



Evaluation of the safety of mixed tocopheryl phosphates (MTP)—A formulation of α -tocopheryl phosphate plus α -di-tocopheryl phosphate

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Abstract

The safety of a formulation of mixed tocopheryl phosphates, (MTP) was evaluated in a series of toxicological tests in vivo using rats, mice and rabbits and in vitro using bacterial and mammalian cell cultures. The tests conducted included an oral LD₅₀ study, three 28-day oral repeat-dose studies, two dermal toxicity tests, an ocular irritation test, mutagenic potential tests, and chromosomal aberrations tests. MTP consists of mono α -tocopheryl phosphate (TP) and di-tocopheryl phosphate (T₂P) and is intended for use as a dietary supplement and for dermal applications in humans and animals. The dermal and oral LD₅₀ values of MTP were determined to be >1130 mg/kg bw (918 mg tocopherol equivalents/kg bw) in rabbits and rats, respectively. MTP was not a dermal or eye irritant in rabbits and showed no allergenic potential in mice. In the mutagenicity and genotoxicity studies, MTP did not increase the number of revertants in *Salmonella typhimurium* or *Escherichia coli* and did not induce chromosomal aberrations in cultured Chinese hamster ovary (CHO) cells. When administered daily for 28 days by gavage at doses up to 955 mg/kg bw/day (780 mg tocopherol equivalents/kg bw/day), MTP produced no consistent, dose-dependent adverse effects in rats.

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1. Introduction

α -Tocopherol, the active form of vitamin E, is used as an ingredient in foods and personal care products and is widely regarded as one of the safest vitamins that are taken as dietary supplements.

The safety of tocopherols was recently reviewed by the Cosmetic Ingredient Review Expert Panel (CIREP, 2002). Extensive acute toxicity, repeat-dose, ocular and dermal irritation, sensitization, reproductive and developmental studies in animals and clinical studies on irritation, sensitization, and toxicity were evaluated. The Panel concluded that tocopherols were not irritants or sensitizers under the conditions of use and were generally not toxic in animal feeding studies, although very high doses (≥ 2 g/kg body

Abbreviations: ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; CHO = Chinese hamster ovary; GGT = gamma glutamyl transferase; PCV = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MTP = mixed tocopheryl phosphates; TP = mono- α -tocopheryl phosphate; T₂P = α -di-tocopheryl phosphate; LD₅₀ = 50% lethal dose concentration; RDA = recommended daily allowance; UL = upper intake level; RO = reverse osmosis; dpm = disintegrations per minute; APTT = activated prothrombin time; CPK = creatine phosphokinase; LDH = lactate dehydrogenase; SPF = specific pathogen free; FOB = functional observation battery; ANOVA = analysis of variance; TE = α -tocopherol equivalents calculated for MTP. Vital ET is a trademark of the ISP Group and Vital Health Sciences.

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weight/day) in animals produced hemorrhagic effects. The Institute of Medicine (IOM) has published Dietary Reference Intakes for Vitamin E with a recommended daily intake (RDA) for adults of 15 mg (34.9 μmol)/person/day of *RRR*- α -tocopherol and a tolerable upper intake level (UL) for adults of 1000 mg (2326 μmol)/person/day of any form of supplementary α -tocopherol (IOM, 2000). A review of the safety of vitamin E that was conducted very recently (Hathcock et al., 2005) found that the tolerable upper intake level is also 1000 mg/day for *RRR*- α -tocopherol, or the molar equivalents of its esters. In addition, no risk was identified when the vitamin is used at this level by the general population.

The monophosphate ester of α -tocopherol, α -tocopheryl phosphate, and the di-ester, α -di-tocopheryl phosphate are contained in the product MTP, which is currently being examined for use as a dietary supplement. These phosphorylated forms of tocopherol are not yet classified by the IOM as being dietary supplements for vitamin E. However, it can be said that α -tocopheryl phosphate is a naturally occurring form of α -tocopherol (Ogru et al., 2003; Gianello et al., 2005; Negis et al., 2005), and there are some in vitro biological data from tests with MTP that show it has anti-inflammatory properties (Munteanu et al., 2004).

The present paper describes the results of subjecting the mixture MTP, to a group of acute toxicity, irritation, sensitization, mutagenicity, genotoxicity, and repeat-dose toxicity tests.

2. Methods

The mixture MTP was produced by phosphorylating natural *d*- α -tocopherol with P_4O_{10} , as described previously (Munteanu et al., 2004). The final composition typically contained TP, α -di-tocopheryl phosphate and α -tocopherol in the ratio 1:0.49:0.08 (w/w/w). Additional components include inorganic phosphate and water at less than 5% w/w. The MTP was prepared for oral gavage in a medium chain triglyceride (MCT; Captex 355 Low C6, Abitech Corp, USA), or, for dermal, other testing, the tocopheryl phosphates were formulated as an aqueous gel (Vital ETTM), and contained excipients. Test formulations and stabilities were measured by RP-HPLC and P-NMR.

2.1. Acute oral toxicity study

Five male and 5 female Wistar albino rats were given a single oral dose of Vital ET containing 1130 mg MTP/kg body weight (918 mg TE/kg bw) in distilled water (by gavage and observed at 1, 2 and 4 h post-dose and once daily for 14 days for toxicity and pharmacological effects and twice daily for mortality. Body weights were recorded prior to dosing, weekly after dosing and at the end of the study. All surviving animals were killed by CO_2 asphyxiation and examined for gross pathology. The study met the GLP of the EPA, 40 CFR 160 and 792, FDA 21

CFR 58 and as specified in The Testing of Chemicals, published by the OECD, 1997.

2.2. Acute dermal toxicity study

A dose of 1130 mg MTP/kg body weight (918 mg TE/kg bw) in paste form was applied to the clipped dorsal trunk (10% of body surface area) of New Zealand rabbits (5/sex) and was kept in direct skin contact for 24 h using surgical gauze. After 24 h, the skin was gently washed with distilled water to remove residual test material. The skin test sites were evaluated at 24 h, 7 days and 14 days using the Draize scoring method for dermal irritation and were assessed for signs of ulceration, necrosis, or any tissue destruction. In addition, the rabbits were observed at 1, 2 and 4 h post-dose and once daily for 14 days for toxicity and pharmacological effects and twice daily for mortality. Body weights were recorded prior to dosing, weekly after dosing and at the end of the study. At the end of the study, all surviving animals were killed by CO_2 asphyxiation and examined for gross pathology. The study met the GLP of the EPA, 40 CFR 160 and 792, FDA 21 CFR 58 and as specified in The Testing of Chemicals, published by the OECD, 1997.

2.3. Dermal irritation study

Vital ET at a dose of 0.5 g/site (containing 88–101 mg MTP/kg body weight; 71–82 mg TE/kg bw) was applied in paste form under gauze to the clipped dorsal trunk (10 cm \times 10 cm) of 1 male and 2 female New Zealand rabbits in a semi-occlusive manner. After 4 h, the gauze was removed and the skin was gently washed with distilled water to remove residual test material. The skin test sites were evaluated at 60 min after patch removal and again at 24, 48, and 72 h using the Draize scoring method for erythema and oedema and were assessed for signs of ulceration, necrosis, or any tissue destruction. Body weights were recorded prior to treatment and at study termination. The general health of the animals was observed at each time period. At the end of the study, all surviving animals were killed by CO_2 asphyxiation. The study met the GLP of the EPA, 40 CFR 160 and 792, FDA 21 CFR 58 and as specified in The Testing of Chemicals, published by the OECD, 1997.

2.4. Eye irritation study

Vital ET (0.1 ml containing 47.5 mg MTP; 38.4 mg TE/kg bw) was applied into the conjunctival sac of one eye of New Zealand rabbits (1 male and 2 females). After instillation, the eyelids were held together for approximately 1 s to ensure adequate dispersal of the test article. The contralateral eye of each animal was maintained as a control. The eyes were examined using a high-intensity light and scored by the Draize method at 1, 24, 48 and 72 h post-dose. Sodium fluorescein dye was used at the 24 h time-point

and the eyes were examined with an ultraviolet light source. Body weights were recorded prior to treatment. The general health of the animals was observed at each time period. At the end of the study, all surviving animals were killed by CO₂ asphyxiation. The study met the GLP of the EPA, 40 CFR 160 and 792, FDA 21 CFR 58 and as specified in The Testing of Chemicals, published by the OECD, 1997.

2.5. Allergenic potential

The murine local lymph node assay was used to assess the allergenic potential of topical Vital ET. Groups of female CBA/J mice (5/group) were left untreated (naïve control), treated with reverse osmosis (RO) water (RO control), treated with acetone/olive oil (vehicle control for hexylcinnamaldehyde), treated with 5, 10, or 25% (w/v) Vital ET in RO water (containing 1.13, 2.26, or 5.65 mg MTP, respectively; or 0.92, 1.84, or 4.60 mg TE, respectively), or treated with 25% (w/v) hexylcinnamaldehyde in acetone/olive oil (positive control). Treatments were applied at a volume of 25 µl behind the dorsal aspect of each ear daily for three consecutive days. Animals were untreated on days 4 and 5, and on day 6 they were given a single intravenous injection of H³-thymidine. Approximately 5 h post-H³-thymidine injection, the animals were killed and the auricular lymph nodes were removed intact and pooled for each individual mouse. Single cell suspensions were prepared, the radiolabeled macromolecules were acid-precipitated, and the samples were counted [disintegrations per minute (dpm)] using liquid scintillation counting. During the study period, the mice were observed generally for mortality, abnormalities, and signs of pain or distress and the treatment sites were observed for any significant alterations in appearance. The dpm values were analysed statistically and the stimulation index (mean dpm/mean dpm of vehicle control) was calculated. The study met the GLP of the FDA 21 CFR 58 and the OECD Principles of GLP, EMV/MC/CHEM (98) 17, and with any applicable amendments with the exception that dose analysis was not done. This study was also conducted in accordance with the Nuclear Regulatory Commission regulations, License No. 48-11805-02.

