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Assessment of Oral Toxicity and Safety of Pentamethylchromanol (PMCol), A Potential Chemopreventative Agent, in Rats and Dogs

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Abstract

2,2,5,7,8-pentamethyl-6-chromanol (PMCol) was administered by gavage in rats for 28 days at dose levels of 0, 100, 500, and 2000 mg/kg/day. PMCol administration induced decreases in body weight gains and food consumption, hepatotoxicity (increased TBILI, ALB, ALT, TP; increased relative liver weights; increased T4 and TSH), nephrotoxicity (increased BUN and BUN/CREAT, histopathology lesions), effect on lipid metabolism (increased CHOL), anemia, increase in WBC counts (total and differential), coagulation (FBGN↑ and PT↓) and hyperkeratosis of the nonglandular stomach in the 2000 mg/kg/day dose group (in one or both sexes). In the 500 mg/kg/day dose group, toxicity was seen to a lesser extent. In the 100 mg/kg/day dose group, only increased CHOL (females) was observed. To assess the toxicity of PMCol in male dogs it was administered orally by capsule administration for 28 days at dose levels of 0, 50, 200 and 800 mg/kg/day (4 male dogs/dose group). PMCol treatment at 800 mg/kg/day resulted in pronounced toxicity to the male dogs. Target organs of toxicity were liver and thymus. Treatment at 200 mg/kg/day resulted in toxicity consistent with slight adverse effect on the liver only. The results of the safety pharmacology study indicate that doses of 0, 50, 200 and 800 mg/kg administered orally did not have an effect on the QT interval, blood pressures and body temperatures following dosing over a 24-hour recording period. Under the conditions of this study, the no-observed-adverse effect level (NOAEL) for daily oral administration of PMCol by gavage for 28 days to male rats was 100 mg/kg/day and 50 mg/kg in male dogs. In female rats, the NOAEL was not established due to statistically significant and biologically meaningful increases in CHOL level seen in the 100 mg/kg/day dose group. The results of these studies indicated that administration of PMCol at higher dose levels resulted in severe toxicity in dogs and moderate toxicity in rats, however, administration at lower levels is considered to be less likely to result in toxicity following 28 days of exposure. Sex-related differences were seen in rats. Male rats appeared to have greater

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sensitivity to nephrotoxicity, while female animals had a greater incidence of hepatotoxicity and changes in hematological parameters evaluated, especially at a dose of 500 mg/kg/day, which correlated to the higher plasma drug levels in female rats. It appeared that dogs were generally more sensitive than rats to oral administration of PMCol. Further examination of the potential toxic effects of PMCol in longer term studies is required prior to understanding the full risks of PMCol administration as a chemopreventative agent.

Keywords

chemoprevention; vitamin E; pentamethylchromanol; toxicity; oral; rat; beagle dog

I. INTRODUCTION

The prevention of cancer by chemopreventive agents is preferable to the use of chemotherapy against the established disease. Antioxidants, such as vitamin E, are being investigated for efficacy in cancer prevention. There are indications that it may be efficacious against bladder (Liang *et al*, 2008) and mammary cancer (Shet *et al*, 2008; Sharhar *et al*, 2008; Suh *et al*, 2007). Vitamin E consists of a head (chroman ring) which carries the active antioxidant group, and a phytol tail. Chromanol-type compounds act as an antioxidant in biological systems by reduction of oxygen-centered radicals (Gregor *et al*, 2005, Tyurina *et al*, 1995). The chroman ring (Figure 1), 2,2,5,7,8-pentamethyl-6-chromanol (PMCol), has shown antiandrogen activity in prostate carcinoma cells and is thus considered a potent chemopreventive agent of androgen dependent cancers such as prostate cancer (Thompson and Wilding, 2003). An advantage of PMCol over vitamin E as a chemopreventative agent is its water soluble properties, as well as purported androgen antagonist activity. Absence of the phytol chain confers a marked increase in water solubility of PMCol (Thompson and Wilding, 2003). This provides a major advantage in the evaluation of its chemopreventative effects, given that *in vitro* work in cellular suspensions and in growth plates requires the test agents to be in solution. In the presence of PMCol, the androgen-stimulated biphasic growth curve of LNCaP human prostate carcinoma cells was shifted to the right, indicating a reduction in cancer cell growth. Authors reported that the PMCol-induced growth shift was similar to that produced by treatment with the pure antiandrogen bicalutamide (i.e., Casodex), indicative of androgen receptor (AR) antagonist activity. The concentration of PMCol which provides AR antagonist activity is lower than that which shifts the LNCaP cell growth curves. Additional evidence of the antioxidant activity of PMCol is the protection of erythrocytes from hemolysis when exposed to the free radical generator (Koga *et al*, 1998). Results of further testing indicate that PMCol seems to be slightly anchored within membranes because of the lack of a hydrophobic side chain (Koga *et al*, 1998). Treatment of human hepatocytes with 2, 20, 100, or 200 μ M PMCol resulted in no significant induction responses of *CYP1A2*, *CYP2B6*, and *CYP3A4* enzymes (Jackson *et al*, 2008). The safety of vitamin E has been previously investigated and the toxicity to several organ systems identified at high doses in rats (Abdo *et al*, 1986). The safety of PMCol had not been previously established, which is a necessary step in the progression of this compound for use as a potential chemopreventative agent.

II. ANIMALS, MATERIALS AND METHODS

Test Article

Pentamethylchromanol (2,2,5,7,8-Pentamethyl-6-chromanol, PMCol), a white powder, was supplied by the DCP Repository c/o Fisher BioServices, Germantown, MD. In all studies, the purity was determined by HPLC prior to the initiation of dosing and after completion of the in-life phase which confirmed test article stability over the course of the study.

Preparation and Analysis of Test Substances

In the studies in rats, test article formulations were prepared in 1% aqueous methylcellulose (vehicle). The test article formulations were tested for concentration prior to being used for administration. Stability and homogeneity of the formulations were demonstrated for at least 11 days at the concentrations administered. In the dog studies, neat test article was administered orally in gelatin capsules.

Test System

In the four week oral toxicity/FOB study, 103 male and 103 female Crl:CD (SD) 8 weeks old Virus Antibody Free (VAF) rats were obtained from Charles River Breeding Laboratories (Portage, MI). For the 4-week oral toxicity study in dogs, eighteen male Beagle dogs were received from Marshall Farms (North Rose, NY). The dogs were 5–6 months old and weighed 4.8 – 6.5 kg upon initiation of treatment. In the cardiac safety pharmacology study, four male Beagle dogs were received from Marshall Farms (North Rose, NY). The dogs had an implantable battery operated telemetry device from Data Sciences International (DSI, St. Paul, MN) implanted following release from quarantine at UIC. The device was positioned subcutaneously in the dogs with one lead catheter placed on the apex of the heart and another embedded in the left pectoral muscle for measuring heart rate and electrocardiogram parameters. A fluid-filled cannula with an attached pressure transducer was positioned in the abdominal aorta for measurement of arterial blood pressure. At dosing initiation, the animals were approximately 2 years old and weighed 6.5 – 9.4 kg.

