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In vitro and *in vivo* safety evaluation of Nephure™

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ABSTRACT

Nephure™ is a proprietary oxalate decarboxylase (OxDC) enzyme being developed as a food ingredient. In this study, the safety of Nephure™ was evaluated in a bacterial mutagenicity assay and in a sub-chronic (13-week) oral toxicity study in rats. Nephure™ did not show any mutagenic properties in the mutagenicity assay. In the 13-week sub-chronic oral toxicity study in which 10 Sprague Dawley rats per sex were administered 0, 118, 235 and 475 mg/kg bw/day (8260, 16450 and 33,250 Units/kg bw/day, respectively) of Nephure™ by gavage, male and female rats did not show any test article-related clinical observations or effects on body weight, body weight gain, food consumption, food efficiency, ophthalmology, functional observational battery parameters or motor activity. Furthermore, there were no changes in coagulation, clinical chemistry, urinalysis or hematology parameters, macroscopic/microscopic findings or organ weights that could be attributed to the test article. Based on these results, Nephure™ was not mutagenic and the no-adverse-effect level (NOAEL) in the 13-week study was determined to be 475 mg/kg bw/day (33,250 Units/kg bw/day). Evaluation of the estimated consumption of Nephure™, generation of the metabolite formate, and the current safety studies resulted in a conclusion of a tolerable upper limit of 3450 Units of OxDC activity/day (57.5 Units activity/kg bw/day), when Nephure™ is added to food to decrease dietary oxalate.

1. Introduction

Oxalate is normally produced in plants, primarily in the leaves, nuts, fruits, and bark. The content of oxalate is high in many plant foods, including vegetables, cereal grains, nuts, beans and beverages. For example, oxalate concentrations (mg/100 g) in fresh spinach, dried soy beans, dried peanuts, and dried wheat bran are 400–900 mg, 82–214 mg, 96–705 mg and 457 mg, respectively (Massey, 2007). Daily oxalate intake is typically 80–120 mg/day; however, it can range from 44 to 350 mg/day in individuals who eat typical Western diets (Holmes and Kennedy, 2000). Most plant-based foods contain various quantities of oxalate. Oxalate content can also vary widely within the same plant species due to growth seasons, soil and water conditions (Massey, 2007; Holmes and Kennedy, 2000).

Although oxalate can be catabolized in bacteria, fungi and plants, humans do not produce enzymes that can degrade oxalate (Chakraborty et al., 2013). Oxalic acid-degrading bacteria have been found in feces of humans (Abratt and Reid, 2010); however the extent to which they degrade oxalic acid in the gastrointestinal (GI) tract of humans is either unknown or inconclusive. One gut bacteria that has been investigated as a therapeutic is *Oxalobacter formigenes*. This organism uses oxalate as its major energy and carbon source. Anywhere from 1 to 22% of ingested oxalate is absorbed in humans, depending on source, dose and state (fed or fasting) (Noonan and Savage, 1999). Oxalate is a known chelating agent for metal cations and dietary oxalate may form magnesium, iron or calcium salts in food or the GI tract (Asplin 2002; Israr et al. 2013). Consequently, unabsorbed oxalate can form salts with the aforementioned minerals in the GI tract, promoting mineral excretion in feces and reducing the potential for essential mineral absorption

Abbreviations: ADI, acceptable daily intake; ANOVA, analysis of variance; AOAC, association of official analytical chemists; bw, body weight; FDA, US Food and Drug Administration; FOB, functional observation battery; GI, gastrointestinal; GLP, good laboratory practice; MA, motor activity; NOAEL, no-observed-adverse-effect level; OECD, Organization for Economic Co-operation and Development; OxDC, oxalate decarboxylase; PSL, Product Safety Labs; USDA, United States Department of Agriculture; USP, United States Pharmacopeia; wt, weight.

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(Noonan and Savage, 1999).

Oxalate may be absorbed throughout the entire GI tract, beginning at the stomach (Hautmann, 1993; Chen et al., 2003). The concentration of oxalate in serum of humans consuming typical Western diets is between 1 and 6 μM; much lower than the saturation concentration (Porowski et al., 2008). However, due to water re-absorption in the kidney, oxalate concentration in urine increases over 100 times, usually in the range of 0.1–0.5 mM (Sakhaee, 2009). Urinary calcium concentration is normally about 10 times that of oxalate concentration (Pak et al., 2004). Therefore, urinary calcium oxalate is often supersaturated, which is usually described by the supersaturation ratio, a number being approximately the ratio of the salt concentration in the urine to its solubility. When a salt concentration in a solution is higher than its solubility, salt will start to precipitate at a certain concentration, which is called the upper limit of metastability (Coe et al., 2005). The upper limit of calcium oxalate metastability varies with urine conditions such as other ions, macromolecules and urine pH. By reducing oxalate and/or increasing citrate intake and/or encouraging at least 2 L of urine output per year the salt concentration in urine can be kept below the upper limit of metastability; thus, allowing for normal variations of urine ion, macromolecule and pH conditions without introducing an abnormal level of calcium oxalate precipitate formation. It is believed that calcium oxalate supersaturation and lack of hydration are the drivers of calcium oxalate stone formation, although the process is complex, not completely clear and may involve several other critical factors including urinary macromolecules, bile acids and renal tubular damage (Tsujihata, 2008; Porowski et al., 2008; Coe et al., 2005; Matlaga et al., 2007; Saso et al. 2001).

Typical strategies to limit oxalate exposure are dietary avoidance of oxalate and increased calcium ingestion to saturate calcium-binding sites on oxalate and promote oxalate excretion (Lieske et al., 2005, 2010). Oxalate avoidance is difficult because oxalate is present in many different foods and levels of oxalate in a single food are variable (Holmes and Kennedy, 2000). Therefore, it is challenging to find foods that keep oxalate ingestion low without compromising nutritional balance. Bong and coworkers recently demonstrated that calcium chloride can successfully remove a significant portion of soluble oxalate from processed spinach (Bong et al., 2017). Herein, an enzyme approach is presented that is effective at reducing/removing both soluble and insoluble oxalate from a variety of foods and beverages.

Oxalate decarboxylase (EC 4.1.1.2 and CAS Registry No. 9024-97-9), an oxalate metabolizing enzyme, requires a catalytic amount of oxygen to convert oxalate to formate and carbon dioxide and is found in bacteria and fungi. After screening thousands of homologs, the oxalate decarboxylase Nephure™ was discovered to have ideal properties for degradation of oxalate in food over a wide pH range, including the acidic pH of the stomach. In order to be used as a food ingredient, it is important to show that this particular enzyme is safe to consume. Therefore, an *in vitro* bacterial mutagenicity study and a 13 week sub-chronic rat study were conducted to evaluate the potential toxicity of Nephure™. All studies were performed according to OECD principles and following Good Laboratory Practices (GLP).

2. Materials and methods

2.1. Identity, properties and manufacturing

2.1.1. Identity

Nephure™ is a highly soluble (>212 g/L) manganese-containing trimeric oxalate decarboxylase (OxDC) enzyme with a single subunit molecular weight of 40.4 kDa as determined by Electrospray Ionization Mass Spectrometry (ESI-MS). The enzyme demonstrates absolute specificity for oxalate as demonstrated from an absence of activity with other carboxylic acids of similar structure (oxamic, phthalic, furmaric, glutaric, 2-ketoglutaric, formic, acetic oxaloacetic, malic, citric, glycolic, succinic malonic and pyruvic acid). Furthermore, the enzyme has a high melting point, 65 °C, and is active over a wide pH range (1.5–6.0). Optimal activity at pH 2.0 and 37 °C is 90 μmol/min/mg.

Using CLUSTAL 2.1 software, multiple sequence alignment comparisons of the wild-type OxDC gene from *Synechoccus elongates* (PCC6301) with the gene expressed in *Escherichia coli* BW25113 were conducted (Larkin et al., 2007). Although the codon usage was optimized for gene expression in *E. coli*, the amino acid sequences of both encoded proteins were identical.

2.1.2. Method of manufacture

2.1.2.1. Raw materials. The raw materials used in the fermentation and recovery process included de-mineralized water, yeast extract, peptones, minerals including manganese chloride, pH regulators, kanamycin, sugar-based carbon sources and anti-foaming aids; all standard ingredients used in the enzyme industry. Anti-foaming additives were used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of the antifoams if used in the product was less than 1%.

2.1.2.2. Cell line. The OxDC gene was synthesized using DNA sequence information from the gene encoding OxDC from *Synechoccus elongates* (PCC6301) available from NCBI Genebank: BAD79907.1 (native, full length). The gene was codon-optimized for *E. coli* expression. The codon-optimized gene was inserted into an expression plasmid, which was transformed into *E. coli* BW25113 (K-12 origin). The source organism (*E. coli* K12) is a non-pathogenic strain with a long history of safe use (OECD, 1986; EPA, 1997), and the source of the OxDC gene template (*Synechococcus elongates* PCC 6301) is categorized as a Biosafety Level 1 organism (Joint Genome Institute, 2015); such organisms are recognized as not causing disease in adult humans. Since the Nephure™ gene is produced synthetically there is no risk of transferring unknown pathogenic or toxicogenic determinants from *Synechococcus elongates* to humans.