2.6. Mutagenicity and genotoxicity studies

2.6.1. Reverse mutation

Vital ET (dissolved in water) was assayed for mutation using the Ames test (Ames et al., 1975) in 4 histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and 1 tryptophan-requiring strain of *Escherichia coli* (WP2 uvrA), both in the presence and absence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9). Triplicate plates with and without S-9 were used. Negative solvent (water) and positive (i.e., 2-nitrofluorene, sodium azide, 9-aminoacridine, 4-nitroquinoline 1-oxide, benzo[a]pyrene, and 2-aminoanthracene) controls were included in

quintuplicate and triplicate, respectively, with and without S-9. For the platings, 0.1 ml bacterial culture, 0.5 ml test article solution, and 0.5 ml 10% S-9 mix or buffer solution were added to 2.5 ml molten agar at 46 ± 1 °C. The molten agar was rapidly mixed and poured on to Vogel–Bonner E agar plates. When set, the plates were inverted and incubated for 3 days at 37 ± 1 °C. Following incubation, the plates were examined for evidence of toxicity to the background bacterial lawn and, where possible, revertant colonies were counted. An increase in the number of revertants was considered significant if it was concentration related and at least 2 times the mean negative control counts for strains TA98, TA100 and WP2 uvrA and at least 3 times the mean negative control counts for strains TA1535 and TA1537.

Initially, MTP in Vital ET was tested as described above in strain TA100 at concentrations of 0.9, 4.5, 23, 113, 565, and 2825 µg MTP/plate (or 0.73, 3.7, 18.4, 91.8, 458.8 and 2294 µg TE/plate, respectively). Following the results of this initial concentration-finding experiment, the other 3 *S. typhimurium* strains and the *E. coli* strain were tested under the same conditions and concentrations.

A second experiment was conducted using the same test strains and experimental conditions but included a pre-incubation step and used plate concentrations of 88.3, 176.6, 353.1, 706.2, 1412.5 and 2825 µg MTP/plate (or 71.7, 143.4, 286.8, 573.5, 1146.9 and 2293.9 µg TE/plate, respectively). Prior to the addition to the 2.5 ml molten agar, the bacterial solution was well mixed and incubated for 1 h at 37 ± 1 °C. The plating procedure was identical to that described above. This study was conducted in compliance with the UK Statutory Instrument 1999 No. 3106, The GLP Regulations 1999 and the OECD Principles of GLP (rev 1997, issue Jan 1998) ENV/MC/CHEM (98) 17.

2.6.2. Chromosomal aberrations

Vital ET was tested for chromosomal aberrations using duplicate cultures of Chinese hamster ovary (CHO) cells in 2 independent experiments. In both experiments, Vital ET was suspended in 1% carboxy methyl cellulose and treatments were made with and without metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9). Cultures were maintained in 75 cm² tissue culture flasks and incubated at 37 °C. S-9 was added to cultures requiring metabolic activation followed by addition of the respective test solution. Cultures without metabolic activation received KCl in place of S-9 at a volume equivalent to the amount used in S-9 preparation. Negative [solvent (1% carboxy methyl cellulose) and untreated] controls were included in both experiments. Similarly, cyclophosphamide and 4-nitroquinoline 1-oxide were used as positive controls in the presence and absence of S-9, respectively.

Two hours prior to harvest, colchicine was added to arrest dividing cells in metaphase. The suspension from each flask was centrifuged at 200 × g for 5 min to pellet the cells, which were resuspended in 4 ml hypotonic (0.075 M) KCl and incubated at 37 °C for 5 min. Cells were

fixed using methanol/glacial acetic acid (3:1; v/v) and the fixative was changed by centrifugation and resuspension several times until the cell pellets were clean. Several drops of 45% (v/v) aqueous acetic acid were added and the cells were transferred to microscopic slides. The cells were stained with Giemsa stain for 5 min and then rinsed, dried and mounted with cover slips. Cell numbers were determined using a Coulter counter. One hundred metaphase/slide were analysed for chromosomal aberrations. Classification of structural aberrations was conducted using the scheme described by ISCN (1995). The test item was considered positive if (1) the proportions of cells within structural aberrations at one or more concentrations exceeded the normal range in both replicate cultures, and (2) a statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurred at these concentrations.

In the first experiment, treatment with and without S-9 was for 3 h followed by a 17-h recovery period prior to harvest. The concentrations of MTP in the Vital ET tested were 13.4, 26.1, 32.6 and 40.8 µg MTP/ml (or 10.9, 21.2, 26.5 and 33.1 µg TE/ml, respectively) with S-9 and 26.1, 32.6, and 40.8 µg MTP/ml without S-9 (or 21.2, 26.5 and 33.8 µg TE/ml, respectively).

In the second experiment, treatment with S-9 was for 3 h followed by a 17-h recovery period prior to harvest and the concentrations of MTP in Vital ET tested were 15.5, 19.4 and 37.9 µg MTP/ml (or 12.6, 15.8 and 30.8 µg TE/ml, respectively). Treatment without S-9 was continuous for 20 h and the MTP concentrations tested were 3.7, 17.8 and 67.8 µg MTP/ml (or 3.0, 14.5 and 55.1 µg TE/ml, respectively). This study was conducted in compliance with the UK Statutory Instrument 1999 No. 3106, The GLP Regulations 1999 and the OECD Principles of GLP (rev 1997, issue Jan 1998) ENV/MC/CHEM (98) 17.

2.7. Repeat-dose toxicity studies

Three repeat-dose toxicity studies involving 28-day exposures were conducted using rats and are described below.

2.7.1. Study 1

In a non-GLP study, four groups of male and female Sprague-Dawley OB SPF rats (10–11/sex/group; see Table 1), aged 8–9 weeks were administered 0 (vehicle control, MCT oil), 368, 735 or 982 mg MTP/kg body weight/day. These doses have calculated values of 0, 300, 600 and 800 mg TE/kg body weight/day, respectively. The average doses administered by oral gavage of the oils were determined by the differences in syringe weights before and after the administration (emptied syringe weights subtracted from loaded weights). On average for males and females combined, over the 28-day treatment period the following doses were administered: 0, 354, 707 and 956 mg/kg body weight/day (or 289, 576 and 779 mg TE/kg body weight/day, respectively). The administered doses were within

Table 1
Experimental design for the repeat-dose studies

Group	Treatment ^{a,b} (mg/kg bw/day)	No. of animals ^b
Study 1 (28-day exposure)		
A (control)	0	10 (M); 10 (F)
B (low)	350 (M; TE 285); 357 (F; TE 291)	10 (M); 11 (F)
C (mid)	708 (M; TE 577); 705 (F; TE 575)	11 (M); 11 (F)
D (high)	927 (M; TE 756); 984 (F; TE 802)	10 (M); 10 (F)
Study 2 (28-day exposure, 14-day recovery)		
A (control)	0	5 (M); 5 (F)
D (high)	869 (M; TE 708); 788 (F; TE 642) ^c	5 (M); 4 (F)
Study 3 (28-day exposure)		
1 (control)	0	5 (M); 5 (F)
2 (low)	56.5 (TE 45.9)	5 (M); 5 (F)
3 (mid)	282.5 (TE 229.4)	5 (M); 5 (F)
4 (high)	565 (TE 458.8)	5 (M); 5 (F)

^a Average gavage doses of MTP (TE = tocopherol equivalents).

^b M = male; F = female.

^c These high doses were lower than Study 1 due to a lower weekly average dose during the second week of treatment.

3.8%, 3.8% and 2.6% of the target doses, respectively for the low, medium and high doses.

The test article was mixed in MCT, with the amounts administered to all treatment groups kept to approximately 0.2 ml per 100 g body weight. The rats were given standard laboratory rodent chow and water ad libitum and were housed 1–5 rats per cage on a 12-h light/dark cycle at a room temperature of 22 ± 2 °C.

Animals were observed daily for general clinical signs and individual body weights were recorded prior to treatment and weekly thereafter. Food and water was provided ad libitum, with food consumption recorded weekly, and water consumption monitored but not recorded. Behavioural (i.e., awareness, mood, motor activity, and motor coordination) and neurological (i.e., central excitation, muscle tone, body posture, and reflexes) observations also were recorded prior to treatment and weekly thereafter. Twenty-four hours following the last treatment, the animals were euthanized by CO₂ asphyxiation, blood samples were taken by heart puncture for haematology (i.e., red cell count, haemoglobin, PCV, MCV, MCH, MCHC, white cell count, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, prothrombin time, APTT, and fibrinogen) and clinical chemistry (i.e., sodium, potassium, chloride, bicarbonate, urea, creatine, calcium, phosphorus, protein, albumin, globulin, total bilirubin, alkaline phosphatase, ALT, GGT, AST, CPK, LDH, cholesterol, phospholipids, and triglycerides) analyses. Urinalyses (i.e., pH, specific gravity, protein, glucose, blood, ketones, bilirubin, urobilinogen, white blood cells, red blood cells, epithelials, fat globules, and casts) were conducted on urine samples taken 3 days prior to the final treatment day, with the rats placed unfasted in metabolic cages for 16 h.