Animals were housed in the UIC AAALAC Intl.-accredited animal facility and their use was approved by the facility Institutional Animal Care and Use Committee. Each rat was singly housed in microisolator polycarbonate cages in a temperature and humidity controlled room on a 14 hour light/10 hour dark cycle. Certified Rodent Chow No. 5002 (PMI Feeds Inc., St. Louis, MO), meal form, was provided *ad libitum* from arrival until termination. Autoclaved tap water in water bottles was provided *ad libitum*. Dogs were singly housed in runs in a temperature and humidity controlled room with a 12 hour light/12 hour dark cycle. Dogs were fed Harlan Teklad Certified Diet No. 2025C (Harlan Teklad, Madison, WI).

Body weights were recorded at randomization, on day 1 for the respective stagger-start, once a week thereafter, and at necropsy. All animals were observed once daily for clinical signs of toxicity, approximately 1 – 2 hours after dosing. All animals were subjected to a physical examination in week -1, day 1 and once a week thereafter. Food consumption for all rats was measured once a week commencing in week -1. Food consumption for all dogs was measured over an approximate 24-hour period once a week.

Clinical Pathology

Blood samples for clinical chemistry, hematology and coagulation evaluations were collected from fasted dogs and non-fasted rats. Hematology parameters were measured using an Advia 120 Hematology Analyzer (Bayer HealthCare, Tarrytown, NY) by using standardized methods. Tests included Hemoglobin (HGB), Hematocrit (HCT), Erythrocyte Count (RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Leukocyte Count (WBC), Platelet Count (PLT), Reticulocyte Count (RETIC), Leukocyte Differential Count, Nucleated RBCs, and RBC Morphology. The following coagulation parameters were measured on an MLA 900 coagulation machine: Activated Partial Thromboplastin Time (APTT); Prothrombin Time (PT); Fibrinogen. The following clinical chemistry parameters were measured using a Hitachi 912 Clinical Chemistry Analyzer using standardized methods: Glucose, Urea Nitrogen (BUN), Inorganic Phosphorus (IP), Creatinine (CREAT), Total Protein (TP), Albumin (ALB), Calcium (CA), Aspartate Aminotransferase (AST/GOT), Alanine

Aminotransferase (ALT/GPT), Alkaline Phosphatase (ALP), Total Bilirubin (TBILI), Creatine Kinase (CK), sodium, potassium, and chloride. Assays for Follicle-Stimulating Hormone (FSH), Thyroid-stimulating Hormone (TSH), Prolactin (PROL), Thyroxine (T4) and Triiodothyroxine (T3) performed using species-specific RadioImmunoassays (RIA) by Doug Neptune of Antech Diagnostics GLP (Morrisville, NC).

Plasma Drug Levels

Plasma drug level analysis was performed at different facilities for the rat and dog studies, using similar methodologies. Samples and standards for rat and dog plasma drug level analysis were assayed using an Aquasil C18 100 × 2 mm size LC column equipped with a Security Guard C18 guard column. A 10 µL injection was assayed under a gradient profile at a constant flow rate of 400 µL/min. Mobile phase A consisted of 5 mM ammonium acetate with 0.1% TFA while mobile phase B consisted of acetonitrile with 0.1% TFA. The gradient profile began at 10% B for 0.5 minutes and was increased linearly to 90% B over 6.5 minutes. After a 1 minute hold at 90% B, the mobile phase composition was changed in a step to 10% B and reequilibrated for 2.9 minutes before the next injection. Detection for rat plasma samples was performed using an Applied Biosystems 4000 QTRAP LC-MS/MS (Forest City, CA) equipped with a TurboIon spray source operating at 450 °C at 5kV in the positive ion mode for the rat plasma samples. Quantitation of the parent compound in dog plasma samples was achieved using a Waters 2795 HPLC Separations Module with Micromass Quattro microTM API Triple Quadrupole Mass Spectrometer (Milford, MA) and MassLynx Software (version 4.1) in the mixed reaction mode (MRM) using the following m/z ion transitions: 221.3 – 165.3 for PMCol and 177.1 – 105.1 for the internal standard (4-methylumbelliferone). Calculations were performed using Analyst software (Version 1.4.1).

EXPERIMENTAL DESIGNS

RAT TOXICITY STUDIES: The 28-day oral dose study in rats was performed to assess general toxicity of PMCol in rats (n=20/sex/dose group) at doses of 0, 100, 500, and 2000 mg/kg following daily gavage administration. The animals were randomized by sex into one of the four dose groups using a computer-generated randomization procedure on the basis of body weight. Additionally, 5 animals per sex were not dosed and used only for hormone analysis. The study consisted of two subsets of animals. Subset 1 was used for measurement of clinical pathology and plasma drug levels analyses and subset 2 was used for measurement of Functional Observational Battery (FOB).

The test article was administered once daily by gavage at a dosing volume of 10 mL/kg/day. Control animals were dosed with the test article vehicle (1% aqueous methylcellulose) at the same dosing volume. The sham animals were not dosed. All rats in the FOB-tested subgroups were evaluated in a FOB at baseline and in week four, 100 to 140 minutes after PMCol administration. The following parameters were assessed: general appearance, home cage behavior (e.g., tremors, posture, convulsions), small novel environment behavior (e.g., rearing, sniffing, head searching, stereotypy, elimination, grooming), arousal/anxiety when handled, cranial nerve function (e.g., direct and consensual pupillary light reflex, palpebral reflex), spinal reflexes, proprioception/balance, grip strength, gait coordination and reactivity to sensory stimulation (e.g., visual, auditory, tactile, pain). The FOB parameters were determined in an open field test over a 2 minute observation period. Supported rears were determined by counting the number of times the rats raise their front paws off the ground and use the side of the containment box to support themselves. Freezing time was measured as the total length of freezing episodes in seconds. The open field containment box was divided into squares (7 cm × 7 cm) and a count was made for each time a rat passed into an adjacent square. Latency time was assessed by measuring the amount of time a rat took to leave the middle square of the box upon being placed in the box. Blood samples

were collected in week 4 for measurement of clinical pathology parameters, plasma drug levels, and hormone levels. The necropsy procedure included collection of 39 tissues/organs (Table 1). Microscopic findings were collected on all tissues in the control and high dose groups and target organs (kidneys, stomach and esophagus) in the low and mid dose groups. Organ weights were conducted on select tissues (Table 1) and presented as percent of brain weight for statistical comparison.

DOG TOXICITY STUDIES: Dose levels chosen were 0, 50, 200, and 800 mg/kg/day for the 28-day dog toxicity study. Each group contained four male dogs. Animals were dosed once a day using gelatin capsules by oral administration. The control animals received empty gelatin capsules. The specific quantity of administered test article was based each animal's most recent scheduled body weight. Thirty-second to one minute recordings from leads I, II, III, aV_F, aV_L and aV_R were collected during the quarantine/pretest period and in week 4. Analysis of the electrocardiograms (figure 2) included heart rate and rhythm, duration of the P wave, P-R complex interval, QRS complex interval, QT interval, and P wave and QRS complex amplitudes. Hematology, coagulation, and clinical chemistry parameters were measured twice during the quarantine/pretest period and in week 4 for all animals.