2.1.2.3. Fermentation, purification and drying. The production of Nephure™ originates from a pure culture submerged fed-batch fermentation of a non-pathogenic *E. coli* carrying an expression plasmid. Manganese chloride is added in the process to ensure expression of an active enzyme. All equipment is carefully designed, constructed, operated, cleaned and maintained as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken to ensure absence of foreign microorganisms and to confirm strain identity.

The recovery process is a multi-step operation designed to separate the desired enzyme from the microbial biomass and partially purify, concentrate, and stabilize the enzyme. Following fermentation, the enzyme is first liberated from the production strain by cell disruption. The homogenized biomass is then treated with buffered solutions, and residual solids are removed by a series of sedimentation and filtration processes designed to remove debris greater than the molecular weight of Nephure™. Nuclease is added to remove the remaining remnants of plasmid DNA. The resulting filtrate is then concentrated using ultra-filtration and diafiltration. Finally, the retentate is filtered through a 0.22 μm filter to sterilize. This resulting enzyme concentrate was the test article used in the studies found herein.

2.1.3. Specifications

Appropriate food grade specifications have been established for Nephure™ (see Table 1). The specifications are consistent with the purity and microbial limits established for enzyme preparations in the FCC, 8th Edition and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2003; FCC, 2012). All methods of analysis utilized are internationally recognized or have been validated (see Table 1).

2.1.4. Physical and technical effect

Nephure™ specifically acts on oxalate by converting oxalate to formate and carbon dioxide. The Nephure™ preparation is to be added to food in order to remove some or all of the available oxalate.

To demonstrate the enzyme's effectiveness, a variety of juices, beers, and canned foods have been analyzed for oxalate content and the ability of Nephure™ to extract and degrade oxalate. Liquid foods/beverages were tested by adding Nephure™ at various concentrations and incubating at 37 °C for three hours. Solid foods were first resuspended prior to adding Nehpure™. Samples were then analyzed using a Dionex IC 2000 system (ion chromatography) with an AS11-HC column using a potassium hydroxide eluent (Kaviraj et al., 2015).

2.2. Safety evaluation

2.2.1. Bacterial reverse mutation assay

2.2.1.1. Guidelines. The bacterial reverse mutation test was conducted in accordance with the following guidelines: US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C.1.a. Bacterial Reverse Mutation Test (Revised 2007) and ICH S2 (R1) Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (2012).

2.2.1.2. Study design. The mutagenicity of Nephure™ (average activity of 70 Units/mg) was evaluated by measuring its ability to induce reverse mutations in *Salmonella typhimurium* strains TA1535, TA 1537, TA98, TA 100 and *Escherichia coli* strain WP2 uvrA, purchased from Molecular Toxicology, Inc. (Boone, NC). Each assay (plate incorporation and pre-incubation) was conducted in the presence and absence of metabolic activation with S9 mix prepared from the S9 microsomal fraction of rat liver. The S9 microsomal liver fraction was prepared from male Sprague-Dawley rats induced with phenobarbital and benzoflavone. The test substance was suspended in 50 mM arginine buffer (pH 9.5) and mixed thoroughly with sterile water using a vortex and added to plates to achieve concentrations of 5000, 1580, 500, 158, 50, 15.8, 5.0, and 1.58 μg/plate. The positive controls in the absence of S9 mix were daunomycin (60 μg/mL) for *S. typhimurium* TA98, sodium azide (15 μg/mL) for *S. typhimurium* TA100 and TA1535, ICR 191 acridine (10 μg/mL) and methyl methanesulfonate (25 μg/mL) for *E. coli* WP2uvrA. The positive control for all bacterial strains in the presence of S9 mix was 2-aminoanthracene (100 μg/mL) and the negative control for all strains in the presence or absence of S9 mix was sterile water. The source of the positive control mutagens and the S9 microsomal liver fraction was Molecular Toxicology, Inc. (Boone, NC). Plates were prepared in triplicate. Supplemental testing was performed for strain TA100 to verify bacterial titer.

The results of the study were considered to be positive if there was an increase in the mean number of revertants $\geq$3 times the negative control value for strains TA1535 and TA1537 and $\geq$ 2 times the negative control value for strains TA98, TA100 and WP2 uvrA. Criteria for validity were normal appearance of the bacterial lawn, substantial increases in numbers of revertant colonies with the positive controls, and mean revertant colony counts of vehicle controls within laboratory historical control and/or published values.

2.2.2. Thirteen week subchronic dietary study

2.2.2.1. Guidelines. The subchronic oral toxicity study was conducted in accordance with Good Laboratory Practice (GLP), OECD Guidelines for Testing of Chemicals, Section 4, No. 408, Health Effects, Repeated Dose 90-Day Oral Toxicity Study in Rodents (1998) and US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C.4.a Subchronic Toxicity Studies with Rodents (Revised 2003).

2.2.2.2. Animals. Eighty Crl:CD® IGS Sprague Dawley rats (40 of each sex) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Upon arrival at Product Safety Labs (Dayton New Jersey) the animals were individually housed in suspended stainless steel cages within a room that was maintained under a 12-hour light/dark cycle, 18–23°C and 39–69% relative humidity. Litter paper placed underneath the cages was changed at least three times per week. Rats were provided 2016C Envigo Teklad Certified Global Rodent Diet® (Indianapolis, IN) and filtered tap water *ad*

Table 1
Specifications of Nephure™.

Analysis	Method	Specification
Appearance	Visual	Yellowish to Brownish Liquid
Oxalate-degrading activity (Units (μmol/min)/mg substance)	Internal Method	>40
Total protein concentration (mg/ml)	Bradford Assay	Report Result
Residual plasmid (CFU)	Internal Method	0
Moisture (%)	AOAC 930.15	≤99
Ash (%)	AOAC 923.03	≤0.6
Kanamycin (ng/mg protein)	USDA, LC-MS-MS [f]	<100
Heavy metals		
Arsenic (ppm)	AOAC 986.15 (mod)	<1.5
Lead (ppm)	AOAC 986.15 (mod)	<1
Cadmium (ppm)	AOAC 986.15 (mod)	<0.5
Mercury (ppm)	AOAC 986.15 (mod)	<0.5
Microbial		
Total aerobic (CFU/ml)	USP <61>	<50,000
Escherichia coli (*per* ml)	USP <62>	Absent
Salmonella spp. (*per* 10 mL)	USP <62>	Absent
Yeast and mold (CFU/ml)	USP <61>	<5000

CFU = colony forming units; mod = modified; USP = United States Pharmacopoeia.

libitum throughout the study except for the night before termination, when food was withdrawn. The diet contained 818 ppm oxalic acid as analyzed by AOAC Official Method 986.13 (modified). The animals weighed 215–249 g (male) and 158–186 g (female) after a seven-day acclimation period, and were approximately seven to eight weeks of age at initiation of dosing. The rats were randomly distributed (10/sex/dose), among the three dose groups (118, 235 and 475 mg/kg bw/day) and a control group. The doses used were based on the results of a previous fourteen-day rat pilot study (125, 250 and 500 mg/kg bw/day), using the same strain and species as used in the 90-day study. The high dose was considered a practical dose with the test-article formulation and test system.

Serum samples from three animals that were housed with study animals but were not part of the study were evaluated for the absence of common rat pathogens (Rat *parvovirus*, Toolan's H-1 Virus, Kilham Rat Virus, Rat Minute Virus, *Parvovirus* NS-1, Rat *Coronavirus*, Rat *Theilovirus*, and *Pneumocystis carinii*) on the last day of the test period. Because the sentinel samples were negative for all pathogens evaluated, the study animals were deemed healthy and reasonably free of common rat pathogens.

2.2.2.3. Study design. Dosing solutions were prepared daily by diluting Nephure™ (average activity 70 Units/mg) in 50 mM arginine (pH 9.5) to produce dosing solutions of 1.18, 2.35 and 4.75%. The solutions were stirred at ambient temperature until a visually homogeneous mixture was achieved and were maintained on a magnetic stir plate during dose administration. The solutions were administered to the rats via oral gavage at a dosage volume of 10 mL/kg bw, to provide doses of 118, 235 and 475 mg/kg bw/day (8260, 16450 and 33,250 Units/kg bw/day, respectively). The control group received vehicle (50 mM arginine pH 9.5) only, at the same dose volume as the test animals. Absolute volumes provided were calculated based on the most recent weekly body weights and were adjusted each week to maintain the targeted dose level for all rats. Dosing was at approximately the same time each day (8AM ± 2 h; the first study activity of the day after formulation) except for the dates of hematology and/or clinical chemistry sample collection and the day of Functional Observational Battery (FOB) testing. The 8AM dosing schedule was designed to maximize formation of formate from ingested food, as rats eat at night and consume their last meal at 5–6 AM (Ulman et al., 2008).