On day 29, the animals were euthanized by CO₂ asphyxiation, organs (i.e., liver, spleen, intestine, kidney, heart, testes, and brain) were removed and weighed, a necropsy was conducted by a veterinary pathologist, and the macro-

scopic and microscopic findings were reported. The post-mortem and pathology were conducted under GLP.

Data were analysed using a 2-way ANOVA with Treatment Group (control, low, medium and high dose) and Sex (male and female) as the between-subjects factors. If the ANOVA indicated a statistical effect, mean values were compared using Fisher's test of least significant difference. Data were analysed using SPSS for Windows (SPSS Inc., Release 11.0.0).

2.7.2. Study 2

In a second non-GLP study, two groups of male and female Sprague-Dawley OB SPF rats (4–5/sex/group; see Table 1) aged 8–9 weeks were administered an average of 0 (vehicle control) or 869 mg MTP/kg bw/day (708 mg TE/kg/day) for males and 0 (vehicle control) or 788 mg MTP/kg bw/day (642 mg TE/kg/day) for females by gavage for 28 days followed by a 14-week recovery period. All other study parameters were identical to Study 1.

2.7.3. Study 3

In this study, 4 groups of Wistar rats (5/sex/group) of similar weight (261–322 g and 186–236 g for males and females, respectively) were administered 0 (vehicle control), 56.5, 282.5 or 565.0 mg MTP/kg bw/day (45.9, 229.5 or 458.8 mg TE/kg/day, respectively) prepared as Vital ET in distilled water by gavage for 28 days (Table 1). The dosage volume was 4.0 ml/kg bw. The rats were housed one per cage on a 12-h light/dark cycle with a room temperature of 21.1–22.2 °C (humidity 16–68%). Certified Purina Rodent Chow Diet #5002 and fresh water were provided ad libitum.

Animals were observed twice daily for morbidity and mortality and once daily for toxicity and pharmacological effects. Body weights were recorded on day of receipt, after 1 week of acclimatization, immediately pre-test, weekly, at death and at termination in the survivors. Feed consumption was determined weekly. On study days 23 and 24, animals underwent a functional observational battery (FOB) test to assess neurotoxicity and neurobehavioral changes.

On study day 29, following an overnight fast, animals were anesthetized with ether and blood was collected from the aorta for haematological (i.e., red blood cell count, haemoglobin, haematocrit, MCV, MCH, MCHC, white blood cell count, differential leukocyte count, platelet count, and prothrombin time) and clinical chemistry (i.e., magnesium, sodium, ALT, sorbitol dehydrogenase, blood urea nitrogen, total bilirubin, calcium, phosphorous, AST, albumin, globulin, total cholesterol, triglycerides, chloride, potassium, alkaline phosphatase, GGT, creatinine, glucose, and total protein) analyses. After blood collection, all surviving animals were terminated by exsanguination and underwent gross necropsy. The adrenals, kidneys, liver, testes (with epididymides), thymus, spleen, brain, and heart were removed, weighed, and preserved in 10% neutral formalin. Additional tissues and organs (i.e., oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, pitu-

itary, peripheral nerves, spinal cord, thyroids/parathyroids, pancreas, lung and trachea, thoracic aorta, sternal bone marrow, lymph nodes, urinary bladder, seminal vesicles, uterus, ovaries and vagina, prostate, and all gross lesions and masses) also were preserved in 10% neutral buffered formalin. All preserved tissues from the control and high-dose group were examined microscopically by a veterinary pathologist. The study met the GLP of the EPA, 40 CFR 160 and 792, FDA 21 CFR 58 and as specified in The Testing of Chemicals, published by the OECD, 1997 with the following exception: the test article characterisation was not performed in full compliance with GLPs.

An Analysis of Variance (ANOVA) was performed on absolute organ weights, body weights, organ/body weight ratios, haematology and clinical chemistry. Parametric data were analysed using ANOVA techniques with the Tukey–Kramer post-hoc test. Non-parametric data were analysed using Kruskal–Wallis analysis of variance with Dunn's post-hoc test. Instat[®] Version 2.0 software was used for statistical analysis.

3. Results

A series of toxicological tests were performed in vivo using rats, mice and rabbits and in vitro using bacterial and mammalian cell cultures. The tests included an oral LD₅₀ study, three 28-day oral repeat-dose studies, two dermal toxicity tests, an ocular irritation test, mutagenic potential tests, and chromosomal aberrations tests. The results are summarized in Tables 2 and 3.

3.1. Acute oral toxicity study

All rats survived to scheduled termination. The only abnormal physical signs noted during the study were some instances of soiling of the anogenital area (1 male at hour 4) and localized alopecia. Localized alopecia of the front limbs, believed to be the result of the feeder design, was reported in 4/10 rats (3 males and 1 female). The remaining animals appeared normal. With the exception of a single female rat losing weight during the second week of the study, all the other rats showed normal body weight changes over the study period. These results indicate that the dermal LD₅₀ of MTP is greater than 1130 mg/kg body weight (918 mg TE/kg bw) in Wistar rats.

3.2. Acute dermal toxicity study

All the rabbits survived to the end of the study. Diarrhoea was noted in 1 male and 1 female on the first day following treatment. Few faeces were reported in 3 females; 2 towards the end of the study and 1 at the beginning of the study. With the exception of a single female rabbit losing weight during the second week of the study, all the other rabbits showed normal body weight changes over the study period. Dermal examination showed some slight to well-defined erythema (4/5 males and 5/5 females) and slight

Table 2
Summary of toxicological studies with MTP

Study	Species/strain	Formulation	Route of admin.	No/sex/group	Dose, dosing regimen	Duration	Results
Oral LD ₅₀	Wistar rat	Vital-ET TM	Gavage	5/sex/group	1130 mg MTP/kg bw (918 mg TE/kg)	One administration; observed 14 days	All animals survived the dose. Instances of anogenital soiling were observed and some alopecia believed to be due to the design of the feeder. No changes in body weight except in a single female
Dermal LD ₅₀	New Zealand white rabbit	Vital-ET TM	Dermal	5/sex/group	1130 mg MTP/kg bw (918 mg TE/kg); single application	24 h; observed 14 days	All animals survived the dose. Instances of diarrhoea and reduced passing of faeces. No changes in body weight except in a single female but still within normal range
Dermal irritation	New Zealand white rabbit	Vital-ET TM	Dermal	1 M 2 F	88–101 mg MTP/kg bw (71–82 mg TE/kg)	4 h; observed 72 h	Erythema was absent to barely perceptible at 60 min with no dermal irritation at 24, 48 and 72 h. There was no change to body weights and no abnormal physical signs
Eye irritation	New Zealand white rabbit	Vital-ET TM	Eye	1 M 2 F	0.1 ml equivalent 47.5 mg MTP (38.4 mg TE)	Observed 72 h	All the rabbits survived to the end of the study and showed no abnormal clinical signs. Treated eyes were normal at all time points
Allergenic potential	CBA/J mouse	Vital-ET TM	Dermal	5/females/group	1.13, 2.26 or 5.65 mg MTP/ml daily (0.92, 1.84 or 4.6 mg TE/ml, resp.); H ³ -thymidine by iv on day 6	3 days	All the mice survived to scheduled termination with no signs of toxicity. Three mice from the 5.65 mg MTP/ml daily group exhibited cranial alopecia and a fourth mouse from this group had a scab/sore on the right ear during the study period. No evidence of skin sensitivity
Repeat-dose Study 1	SD rat	MTP in MCT	Gavage	10–11/sex/group	350–357, 705–708, or 927–984 mg/kg bw/day (285–291, 575–578, 756–802 TE mg/kg/day, resp.)	28 days	All animals survived. The clinical chemistry, haematology, coagulation profile and urinalysis showed that there were no treatment-related differences between the groups. No consistent, dose-dependent changes were detected in body or organ weights, or food consumption

(continued on next page)

Repeat-dose Study 2	SD rat	MTP in MCT	Gavage	4–5/sex/group	869 mg/kg bw/day (M; 708 mgTE/kg/d) 788 mg/kg bw/day (F; 642 mgTE/kg/d)	28 days plus 14-day recovery period	All animals survived. The clinical chemistry, haematology, coagulation profile and urinalysis showed that there were no treatment-related differences between the groups. No consistent, dose-dependent changes were detected in body or organ weights, or food consumption
Repeat-dose Study 3	Wistar rat	Vital-ET™	Gavage	5/sex/group	0, 56.5, 282.5, 565 mg/kg bw/day (45.9, 229.4, 458.8 mgTE/kg/day, resp)	28 days	Two rats died due to gavage error. There were no significant findings in body weights, feed consumption, FOB parameters, haematology, organ weights, and pathology that could be attributed to Vital-ET. Although significant differences were noted in liver/body weight ratios as well as sodium and potassium serum levels in females and ALT serum levels in males, no confirming histopathology was noted

M = male; F = female; TE = tocopherol equivalents.

to moderate oedema (2/5 males and 5/5 females) at 24 h which was cleared up on day 7 and 14. No gross findings upon necropsy were reported. These results indicate that the dermal LD₅₀ of MTP is greater than 1130 mg/kg body weight (918 mg TE/kg bw) in New Zealand rabbits.