Blood samples for toxicokinetic analysis were collected from each dog on day 1 and in week 4 at 0, 1, 2, 3, 4, 6, 9 and 24 hours after dosing. The internal standard storage stability and the long-term stability of plasma samples were determined to be at least 3 weeks. An estimate of free drug concentration was performed on an aliquot of plasma samples using ultrafiltration corrected for non-specific binding on day 1 and week 4 samples with plasma drug levels of at least 100 ng/mL. On day 29, all dogs were sacrificed and necropsied in random order. The necropsy procedure was a thorough and systematic examination and dissection of the animal's viscera and carcass, which included collection of tissues for histopathology (Table 1). Organ weights were conducted on select tissues (Table 1) and presented as percent of brain weight for statistical comparison.

CARDIAC SAFETY PHARMACOLOGY STUDY IN DOGS: The dose levels evaluated in this study were 0, 50, 200, and 800 mg/kg. There were four dogs implanted with telemetry devices, and each dog received a single dose at each dose level, following a Latin-square design. This design allowed a final total of four dogs in each dose group. There was a one-week washout period between dose administrations. Animals were monitored via telemetrized implants for at least one hour prior to and 24 hours following each dose. Dogs were simultaneously monitored for systolic, diastolic and mean arterial blood pressures, body temperature, and electrocardiogram including heart rate, P-R interval, QRS interval, QT interval, QTc interval corrected for heart rate using Fridericia's formula (Fridericia, 1920), and RR interval during the 24 hour monitoring period. The data were collected and analyzed using the Ponemah Physiology Platform system (version 4.70).

Statistical Analyses

For each sex, one-way analysis of variation tests were conducted on body weight, rat food consumption, blood pressure, ECG measurements, body temperature, hematology, clinical chemistry, coagulation, urinalysis (pH and specific gravity) and organ weight data. Organ weight analysis considered organ weights relative to brain weights. If a significant F ratio was obtained ($p \leq 0.05$), Dunnett's t test was used for pair-wise comparisons to the control group. Statistical analysis of the FOB measurements for all parametric, not-ordinal data (i.e., # grooming episodes, # of squares, # fecal boluses, # urine spots, # sniffing movements, # head searches, etc.) was performed using analysis of variance, with post-hoc comparison made using Dunnett's test. For all non-parametric, ordinal data (i.e., tremors, vocalizations,

diarrhea, etc.) the Kruskal-Wallis test was performed with post-hoc comparisons made using the Mann-Whitney U test. In addition, freezing time, supported rears, # squares, and latency time were analyzed for normality using the D'Agostino-Pearson omnibus K2 normality test (GraphPad Prism, version 5.02, La Jolla, CA). The normality test was followed by non-parametric analysis using the Kruskal-Wallis test and Dunn's post comparison test. A minimum of significance level of $p = 0.05$ was used for all comparisons to the control group. Dog food consumption data was analyzed by the Kruskal-Wallis test ($p < 0.05$). Differences in plasma drug levels between males and females within each group were performed using Bonferroni's Multiple Comparison Test. The hormone analysis data were analyzed for normality (Kolmogorov-Smirnov) followed by an ANOVA ($p < 0.05$) and, if significant, a comparison of groups by the Kolm-Sidak test. If the test for normality failed, the ANOVA was based on Kruskal-Wallis ANOVA on Ranks ($p < 0.05$) and, if significant, Dunnett's comparison of groups. Values below the limit of detection were converted to one half ($1/2$) of the limit for statistical evaluation including the calculation of Mean and standard deviation. Values presented throughout this manuscript are in mean $\pm$ standard deviation.

III. RESULTS

RAT TOXICITY STUDIES

An initial pilot study in rats at doses of 0, 50, 150, 500 and 2000 mg/kg/day (5/sex/group) was conducted to assess toxicity following 14 days of daily oral (gavage) administration of PMCol. Decreases in body weight gains and food consumption, increases in BUN (confirmed by mild nephropathy), RETIC and in APTT were seen in the 2000 mg/kg/day group. Relative liver weights were significantly increased in the 500 and 2000 mg/kg/day dose groups. Pituitary gland weights were significantly decreased in all test article-treated groups; decreases were most pronounced in the 2000 mg/kg/day dose group. Due to a significant decrease in pituitary weights observed in the 14-day rat toxicity study, as well as the known changes in thyroid hormones resulting from vitamin E administration at high doses (Abdo *et al.*, 1986), thyroid hormone analysis was conducted in the 28-day toxicity study in rats. The results of this pilot study (not presented in detail) were used to select doses for a toxicity study following 28-days of daily oral (gavage) administration in male and female rats, the results of which are presented in detail in this manuscript.

A summary of test article-related responses is found in Table 2. There were no test article-related clinical signs of toxicity seen at any dose level examined in this study. Body weight gains were statistically significantly decreased in the males in the 2000 mg/kg/day (22 ± 13.8 g) dose group on day 8 when compared to the control group (35 ± 7.4 g). Total body weight gains were also lower in the 2000 mg/kg dose group (100 ± 19.2 g) compared to the control group (111 ± 25.3 g), but not statistically significantly. In the 2000 mg/kg/day dose group females, body weight gains were statistically significantly increased on day 15 (18 ± 6.6 g) when compared to the control group (13 ± 5.6 g). Total body weight gains were not statistically significantly changed in any dose group when compared to the control group animals. In the 2000 mg/kg/day dose group males, food consumption was lower (19 ± 2.9 g) on days 1 – 8 when compared to the control group (22 ± 1.4 g). This decrease in food consumption corresponded to the decrease in body weight gains. There were also statistically significant dose-dependent decreases in food consumption in the 2000 and 500 mg/kg/day female dose groups on days 1 – 8 (13 ± 1.3 g and 14 ± 1.1 g, respectively) when compared to the control group (16 ± 1.5 g). There were no ophthalmic changes in any dose groups administered PMCol or in the control group.

Evaluation of FOB parameters revealed slight excitation, which can be seen in the selected FOB parameters (Supplemental Table 1). This was indicated by a statistically significant increase in supported rears in the 2000 mg/kg/day male dose group (2 ± 1.5) when compared

to the control group (0.5 ± 0.71). This was considered treatment-related due to the fact that other biologically relevant changes in the open field activity in a novel environment were observed, such as a dose-dependent decrease in freezing time. For instance, in the 2000 mg/kg/day dose group males mean latency time and freezing time were decreased and mean squares count was increased, though not statistically significantly. It was proposed that due to the high variability of these parameters that the statistical differences may not have been apparent with parametric statistical analysis. The D'Agostino and Pearson omnibus normality test was used to determine normal distribution of these parameters. Mean latency, mean squares count, and freezing time were found to be not normally distributed; however, supported rears were found to be normally distributed. These parameters were subsequently analyzed using the Kruskal-Wallis test followed by the Dunn's post-hoc test and the results of the parametric analysis were confirmed. No other statistically or biologically relevant changes were seen in the 500 and 100 mg/kg/day dose groups (male and female) or the 2000 mg/kg/day female dose group when compared to the control group.

Biologically relevant changes in serum chemistry values in the liver and kidney were seen in week 4 (Table 3). Hepatotoxicity was seen at 2000 mg/kg/day in the statistically significant increases in mean ALT (66 ± 8.7 IU/L), TBILI, CHOL, and BUN when compared to the control group. Kidney values affected included statistically significant increases in BUN and decreases in IP. In addition, there were dose-dependent increases in ALB, which may be the result of dehydration. There were dose-dependent decreases in ALP levels, which were statistically significant in the 2000 mg/kg/day dose group. Dose-dependent decreases in IP were statistically significant in the 500 and 2000 mg/kg/day dose groups. There was a dose-dependent, though not significant, increase in mean serum TP levels. In general, female rats were more sensitive to PMCol administration than male rats. This was evident by greater changes in the previously mentioned blood chemistry parameters, as well as statistically significant increases in BUN/CREAT ratio levels.