For homogeneity testing, samples were taken on Days 1, 8, 15, 22 and 70 from the top, middle and bottom of dosing containers while the preparations were stirring. Homogeneity samples were also used to verify concentration. On Days 43, 50, 56, 57, 66, 67, 69, 90 and 96, samples were collected for concentration verification. Samples were immediately frozen and shipped to Captozyme, Inc (Gainesville, FL) for analysis. Homogeneity and concentration verification were evaluated using the Bradford assay (Bradford, 1976).

Body weights were recorded prior to test initiation, weekly during the study, and just prior to study termination. Body weight gain was calculated for selected intervals and for the overall study. Food consumption was recorded weekly and food efficiency (mean daily body weight gain/mean daily food consumption) was calculated. Ophthalmologic evaluations were conducted on all animals during acclimation and just prior to test termination (Day 93). The animals were observed at least twice daily for mortality and once per day for signs of gross toxicity. Detailed clinical observations were performed daily and included changes in the skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions, and autonomic activity. In addition, changes in gait, posture, and response to handling, as well as the presence of clonic or tonic movements, stereotypies, or bizarre behavior were also noted. A complete FOB was carried out during Week 11 of Nephure™ administration. Rats were examined in random order with the observer not knowing what treatment groups from which the rats belonged. The FOB included numerous home cage, handling, open field and sensory observation parameters. Motor activity was also evaluated on all animals.

Clinical pathology was performed on all animals at the end of the dosing phase with blood samples for hematology and clinical chemistry being collected via sublingual bleeding under isoflurane anesthesia. Approximately 500 μL of blood was collected in a pre-calibrated tube containing K_2EDTA for hematology assessments and 1000 μL was collected into a tube containing no preservative for clinical chemistry. These samples were centrifuged in a refrigerated centrifuge and the resulting serum collected and stored on ice. Blood samples used to determine the prothrombin and partial thromboplastin time (coagulation) were collected via the inferior vena cava under isoflurane anesthesia at terminal sacrifice. Approximately 1.8 mL of blood was collected in a pre-calibrated tube containing 3.2% sodium citrate, centrifuged and the resulting plasma collected and stored at −80°C. At terminal sacrifice, all rats were humanely euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia. Urinalysis including color and clarity, specific gravity, pH, protein, glucose, ketones, bilirubin, occult blood, nitrites, urobilinogen, volume microscopy of sediment and leukocytes was also conducted during Week 13.

A complete necropsy was conducted on all animals at termination of the study and the following tissues and organs were collected: accessory genital organs, adrenals, aorta, bone (femur), bone marrow (from femur and sternum), brain (medulla/pons, cerebellar, and cerebral cortex), cecum, cervix, colon, duodenum, epididymides, esophagus, eyes, Harderian gland, heart, ileum with Peyer's patches, jejunum, kidneys, larynx, liver, lungs, lymph node mandibular, lymph node mesenteric, mammary gland, nasal turbinates, nose, optic nerve, ovaries, oviducts, pancreas, parathyroid, peripheral nerve (sciatic), pharynx, pituitary, rectum, salivary glands (sublingual, submandibular and parotid), skeletal muscle, skin, spinal cord (cervical, mid thoracic and lumbar), spleen, sternum, stomach, testes, thymus, thyroid, trachea, urinary bladder, uterus and vagina. Weights were recorded for the following organs: adrenals, brain, epididymides, heart, kidneys, liver, ovaries and oviducts, spleen, testes, thymus and uterus. All tissues were fixed in neutral buffered formalin, except for the eyes (including optic nerve) and testes, which were fixed in modified Davidson's fixative (Moore and Barr, 1954). Histopathology was conducted on all animals in the control and high dose groups from hematoxylin-eosin stained paraffin sections of the tissues listed.

2.2.2.4. Statistical methods. Body weights, body weight changes, food consumption, and organ weights were evaluated for homogeneity of variances and normality by Bartlett's test (Bartlett, 1937). Where Bartlett's test indicated homogeneous variances, treated and control groups were compared using a one-way analysis of variance (ANOVA). When one-way analysis of variance was significant ($p < 0.05$), a comparison of the treated groups to control by Dunnett's test (Dunnett, 1964, 1980) for multiple comparisons was performed. Where variances were considered significantly different by Bartlett's test, groups were compared using a Kruskal-Wallis non-parametric analysis of variance (Kruskal and Wallis, 1952). When non-parametric analysis of variance was significant, comparison of treated groups to control was performed using Dunn's test (Dunn, 1964). Statistical analysis was performed on all quantitative data for in-life and organ weight parameters using Provantis® version 9. Motor activity data were analyzed using a Two-Way Repeated Measures ANOVA (INSTAT Biostatistics, Graph Pad Software, San Diego, CA). Clinical pathology results were analyzed using Levene's test for homogeneity and Shapiro-Wilk

test for normality. When the preliminary test was not significant, a one-way analysis of variance was performed followed with a Dunnett's test.

3. Results

3.1. Technical effect (oxalate removal from food and beverages)

Most foods tested were within a pH range where Nephure™ shows activity (Table 2). Nephure™ was able to remove a significant portion of both soluble and insoluble oxalate from the foods outlined in Table 2.

3.2. Bacterial reverse mutation assay

Nephure™ did not induce any significant or dose-dependent increases in the numbers of revertant colonies in any strain tested in the absence or presence of S9 mix, in either of the experiments (Table 3). No signs of precipitation or contamination were noted in any of the strains. Signs of toxicity were observed for *E. coli* at doses ≥50 μg/plate in the plate incorporation or pre-incubation experiments in absence or presence of S9 with evidence of decrease revertant counts, microcolony formation and absence of lawn. To further investigate toxicity supplemental testing was performed for this strain using five additional dose levels in a range of 0.5–7.5 μg/plate. Eight dose levels without precipitation or plate contamination were evaluated for all strains, therefore bacterial mutagenicity was adequately assessed.

The mean revertant colony counts for each strain treated with the vehicle were close to or within the expected range, considering the laboratory historical control range and/or published values (Mortelmans and Zeiger, 2000; Gatehouse, 2012). The positive control substances caused the expected substantial increases in revertant colony counts in both the absence and presence of S9 in each phase of the test confirming the sensitivity of the test and the activity of the S9 mix. Therefore, each phase of the test was considered valid.

3.3. 13-Week subchronic study

3.3.1. Analysis of test substance

The test substance was determined to be stable under the conditions of storage over the course of this study. Nephure™ was stable throughout the duration of the study as determined from activity and SDS-PAGE analysis. All homogeneity samples had a % RSD of 0.4–6.9%; when combined with the concentration verification samples, the %RSD was 1.6–13.3%. Based on the overall stability, homogeneity and concentration verification analysis, animals were considered to have received target dose concentrations of Nephure™ within an acceptable margin of variation.

Table 2
Oxalate decrease in various food products per serving by the addition of Nephure™.

	Foodstuff	pH	Oxalate removed		
			mg/serving	% of total	% Daily average
Juices	Pink lemonade	2.7	1.8	32%	1.2%
	Carrot	6.3	9.1	66%	6.1%
	Blueberry banana almond milk	3.9	8.4	58%	5.6%
Teas					
	Peach tea	2.9	4.8	100%	3.2%
	Bottled tea	3.8	7.8	87%	5.2%
Beers					
	Pilsner	4.2	0.7	100%	0.5%
	Belgian white ale	4.3	1.3	89%	0.8%
	Blonde ale	4.2	4.4	95%	2.9%
	Amber ale	4.2	5.4	88%	3.6%
	Brown ale	3.9	2.3	93%	1.5%
Milks					
	Almond milk	7.7	1.1	100%	0.8%
	Coconut milk	8.7	0.2	77%	0.1%
	Soymilk	8.2	11.8	94%	7.9%
	Chocolate milk	6.8	1.4	77%	0.9%
Soups					
	Clam Chowder	6.1	15	87%	9.8%
	Lentil	5.6	70	88%	46.5%
	Chunky	5.3	7	82%	5.0%
Canned fruit					
	Mandarin oranges	3.5	41	91%	27.3%
	Pear halves	3.7	6	61%	4.3%
	Sliced peaches	3.9	70	95%	46.7%
	Pineapple slices	3.6	11	94%	7.5%
Canned vegetables					
	Carrots	5.0	11	78%	7.1%
	Olives	3.37	2	56%	1.4%
	Green beans	5.1	6	79%	4.3%
	Corn	6.3	5	93%	3.3%
	Veggie blend	5.8	21	72%	14.1%
	Spinach	5.7	146	79%	97.2%
	Diced tomatoes with green chiles	3.8	5	73%	3.4%
	Diced tomatoes	3.88	11	94%	7.2%
Sauces					
	Marinara	4.0	0.	38%	0.5%
	Tomato sauce	4.1	0.5	42%	0.3%

% Daily Average = The % daily average oxalate intake.

Table 3
Results of the bacterial reverse mutation assay.

