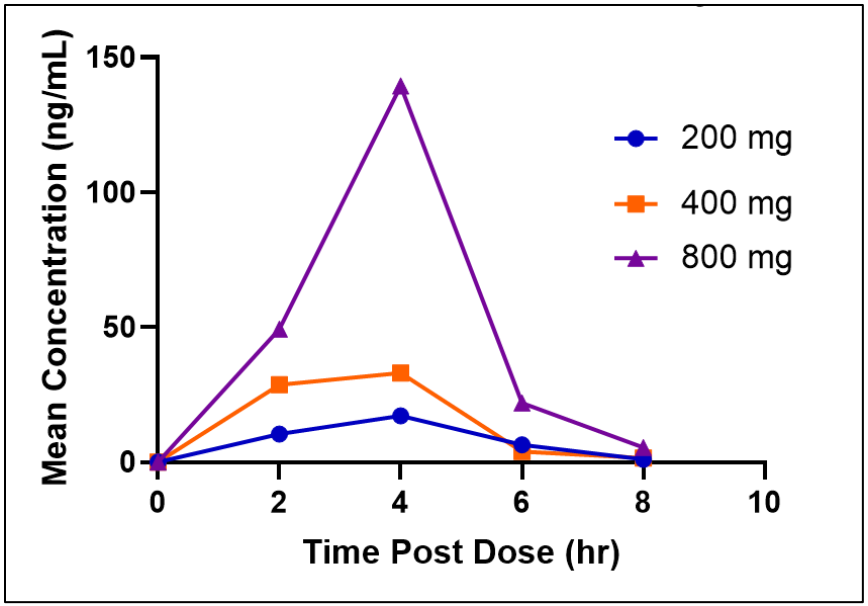

Figure 6. PG pharmacokinetic profile in a single ascending dose clinical study (NCT03362593) with oral administration of enteric coated MEDI7219 tablets, resulting in PG doses of 200, 400, or 800 mg. LLOQ of PG is 0.02 ng/mL. The T_{max} of PG was reached around 3-4 hours post-dose.