Evaluation of the hematology data revealed apparent hemolytic anemia. This observation included statistically significant decreases in RBC, HGB, and HCT levels as well significant increases in RETICS (Table 3). There was also a dose-dependent increase in mean PLT counts, which was statistically significant in the 2000 mg/kg/day dose group. No significant changes in hematology parameters were seen in the 100 mg/kg/day dose group. Changes were also seen in WBC differentials, including statistically significant increases in MONO and LYMPH (females only) counts. However, these changes in white blood cell differentiation were within normal limits and were not considered to be biologically relevant. However, leukocytosis was present in both male and female rats in the 2000 mg/kg/day dose group. Incidences of hemoglobin concentration variance and hyperchromia were observed in both male and female animals in the mid and high dose groups receiving PMCol, but were not seen in the control group receiving the vehicle.

In males, there was a dose-dependent increase in FBGN, which was statistically significant in the 2000 and 500 mg/kg/day dose groups when compared to the control group. There was a dose-dependent decrease in PT, which was statistically significant in the 2000 and 500 mg/kg/day dose groups when compared to the control group (Table 3). No significant changes in coagulation parameters were seen in the 100 mg/kg/day dose group.

Plasma Hormone Levels—There were no significant changes in any PMCol-treated male dose groups when compared to the control group. In females, there was a dose-dependent increase in T4 concentrations in all dose groups administered PMCol when compared to the 0 mg/kg/day dose group (Figure 3, Panel A). This was statistically significant in the 500 and 2000 mg/kg/day dose groups. There was also a dose-dependent

increase in plasma thyroid-stimulating hormone, which was statistically significant in the 2000 mg/kg/day female dose group (77.5%) when compared to the vehicle control group.

Plasma Drug Levels—In males and female rats, mean plasma drug levels at 2 hr post dosing were increased with an increasing dose of PMCol (Figure 4). In the 100 mg/kg/day male dose group, PMCol could only be determined in one animal (102 ng/mL). In the 500 and 2000 mg/kg/day male dose group, PMCol mean concentrations were 394.0 ± 161.6 ng/mL and 826.8 ± 211.6 ng/mL, respectively. In the 100 mg/kg/day female dose group, PMCol peaks (from 66.3 ng/mL to 190 ng/mL; mean concentration was 101.8 ± 42.3) were determined in seven animals. In the female 500 mg/kg/day dose group, PMCol concentrations ranged from 299 ng/mL to 781 ng/mL and mean concentration was 480.5 ± 178.2 ng/mL. In the 2000 mg/kg/day dose group females, the test article concentrations ranged from 499 ng/mL to 2610 ng/mL and mean concentration was 1247.9 ± 587.4 ng/mL. Mean plasma drug levels in females were higher than in males in the 500 and 2000 mg/kg/day dose groups 2 hours post administration in week 4, which was statistically significant in the 2000 mg/kg/day dose group.

Organ Weights—In males, dose-dependent increases were seen in mean liver weights when corrected by percent of brain weights, which was significant in the 500 ($987 \pm 108\%$) and 2000 ($1174 \pm 110\%$) mg/kg/day dose groups when compared to the control group ($737 \pm 95\%$). Kidney weights were also significantly increased in the 2000 mg/kg/day male dose group ($168 \pm 26\%$) when compared to the control group ($151 \pm 16\%$). In females, dose-dependent increases were seen in mean liver weights, significantly increased in the 500 ($661 \pm 53\%$) and 2000 ($903 \pm 65\%$) mg/kg/day dose groups when compared to the control ($474 \pm 55\%$). In females, the spleen weights were also statistically significantly increased when corrected by percent of brain weight in the 500 ($29.7 \pm 4.9\%$) and 2000 ($33.2 \pm 4.3\%$) mg/kg/day dose groups when compared to the control group ($25.7 \pm 3.7\%$).

Pathology—A summary of the histopathology findings is included in Table 4. In the kidney, there was an increase in the incidence and severity of apoptosis in the tubular cells and an increase in chronic inflammation in the 2000 mg/kg/day male dose group when compared to the male control group. Apoptosis was characterized by the presence in the lumen of cells from the lining of the tubules which had been contracted with condensed chromatin, especially in tubules undergoing regeneration. There was an increase in the incidence and severity of tubular regeneration in the 500 and 2000 mg/kg/day dose group males when compared to the control males. Regeneration was characterized by the presence of increased basophilic cytoplasm with occasional mitotic figures in cells lining the tubules or clusters of tubules within the cortex of the kidney. A total of at least five clusters of tubules lined by basophilic cells were required to be present before a grade of minimal was assigned. A severity of mild was assigned when larger multiples of clusters were obvious at low magnification (4 $\times$ objective). Moderate severity was assigned when there were broad radiating regions of tubules with basophilic cytoplasm.

28-DAY DOG TOXICITY STUDY

Dose levels were chosen based on the results of a previous 14-day oral toxicity study. In that study, four male dogs were administered PMCol, one each at 0, 250, 500 and 2000 mg/kg/day. The high dose of 2000 mg/kg/day was reduced to 1000 mg/kg/day on day 5 due to recurrent vomiting, weight loss and loss of appetite in this dog. This dog was sacrificed moribund on day 13 of the study for humane concerns as a result of the inappetence and progressive weight loss. Biologically relevant changes in clinical chemistry and hematological parameters were observed in the 250, 500 and 2000/1000 mg/kg/day dogs. Due to a significant decrease in pituitary weights observed in the 14-day rat toxicity study,

as well as the known changes in thyroid hormones resulting from vitamin E administration at high doses (Abdo *et al.*, 1986), thyroid hormone analysis was conducted in the 28-day toxicity study in dogs. No histopathological effects were observed at 250 and 500 mg/kg/day dose levels. Histopathological findings that were considered test article-related were limited to the dog dosed at 2000/1000 mg/kg/day. These findings included a small focus of C-cell hyperplasia in the thyroid gland, moderate alveolar edema of the lung, microvesicular fatty changes in midzonal hepatocytes (characterized by the presence of multiple small round vacuoles in the cytoplasm which did not displace the nucleus) and atrophy of centrilobular hepatocytes in the liver, moderate atrophy of the thymus, and an overall decrease in cellularity of the bone marrow. Further evaluation of bone marrow indicated moderately increased total myeloid cell count and myeloid/erythroid ratio compared to the control dog. Lower erythroid counts (relative to elevated myeloid counts) were also noted.

A summary of test article related-findings from the 28-day dosing study in dogs are presented in Table 5. Decreased food consumption was seen in the 800 mg/kg/day dose group, as well as decreased activity, changes in stool consistency, dehydration, diarrhea, emesis, and ocular discharge. Mean body weight gains for the 800 mg/kg/day dose group were generally lower, significantly on day 22, when compared to the control group, which resulted in significantly lower total body weight gains (Supplemental Table 2). There were no significant treatment-related differences in food consumption between PMCol dose groups and the control group, although food consumption in the 800 mg/kg/day dose group was typically decreased during the study when compared to the control group (Supplemental Table 2). This finding was considered treatment-related. There were no treatment-related changes in electrocardiographic parameters or selected organs weighed at necropsy in any dose groups.