Test substance	Dose (μg/plate)	Average number of revertant colonies/plate (n = 3)									
		TA98		TA100		TA1535		TA1537		WP2uvrA	
		Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
Nephure™ with S9	0	34	32	115	112	16	11	17	19	51	50
	0.5									49	49
	0.75									54	48
	1.00									53	52
	1.58	33	34	116	108	15	15	16	15	55	52
	2.5									53	52
	5	31	37	105	114	12	14	15	18	56	53
	7.5									52	46
	15.8	28	36	116	116	14	12	18	19	58	49
	50	37	34	107	120	13	14	15	13	55	8*
	158	33	36	111	111	15	13	14	18	31*	2*
	500	33	35	108	114	15	11	17	21	2*	MC/NL
	1580	30	35	113	108	13	14	18	21	MC/NL	MC/NL
	5000	37	34	96	106	12	14	23	17	MC/NL	MC/NL
Positive control 2-AA	10	3351	3507	3095	3108	330	453	455	446	116	108
Nephure™ without S9	0	22	35	106	112	22	21	14	18	45	44
	0.5									37	44
	0.75									47	40
	1.00									39	46
	1.58	29	23	109	111	15	20	17	21	50	45
	2.5									42	47
	5	21	30	119	100	18	23	14	16	45	40
	7.5									48	46
	15.8	30	23	121	118	20	22	19	19	48	46
	50	23	33	106	106	18	20	15	22	50	45
	158	28	26	102	105	19	21	15	17	43	37
	500	29	28	105	105	18	20	20	21	33*	42
	1580	26	30	103	107	15	15	16	14	13*	26*
	5000	28	27	110	114	19	17	15	19	0*	8*
Positive controls											
ICR 191 Acridine	1							2579	4412		
NaN_3 without S9	1.5			621	585	554	645				
Daunomycin	6	723	352								
MMS	2.5									708	330

Nephure™ was tested using the standardized plate incorporation assay (Expt. 1) and the pre-incubation method (Expt. 2). 2-AA = 2-aminoanthracene; MC = microcolonies; MMS = methyl methanesulfonate; NaN_3 = sodium azide; NL = no lawn; S9 = S9 microsomal liver fraction.
* Toxicity observed.

3.3.2. *In-life observations*

There were no mortalities during the course of the study or clinical observations attributed to oral administration of Nephure™. All clinical observations were considered incidental and regarded as toxicologically insignificant.

The functional behavioral results of the test groups of male and female rats performed during Week 12 were considered comparable to the control groups. Any changes in quantitative measurements or increases in incidence of open field measurements were minimal and not associated with a constellation of findings that would support a toxicologically significant behavioral change. Mean quantitative measurements for forelimb/hindlimb grip strength and hindlimb foot splay were comparable to control values for both female and male rats. Motor Activity (i.e., mean total movements) results were comparable in male and female rats for all groups (data not shown).

There were no changes in body weight or body weight gain in male and female rats attributable to the administration of the test substance (Fig. 1). Mean weekly body weights and mean daily body weight gain for treated male rats were comparable to control males throughout the study. Likewise, mean daily body weight gain for treated female rats was generally comparable to control females throughout the study, with the exception of a statistically significant decrease ($p < 0.05$) in the 475 mg/kg bw/day group on Days 28–35. This statistically significant change in weight gain was considered transient, spurious in nature, incidental and regarded as toxicologically insignificant.

There were no changes in food consumption (Table 4) and food efficiency (data not shown) attributable to the administration of the test substance. Mean daily food consumption and mean food efficiency for treated male rats were comparable to control males throughout the study. Likewise, mean daily food consumption and efficiency for treated female rats were generally comparable to control females throughout the study. Statistically significant decreases ($p < 0.05$) in food efficiency of female rats occurred in the 235 mg/kg bw/day group on Days 70–77 and in the 475 mg/kg bw/day group on Days 28–35. The statistically significant transient changes in food efficiency were considered spurious in nature, incidental and regarded as toxicologically insignificant.

3.3.3. *Clinical pathology observations*

There were no changes in hematology, coagulation (Table 5), clinical chemistry (Table 6) or urinalysis (Table 7) parameters attributed to Nephure™ administration. Changes in hematology were limited to increased white blood cell counts and absolute lymphocyte counts in female rats in the 235 and 475 mg/kg bw/day groups ($p < 0.05$). White blood cell counts in these groups were 7.88 × 10^3/μL and 8.27 × 10^3/μL, respectively; within the historical range of the laboratory values of 2.41–14.79 × 10^3/μL. Absolute lymphocyte counts were 6.35 × 10^3/μL and 6.71 × 10^3/μL,

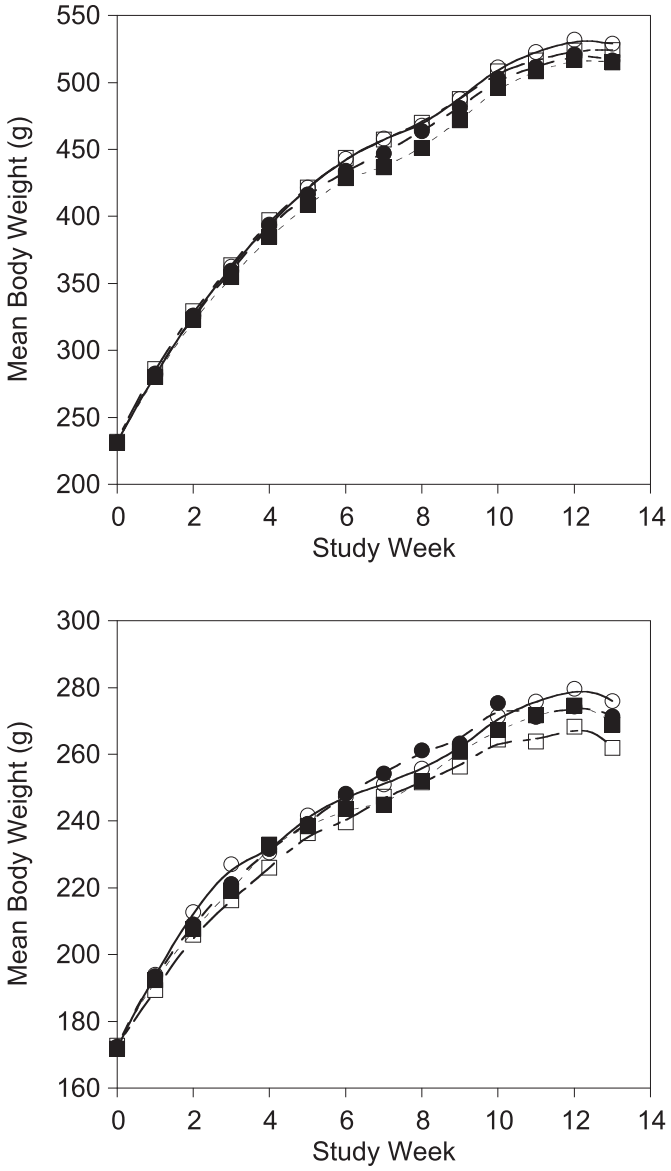

Fig. 1. Mean body weights (g) of male (top) and female (bottom) rats provided Nephure™ at daily doses of 0 (open circle), 118 (open square), 235 (sold circle) and 475 (solid square) mg/kg bw for 13 weeks.

respectively; also within the historical laboratory range values of 1.80–12.84 × 10^3/μL. The changes in white blood cell and lymphocyte counts did not correlate with organ weight or microscopic observations and were thus interpreted to be of little toxicological significance and not adverse. There was a statistically significant increase in cholesterol in females at the 235 mg/kg bw dose that was judged not to be toxicologically relevant because it was only observed at the mid-dose and the value was within normal published values of 98 ± 19 mg/dL in female control Sprague-Dawley rats of similar age (Lee et al., 2012).

3.3.4. Organ weights

There were no effects of the test material on organ weight. Mean absolute organ weights and organ weight ratios relative to brain or body weight of treated animals were generally comparable to control animals (Table 8). Statistically significant differences included an increase ($p < 0.05$) in mid dose male spleen-to-body weight ratio and a decrease ($p < 0.01$) in high dose female absolute brain weights. Non-dose dependent, statistically significant increases in spleen-to-body weight ratios measured on Day 96 in mid dose male rats and decreased brain weights measured on Day 97 in high dose female rats administered Nephure™ were without histologic correlates and were not directly correlated with any other study parameters, and were therefore considered not to be toxicologically significant.

3.3.5. Gross and microscopic pathology

There were no macroscopic or microscopic findings related to exposure of the test substance. The observed macroscopic observations at termination were of sporadic incidence and showed no trends/patterns to suggest a relationship to the administration of Nephure™. Two animals (one per sex) given 475 mg/kg bw/day Nephure™ had microscopic observations involving the esophagus that were attributed to gavage procedures. These findings were focal to multifocal, mural necrosis of muscle in association with chronic inflammation. In addition, one female given 235 mg/kg bw/day Nephure™ had a mammary gland adenocarcinoma in the thoracic region. This was considered to be an incidental neoplasm unrelated to administration of the test substance based on its occurrence in a single mid-dose female.

Table 4
Mean daily food consumption (g/day) of male and female rats administered Nephure™ for 13 weeks.