3.3. Dermal irritation study

All the rabbits survived to the end of the study and showed no abnormal clinical signs. Body weight changes were normal over the study period. Erythema was barely perceptible in the male rabbit at 60 min and was not present at any other time points or in the females. There was no oedema in any of the animals. MTP at 88–101 mg MTP/site (71–82 mg TE/site) was not a dermal irritant in this study.

3.4. Eye irritation study

All the rabbits survived to the end of the study and showed no abnormal clinical signs. Treated eyes were normal at all time points. MTP was not an eye irritant in this study.

3.5. Allergenic potential

All the mice survived to scheduled termination with no signs of toxicity. MTP-treated mice showed no changes in mean body weights as compared with controls. Three mice from the application group of 5.65 mg MTP/25 µl (4.6 mg TE/25 µl) group exhibited cranial alopecia and a fourth mouse from this group had a scab/sore on the right ear during the study period.

MTP concentrations of 1.13, 2.26 and 5.65 mg MTP/25 µl (0.92, 1.84 and 4.6 mg TE/25 µl) resulted in stimulation indices of 0.8, 0.7, and 1.8, respectively; whereas mice treated with 25% hexylcinnamaldehyde had a stimulation index of 19.2. The mean dpm values for MTP-treated mice did not differ statistically from mice treated with water. Conversely, mice treated with hexylcinnamaldehyde had dpm values significantly higher than their vehicle controls. Based on the results of this study, MTP was not a skin sensitizer in mice.

3.6. Mutagenicity and genotoxicity studies

3.6.1. Reverse mutation

There was no evidence of cytotoxicity in any of the experiments. In the second experiment, which included a pre-incubation step, precipitation was noted at the highest concentration. The mean numbers of revertant colonies on negative control plates all fell within normal historical control ranges of the performing laboratory, and were significantly elevated by positive control treatments (data not shown). In all the experiments, MTP treatment did not significantly increase the number of revertants in any test strain in a concentration dependent or reproducible

Table 3
Summary of mutagenicity and genotoxicity studies with MTP

Study	Species/strain	Formulation	Study design	Concentration/Dose	Results
Ames assay	<i>Salmonella typhimurium</i> TA 98, TA100, TA1535, TA1537 <i>Escherichia coli</i> WP2uvrA	Vital-ET™	In vitro incubation in the presence and absence of S9 metabolic activation (with pre-incubation) using the plate incorporation method	88.3, 176.6, 353.1, 706.2, 1412.5 or 2825 µg MTP/ plate (71.7, 143.4, 286.8, 573.5, 1146.9 or 2293.9 µg TE/plate, respectively)	No increase in the number of revertants
Chromosomal aberrations	Chinese Hamster Ovary cells	Vital-ET™ formulated in 1% carboxy methyl cellulose	Cultures were incubated with formulation for 3 h (+17 recovery) with S-9 metabolic activation and 20 h without; 2 independent experiments were conducted	Exp. 1: without S-9 (3 h); 26.1, 32.6 or 40.8 µg MTP/ml (21.2, 26.5 or 33.1 µg TE/ml, respectively); with S-9 (3 h): 13.4, 26.1, or 32.6 µg MTP/ml (10.9, 21.2 or 26.5 µg TE/ml, resp.) Exp. 2: with S-9 (3 h): 15.5, 19.4, or 37.9 µg MTP/ml (12.6, 15.8 or 30.8 µg TE/ml, resp.); without S-9 (20 h): 3.7, 17.8, or 67.8 µg/ml (3, 14.5 or 55.1 µg TE/ml, resp.)	No increase in the induction of structural and numerical chromosomal aberrations

manner (data not shown). MTP was not mutagenic in this study.

3.6.2. Chromosomal aberrations

The highest concentrations tested in both experiments with and without S-9 induced a 40–57% reduction in cell number. The highest concentration of 67.8 µg MTP/ml (55.1 mg TE/ml) in the second experiment was in excess of the solubility limit of MTP in culture medium. Treatment of CHO cultures with MTP in the presence and absence of S-9 (both experiments) resulted in frequencies of cells with structural aberrations similar to those of concurrent vehicle controls (data not shown). Numbers of aberrant cells (excluding gaps) in the majority of MTP-treated cultures were within the historical control range of the performing laboratory. The only exception was in a single culture at the mid concentration (19.4 µg MTP/ml; or 15.9 µg TE/ml) with S-9 in the second experiment, where the frequency of cells with structural aberrations marginally exceeded the normal range. This increase was not observed in the replicate culture at this concentration or in any other MTP-treated cultures and, therefore, was considered spurious and of no biological significance.

The frequency of cells with numerical aberrations in CHO cultures treated with MTP (both experiments) was similar to those of concurrent vehicle controls (data not shown); however, sporadic increases in endoreduplicated cells were observed following treatment with S-9 (both experiments). These increases were considered of no biological relevance since they were small and the group mean frequencies of cells with numerical aberrations were within the historical control range of the laboratory. In this study, MTP did not induce chromosomal aberrations in cultured CHO cells when tested up to its limit of cytotoxicity or in excess of its solubility limit in culture medium.

3.7. Repeat-dose toxicity studies

3.7.1. Study 1

All the animals survived to scheduled termination without any clinical signs, of toxicity. Behavioural and neurological observations were normal (data not shown). There was no dose-dependent change in feed consumption on a per cage basis (data not shown). Within each group, the rats were started at differing times over a 3-week period; however, the initial age and body weights of the animals were similar. Mean body weights did not differ statistically between treated and control groups (Table 4).

Oral administration of MTP did not affect any of the haematological parameters, including clotting times, tested (Table 5). In many samples, no basophils were reported so statistical analyses of these data are weak. Similarly, platelet counts were often missing actual numerical values and were described as “adequate” in many samples. As shown in Table 6, MTP exposure had no effect on serum albumin, total bilirubin, sodium, bicarbonate, potassium, alkaline phosphatase, cholesterol, AST, creatinine kinase, triglycer-

Table 4
Mean body weights (g)—28-day Study 1^a

	Study day					
	0	7	14	21	22	28
Group A						
Male (<i>n</i> = 10)	276.7 ± 16.9	300.7 ± 23.77	342.6 ± 31.18	–	371.8 ± 34.51	363.5 ± 35.73
Female (<i>n</i> = 10)	194.7 ± 7.63	195.0 ± 14.69	213.4 ± 14.75	–	230.3 ± 21.01	219.7 ± 17.85
Group B						
Male (<i>n</i> = 10)	260.1 ± 26.06	283.2 ± 24.17	329.5 ± 28.54	–	363.3 ± 28.39	346.4 ± 32.71
Female (<i>n</i> = 11)	196.45 ± 9.34	206.73 ± 14.16	228.27 ± 14.91	240.18 ± 16.78	–	230.73 ± 17.37
Group C						
Male (<i>n</i> = 11)	269.0 ± 24.79	294.45 ± 30.18	332.0 ± 29.42	363.09 ± 36.13	–	355.82 ± 35.52
Female (<i>n</i> = 11)	198.0 ± 13.62	210.27 ± 15.58	227.0 ± 17.67	235.82 ± 22.18	–	227.18 ± 20.39
Group D						
Male (<i>n</i> = 10)	274.5 ± 27.14	302.8 ± 21.33	331.0 ± 29.08	–	349.1 ± 20.73	350.0 ± 31.89
Female (<i>n</i> = 10)	199.3 ± 16.19	206.1 ± 21.26	222.3 ± 23.60	–	235.1 ± 21.51	223.2 ± 24.15

^a Mean ± standard deviation.