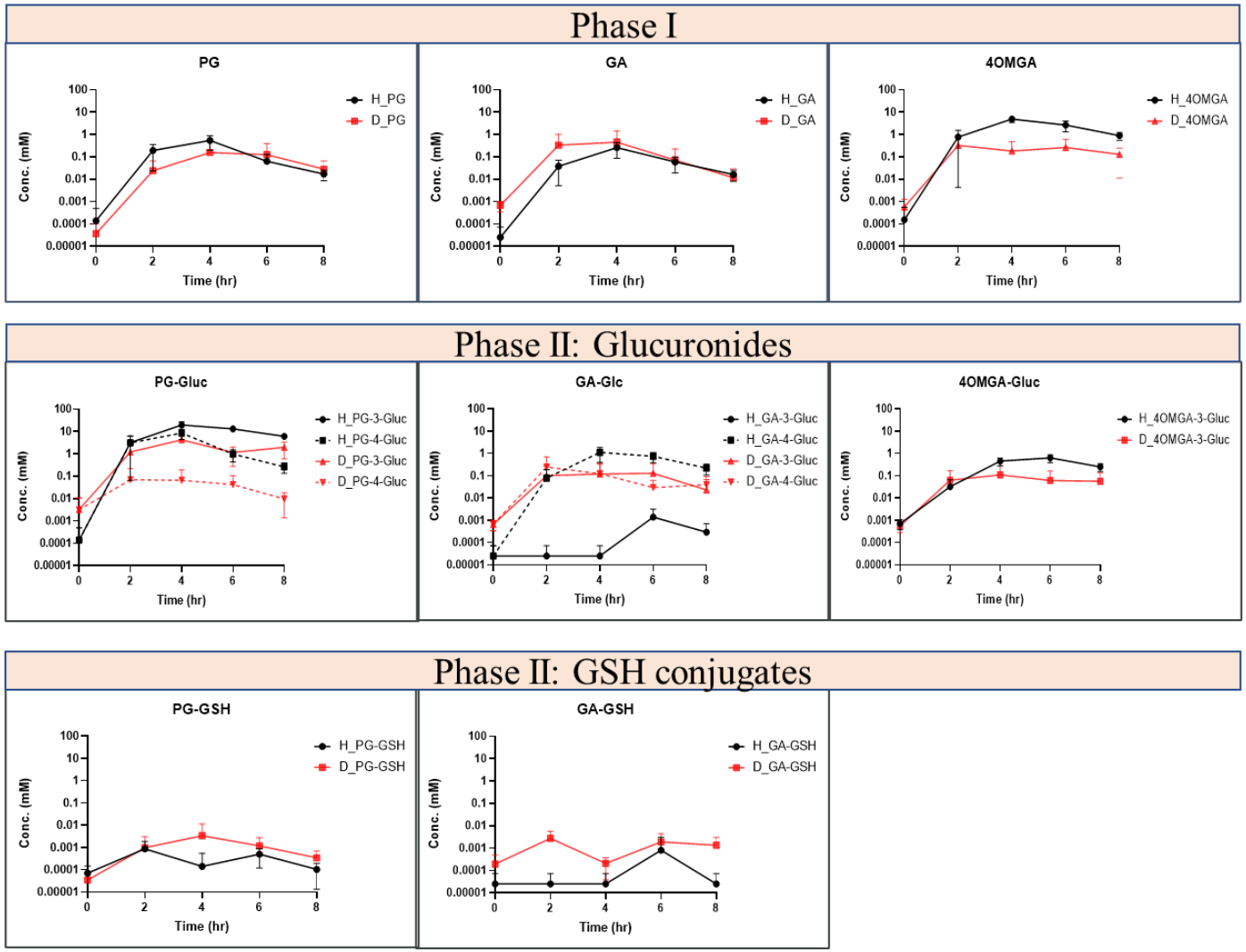

Figure 7. Exposure comparison of PG and phase-I and phase-II metabolites in dog and human plasma. Human (H) data is in black. Dog (D) data is in red. Concentration (mM) is in log10 scale. Data was normalized to PG dose (mg/kg/day). N=8 for human (PG

dosed at 800 mg with MEDI7219 tablets). N=6 for dogs (PG dosed at 200 mg/kg/day). Below LLOQ values were set to ½ LLOQ for the calculation. (Compound abbreviations in figures are summarized in Table 2)

Table 1a. Dosing design of 4-week dog toxicology study							
Group	1	2	3	4	5	6	7
Component A*	+	+	+	-	-	-	-
Component B**	+	-	+	+	-	+	-
Propyl Gallate	+	+	-	+	+	-	-
Table 1b. Key toxicology findings							
# Animals with Nephropathy, bilateral	3/3	3/3	0/3	3/3	3/3	0/3	0/3
Grade 2	1	3	-		2	-	-
Grade 3	2	-	-	2	1	-	-
Grade 4	-	-	-	1	-	-	-

Table 1. Four-week investigative repeat-dose toxicology study with enteric coated excipient tablets in beagles. n=3. Target dose levels of PG was at 200 mg/kg/day. a. Each group received one of 7 different tablet formulations with various excipient combinations of PG and two other components (*Component A is a pH stabilizing reagent. **Component B is a different permeation enhancer in the MEDI7219 formulation). b. Nephropathy toxicity was only found in animals dosed with enteric coated tablets containing PG.

Compound	LLOQ in human plasma (ng/mL)	LLOQ in dog plasma (ng/mL)
Propyl gallate (PG)*	0.02	0.04
Gallic acid (GA)*	1	2
4-O-methyl gallic acid (4OMGA)*	5	3
Propyl gallate-glutathione (PG-GSH)	1	
Gallic acid-glutathione (GA-GSH)	10	
Propyl gallate-3-glucoronide (PG-3-Gluc)	0.2	
Propyl gallate-4-glucoronide (PG-4-Gluc)	0.2	
Gallic acid-3-glucoronide (GA-3-Gluc)	10	
Gallic acid-4-glucoronide (GA-4-Gluc)	10	
4-O-methyl gallic acid-3-glucoronide (4OMGA-3-Gluc)	8	

Table 2. Summary of LLOQ of each analyte. *Assays of these analytes were validated in both human and dog plasma.