Days relative to start date	Males (n = 10)				Females (n = 10)			
	Control	118 mg/kg	235 mg/kg	475 mg/kg	Control	118 mg/kg	235 mg/kg	475 mg/kg
0→7	26.27 ± 2.08	26.00 ± 1.67	26.33 ± 2.14	25.16 ± 1.73	18.20 ± 1.42	16.16 ± 2.08	18.13 ± 1.66	18.16 ± 3.51
7→14	28.31 ± 2.37	27.39 ± 1.90	27.89 ± 2.07	27.17 ± 1.78	18.56 ± 1.43	18.23 ± 1.78	18.64 ± 1.02	18.20 ± 1.49
14→21	28.11 ± 1.87	27.17 ± 2.18	27.37 ± 2.43	26.31 ± 1.39	19.47 ± 1.83	18.09 ± 1.93	18.56 ± 0.96	18.69 ± 1.28
21→28	28.79 ± 2.06	27.64 ± 2.11	27.86 ± 2.29	26.79 ± 1.21	18.36 ± 1.64	18.64 ± 1.98	19.27 ± 1.07	19.29 ± 2.02
28→35	29.41 ± 2.50	28.26 ± 2.50	28.23 ± 2.32	26.86 ± 0.98	19.24 ± 1.06	18.79 ± 1.60	19.27 ± 1.26	18.36 ± 1.65
35→42	28.50 ± 2.38	27.76 ± 2.71	26.94 ± 1.84	26.77 ± 1.26	18.94 ± 1.34	18.56 ± 1.77	19.51 ± 1.19	18.37 ± 1.46
42→49	28.21 ± 2.34	26.89 ± 2.39	26.53 ± 2.65	25.03 ± 2.74	18.97 ± 1.81	18.86 ± 1.72	18.99 ± 1.15	17.76 ± 2.29
49→56	27.03 ± 2.23	26.91 ± 2.64	27.20 ± 2.39	26.64 ± 0.99	18.94 ± 1.70	18.70 ± 1.68	19.41 ± 1.66	18.69 ± 1.84
56→63	29.30 ± 2.10	28.09 ± 2.69	28.24 ± 2.65	27.50 ± 1.31	18.74 ± 0.91	18.93 ± 2.57	18.53 ± 1.65	17.93 ± 2.26
63→70	25.97 ± 2.18	24.63 ± 2.11	24.33 ± 2.28	24.64 ± 1.08	15.94 ± 0.66	15.91 ± 3.03	16.14 ± 1.03	15.70 ± 1.62
70→77	29.00 ± 2.51	27.39 ± 2.15	26.90 ± 2.89	27.20 ± 1.01	18.56 ± 2.23	17.61 ± 2.36	17.43 ± 1.04	18.20 ± 3.95
77→84	29.41 ± 2.99	27.74 ± 2.47	27.71 ± 2.49	27.50 ± 1.50	20.29 ± 3.09	18.89 ± 2.55	18.16 ± 0.62	18.11 ± 2.53
84→91	26.83 ± 2.48	26.54 ± 2.72	25.14 ± 2.22	25.39 ± 1.10	18.09 ± 1.81	16.79 ± 2.75	17.46 ± 1.71	16.29 ± 1.40
0→91	28.09 ± 1.97	27.11 ± 2.11	26.97 ± 2.13	26.38 ± 0.87	18.64 ± 0.80	18.01 ± 1.77	18.42 ± 0.86	17.98 ± 1.81

Results are reported as mean ± standard deviation. No statistically significant differences from control were noted at $p < 0.05$.

Table 5
Hematology and coagulation parameters in rats administered Nephure™ for 13 weeks.

	Males (n = 10)[a]				Females (n = 10) [a]			
	Control	118 mg/kg	235 mg/kg	475 mg/kg	Control	118 mg/kg	235 mg/kg	475 mg/kg
Hematology								
RBC (10^{12}/L)	8.66 ± 0.34	8.72 ± 0.47	8.46 ± 0.39	8.61 ± 0.58	8.17 ± 0.15	8.15 ± 0.26	8.18 ± 0.26	8.09 ± 0.35
HGB (g/dL)	16.3 ± 0.6	16.7 ± 0.7	15.8 ± 0.5	15.9 ± 1.0	15.7 ± 0.3	15.7 ± 0.5	15.5 ± 0.5	15.6 ± 0.9
HCT (%))	47.5 ± 1.7	47.1 ± 2.2	45.8 ± 1.2	46.4 ± 2.7	44.8 ± 1.0	44.4 ± 1.4	44.2 ± 1.3	44.4 ± 2.6
MCV (fl)	54.8 ± 1.2	54.1 ± 2.1	54.2 ± 1.5	53.9 ± 1.4	54.8 ± 1.1	54.5 ± 1.1	54.0 ± 1.9	54.9 ± 1.4
MCH (pg)	18.8 ± 0.5	18.7 ± 0.8	18.7 ± 0.6	18.5 ± 0.5	19.3 ± 0.6	19.3 ± 0.5	18.9 ± 0.8	19.2 ± 0.5
MCHC (g/dL)	34.4 ± 0.6	34.5 ± 0.3	34.5 ± 0.5	34.3 ± 0.3	35.1 ± 0.5	35.4 ± 0.4	35.0 ± 0.6	35.0 ± 0.3
RDW (%)	13.6 ± 1.2	13.9 ± 1.2	14.1 ± 1.5	13.9 ± 1.2	11.5 ± 0.4	11.3 ± 0.3	11.7 ± 0.6	11.5 ± 0.4
PLT (10^9/L)	1042 ± 153	1009 ± 75	1071 ± 159	1076 ± 91	1013 ± 85	1030 ± 153	1078 ± 107	1006 ± 124
WBC (10^9/L)	12.35 ± 1.97	12.92 ± 2.88	14.10 ± 4.24	12.02 ± 2.22	6.13 ± 2.53	6.88 ± 1.94	7.88 ± 1.29*	8.27 ± 1.13*
ANEU (10^9/L)	1.61 ± 0.57	1.77 ± 0.47	1.52 ± 0.42	1.41 ± 0.32	0.89 ± 0.44	0.95 ± 0.35	1.05 ± 0.36	1.08 ± 0.37
ALYM (10^9/L)	10.02 ± 1.84	10.33 ± 2.40	11.79 ± 3.67	9.94 ± 2.16	4.82 ± 2.16	5.58 ± 1.65	6.35 ± 1.06*	6.71 ± 091*
AMON (10^9/L)	0.36 ± 0.11	0.42 ± 0.16	0.37 ± 0.15	0.35 ± 0.13	0.21 ± 0.12	0.16 ± 0.03	0.23 ± 0.06	0.25 ±0.08
AEOS (10^9/L)	0.18 ± 0.03	0.21 ± 0.06	0.20 ± 0.06	0.17 ± 0.04	0.13 ± 0.05	0.13 ± 0.05	0.17 ± 0.10	0.15 ± 0.04
ABAS (10^9/L)	0.07 ± 0.02	0.07 ± 0.04	0.10 ± 0.05	0.07 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
ALUC (10^9/L)	0.11 ± 0.05	0.12 ± 0.07	0.11 ± 0.05	0.09 ± 0.04	0.05 ± 0.03	0.05 ± 0.02	0.06 ± 0.04	0.05 ± 0.02
ARET (10^9/L)	221.0 ± 58.1	214.1 ± 49.2	255.8 ± 98.8	219.6 ± 33.8	157.1 ± 28.8	181.2 ± 43.9	169.8 ± 37.8	157.1 ± 26.9
Coagulation								
PT (sec)	10.5 ± 0.3	10.6 ± 0.2	10.4 ± 0.2	10.3 ± 0.2	9.9 ± 0.2	9.9 ± 0.2	9.7 ± 0.2	9.8 ± 0.2
APTT (sec)	23.6 ± 2.6	22.2 ± 2.3	21.1 ± 2.5	21.5 ± 2.1	19.6 ± 2.9	19.2 ± 2.0	18.2 ± 1.5	17.9 ± 2.3

Results are reported as mean ± standard deviation; * Statistically significant from control at $p < 0.05$.
ABAS = Absolute basophils; AEOS = Absolute eosinophils; ALUC = absolute large unstained cells; ALYM = absolute lymphocytes; AMON = absolute monocytes; ANEU = absolute neutrophils (all forms); APTT = activated partial thromboplastin time; ARET = absolute reticulocytes; HCT = hematocrit; HGB = hemoglobin; MCH = mean corpuscular (cell) hemoglobin; MCHC = mean corpuscular (cell) hemoglobin concentration; MCV = mean corpuscular (cell) volume; PLT = platelet count; PT = prothrombin time; RBC = red blood cell count; RDW = red cell distribution width; WBC = white blood cell count.
[a] Number of samples in male control group and female 475 mg/kg group = 9 for all analyses except PT and APTT due to clotting of one sample (male group) or insufficient amount of one sample for analysis (female group).

Table 6
Clinical chemistry parameters in rats administered Nephure™ for 13 weeks.