Table 5
Mean haematological values—28-day Study 1^{a,b}

	Group A		Group B	
	Male	Female	Male	Female
MCH (pg)	19.4 ± 0.7 (<i>n</i> = 9)	19.8 ± 0.6	19.5 ± 0.7	19.0 ± 0.5
MCHC (g/l)	317 ± 5 (<i>n</i> = 9)	314 ± 6	315 ± 6	315 ± 5
White cell count (×10 ⁹ /l)	13.3 ± 3.8 (<i>n</i> = 9)	5.5 ± 2.7	8.7 ± 2.8	7.8 ± 3.4
Neutrophils (×10 ⁹ /l)	7.8 ± 5.7 (<i>n</i> = 9)	12.3 ± 11.9	9.7 ± 4.7	7.9 ± 3.7
Lymphocytes (×10 ⁹ /l)	88.1 ± 6.5 (<i>n</i> = 9)	83.9 ± 11.9	86.4 ± 6.0	86.8 ± 5.3
Monocytes (×10 ⁹ /l)	2.89 ± 1.96 (<i>n</i> = 9)	3.00 ± 2.26	2.50 ± 2.12	3.91 ± 2.77
Eosinophils (×10 ⁹ /l)	0.89 ± 0.78 (<i>n</i> = 9)	0.50 ± 0.71	0.90 ± 0.57	1.36 ± 1.96
Basophils (×10 ⁹ /l)	0.33 ± 0.50 (<i>n</i> = 9)	0.30 ± 0.48	0.50 ± 0.53	0
Red cell count (×10 ¹² /l)	8.36 ± 0.50 (<i>n</i> = 9)	7.44 ± 0.40	8.35 ± 0.41	7.83 ± 0.36
Haemoglobin (g/l)	163.0 ± 9.5 (<i>n</i> = 9)	145.6 ± 8.6	162.0 ± 4.8	149.5 ± 7.4
PCV	0.51 ± 0.03 (<i>n</i> = 9)	0.46 ± 0.03	0.52 ± 0.02	0.48 ± 0.02
MCV (fL)	61.7 ± 1.6 (<i>n</i> = 9)	62.6 ± 1.9	61.9 ± 1.7	60.7 ± 1.6
Platelet count (×10 ⁹ /l)	1115 ± 82 (<i>n</i> = 9)	1175 ± 159	1074 ± 108 (<i>n</i> = 9)	1058 ± 85 (<i>n</i> = 6)
Prothrombin time (s)	8.9 ± 0.5 (<i>n</i> = 8)	8.4 ± 0.7 (<i>n</i> = 9)	8.4 ± 1.3	8.7 ± 2.5
Fibrinogen (g/l)	2.04 ± 0.33 (<i>n</i> = 8)	1.70 ± 1.09 (<i>n</i> = 9)	2.12 ± 0.70	1.45 ± 0.73
APTT (s)	15.2 ± 2.1 (<i>n</i> = 8)	14.0 ± 4.4 (<i>n</i> = 9)	9.0 ± 0.8	9.6 ± 4.2
Group C				
Group D				
	Male	Female	Male	Female
MCH (pg)	19.9 ± 0.9	19.6 ± 0.8	19.4 ± 0.5	19.7 ± 1.1
MCHC (g/l)	320 ± 8	320 ± 9	315 ± 3	317 ± 6
White cell count (×10 ⁹ /l)	11.4 ± 5.0	9.6 ± 4.1	9.6 ± 6.6	7.6 ± 3.5
Neutrophils (×10 ⁹ /l)	9.7 ± 4.1	7.7 ± 3.9	13.0 ± 7.0	8.2 ± 3.1
Lymphocytes (×10 ⁹ /l)	85.0 ± 5.4	87.7 ± 4.8	81.9 ± 8.2	85.5 ± 3.5
Monocytes (×10 ⁹ /l)	3.82 ± 3.43	3.55 ± 2.42	4.40 ± 2.37	5.40 ± 1.58
Eosinophils (×10 ⁹ /l)	0.91 ± 0.54	0.82 ± 0.75	0.70 ± 0.67	0.90 ± 0.57
Basophils (×10 ⁹ /l)	0.55 ± 0.52	0.18 ± 0.40	0	0
Red cell count (×10 ¹² /l)	8.30 ± 0.42	7.94 ± 0.69	8.25 ± 0.54	7.92 ± 0.25
Haemoglobin (g/l)	163.6 ± 4.4	154.8 ± 10.6	158.5 ± 8.9	155.2 ± 6.6
PCV	0.51 ± 0.02	0.49 ± 0.04	0.50 ± 0.03	0.49 ± 0.02
MCV (fL)	61.6 ± 3.0	61.1 ± 2.0	61.1 ± 1.5	61.9 ± 2.5
Platelet count (×10 ⁹ /l)	1024 ± 143 (<i>n</i> = 9)	1043 ± 119 (<i>n</i> = 7)	1071 ± 167 (<i>n</i> = 6)	1137 ± 105 (<i>n</i> = 6)
Prothrombin time (s)	8.4 ± 0.7 (<i>n</i> = 9)	8.0 ± 0.4 (<i>n</i> = 10)	8.8 ± 0.5	7.9 ± 0.5
Fibrinogen (g/l)	2.32 ± 0.54 (<i>n</i> = 9)	1.40 ± 0.27 (<i>n</i> = 10)	2.71 ± 1.25	1.67 ± 0.46
APTT (s)	9.4 ± 2.1 (<i>n</i> = 9)	10.4 ± 3.1 (<i>n</i> = 10)	10.2 ± 2.9	7.5 ± 0.9

^a Unless otherwise stated, all the animals in the group were tested.

^b Mean ± standard deviation.

Table 6
Mean clinical chemistry values—28-day Study 1^{a,b}

	Group A		Group B	
	Male	Female	Male	Female
Albumin (g/l)	43.3 ± 2.0	45.6 ± 5.5	42.6 ± 3.0	44.0 ± 5.8
Total bilirubin (µmol/l)	1.9 ± 0.3	2.1 ± 0.3	1.5 ± 0.5	2.2 ± 0.6
Total protein (g/l)	63.5 ± 1.6	66.3 ± 2.5	62.2 ± 3.2**	61.3 ± 6.9**
Sodium (mmol/l)	148.5 ± 1.2	148.2 ± 2.7	151.0 ± 3.9	146.7 ± 5.2
Bicarbonate (mmol/l)	32.8 ± 2.0	33.0 ± 3.7	33.7 ± 2.9	30.4 ± 2.2
Potassium (mmol/l)	6.2 ± 0.7	6.3 ± 0.7	6.1 ± 0.5	6.4 ± 0.8
alkaline phosphatase (U/l)	213.8 ± 35.7	150.5 ± 68.2	218.6 ± 53.9	138.5 ± 51.0
Cholesterol (mmol/l)	1.4 ± 0.2	1.8 ± 0.4	1.4 ± 0.4 (n = 9)	1.6 ± 0.5
Lactate dehydrogenase (U/l)	375.2 ± 184.7	588.2 ± 205.1	286.6 ± 96.5** (n = 8)	365.9 ± 196.7** (n = 9)
Glucose (mmol/l)	10.6 ± 4.9	5.3 ± 3.5	7.4 ± 2.1** (n = 9)	7.6 ± 3.0
ALT (U/l)	25.6 ± 4.4	26.8 ± 4.6	30.1 ± 5.0**	29.4 ± 6.0**
AST (U/l)	84.8 ± 16.5	105.8 ± 15.6	99.4 ± 40.2	104.3 ± 19.4
GGT (U/l)	2.7 ± 0.9	2.6 ± 1.1	3.4 ± 5.2 (n = 9)	2.9 ± 1.7
Phosphorous (mmol/l)	4.2 ± 0.5	4.2 ± 0.3	4.1 ± 0.7 (n = 9)	3.4 ± 0.7 (n = 9)
Calcium (mmol/l)	3.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	2.9 ± 0.2
Creatine kinase (U/l)	275.2 ± 132.4	438.2 ± 124.9	331.8 ± 382.0	342.0 ± 139.2
Creatinine (mmol/l)	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01** (n = 9)	0.05 ± 0.01** (n = 10)
Triglycerides (mmol/l)	0.8 ± 0.3	0.6 ± 0.1	0.7 ± 0.2 (n = 9)	0.5 ± 0.1 (n = 10)
Urea (mmol/l)	5.1 ± 0.9	6.3 ± 1.4	5.2 ± 0.7 (n = 8)	5.6 ± 1.0 (n = 10)
Chloride (mmol/l)	100.5 ± 1.1	99.4 ± 2.3	100.2 ± 1.5	102.4 ± 1.9*
	Group C		Group D	
	Male	Female	Male	Female
Albumin (g/l)	41.1 ± 2.5	46.3 ± 2.9	40.4 ± 3.2	44.7 ± 3.0
Total bilirubin (µmol/l)	1.5 ± 0.5	2.2 ± 0.6	1.8 ± 0.8	2.3 ± 0.5
Total protein (g/l)	61.1 ± 3.0**	63.4 ± 4.2**	59.4 ± 3.3**	60.6 ± 3.2**
Sodium (mmol/l)	149.5 ± 3.6	149.5 ± 3.6	150.1 ± 2.0	147.7 ± 2.6
Bicarbonate (mmol/l)	33.9 ± 3.9	32.4 ± 4.1	34.0 ± 3.9	29.1 ± 1.7
Potassium (mmol/l)	6.0 ± 0.4	6.3 ± 0.4	6.4 ± 0.8	6.6 ± 0.4
Alkaline phosphatase (U/l)	210.0 ± 35.0	142.1 ± 37.9 (n = 9)	192.3 ± 39.4	119.6 ± 18.5
Cholesterol (mmol/l)	1.3 ± 0.2 (n = 10)	1.4 ± 0.5	1.2 ± 0.3	1.3 ± 0.5
Lactate dehydrogenase (U/l)	320.5 ± 182.8	369.4 ± 264.7	381.1 ± 217.7	651.8 ± 157.0
Glucose (mmol/l)	6.9 ± 1.6**	6.8 ± 2.0	5.9 ± 1.5**	7.3 ± 1.8 (n = 9)
ALT (U/l)	35 ± 5***	31 ± 4***	39 ± 6***	37 ± 7*** (n = 9)
AST (U/l)	100 ± 55	106 ± 28	93 ± 20	118 ± 31
GGT (U/l)	2.5 ± 1.16 (n = 10)	4.8 ± 5.6	1.2 ± 1.5 (n = 9)	1.0 ± 1.1
Phosphorous (mmol/l)	3.5 ± 1.2*	3.7 ± 0.6*	4.1 ± 0.4	3.7 ± 0.5
Calcium (mmol/l)	2.9 ± 0.1	3.1 ± 0.1	2.9 ± 0.0*	2.9 ± 0.1
Creatinine kinase (U/l)	374.5 ± 471.4	357.0 ± 191.5	274.2 ± 190.8	501.3 ± 288.0
Creatinine (mmol/l)	0.05 ± 0.01**	0.05 ± 0.01** (n = 10)	0.05 ± 0.01	0.06 ± 0.01
Triglycerides (mmol/l)	0.7 ± 0.3	0.6 ± 0.2 (n = 10)	0.7 ± 0.3	0.7 ± 0.3
Urea (mmol/l)	4.9 ± 0.8	5.9 ± 1.1 (n = 10)	4.7 ± 1.8 (n = 9)	5.4 ± 2.0 (n = 9)
Chloride (mmol/l)	100.2 ± 1.8	100.6 ± 2.2	99.5 ± 1.6	100.2 ± 1.3

* $p < 0.05$ compared to controls.