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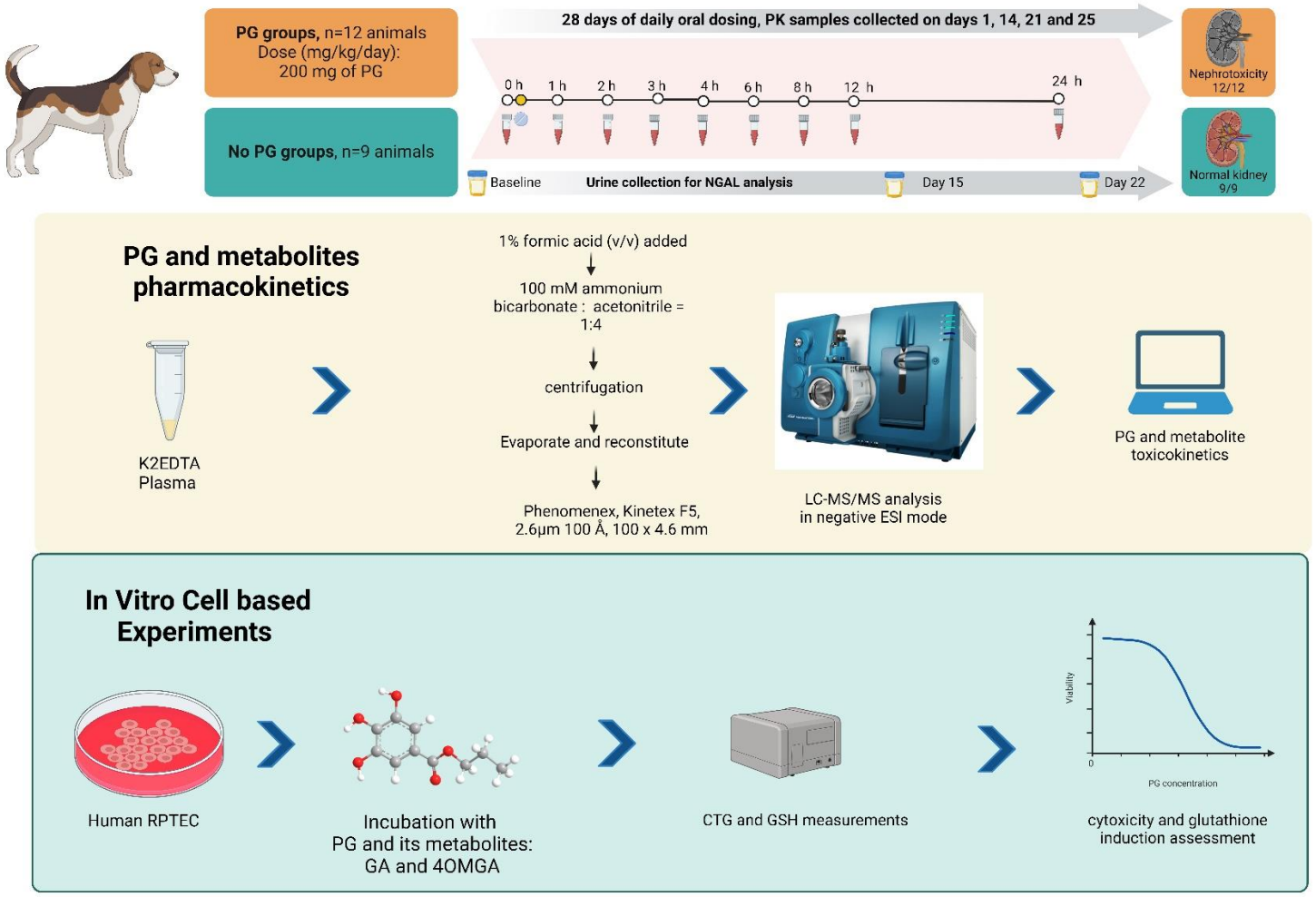
Author contributions:

S.M., B.T.H. and A. I. R. contributed to research conception. S. M., J Y, YH conducted experiments. all authors contributed to manuscript writing. Analysis: SM, AIR, JY, YH. Methodology: SM, JY, YH. Supervision: BTH, AIR, RF, LKR and ML. Funding acquisition: BTH and AIR.

Graphical Abstract:



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