	Males (n = 10)				Females (n = 10)			
	Control	118 mg/kg	235 mg/kg	475 mg/kg	Control	118 mg/kg	235 mg/kg	475 mg/kg
AST (U/L) †	81 ± 16	85 ± 13	82 ± 18	85 ± 14	96 ± 56	69 ± 6	78 ± 24	88 ± 36
ALT (U/L)	36 ± 6	37 ± 7	34 ± 12	34 ± 4	43 ± 23	31 ± 6	35 ± 8	45 ± 23
SDH (U/L)†, ϒ	8.5 ± 3.7	5.9 ± 3.4	6.7 ± 2.4	6.1 ± 3.1	8.7 ± 4.6	6.6 ± 1.5	7.7 ± 3.6	8.6 ± 4.3
ALKP (U/L)	82 ± 10	82 ± 15	86 ± 12	83 ± 12	45 ± 11	46 ± 7	49 ± 21	45 ± 12
BILI (mg/dL)	0.16 ± 0.02	0.16 ± 0.03	0.16 ± 0.04	0.16 ± 0.03	0.17 ± 0.04	0.17 ± 0.03	0.17 ± 0.03	0.18 ± 0.03
BUN (mg/dL)	14 ± 2	13 ± 2	13 ± 1	13 ± 2	15 ± 1	15 ± 2	15 ± 2	15 ± 2
CREA (mg/dL)	0.30 ± 0.04	0.30 ± 0.04	0.29 ± 0.02	0.29 ± 0.03	0.33 ± 0.05	0.37 ± 0.03	0.34 ± 0.04	0.36 ± 0.04
CHOL (mg/dL)	72 ± 20	67 ± 11	71 ± 21	73 ± 24	81 ± 15	84 ± 14	104 ± 17*	92 ± 18
TRIG (mg/dL)	87 ± 38	74 ± 20	71 ± 26	62 ± 12	50 ± 20	40 ± 5	57 ± 25	45 ± 11
GLUC (mg/dL)	112 ± 16	116 ± 22	112 ± 16	113 ± 16	117 ± 14	120 ± 14	130 ± 14	117 ± 9
TP (g/dL)	6.4 ± 0.3	6.5 ± 0.2	6.4 ± 0.2	6.4 ± 0.5	7.0 ± 0.6	7.0 ± 0.4	7.3 ± 0.5	7.2 ± 0.4
ALB (g/dL)	3.1 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	3.1 ± 0.2	3.6 ± 0.4	3.7 ± 0.2	3.8 ± 0.3	3.7 ± 0.3
GLOB (g/dL)	3.3 ± 0.3	3.3 ± 0.2	3.3 ± 0.2	3.2 ± 0.3	3.4 ± 0.2	3.3 ± 0.2	3.5 ± 0.2	3.5 ± 0.2
CALC (mg/dL)	10.5 ± 0.5	10.5 ± 0.3	10.5 ± 0.3	10.4 ± 0.3	10.7 ± 0.6	10.6 ± 0.3	11.0 ± 0.4	10.9 ± 0.4
IPHS (mg/dL) †	6.8 ± 0.5	6.6 ± 0.4	6.6 ± 0.4	6.8 ± 0.5	5.1 ± 1.1	4.8 ± 0.7	4.9 ± 0.7	5.4 ± 0.8
Na (mmol/L)	143 ± 2.5	143.4 ± 2.1	143.8 ± 1.9	142.9 ± 1.5	141.5 ± 1.1	141.4 ± 1.6	140.5 ± 0.6	141.2 ± 1.9
K (mmol/L)	5.05 ± 0.27	5.06 ± 0.42	5.12 ± 0.36	5.18 ± 0.31	4.56 ± 0.34	4.50 ± 0.25	4.65 ± 0.67	4.64 ± 0.66
Cl (mmol/L)	102.1 ± 1.8	102.9 ± 1.5	102.9 ± 2.1	102.5 ± 1.0	102.3 ± 1.7	102.8 ± 1.5	101.8 ± 1.4	102.0 ± 1.5
HCO_3 (mmol/L)	22.4 ± 1.1	21.6 ± 0.9	22.0 ± 0.7	22.3 ± 1.0	20.4 ± 1.2	20.5 ± 1.4	20.2 ± 1.1	20.0 ± 1.5

Results are reported as mean ± standard deviation.
†Number of samples for males: Control: 9; 118 mg/kg bw/day: 8; 235 mg/kg bw/day: 9; 45 mg/kg bw/day: 8. For samples where the number does not equal 10, analyses were not performed due to sample hemolysis. ϒ Number of samples for females in the 235 mg/kg bw/day group is 9 due to an invalid result in one sample; all other groups 10.
* Statistically significant from control at $p < 0.05$.
ALB = albumin; ALKP = alkaline phosphates; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BILI = total bilirubin; BUN = urea nitrogen; CALC = calcium; CHOL = cholesterol; Cl = chloride; CREA = creatinine; GLOB = globulin; GLUC = glucose; HCO_3 = bicarbonate; IPHS = inorganic phosphorus; K = potassium; Na = sodium; SDH = sorbitol dehydrogenase; TP = total protein: TRIG = triglycerides.

4. Discussion

4.1. Toxicology studies

Nephure™ is a proprietary oxalate decarboxylase (OxDC) enzyme being developed as a food ingredient to degrade oxalic acid in food, reducing the concentration of oxalate available to cause anti-nutritional or toxicological effects. In order to be used in food, it must be demonstrated safe for use. Nephure™ did not show any mutagenic properties in a valid *in vitro* bacterial reverse mutation assay, with or without metabolic activation. Furthermore, results of an OECD Guideline No. 408 toxicity study in rats indicated that the 13-week oral (gavage) no observable adverse effect level (NOAEL) in rats was 475 mg/kg bw/day, the highest dose administered.

In the 13-week toxicity study, there were no clinical observations or changes in ophthalmology, body weight, body weight gain,

Table 7
Urinalysis parameters in rats administered Nephure™ for 13 weeks.

	Males (n = 10)				Females (n = 10)			
	Control	118 mg/kg	235 mg/kg	475 mg/kg	Control	118 mg/kg	235 mg/kg	475 mg/kg
UVOL (mL)	11.6 ± 4.4	15.4 ± 6.9	11.7 ± 4.7	7.2 ± 5.0	8.7 ± 5.9	7.8 ± 4.1	6.8 ± 4.1	7.6 ± 5.2
pH	6.7 ± 0.4	6.8 ± 0.3	6.6 ± 0.4	6.5 ± 0.4	6.6 ± 0.5	6.5 ± 0.3	6.5 ± 0.3	6.8 ± 0.6
SG	1.032 ± 0.015	1.024 ± 0.0.10	1.029 ± 0.015	1.040 ± 0.0.025	1.032 ± 0.018	1.025 ± 0.011	1.028 ± 0.012	1.028 ± 0.020
URO (EU/dL)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.3
UMTP (mg/dL)	113 ± 66	87 ± 47	106 ± 66	141 ± 110	35 ± 26	28 ± 14	34 ± 18	44 ± 41

Results are reported as mean ± standard deviation.
EU = Ehrlich Unit; SG = specific gravity; UMTP = protein; URO = urobilinogen; UVOL = volume.

Table 8
Mean absolute organ weight and organ relative to body weight.