Clinical Pathology—There were dose-dependent decreases in serum TP and ALB which were statistically significant in the 800 mg/kg/day dose group (TP=4.1 ± 0.34 g/dL; ALB=2.5 ± 0.50 g/dL) when compared to the control group (TP=5.1 ± 0.38; ALB=3.4±0.29). In the 800 mg/kg/day dose group, there was also a statistically significant decrease in serum calcium levels (9.1%) when compared to the control group.

There was a nonsignificant, dose-dependent increase in serum ALP in all groups administered PMCol when compared to the control group, however only the 200 (200 ± 46 IU/L) and 800 (255 ± 140 IU/L) mg/kg were considered to be biologically relevant increases over the control group (115 ± 18 IU/L). ALT levels were slightly increased in the 800 mg/kg/day dose group (43 ± 19 IU/L) when compared to the control group (27 ± 6.6 IU/L).

In the 800 mg/kg/day dose group, the platelet count (739 ± 250 × 10³/mL) was significantly increased when compared to the control group (366 ± 79.5 × 10³/mL). There were nonsignificant dose-dependent increases in NEUT and LYMPH counts over the control group. There were no other effects of PMCol on clinical pathology parameters.

Hormone Analysis—In week 4, there were dose-dependent decreases in serum triiodothyronine (T3) and thyroxine (T4) levels (Figure 3). Changes in the 800 mg/kg/day dose group were statistically significant; mean group T3 (13.3 ± 11.26 ng/dL) and T4 (0.35 ± 0.0 ng/dL) levels were decreased 78% and 67%, respectively, when compared to the control group (T3 = 69.0 ± 12.73 ng/dL; T4 = 2.10 ± 0.327 ng/dL). In the 800 mg/kg/day dose group, only two dogs had T3 values above detectable limits; all dogs had T4 values below detectable limits. The lower albumin levels may have resulted in lower T4 and TSH levels due to less circulating bound hormones. The TSH levels were not increased as expected with a decrease in thyroid hormones indicating a lack of response. This does not indicate a toxic effect on the pituitary function. All FSH levels were below detectable limits.

at the pretest time-point and only 4 animals had detectable values in week 4. These low levels were probably due to the young age of the dogs and pituitary toxicity was indeterminate. There were no significant changes in prolactin levels.

Histopathology—A summary of histopathology findings can be found in Table 6. In the 800 mg/kg/day dose group, lesions were found in the liver and thymus. In the liver, there was an increase in incidence of microvesicular vacuolation of midzonal to periportal hepatocytes when compared to the control group. In the thymus, there were increases in incidence of atrophy when compared to the control group, which varied from minimal to moderate. There were no histologic test article-related findings in the 50 or 200 mg/kg/day dose groups. There were no differences in bone marrow cellularity or differential cell counts in any dose groups.

Plasma Drug Levels—It was not possible to estimate pharmacokinetic parameters in this study, due to the fact that the majority of plasma samples collected were below the limit of quantitation (BQL, 10 ng/mL). In the 50 mg/kg/day dose group, PMCol levels were BQL at all time points collected on day 1 and week 4. PMCol levels were only determined in one to three animals at time points ranging from 1 to 6 hours in the 200 mg/kg/day group and from 1 to 8 hours in the 800 mg/kg/day group. However, it appears that mean plasma drug levels were increased with increasing dose of PMCol on day 1. The maximum concentrations of PMCol were observed 3 hours post-dosing. The week 4 data did not allow comparison between groups to be made due to the fact that PMCol was only determined in three animals at low levels near the lower limit of quantitation. Analysis of free (unbound) PMCol in plasma was performed in five samples on day 1 which were found to have the highest PMCol concentrations. *In vivo* protein binding corrected for non-specific binding was established at a level of at least 82.5%. Free PMCol levels were BQL in these samples. The long-term stability of PMCol in the biological matrix and the internal standard at the intended storage conditions (−75 to −86 °C) was evaluated, and determined to be stable for the duration of the study sample storage.

CARDIAC SAFETY PHARMACOLOGY DOG STUDY

There was no mortality or clinical signs of toxicity at the dose levels evaluated in this study. There were no significant dose-related changes in body weights, food consumption or body temperature parameters. Systolic, diastolic, and mean arterial pressures were monitored in the dogs for 1 hour prior to dosing and over a 24-hour period following each dose. For a one-hour period prior to dosing and for 24-hours following dosing, ECG waveforms were recorded continuously. The intervals presented were calculated for each measured heart beat and then averaged over 10 minute periods and compared statistically to the control animals at each period. These data were evaluated qualitatively and quantitatively for changes in specific intervals (PR, QRS interval, QT interval, QTc interval, and RR interval); along with changes in heart rate. Fridericia's QT correction factor, QTcf, (Fridericia, 1920) was utilized in this study to adjust the QT interval for changes in heart rate. There were no changes in ECG intervals or blood pressure values at the doses evaluated in this study.

IV. DISCUSSION

Antioxidants, such as vitamin E, are being investigated for efficacy in cancer prevention. There are indications that it may have efficacy against bladder (Liang *et al*, 2008) and mammary cancer (Shet *et al*, 2008; Sharhar *et al*, 2008; Suh *et al*, 2007). The studies presented in this manuscript were carried out to determine the toxicity profile of PMCol in rats and dogs. In general, toxicity in rats was greater than that seen in dogs in the studies performed, which was apparently due to the lower dose in dogs on mg/m² basis in addition

to apparent lower bioavailability in dogs. Mild hepatotoxicity at higher dose levels was observed in rats. Hepatotoxicity of PMCol was exhibited in both male (TBILI, ALB, and ALT levels were significantly elevated at 2000 mg/kg/day) and female (TBILI, ALB, and TP levels were significantly elevated at 500 and 2000 mg/kg/day) rats. However, the apparent hepatotoxicity observed in the clinical pathology parameters evaluated did not cause concomittant changes in liver morphology. In studies conducted by other investigators on vitamin E, rats had increased lipid content in the liver (Wheldon *et al*, 1983). Body weight gains were significantly decreased on day 8 in the 2000 mg/kg/day dose group males, which was due to significantly decreased food consumption during the first week of dosing in rats receiving 2000 mg/kg/day. These findings are likely due to the gastroirritation observed following histopathological evaluation of the rats receiving 2000 mg/kg/day, and may not be related to the mild hepatotoxicity observed. In male rats, administration of PMCol caused a dose-dependent decrease in prothrombin time (PT), which was significant in the 500 and 2000 mg/kg/day dose groups. Fibrinogen concentrations were also dose-dependently increased, significantly in the 500 and 2000 mg/kg/day dose group males. In females, PT was dose-dependently decreased, significantly in the 100 mg/kg/day dose group. Thus PMCol appears to influence glycogen and protein (e.g., albumin and fibrinogen) synthesis metabolic pathways in the liver, likely a result of the mild hepatotoxicity.