** $p < 0.01$ compared to control.

*** $p < 0.001$ compared to control.

^a Unless otherwise stated, all the animals in the group were tested.

^b Mean ± standard deviation.

ides, and urea. Total protein was significantly decreased in all treated groups compared to controls. Lactate dehydrogenase and creatinine showed a statistically significant, non-dose-dependent decrease at the lowest dose. Chloride was significantly increased only in females given the lowest dose. GGT and calcium were significantly decreased in animals given the highest dose. Phosphorous was significantly decreased at the middle dose. The only dose-dependent changes in serum biochemistry were a decrease in glucose

values in males at all doses and an increase in ALT in all treated animals compared to controls. There was no statistically significant effect of MTP treatment on serum phospholipids (data not shown). With the exception of significantly decreased urinary pH in high-dose animals compared to controls, urinalyses did not reveal any uncommon findings (data not shown).

There were no significant changes in absolute organ weights (liver, spleen, heart, intestine, kidney, brain, and

Table 7
Mean^a organ weights (g) and ratio organ weight to body weight (%)—28-day Study 1

	Group 1		Group 2		Group 3		Group 4	
	Male (n = 10)	Female (n = 10)	Male (n = 10)	Female (n = 11)	Male (n = 11)	Female (n = 11)	Male (n = 10)	Female (n = 10)
Absolute (g)								
Liver	11.59 ± 1.36	6.41 ± 0.84	11.50 ± 1.67	6.84 ± 1.19	11.11 ± 1.67	7.26 ± 0.90	11.51 ± 1.59	7.18 ± 0.82
Spleen	0.73 ± 0.11	0.58 ± 0.21	0.60 ± 0.09	0.55 ± 0.14	0.67 ± 0.12	0.47 ± 0.08	0.71 ± 0.20	0.51 ± 0.07
Intestine	22.46 ± 4.03	17.05 ± 3.13	22.41 ± 4.22	16.97 ± 1.97	23.10 ± 4.08	18.60 ± 1.51	23.66 ± 2.63	18.04 ± 1.88
Kidney	3.03 ± 0.30	1.78 ± 0.18	2.92 ± 0.39	1.81 ± 0.32	2.91 ± 0.33	1.74 ± 0.12	2.86 ± 0.16	1.76 ± 0.19
Brain	1.91 ± 0.17	1.65 ± 0.34	1.83 ± 0.14	1.73 ± 0.20	1.86 ± 0.15	1.72 ± 0.08	1.82 ± 0.11	1.69 ± 0.12
Heart	1.50 ± 0.21	0.97 ± 0.23	1.25 ± 0.21*	0.93 ± 0.19*	1.33 ± 0.21	0.91 ± 0.16	1.29 ± 0.11*	0.87 ± 0.09*
Testes ^b	5.04 ± 0.40		4.78 ± 0.48		4.67 ± 0.36		4.64 ± 0.37	
Ratio organ weight to body weight (%)								
Liver	3.2 ± 0.2	2.9 ± 0.4	3.3 ± 0.4	3.0 ± 0.4	3.1 ± 0.2	3.2 ± 0.3	3.3 ± 0.2	3.2 ± 0.2
Spleen	0.20 ± 0.03	0.26 ± 0.09	0.18 ± 0.03	0.24 ± 0.06	0.19 ± 0.03	0.21 ± 0.03	0.20 ± 0.06	0.23 ± 0.03
Intestine	6.1 ± 0.7	2.9 ± 0.4	6.5 ± 1.4	3.0 ± 0.5	6.5 ± 0.7	3.2 ± 0.3	6.8 ± 0.4	3.2 ± 0.1
Kidney	0.84 ± 0.04	0.81 ± 0.09	0.84 ± 0.1	0.79 ± 0.12	0.82 ± 0.06	0.77 ± 0.08	0.77 ± 0.2	0.79 ± 0.06
Brain	0.53 ± 0.06	0.76 ± 0.17	0.53 ± 0.07	0.76 ± 0.11	0.52 ± 0.04	0.76 ± 0.07	0.52 ± 0.04	0.77 ± 0.10
Heart	0.41 ± 0.04	0.44 ± 0.09	0.36 ± 0.07	0.40 ± 0.08	0.38 ± 0.06	0.40 ± 0.05	0.37 ± 0.02	0.0039 ± 0.05
Testes ^b	1.39 ± 0.11		1.39 ± 0.19		1.32 ± 0.11		1.33 ± 0.14	

* $p \leq 0.05$ compared to controls.

^a Mean ± standard deviation.

^b With epididymides.

testes) when treated animals were compared with controls (Table 7). Evaluation of organ weights relative to body weights also showed no statistically significant differences.

The macroscopic and microscopic findings during the post-mortem examination revealed no inherent toxicity of orally administered MTP. Several animals in all the test groups (3/20 in Group A, 3/21 in Group B, 1/22 in Group C, and 1/20 in Group D) showed microscopic changes (e.g., systemic pyrogranulomatous inflammation, periportal granulomatous hepatitis, and non-suppurative pericarditis and interstitial pneumonia), related to dosing injuries and subsequent systemic inflammation. Several other lesions (e.g., multifocal granulomatous inflammation of the skeletal muscle, eosinophilic pneumonia, focally disseminate peribronchiolar granulomatous pneumonia, multifocal endogenous lipid pneumonia, focal mineralization of the liver, mild focal ulcerative gastritis, and focal non-suppurative myocarditis) not attributable to dosing injuries were observed at a low incidence sporadically and without a

dose-dependent trend throughout all the test groups. The conclusion of this study was that there was no significant pathology that could be related to the test substance.

3.7.2. Study 2

All the animals survived to scheduled termination without any clinical signs, of toxicity. Behavioural and neurological observations were normal (data not shown). Within each group, the rats were started at differing times varying as much as 2.5 weeks; however, the initial age and body weights of the animals were similar. Over the course of the study, mean body weights of the treated male animals were significantly lower than controls; this appears to be the result of one rat in the control group having a starting weight within the normal range, but finishing at day 42 with a heavier than the average weight (Table 8). The individual weights are as follows, with the rat having the aberrant weight listed in italics: starting weights for Group A were 223, 252, 246, 252, 256, and for Group D

Table 8
Mean body weights (g)—28-day Study 2^a

Week no.	Group A		Group D	
	Female	Male	Female	Male
0	195.80 ± 7.36	245.80 ± 13.24	197.50 ± 6.24	240.20 ± 13.39
1	—	289.00 ± 4.24 ^b	216.00 ^c	280.67 ± 3.79 ^d
2	232.40 ± 4.28	336.80 ± 5.93	230.75 ± 7.37	324.20 ± 11.21
3	245.00 ± 4.90	370.40 ± 7.37	238.25 ± 10.87	344.20 ± 21.78
6	261.80 ± 6.94	429.40 ± 25.98	255.00 ± 7.12	397.60 ± 19.74*

* Indicates statistical differences between groups at week 6.

^a Mean ± standard deviation.

^b $n = 2$.

^c $n = 1$.

^d $n = 3$.

were 231, 238, 226, 260, 246; final weights for Group A were 471, 431, 427, 401, 417, and for Group D were 412, 365, 407, 393, 411. Oral MTP exposure followed by a recovery period, had no effect on haematological and clinical chemistry parameters with the exception of a statistically significant decrease in albumin values in treated females and a statistically significant increase in glucose values in treated rats of both sexes (Table 9). With the exception of significantly decreased urinary pH in high-dose males and significantly increased urinary pH in high-dose females compared to corresponding controls, urinalyses did not reveal any uncommon findings (data not shown).

There were no significant changes in absolute organ weights (liver, spleen, intestine, kidney, brain, heart, and

testes weight) when treated animals were compared with controls (Table 10).

As in the previous study, no adverse macroscopic and microscopic findings were attributable to the test substance. The most common finding seen in several animals in both the control and treated groups were cardiac changes (i.e., myocardial myofibre necrosis) and atelectasis of the lungs related to pneumothorax. These changes were related to blood collection. Two animals (1 control male, 1 treated female) showed lesions consistent with dosing injuries and subsequent systemic inflammation. One control male had a small focus of eosinophilic granulomatous inflammation in the heart. The study veterinary pathologist concluded that there was no significant pathology that could be related to the test substance.