	Males (n = 10)				Females (n = 10)			
	Control	118 mg/kg	235 mg/kg	475 mg/kg	Control	118 mg/kg	235 mg/kg	475 mg/kg
Body weight (g)	512.6 ± 38.8	508.8 ± 39.4	501.7 ± 38.6	499.0 ± 22.2	265.5 ± 15.3	254.7 ± 26.5	260.9 ± 14.7	259.7 ± 22.7
Adrenal Glands (g)	0.0667 ± 0.0043	0.0669 ± 0.0096	0.0603 ± 0.0058	0.0619 ± 0.0060	0.0700 ± 0.0103	0.0645 ± 0.0137	0.0643 ± 0.0130	0.0647 ± 0.0092
Adrenal/TBW	0.1307 ± 0.0118	0.1321 ± 0.0214	0.1209 ± 0.0159	0.1241 ± 0.0110	0.2641 ± 0.0393	0.2559 ± 0.0582	0.2480 ± 0.0576	0.2491 ± 0.0273
Brain (g)	2.256 ± 0.105	2.283 ± 0.089	2.256 ± 0.075	2.272 ± 0.086	2.093 ± 0.091	2.006 ± 0.084	2.007 ± 0.108	1.968 ± 0.058**
Brain/TBW	4.417 ± 0.299	4.510 ± 0.370	4.517 ± 0.328	4.563 ± 0.291	7.904 ± 0.516	7.939 ± 0.719	7.715 ± 0.613	7.637 ± 0.794
Epididymides (g)	1.495 ± 0.192	1.539 ± 0.146	1.533 ± 0.146	1.527 ± 0.167				
Epididymides/TBW	2.9271 ± 0.4033	3.0404 ± 0.3644	3.0676 ± 0.3847	3.0665 ± 0.3788				
Heart (g)	1.492 ± 0.106	1.476 ± 0.150	1.483 ± 0.099	1.464 ± 0.091	0.937 ± 0.084	0.942 ± 0.145	0.991 ± 0.108	0.911 ± 0.088
Heart/TBW	2.915 ± 0.158	2.902 ± 0.231	2.959 ± 0.108	2.937 ± 0.180	3.531 ± 0.268	3.695 ± 0.408	3.812 ± 0.506	3.509 ± 0.159
Kidneys (g)	3.255 ± 0.318	3.258 ± 0.327	3.433 ± 0.319	3.239 ± 0.255	1.916 ± 0.172	1.748 ± 0.221	1.969 ± 0.245	1.749 ± 0.137
Kidneys/TBW	6.357 ± 0.518	6.403 ± 0.365	6.844 ± 0.385	6.490 ± 0.414	7.225 ± 0.606	6.869 ± 0.554	7.577 ± 1.142	6.753 ± 0.456
Liver (g)	12.892 ± 1.876	12.713 ± 1.422	12.851 ± 1.664	12.089 ± 1.062	7.447 ± 1.114	6.856 ± 1.062	7.453 ± 0.484	7.144 ± 0.826
Liver/TBW	25.071 ± 2.280	24.970 ± 1.891	25.590 ± 2.362	24.223 ± 1.742	28.007 ± 3.504	26.876 ± 2.642	28.664 ± 2.702	27.533 ± 2.495
Spleen (g)	0.865 ± 0.149	0.857 ± 0.131	0.991 ± 0.191	0.863 ± 0.084	0.495 ± 0.049	0.531 ± 0.079	0.548 ± 0.056	0.512 ± 0.075
Spleen/TBW	1.687 ± 0.257	1.681 ± 0.196	1.979 ± 0.380*	1.731 ± 0.165	1.867 ± 0.183	2.090 ± 0.258	2.104 ± 0.230	1.971 ± 0.233
Testes (g)	3.568 ± 0.203	3.538 ± 0.284	3.490 ± 0.431	3.537 ± 0.184				
Testes/TBW	6.982 ± 0.465	6.987 ± 0.745	6.992 ± 1.017	7.093 ± 0.339				
Thymus (g)	0.2943 ± 0.0891	0.2728 ± 0.0651	0.2861 ± 0.0595	0.2896 ± 0.0610	0.2537 ± 0.0793	0.2583 ± 0.0722	0.2392 ± 0.0705	0.2660 ± 0.0518
Thymus/TBW	0.5753 ± 0.1755	0.5355 ± 0.1184	0.5702 ± 0.1137	0.5846 ± 0.1453	0.9479 ± 0.2608	1.0049 ± 0.2398	0.9150 ± 0.2597	1.0244 ± 0.1824
Ovaries with Oviducts (g)					0.1226 ± 0.0164	0.1138 ± 0.0154	0.1240 ± 0.0225	0.1232 ± 0.0139
Ovaries with Oviducts/TBW					0.4622 ± 0.0585	0.4479 ± 0.0484	0.4777 ± 0.0995	0.4754 ± 0.0482
Uterus (g)					0.692 ± 0.242	0.687 ± 0.169	0.973 ± 0.861	0.853 ± 0.322
Uterus/TBW					2.606 ± 0.892	2.697 ± 0.567	3.809 ± 3.560	3.325 ± 1.363

Results are reported as mean ± standard deviation; * Statistically significant from control at $p < 0.05$; ** Statistically significant from control at $p < 0.01$.
Relative organ weights (Ratios) presented in the table are times 1000. TBW = Terminal Body Weight.

food consumption, food efficiency, FOB or motor activity attributable to Nephure™. There were no test material-related changes in hematology, coagulation, clinical chemistry, or urinalysis parameters. Changes in hematology were limited to increased white blood cell counts and absolute lymphocyte counts in mid (235 mg/kg bw/day) and high dose female rats. However, all of the results that achieved statistical significance were well within historical control ranges, and the changes were small in magnitude and did not correlate with organ weight or microscopic observations. Thus, the hematological changes were interpreted to be of little toxicological significance and not adverse.

There were no macroscopic or microscopic findings or organ weight changes attributed to Nephure™ administration. Sporadic pathological findings were considered incidental as they showed no trends or patterns to suggest a relationship to administration of Nephure™ and were observed at similar rates in Sprague-Dawley rats of similar age and gender (Germann et al., 1998, Sugimoto et al., 2000; Percy and Barthold, 2007; McInnes, 2012). One female rat administered 235 mg/kg bw/day Nephure™ had a mammary gland adenocarcinoma in the thoracic region. This was also considered to be unrelated to the test material as it was isolated in nature and occurred at the middle dose. Mammary gland adenocarcinomas have been reported in Sprague-Dawley rats as young as twelve weeks of age (Kuzutani et al., 2012). The age-dependent frequency of mammary adenocarcinoma findings makes it difficult to give robust quantitative statements. However, based upon experience at PSL adenocarcinomas are equally likely to be observed in control groups as they are treatment groups. Therefore, one or two adenocarcinomas in treatment groups are not considered to be test substance-related. The statistically significant increase in mid dose male spleen-to-body weight ratio and decrease in high dose female absolute brain weight did not correlate with any histopathological or clinical pathology findings, and there was no effect of the test material on any test of neurological function. It is recognized that organ weight changes in and of themselves, without macroscopic or microscopic correlation, are not necessarily treatment-related or adverse (Sellers et al., 2007).

4.2. Potential human exposure

To calculate the estimated daily intake (EDI) of Nephure™ for individuals consuming the food groups selected for the addition of Nephure™, data from the U.S. Department of Agriculture's What We Eat In America (WWEIA) national food intake survey (NHANES, released every two years as one dataset) was used to determine Nephure™ intake. The NHANES dataset utilizes two days of dietary

Table 9
Foods selected for Nephure™ addition[a].

Food category	Intended use level (mg/serving)
Canned Pineapple	3
Canned Cherries	2
Strawberry Jelly	2
Canned Baked Beans	2
Canned Spinach	30
Canned Tomato	2
Canned Rhubarb	30
Canned Pickled Beets	30
Canned Sweet Potato	4
Mashed Potatoes	3
Brownies	4
Candies with Nuts (*i.e.* Snickers®)	4
Chocolate Syrup	4
Fudge Sauce	3
Chocolate Chip Cookies	2
Milk Chocolate Candies	2
Carrot Juice	3
Hot Chocolate	8
Lemonade (frozen from conc.)	2
Tea, Brewed	2
Tomato Juice	2
Prune Juice	2
Cranberry Juice	2
Chocolate Milk	2
Beer (Regular)	2
Beer (Light)	2
Miso	5
Peanut Butter	2
Creamer Suisse Chocolate	4
Mustard	2
All-Purpose Flour	2
Brown Rice Flour	8
Cocoa Powder	8
Cornmeal	8
Soy Flour	10
Wheat Flour, Whole Grain	3
Clam Chowder	2
Lentil Soup	4
Miso Soup	15
Cream of Wheat	2
Corn Grits	10
Pancakes (dry mix)	4

[a] A standard of identity may exist for some of these foods, which prohibits the addition of ingredients to the food not identified as mandated or optional ingredients under the standard. Therefore, addition of Nephure™ to a food for which a standard of identity exists would demand that the food product be named other than that as indicated under the standard of identity or a waiver of that standard be obtained.

intake per respondent; for each day, a detailed description is obtained of the amount and type of each food product consumed, with each food product assigned a specific food code. Utilizing a proprietary computer program, weighted data from the 2011–2012 NHANES data set was evaluated to determine the estimated amount of Nephure™ that may be consumed when added to the selected foods and levels specified in Table 9. The foods correspond to those provided by the USDA, with the exception of brown rice flour and soy flour which were not present in the NHANES survey. As it is likely that these types of flours will be used as replacements for other types of flours in the diet (e.g., wheat or all purpose flour), it is likely that estimates of intake of all purpose and wheat flour will encompass intake from brown rice or soy flour.

If Nephure™ is added to the selected foods at the levels specified in Table 9, the added mean and 90th percentile Nephure™ consumption is estimated at 5.76 mg/day (0.096 mg/kg bw/day assuming a 60 kg person) and 12.38 mg/day (0.206 mg/kg bw/day) respectively, for those individuals that consume these food products (i.e., "eater's only" individuals). The 90th percentile intake level is utilized in safety assessments to indicate high-level consumers of those products that may contain Nephure™. On a gender basis, it is estimated that men will consume 14.77 mg Nephure™/day (0.246 mg/kg bw/day) and women will consume 10.35 mg/day (0.172 mg/kg bw/day) at the 90th percentile. People 20–29 years of age are estimated to consume a higher level of Nephure™ at a mean of 6.54 mg/day (0.109 mg/kg bw/day) and 90th percentile of 14.58 mg/day (0.243 mg/kg bw/day assuming a 60 kg person). For substances that show no gender- or age-specific adverse effects (and there were none for Nephure™), assessments evaluating potential long-term (chronic) intake rely on "eater's only" data at the 90th percentile, to avoid the possibility of underestimating intake by individuals that frequently consume foods that contain the ingredient. Based on a manganese content of 85.4 ppm, the amount of manganese that will be consumed at the 90th percentile level of intake of Nephure™ for the eater's only individuals will be 1.06 μg (17.7 ng/kg bw/day), a nutritionally insignificant amount. Based on an optimal activity of 90 Units (μmol/min)/mg Nephure™, the added mean and 90th percentile Nephure™ consumption in terms of Units of enzymatic activity will be 518.4 Units/day (8.64 Units/kg bw/day) and 1114.2 Units/day (18.57 Units/kg bw/day). Thus, in 90th percentile consumers, addition of Nephure™ to the foods stated in Table 9 could potentially degrade 1114.2 μmol oxalate (98 mg, based on a molecular weight of oxalate of 88.019 g/mol) in one minute.