Nephrotoxicity was also observed at high dose administration of PMCol in rats. In the 2000 mg/kg/day dose group, significant increases in BUN levels (males and females) and in BUN/CREA ratio (females) were seen. Significant decreases in IP concentrations were observed in the 500 and 2000 mg/kg/day males, likely due to decreased reabsorption in the renal tubules. These evident changes in kidney function were confirmed by histopathology. Microscopic evaluation of the kidneys revealed apoptosis and tubular regeneration.

Significant hematological changes were noted in the rats at doses above 500 mg/kg/day, including statistically significant decreases in RBC counts, as well as HBG and HCT levels. These decreases appear to be the result of RBC destruction (i.e., hemolytic anemia), however this cannot be confirmed with these data obtained in this study. There was no evidence of any effect on hematopoiesis in the bone marrow sections evaluated or evidence of extramedullary hematopoiesis in the spleen sections evaluated histopathologically. Abdo *et al*, reported that following 2000 mg/kg/day dietary administration rats showed hemorrhagic diatheses, which resulted in similar decreases in hematological values.

PMCol treatment at 800 mg/kg/day resulted in statistically significant decreases in body weights and body weight gains in dogs, likely due to decreases in food consumption. At 800 mg/kg, clinical chemistry changes including decreased levels of total protein (due to hypoalbuminemia) were seen. Biologically relevant changes consistent with slight toxic injury of the liver were seen in week 4, as increased levels of ALP and ALT. Statistically significant decreases in triiodothyronine (T3) and thyroxine (T4) were seen in dogs dosed at 800 mg/kg/day, which were likely due to liver clearance and decreased albumin concentrations, rather than pituitary function since the thyroid-stimulating hormone levels were not increased. The free forms of the T4 were below the limits of detection, but still may be of sufficient quantity to sustain thyroid-pituitary balance and not stimulate a response by the pituitary. If there were any toxic effects on the pituitary, they were masked by the hepatotoxicity and decreased kidney function in the dogs, as well as the decreased nutritional status of the dogs as seen in the decreased food consumption. Lack of histopathological findings in the pituitary also reduces the potential of direct toxicity. This is an advantage over vitamin E administration, in which significant increases in TSH were observed at doses as low as 125 mg/kg/day following 90 days of administration (Abdo *et al*, 1986). It should however be noted that if the animals were continued on PMCol treatment for a longer period of time, effects on TSH may eventually develop as a result of the effects

on T4. Treatment-related histopathological findings correlated to clinical chemistry changes were limited to the dogs dosed at 800 mg/kg/day. Histopathology lesions included a microvesicular vacuolation of midzonal to periportal hepatocytes and atrophy of the thymus, which was considered normal due to the age of these dogs.

There were significant differences noted in the toxicity profiles between dogs and rats. For example, plasma hormone levels (T4 and TSH) were increased in rats whereas in dogs T4 and T3 were significantly decreased in the 800 mg/kg dose group (Figure 3). Histopathological changes in dogs and rats also differed in that there were no lesions in the liver of rats, but dogs had histopathological changes in the liver consistent with mild toxicity related to administration of PMCol at 800 mg/kg/day.

Analysis of plasma drug levels indicated there was a large difference in peak plasma levels between dogs and rats at the doses administered in the studies (Figure 4, Panel B). At 2 hours post-dosing in week 4, plasma levels in rats were approximately 400 ng/mL (500 mg/kg/day) or greater, where dogs were mostly below 10 ng/mL at a dose of 800 mg/kg/day at this same time point. This is probably due to a lower bioavailability in dogs versus rats or possible differences in metabolism; however, differences in the preparation of the dosage formulation between the rats and dogs could play a role. The majority of PMCol in dogs appeared to be protein bound, which likely played a role in the lower toxicity observed in dogs. Results of this analysis indicate that the free fraction of PMCol was determined to be at less than 15% of the total circulating PMCol. However, this was based on a limited number of samples. PMCol is rapidly metabolized, as multiple peaks were observed in the LC/MS/MS chromatograms in addition to the parent compound during the analyses of both rat and dog plasma samples. When comparing dose administered to body weight (mg/kg), dogs were more sensitive than rats. This can be seen by the results of the 14-day toxicity study in dogs in which a dose of 2000 mg/kg resulted in moribund sacrifice of this dog. This was following a reduction in the dose to 1000 mg/kg after four days of administration at 2000 mg/kg. However, when an adjustment is made for relative body surface area to the dose levels (see Table 2 and 3), it appears that rats were more sensitive than dogs. This is likely due to the increased levels of PMCol parent compound in circulation exhibited by rats over dogs; however, differences in absorption, metabolism, and distribution may also have a role in the differences observed in toxicity between dogs and rats.

The results of these studies indicated that administration of PMCol at higher dose levels resulted in severe toxicity in dogs and moderate toxicity in rats, however, administration at lower levels is considered to be less likely to result in toxicity following 28 days of exposure. Due to the low levels of drug detected in the plasma of dogs, an unequivocal determination of toxicity as it relates to exposure cannot be made. However, even at these low exposure levels toxicity is evident in dogs at the higher dose level. In addition, obvious metabolite formation observed during plasma analysis confirmed exposure. It was not possible to identify and measure the metabolites within the scope of this study. Sex-related differences were seen in rats. Male rats appeared to have greater sensitivity to nephrotoxicity, while female animals had a greater incidence of hepatotoxicity and changes in hematological parameters evaluated, especially at a dose of 500 mg/kg/day, which was likely due to the higher plasma drug levels in female rats. It appeared that dogs were generally more sensitive than rats to oral administration of PMCol. Further examination of the potential toxic effects of PMCol in longer term studies is required prior to understanding the full risks of PMCol administration as a chemopreventative agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Pentamethylchromanol (PMCol) chemical structure

Figure 2.
A diagram illustrating the intervals of a single waveform collected during an electrocardiogram.

Panel A. T4 and TSH in Female Rats

Panel B. T3 and T4 in Male Dogs

Figure 3.
Mean serum hormone levels ($\pm$ SD) altered by PMCol following 28-days of daily administration in rats and dogs.

	100 mg/kg/day		500 mg/kg/day		2000 mg/kg/day	
	Mean	SD	Mean	SD	Mean	SD
Males (ng/mL)	102	± 0	394	± 162	827	± 212
Females (ng/mL)	102	± 42	481	± 178	1248	± 588 *

* Statistically significant ($p < 0.05$) when compared to corresponding male dose group

Figure 4.
Comparison of mean plasma concentrations ($\pm$ SD) between male and female rats at 2 hours post-dose following 28 days of daily administration.

Table 1

Tissues collected for histopathology and organ weights in the four week rat and dog toxicity studies

Abnormal lymph nodes
*Adrenal glands
Aorta (thoracic)
*Brain (fore-, mid-, hind-)
Cecum
Colon
Duodenum
Epididymides
Esophagus
Eyes
Femur (with marrow)
Gross lesions
*Heart
Ileum
Jejunum
*Kidneys
*Liver
Lungs/Bronchi (infused with formalin)
Lymph node (mesenteric)
Mammary gland
*Ovaries/Fallopian tubes
Pancreas
*Pituitary
Prostate
Salivary gland (mandibular)
Sciatic nerve
Seminal vesicles ¹
Skeletal muscle
Skin
Spinal cord (cervical and thoracolumbar segments)
*Spleen
Sternum (with marrow)
Stomach
*Testes
Thymus
*Thyroid/parathyroids
*Tissue masses
Tongue ²

Tonsils²

Trachea

Ureters²

Urinary bladder

Uterus (horns and cervix)

Vagina

* Organs weighed at scheduled necropsy

¹ Only collected in rat study.