Table 9
Mean haematological and clinical chemistry values—28-day Study 2^{a,b}

	Group A		Group D	
	Male	Female	Male	Female
Haematology				
MCH (pg)	18.6 ± 0.6	19.6 ± 0.6	18.8 ± 1.1	18.7 ± 0.6 (n = 3)
MCHC (g/l)	309 ± 6	311 ± 4	309 ± 7	312 ± 2 (n = 3)
White cell count (×10 ⁹ /l)	7.9 ± 3.0	8.1 ± 4.9	11.7 ± 3.5	10.1 ± 1.3 (n = 3)
Neutrophils (×10 ⁹ /l)	13.6 ± 2.1	8.6 ± 3.4	11.2 ± 3.4	9.0 ± 6.9 (n = 3)
Lymphocytes (×10 ⁹ /l)	81.8 ± 2.2	87.0 ± 5.5	82.2 ± 5.9	87.0 ± 8.7 (n = 3)
Monocytes (×10 ⁹ /l)	3.40 ± 2.07	3.40 ± 2.51	4.60 ± 3.78	2.33 ± 2.31 (n = 3)
Eosinophils (×10 ⁹ /l)	1.20 ± 0.45	1.00 ± 0.00	1.60 ± 0.55	1.33 ± 0.58 (n = 3)
Basophils (×10 ⁹ /l)	0	0	0.40 ± 0.55	0.33 ± 0.58 (n = 3)
Red cell count (×10 ¹² /l)	8.44 ± 0.59	8.06 ± 0.16	8.35 ± 0.20	8.20 ± 0.43 (n = 3)
Haemoglobin (g/l)	154.6 ± 8.0	155.2 ± 4.6	157.8 ± 5.6	153.7 ± 10.1 (n = 3)
PCV	0.50 ± 0.02	0.50 ± 0.02	0.51 ± 0.02	0.49 ± 0.03 (n = 3)
MCV (fl)	59.6 ± 2.2	61.8 ± 1.9	61.4 ± 3.2	60.3 ± 1.2 (n = 3)
Platelet count (×10 ⁹ /l)	1001 ± 122 (n = 4)	1067 ± 53 (n = 3)	1042 ± 149 (n = 2)	1009 ± 154 (n = 2)
Prothrombin time (s)	8.0 ± 0.2	7.9 ± 0.2 (n = 4)	8.4 ± 0.2	8.7 ± 1.6
Fibrinogen (g/l)	2.44 ± 0.15	1.50 ± 0.29 (n = 4)	1.86 ± 0.38	1.68 ± 0.38
APTT (s)	8.8 ± 1.9	9.3 ± 1.5 (n = 4)	10.0 ± 3.3	9.2 ± 1.6
Clinical chemistry				
Albumin (g/l)	44.4 ± 3.2	54.6 ± 1.5	44.2 ± 1.6	49.8 ± 1.3 ^{***}
Total bilirubin (μmol/l)	2.0 ± 0.7	2.6 ± 0.6	1.4 ± 0.6	2.0 ± 0.8
Total protein (g/l)	66.0 ± 5.8	73.8 ± 2.2	65.6 ± 4.6	70.3 ± 4.0
Sodium (mmol/l)	151.0 ± 3.4	150.4 ± 1.5	151.2 ± 1.6	148.8 ± 2.2
Bicarbonate (mmol/l)	32.2 ± 5.8	28.2 ± 2.8	35.0 ± 5.1	28.5 ± 2.7
Potassium (mmol/l)	6.5 ± 0.4	6.6 ± 0.4	6.2 ± 0.1	7.0 ± 0.7
Alkaline phosphatase (U/l)	201.2 ± 45.4	139.0 ± 41.9	190.6 ± 53.3	137.5 ± 66.5
Cholesterol (mmol/l)	1.8 ± 0.1	1.9 ± 0.3	1.7 ± 0.3	2.1 ± 0.4
Lactate dehydrogenase (U/l)	393.4 ± 266.2	819.8 ± 480.4	476.0 ± 296.4	664.5 ± 300.7
Glucose (mmol/l)	6.36 ± 1.19	7.20 ± 1.13	7.53 ± 1.27 [*]	9.13 ± 2.32 [*]
ALT (U/l)	33 ± 5	35 ± 3	33 ± 3	31 ± 4
AST (U/l)	101 ± 19	115 ± 22	91 ± 16	106 ± 14
GGT (U/l)	11.0 ± 4.2	8.6 ± 3.1	9.0 ± 1.4	7.0 ± 1.7
Phosphorous (mmol/l)	4.10 ± 0.47	3.83 ± 0.51	3.91 ± 0.56	3.63 ± 0.64
Calcium (mmol/l)	3.06 ± 0.15	3.19 ± 0.09	3.07 ± 0.09	3.13 ± 0.18
Creatinine kinase (U/l)	298.60 ± 194.75	630.00 ± 293.54	360.40 ± 205.33	504.25 ± 164.15
Creatinine (mmol/l)	0.05 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.01
Triglycerides (mmol/l)	0.98 ± 0.18	0.90 ± 0.32	1.00 ± 0.42	0.55 ± 0.13
Urea (mmol/l)	6.24 ± 1.50	7.82 ± 0.75	5.70 ± 0.47	7.35 ± 0.45
Chloride (mmol/l)	100.6 ± 2.30	101.00 ± 0.71	101.80 ± 1.30	102.50 ± 2.38

^{*} $p < 0.5$ compared to controls.

^{***} $p < 0.001$ compared to controls.

^a Unless otherwise stated, all the animals in the group were tested.

^b Mean ± standard deviation.

Table 10
Mean absolute organ weights (g)—28-day Study 2^a

	Liver	Spleen	Intestine	Kidney	Brain	Heart	Testes
Group A							
Male (n = 5)	13.81 ± 2.10	0.86 ± 0.15	27.03 ± 3.78	3.45 ± 0.22	1.80 ± 0.26	1.54 ± 0.07	5.61 ± 0.52
Female (n = 5)	9.14 ± 1.04	0.98 ± 1.03	22.84 ± 4.10	2.25 ± 1.15	1.83 ± 0.06	0.71 ± 0.33	
Group D							
Male (n = 5)	13.75 ± 2.45	0.75 ± 0.07	26.93 ± 4.71	3.24 ± 0.34	1.97 ± 0.12	1.45 ± 0.09	5.31 ± 0.51
Female (n = 4)	8.38 ± 0.32	1.17 ± 1.09	22.52 ± 1.09	1.64 ± 0.32	1.69 ± 0.30	0.64 ± 0.34	

^a Mean ± standard deviation.

3.7.3. Study 3

Two rats (1 mid-dose female on day 15 and 1 high-dose female on day 13) died during the study due to gavage error. No other unscheduled deaths occurred. With the exception of one mid-dose male showing instances of dyspnea and one high-dose male showing instances of emaciation, tachypnea, few faeces, piloerection, yellow staining of the anogenital area and dyspnea, no abnormal clinical signs were reported in any test group. There were no statistically significant ($p \leq 0.05$) differences between control and dosed groups in mean body weights (Table 11), mean feed consumption (data not shown), FOB parameters (data not shown), and haematology parameters (Table 12).

The only statistically significant ($p \leq 0.05$) differences between control and dosed groups in clinical chemistry parameters involved serum ALT, potassium and sodium (Table 13). High-dose males, but not females, had a significantly lower ALT value than corresponding controls. Mid- and high-dose females had significantly lower potassium levels than corresponding controls. Low- and mid-dose females had significantly higher sodium levels than corresponding controls. There were no significant differences between mean absolute organ weights of control and dosed groups; however, the mean liver/body weight ratio in high-dose females was significantly higher than that of corresponding controls (Table 14).

Upon necropsy, all animals in the control and low-dose groups appeared normal. At the mid-dose, all animals except the female that died as a result of gavage error appeared normal. Examination of this female revealed yellow material with red fluid in the pleural cavity, a puncture in the oesophagus, lung abnormalities, soiling of the anogenital area and red staining of the nose/mouth area. At the highest dose, 6 of the animals appeared normal. The one female that died as a result of gavage error had white material in the pleural cavity, dark areas on the lungs and spleen, and yellow staining of fat in the reproductive area. One high-dose male had pleural cavity abnormalities and another had fusion of the liver to the diaphragm. One high-dose female had larger than normal adrenals. There were no test substance-related microscopic changes noted in the high-dose animals. All microscopic changes that were observed were typical of those that occur spontaneously and were considered to be unrelated to treatment.

4. Discussion

The product MTP contains 2 phosphorylated forms of α -tocopherol, α -tocopheryl phosphate and α -di-tocopheryl phosphate. The results of the tests on MTP presented here are discussed in terms of what else is known about tocopheryl phosphate and about other formulations of vitamin E.

Table 11
Mean body weights (g)—28-day Study 3^a

	Study day				
	1	8	15	22	28
Group 1 (control)					
Male (n = 5)	294 ± 18.0	328 ± 21.8	351 ± 25.6	371 ± 29.6	384 ± 32.1
Female (n = 5)	216 ± 4.2	233 ± 11.4	244 ± 7.9	260 ± 17.2	270 ± 13.5
Group 2 (56.5 mg/kg bw/day; 45.9 mg TE/kg/d)					
Male (n = 5)	293 ± 17.1	327 ± 19.3	356 ± 21.1	378 ± 28.6	394 ± 24.5
Female (n = 5)	208 ± 12.5	224 ± 4.4	246 ± 9.8	252 ± 10.9	253 ± 7.2
Group 3 (282.5 mg/kg bw/day; 229.4 mg TE/kg/d)					
Male (n = 5)	287 ± 13.6	311 ± 24.0	342 ± 24.5	362 ± 28.1	379 ± 26.1
Female (n = 5)	218 ± 11.6	226 ± 15.0	239 ± 38.8	265 ± 21.9 ^b	271 ± 17.2 ^b
Group 4 (565 mg/kg bw/day; 458.8 mg TE/kg/d)					
Male (n = 5)	284 ± 20.2	314 ± 27.2	328 ± 45.4	355 ± 44.4	370 ± 47.1
Female (n = 5)	209 ± 11.2	224 ± 19.9	241 ± 17.4 ^b	263 ± 21.4 ^b	267 ± 17.3 ^b

^a Mean ± standard deviation.

^b n = 4.