As the enzyme catalyzes the degradation of one mole of oxalate (88.019 g) to one mole of formate (45.01744 g, based on a molecular weight of formate of 45.01744 g/mol), which is a 1.955 g oxalate: 1 g formate ratio, 90th percentile consumers of the foods containing Nephure™ would produce 50 mg formate/day (0.83 mg/kg bw/day) from this source. High blood levels of formate are thought to be responsible for the optic nerve damage and metabolic acidosis that occurs in methanol poisoning. Hallmarks of formate toxicity include decreases in blood potassium and bicarbonate, hyperpnea, and visual impairment (Eells et al., 1996; Mahieu et al., 1989). Certified 2016 Harlan Teklad Global Rodent Diet®, was selected due to its high oxalate content of 880 mg oxalate/kg of diet. Assuming that 100% of the oxalate is degraded to formate the resulting exposure would be 26 and 32 mg formate/kg bw/day for male and female rats, respectively, far exceeding the predicted human exposure. Importantly, there was no effect of Nephure™ on blood potassium or bicarbonate levels and no clinical, ophthalmological or behavioral findings suggestive of formate toxicity.

The current acceptable daily intake (ADI) for formic acid (the acid of formate), as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), is 3 mg/kg bw/day (JECFA, 2003). Some foods naturally contain formic acid and a number of formic acid-containing compounds are permitted by FDA for use as food additives (synthetic flavoring substances and adjuvant).[1] Average daily intake of formic acid from the diet has been estimated by the Select Committee on GRAS Substances (SCOGS) to be < 1 mg/kg bw/day (SCOGS, 1976).

Dietary intake of oxalate has previously been estimated at 44–351 mg/day, with a mean intake of 152 mg/day (Holmes and Kennedy, 2000). The FDA has stated that as an estimation, 90th percentile intake is approximately twice that of the mean (FDA, 2015). Assuming the 90th percentile intake of oxalate at 304 mg (3450 μmoles) *per* person, 155.5 mg (2.59 mg/kg bw/day) formate could be produced if Nephure™ degraded all oxalate that is consumed (using the 1.955 g oxalate: 1 g formate ratio). Thus, if Nephure™ were to be added to food at a level to remove the 90th

[1] Title 21 of the Code of Federal Regulations (CFR) §172.515.

percentile level of dietary oxalate (approximately 38.33 mg, which equates to 3450 Units/day or 57.5 Units/kg bw/day), up to 2.59 mg/kg bw of formate could be produced on a daily basis, which when added to the 1 mg/kg bw/day formate that could currently be ingested from foods is slightly greater than the JECFA ADI of 3 mg/kg bw/day for formic acid, but lower than the doses of formic acid which have been shown to be safe in humans (33.3–66.7 mg/kg bw/day) (InChem, 1973). Although the anticipated amount of Nephure™ consumed from addition to food will be 18.57 Units/kg bw/day, it is anticipated that up to 57.5 Units/kg bw/day can be safely consumed based on the maximum amount of formate that could be generated.

4.3. Kanamycin

As stated above, kanamycin is used during the manufacture of Nephure™ and a residual amount is present in Nephure™. Based on an average kanamycin concentration of 8.7 ng/mg protein in Nephure™, an average protein concentration of 119.5 mg/mL Nephure™, and an average density of 1.00 g/mL, the amount of kanamycin in 1 g of Nephure™ is 1.04 μg (1.04 ppm). At the mean Nephure™ consumption of 5.76 mg/day, approximately 5.99 ng/day (0.10 ng/kg bw/day) kanamycin will be consumed. Kanamycin consumption will increase to 12.87 ng/day (0.21 ng/kg bw/day) at the 90th percentile level of Nephure™ consumption.

Currently, there are no regulations regarding permissible levels of kanamycin in food in the United States. The European Agency for the Evaluation of Medicinal Products (EMEA) has determined that the human ADI for kanamycin with respect to the most sensitive microorganism of the human gut flora (*Peptostreptococcus*) is 480 μg/person (8 μg/kg bw/day) (EMEA, 2003), approximately 38,000 times higher than the daily amount of kanamycin that will be consumed by 90th percentile consumers of Nephure™. Therefore, in the absence of selective pressure on microbial growth there is no risk of developing antibiotic resistance to kanamycin.

Kanamycin is a highly polar, cationic compound with very low oral bioavailability (approximately 1%) in humans (EMEA, 2003). Thus, the amount of kanamycin from Nephure™ that will be absorbed into the bloodstream of 90th percentile consumers is negligible and will not be expected to have a bactericidal effect in any tissues or fluids in the human body.

4.4. Allergenicity

A search of the published literature did not locate any references suggesting that *Synechococcus elongates* and *E. coli* strain BW25113 produce allergenic proteins. While Nephure™ may be considered a protein that is new to the human diet, the probability that it will be allergenic is low based on the fact that under typical circumstances of exposure, only a small number of the total proteins found in foods are allergenic, or known to be associated with food sensitivities (Institute of Medicine and National Research Council, 2004). OxDC enzymes are naturally present in the diet and in bacteria that may inhabit the large intestine of humans without known reports of allergenicity, which suggests that Nephure™ would have low potential to act as an allergen.

Current scientific knowledge suggests that the combined application of certain tests can provide reasonable assurance that a protein has a low probability of acting as an allergen. Tier I tests include evaluating whether the source of the protein is a known allergen and comparing the amino acid sequence of the protein to known allergens (EPA, 2001; FAO/WHO, 2002; Institute of Medicine and National Research Council, 2004; Delaney et al., 2008; FAO/WHO, 2009). These tests have been performed to examine whether Nephure™ could be considered an allergen. The results of these studies (discussed below) indicate that Nephure™ does not possess allergenic activity.

4.4.1. Overall homology

The Allergen Online database version 16 (Accessed May 19, 2016) (FARRP, 2016) was used to conduct a preliminary screen of the Nephure™ protein sequences for relevant matches against known allergens. The database is maintained by the Food Allergy Research and Resource Program (FARRP) of the University of Nebraska. A FASTA3 overall search of Allergen Online was conducted using default settings (E cutoff = 1 and maximum alignments of 20). Low level matches (E value $\geq$ 0.0001) to peanut and soybean proteins sharing approximately 25% identity were identified; however, no matches sharing greater than 50% identity over its length relative to allergens within the database were identified, which indicates that the potential of cross-reactivity of Nephure™ to any allergens is low (Aalberse, 2000).

4.4.2. Epitope homology

An 80 amino acid sliding window (segments 1–80, 2–81, 3–82, *etc.*) was used to scan the amino acid sequences against the allergen database using FASTA to search for matches of 35% identity or more. This 35% identity for 80 amino acid segments is a suggested guideline proposed by Codex for evaluating proteins in genetically modified crops (Goodman et al., 2008; FAO/WHO, 2009). The results of the FASTA3 alignments of all possible 80 amino acid segments of Nephure™ against all putative allergen sequences in the database were all less than the 35% threshold over 80 amino acids. In addition, no sequences were identified with an exact 8-mer match to a known allergen.

5. Conclusion

Nephure™ is an oxalate decarboxylase enzyme formulation that decreases oxalate content in certain processed foods, with an average activity of 70 Units/mg. The results of the studies described in this manuscript show that Nephure™ is not genotoxic and is not toxic to Sprague Dawley rats at the dose levels provided. Under the conditions of the study and based on the toxicological endpoints evaluated, the no-adverse-effect level (NOAEL) for oral administration of Nephure™ was 475 mg/kg/day (33,250 Units/kg bw/day), the highest dose evaluated for both male and female rats. When added to certain foods to decrease oxalate intake, Nephure™ is estimated to be consumed at 12.38 mg/day at the 90th percentile of intake, which equates to approximately 1114.2 Units/day (18.57 Units/kg bw/day). Up to 3450 Units of activity/day (57.5 Units of activity/kg bw/day) can be safely consumed from addition of Nephure™ to food to reduce the intake of dietary oxalate. *S. elongates* and the *E. coli* strain used in this work are not known to produce allergenic proteins, and potential cross-reactivity of the Nephure™ protein sequence with known allergens is low, based on homology screening. The authors conclude that the weight of the toxicological data of the product supports the safety of the substance as a food ingredient.

Conflict of interest

All authors have a financial relationship with the sponsor of the study, Captozyme, Inc.

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