² Only collected in dog study.

Overview of test article-related observations following 28 days of oral administration of PMCol in male and female rats.

Table 2

Dose Level	0 mg/kg/day	100 mg/kg/day (600 mg/m ² /l)	500 mg/kg/day (3000 mg/m ²)	2000 mg/kg/day (12,000 mg/m ²)
Number of Animals per sex	20	20	20	20
Clinical Signs	-	NE	NE	NE
Body Weight Gains	-	NE	NE	↓↓ (M)
Food Consumption	-	NE	↓↓ (F)	↑↑
FOB	-	NE	NE	↑↑ SR (M) ↓MLT (M) ↑MSC (M) ↓FC (M)
Clinical Chemistry	-	↑↑CHOL (F)	↑↑TBILI (F) ↑↑CHOL (F) ↑↑ALB (F) ↑↑TP (F)	↑↑TBILI ↑↑ALP ↑↑IP (M) ↑↑CHOL ↑↑BUN ↑↑ALB ↑↑ALT (M) ↑↑TP (F) ↑↑BUN/CREAT (F)
Hematology	-	NE	↑↑RETIC ↓RBC (F) ↓HGB (F) ↓HCT (F) ↓EOS (F)	↑↑RETIC ↓RBC ↓HGB ↓HCT ↓EOS (F) ↑↑LYMPH (F)
RBC Morphology	-	NE	Slight Hyperchromia (F)	Slight Hyperchromia Marked Hyperchromia (M) Marked Hemoglobin Concentration Variance (M)
Coagulation	-	NE	↑↑FBGN (M) ↓PT (M)	↑↑FBGN (M) ↓PT (M)
Plasma Hormone Analysis	-	NE	↑↑T4 (F)	↑↑T4 (F) ↑↑TSH (F)

Dose Level	0 mg/kg/day	100 mg/kg/day (600 mg/m ² /l)	500 mg/kg/day (3000 mg/m ²)	2000 mg/kg/day (12,000 mg/m ²)
Organ Weights	-	NE	↑↑Liver ↑↑Spleen (F)	↑↑Liver ↑↑Kidney (M) ↑↑Spleen (F)
Pathology	-	NE	KIDNEY -Tubular regeneration (M)	KIDNEY -Tubular regeneration (M) -Apoptosis, tubular epithelium (M) -Inflammation, chronic (M) STOMACH -Hyperkeratosis, nonglandular stomach

Increases in serum chemistries are dose dependent and indicate effects on liver and kidneys. Changes in female serum chemistry levels were found to be more sensitive at the lower dose levels, while at higher dose levels there was little difference between sexes. Statistically significant changes in coagulation were seen only in male rats while statistically significant changes in plasma hormone levels were seen in female rats. Changes in kidney histopathology were restricted to male rats but in histopathology did not correlate with hepatotoxicity seen with changes in serum chemistry values.

NE = No Effect

M = Males

F = Females

↑↑ = Statistically Significant Increase*

↓↓ = Statistically Significant Decrease*

↑ = Biologically Relevant Decrease*

FC = Freezing Counts, FT = Freezing Time, MLT = Mean Latency Time, MSC = Mean Square Count, SR = Supported Rears

ALB = Albumin, ALP = Alkaline phosphatase, ALT = Alanine aminotransferase, BUN = Blood Urea Nitrogen, BUN/CREAT = BUN/Creatinine ratio, CHOL = Cholesterol, GLU = Glucose, IP =

Inorganic phosphorus, TBILI = Total bilirubin, TP = Total protein

EOS = Eosinophil count, HCT = Hematocrit, HGB = Hemoglobin, LYMPH = Lymphocyte count, MONO = Monocyte count, PLT = Platelet count, RBC = Erythrocyte count, RETIC = % Reticulocytes, WBC = Leukocyte count

FBGN = Fibrinogen, PT = Prothrombin time, T4 = Thyroxine, TSH = Thyroid-Stimulating Hormone

/ Reference from Table 3 of Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. July 2005. USFDA.

Table 3
Summary of selected clinical pathology parameters following 4 weeks of daily oral administration of PMCol in male and female rats.

Serum Chemistry Values											
Dose (mg/kg)	Sex	ALP (IU/L)	ALT (IU/L)	TBIL (mg/dL)	CHOL (mg/dL)	BUN (mg/dL)	CREA (mg/dL)	IP (mg/dL)	TP (g/dL)	ALB (g/dL)	
0	M	213 ± 38	48 ± 7.7	0.08 ± 0.019	76 ± 18	15.6 ± 1.7	0.25 ± 0.04	9.7 ± 1.6	6.8 ± 0.4	4.4 ± 0.	
	F	140 ± 24	58 ± 8.3	0.07 ± 0.037	72 ± 7.6	15.8 ± 2.3	0.30 ± 0.03	7.7 ± 0.7	7.1 ± 0.5	4.9 ± 0.4	
100	M	204 ± 48	48 ± 12	0.07 ± 0.022	75 ± 12	15.4 ± 1.6	0.25 ± 0.04	8.7 ± 0.5	6.9 ± 0.5	4.5 ± 0.2	
	F	132 ± 22	63 ± 13	0.09 ± 0.021	90 ± 11	15.3 ± 2.1	0.31 ± 0.030	7.8 ± 1.1	7.3 ± 0.3	4.9 ± 0.3	
500	M	180 ± 35	49 ± 8.4	0.09 ± 0.031	99 ± 21	15.4 ± 1.6	0.25 ± 0.04	8.6 ± 0.9*	4.7 ± 0.3	4.7 ± 0.3	
	F	113 ± 33*	58 ± 8.3	0.10 ± 0.013*	108 ± 19	16.4 ± 2.3	0.30 ± 0.04	7.2 ± 0.8	7.6 ± 0.2*	5.2 ± 0.2*	
2000	M	146 ± 20*	66 ± 7.8*	0.18 ± 0.007*	154 ± 30*	19.2 ± 2.1*	0.29 ± 0.04	8.4 ± 0.4*	7.2 ± 0.5	4.8 ± 0.3*	
	F	104 ± 14*	63 ± 13	0.16 ± 0.016*	148 ± 20*	18.7 ± 2.0*	0.29 ± 0.03	7.9 ± 1.5	8.1 ± 0.3*	5.6 ± 0.2*	

Hematology Values											
Dose (mg/kg)	Sex	RBC (×10 ⁶ /μL)	HCT (%)	HGB (g/dL)	PLT (×10 ³ /μL)	RETIC (%)	WBC (×10 ³ /μL)	PT (sec)	PTT (sec)	FBGN (mg/dL)	
0	M	8.2 ± 0.5	47.4 ± 2.1	16.0 ± 0.7	1035 ± 173	2.4 ± 0.3	18.4 ± 2.5	17.0 ± 0.6	17.5 ± 1.6	265 ± 23	
	F	7.9 ± 0.2	45.0 ± 1.6	15.4 ± 0.7	1083 ± 130	2.8 ± 0.6	13.5 ± 2.7	16.5 ± 0.6	14.9 ± 1.4	235 ± 22	
100	M	8.4 ± 0.3	48.5 ± 2.0	16.3 ± 0.6	1046 ± 181	2.4 ± 0.4	18.6 ± 4.1	16.6 ± 0.5	17.4 ± 2.6	292 ± 32	
	F	7.9 ± 0.2	44.8 ± 1.2	15.4 ± 0.4	1138 ± 152	2.5 ± 0.4	13.5 ± 2.7	16.7 ± 0.4	13.4 ± 2.1	245 ± 60	
500	M	8.0 ± 0.3	46.8 ± 2.4	15.6 ± 0.8	1184 ± 135	3.0 ± 0.5*	17.9 ± 5.0	16.1 ± 0.5	17.3 ± 1.4	315 ± 26*	
	F	7.3 ± 0.3*	41.8 ± 1.7*	14.4 ± 0.6*	1117 ± 135	3.6 ± 0.4*	15.0 ± 3.4	16.3 ± 0.3	14.9 ± 2.4	218 ± 33	
2000	M	7.4 ± 0.2*	42.4 ± 2.2*	14.2 ± 2.2*	1340 ± 236*	4.1 ± 1.0*	21.3 ± 4.4	15.3 ± 0.5*	18.8 ± 1.8	332 ± 30*	
	F	6.7 ± 0.4*	37.6 ± 2.4*	12.7 ± 0.7	1193 ± 105	5.2 ± 0.9*	17.6 ± 3.5*	18.0 ± 8.1	17.0 ± 11	231 ± 34	

Dose-dependent and statistically significant increased levels of alkaline phosphatase, total bilirubin, cholesterol, and albumin were seen in male and female rats, indicating hepatic changes associated with administration of PMCol. In addition, increased levels of alanine aminotransferase were statistically significant in male rats and increased to biologically relevant levels in female rats. Statistically significant increased levels of blood urea nitrogen levels were seen in both male and female rats. Inorganic phosphorus levels in male rats were significantly decreased in a dose-dependent fashion, which is likely the result of renal tubular defects.

Indicates a statistically significant change ($p < 0.05$) from the control group (0 mg/kg).

Review of hematology data indicate anemia developed in the high dose group as a result of PMCol administration, which is observed as decreases in red blood cell counts, hematocrit, and hemoglobin levels, as well as an increase in reticulocytes counts. Decreases in prothrombin time were observed in male animals with a statistically significant change at 2000 mg/kg/day. Fibrinogen levels were statistically increased in male rats at 500 and 2000 mg/kg/day; however, in female rats fibrinogen levels were unchanged.

Table 4

Summary of treatment-related histopathology findings following 28-days of daily administration of PMCol in male and female CD rats.

ORGAN - lesion	Sex	Dose Level				
		0 mg/kg/day	100 mg/kg/day	500 mg/kg/day	2000 mg/kg/day	
KIDNEY - Apoptosis, tubular epithelium -Tubular regeneration -Inflammation, chronic	M	0/20	0/20	0/20	10/20 (0.50)*	
	F	0/20	0/20	0/20	0/20	
	M	0/20	0/20	9/20 (0.55)	15/20 (1.50)	
	F	0/20	0/20	0/20	1/20 (0.05)	
	M	0/20	0/20	2/20 (0.10)	8/20 (0.40)	
	F	1/20 (0.05)	1/20 (0.05)	1/20 (0.05)	2/20 (0.10)	
STOMACH -Hyperkeratosis, nonglandular stomach	M	0/20	0/20	0/20	4/20 (0.20)	
	F	0/20	0/20	0/20	13/20 (1.00)	

The incidence of each lesion is presented as number affected/total number evaluated. Lesions associated with nephrotoxicity were almost exclusive to male rats, while serum chemistry changes indicated both male and female rats exhibited some degree of nephrotoxicity. There was an increase in hyperkeratosis in non-glandular stomach, which was indicated by an increase in the number of retained keratin layers over the nonglandular stomach.

* Severity score in parentheses (determined on a scale of 1 to 4; where 1= mild, 4 = severe).

Table 5
Overview of Toxicity-Related Responses following 28-days of daily administration of PMCol in Beagle dogs.

Dose Level (mg/kg/day)	0	50 (1000 mg/m ² /d)	200 (4000 mg/m ²)	800 (16,000 mg/m ²)
No. Animals	4	4	4	4
Clinical Signs	-	NE	NE	DA LA DH DI OD VR
Total Body Weights and Weight Gains	-	NE	NE	↓ ↓
Food Consumption	-	NE	NE	↓
Clinical Chemistry	-	NE	↑ALP ↓↓TP ↓↓ALB ↓↓CA	↑ALP ↑ALT
Hematology	-	NE	NE	↑↑PLT
RBC Corphology	-	NE	NE	NE
Coagulation	-	NE	NE	NE
Urinalysis	-	NE	NE	NE
Hormone Analysis	-	NE	NE	↓↑T3 ↓↑T4
ECG	-	NE	NE	NE
Ophthalmology	-	NE	NE	NE
Organ Weights (% Brain Weight)	-	NE	NE	NE
Pathology	-	NE	NE	LIVER -microvesicular vacuolation of midzonal to periportal hepatocytes THYMUS - atrophy

Administration of PMCol resulted in several changes in observed parameters that were associated with toxicity in dogs administered 800 mg/kg/day. These included changes in behavior, such as decreased activity, vomiting, diarrhea, ocular discharge, and dehydration. Liver enzymes were increased in high dose group dogs, as well as decreased protein levels that were associated with decreased food consumption. Triiodothyronine and Thyroxine serum levels were statistically significantly decreased following administration of PMCol at a dose of 800 mg/kg/day in male dogs. Pathological changes in

livers in the high dose dogs were characterized by microvesicular vacuolation of midzonal to periportal hepatocytes. In addition, administration of PMCol at 800 mg/kg/day in dogs resulted in thymus atrophy.

NE = No Effect

↑ = Biologically Relevant Increase

↓ = Biologically Relevant Decrease

↑↑ = Statistically Significant Increase

↓↓ = Statistically Significant Decrease

LA = Loss of Appetite, DA = Decreased Activity, DH = Dehydrated, DI = Diarrhea, OD = Ocular Discharge, VR = Vomit in Run

ALB = Albumin, ALP = Alkaline phosphatase, ALT = Alanine aminotransferase, CA = Calcium, TP = Total protein

PLT = Platelet count, WBC = Leukocyte count

T3 = Triiodothyronine, T4 = Thyroxine

Reference from Table 3 of Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. July 2005. USFDA.

Table 6

Summary of histopathology findings following 28-days of oral administration of PMCol in male Beagle dogs.

ORGAN - lesion	Group/Treatment			
	1 0 mg/kg/day	2 50 mg/kg/day	3 200 mg/kg/day	4 800 mg/kg/day
Liver - Microvesicular vacuolation of midzonal to periportal hepatocytes	0/4	0/4	0/4	2/4 (0.50)*
Thymus - Atrophy	0/4	0/4	0/4	3/4 (1.25)

Microscopic evaluation of the liver indicated an increased incidence of pathological changes in dog livers and thymus atrophy in dogs receiving daily administration of 800 mg/kg/day PMCol.

* Severity score in parentheses (determined on a scale of 1 to 4; where 1= mild, 4 = severe).