Table 12
Mean haematological values—28-day Study 3^a

	Group 1 (control)		Group 2 (56.5 mg/kg bw/day; 45.9 mg TE/kg/d)	
	Male (n = 4)	Female (n = 5)	Male (n = 5)	Female (n = 5)
MCH (pg)	18.5 ± 0.5	19.1 ± 0.4	19.0 ± 0.3	19.0 ± 0.5
MCHC (%)	34.2 ± 0.3	35.2 ± 0.4	34.4 ± 0.2	35.2 ± 0.1
White cell count (×10 ³ /μl)	6.7 ± 1.0	5.5 ± 1.3	8.7 ± 3.3	6.8 ± 1.8
Segmented neutrophils (×10 ³ /μl)	0.61 ± 0.12	0.47 ± 0.12	0.63 ± 0.20	0.45 ± 0.1
Lymphocytes (×10 ³ /μl)	5.68 ± 0.83	4.78 ± 1.21	7.68 ± 3.01	6.00 ± 1.68
Monocytes (×10 ³ /μl)	0.23 ± 0.11	0.15 ± 0.02	0.22 ± 0.08	0.20 ± 0.06
Eosinophils (×10 ³ /μl)	0.05 ± 0.01	0.08 ± 0.02	0.05 ± 0.02	0.07 ± 0.03
Red cell count (×10 ⁶ /μl)	8.51 ± 0.47	7.69 ± 0.25	8.16 ± 0.24	7.99 ± 0.31
Haemoglobin (g/dl)	15.7 ± 0.9	14.7 ± 0.7	15.5 ± 0.6	15.2 ± 0.9
Haematocrit (%)	45.9 ± 2.7	41.6 ± 1.8	45.2 ± 1.8	43.1 ± 2.6
Mean corpuscular volume (fl)	54 ± 1.3	54 ± 1.1	56 ± 0.9	54 ± 1.6
Platelet count (×10 ³ /μl)	919 ± 57.7	1012 ± 104.4	909 ± 50.4	1058 ± 51.5
Prothrombin time (s)	27.8 ± 14.6 (n = 5)	21.6 ± 2.8	21.1 ± 4.9	21.0 ± 3.1
	Group 3 (282.5 mg/kg bw/day; 229.4 mg TE/kg/d)		Group 4 (565 mg/kg bw/day; 458.8 mg TE/kg/d)	
	Male (n = 5)	Female (n = 4)	Male (n = 5)	Female (n = 4)
MCH (pg)	19.0 ± 0.3	19.1 ± 0.5	19.1 ± 0.5	19.0 ± 0.5
MCHC (%)	34.5 ± 0.4	35.3 ± 0.4	34.6 ± 0.1	35.4 ± 0.9
White cell count (×10 ³ /μl)	7.2 ± 0.8	6.3 ± 3.0	7.7 ± 2.7	6.3 ± 3.0
Neutrophils (×10 ³ /μl)	0.75 ± 0.16	0.48 ± 0.11	0.96 ± 0.34	0.69 ± 0.25
Lymphocytes (×10 ³ /μl)	6.12 ± 0.23	4.57 ± 1.19	6.32 ± 2.61	5.31 ± 2.61
Monocytes (×10 ³ /μl)	0.23 ± 0.05	0.13 ± 0.06	0.25 ± 0.05	0.19 ± 0.09
Eosinophils (×10 ³ /μl)	0.05 ± 0.02	0.07 ± 0.05	0.08 ± 0.04	0.04 ± 0.02
Red cell count (×10 ⁶ /μl)	8.25 ± 0.36	7.87 ± 0.25	8.18 ± 0.16	7.84 ± 0.20
Haemoglobin (g/dl)	15.6 ± 0.6	15.1 ± 0.2	15.7 ± 0.4	14.9 ± 0.7
Hematocrit (%)	45.3 ± 1.4	42.7 ± 0.5	45.3 ± 1.3	42.2 ± 1.3
Mean corpuscular volume (fl)	55 ± 1.4	54 ± 1.3	55 ± 1.5	54 ± 0.0
Platelet count (×10 ³ /μl)	853 ± 91	1019 ± 87	861 ± 49	1023 ± 139
Prothrombin time (s)	20.8 ± 1.7	17.8 ± 1.0	23.9 ± 7.4	19.2 ± 1.7

^a Mean ± standard deviation.

Overall, vitamin E toxicity is very rare and supplements are widely considered to be safe. The Food and Nutrition Board, Institute of Medicine (IOM, 2000), established the daily tolerable upper intake level (UL) for adults to be 1000 mg of vitamin E, which is equivalent to 1500 IU of natural vitamin E.

The most recent review of the safety of vitamin E was conducted in April 2005 (Hathcock et al., 2005). This was in response to a report early in 2005 that called into question the safety of vitamin E at doses ≥400 IU (Miller et al., 2005). The review revealed that once other factors that were not included in the calculation of the 6% increased risk for the patients, were included then the risk of death became significant only at doses ≥2000 IU vitamin E per day. The study concluded that the evidence is not convincing that vitamin E supplementation up to the UL increases the risk of death due to cardiovascular disease or other causes, and that vitamin E supplements appear to be safe for most adults in amounts less than or equal to 1073 mg of natural α-tocopherol, or the molar equivalent of its esters (Hathcock et al., 2005).

The results of the toxicity tests in the present study allow a certain amount of comparison with other forms of esterified vitamin E. The oral LD50 values for tocopherol,

tocopheryl acetate, tocopheryl nicotinate, tocopheryl succinate, tocophersolan and 75% tocophersolan for rats are 4, 16, 10, 7, 7 and 5 g/kg, respectively. The dermal LD50 values for tocopherol, tocopheryl acetate, 75% tocophersolan are >3, >3, and >2 g/kg, respectively. The oral and dermal LD50 of MTP was found to be greater than 1.13 g/kg. The 28 day repeat dose studies tested MTP up to 0.927–0.984 g MTP/kg/day, without the occurrence of adverse events.

The Cosmetic Ingredients Review Expert Panel (CIREP, 2002) found that the data it reviewed allowed it to conclude that α-tocopherol, tocopheryl acetate, tocopheryl linoleate, tocopheryl linoleate/oleate tocopheryl nicotinate, tocopherol succinate, dioleoyl tocopheryl methylsilanol, potassium ascorbyl tocopheryl phosphate, and tocophersolan are safe as used in cosmetic formulations, with the understanding that the vitamin E used in cosmetic products is of similar grade to that used in foods. Similarly, the results of the tests conducted with MTP and described herein, have shown it to have no irritant, sensitizing, mutagenic, genotoxic, or adverse effects in in vivo and in vitro studies. MTP has very low acute oral and dermal toxicity and was not irritating to the skin or eyes of test animals.

Table 13
Mean clinical chemistry values—28-day Study 3^a

	Group 1 (control)		Group 2 (56.5 mg/kg bw/day; 45.9 mg TE/kg/d)	
	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 5)
Sodium (mmol/l)	146 ± 1	142 ± 1	147 ± 1	144 ± 1*
Potassium (mmol/l)	4.7 ± 0.3	4.7 ± 0.1	4.5 ± 0.3	4.5 ± 0.2
Chloride (mmol/l)	106 ± 1.3	106 ± 0.7	106 ± 1.1	107 ± 1.2
Calcium (mmol/l)	5.3 ± 0.15	5.1 ± 0.15	5.3 ± 0.15	5.3 ± 0.15
Phosphorous (meq/l)	8.5 ± 0.5	7.7 ± 0.4	8.4 ± 0.7	8.1 ± 0.7
Magnesium (mmol/l)	1.2 ± 0.05	1.15 ± 0.05	1.05 ± 0.1	1.1 ± 0.05
ALT (U/l)	30 ± 3	25 ± 6	28 ± 4	27 ± 2
AST (U/l)	133 ± 40	140 ± 18	114 ± 16	127 ± 44
Alkaline phosphatase (U/l)	140 ± 35	85 ± 6	144 ± 25	90 ± 15
GGT (U/l)	1 ± 0	2 ± 1	1 ± 1	2 ± 1
Glucose (mmol/l)	7.4 ± 1.2	6.2 ± 0.8	7.4 ± 0.8	6.2 ± 0.5
BUN (mg/dl)	13 ± 2	13 ± 2	13 ± 1	13 ± 1
Creatinine (mmol/l)	0.05 ± 0	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Cholesterol (mmol/l)	1.5 ± 0.2	1.8 ± 0.4	1.6 ± 0.3	1.6 ± 0.1
Triglycerides (mmol/l)	0.51 ± 0.12	0.36 ± 0.11	0.57 ± 0.17	0.29 ± 0.07
Total bilirubin (µmol/l)	1.71 ± 0	1.71 ± 0	1.71 ± 0	1.71 ± 1.71
Sorbitol dehydrogenase (U/l)	11 ± 4	22 ± 11	11 ± 5	20 ± 5
Albumin (g/l)	43 ± 2	44 ± 1	43 ± 1	45 ± 3
Total protein (g/l)	66 ± 3	63 ± 1	64 ± 2	65 ± 3
Globulin (g/l)	23 ± 2	20 ± 1	21 ± 2	21 ± 2
	Group 3 (282.5 mg/kg bw/day; 229.4 mg TE/kg/d)		Group 4 (565 mg/kg bw/day; 458.8 mg TE/kg/d)	
	Male (n = 5)	Female (n = 4)	Male (n = 5)	Female (n = 4)
Sodium (mmol/l)	146 ± 1	144 ± 2*	146 ± 1	143 ± 1
Potassium (mmol/l)	4.6 ± 0.4	4.2 ± 0.2*	4.5 ± 0.3	4.1 ± 0.1*
Chloride (mmol/l)	106 ± 2.0	106 ± 1.0	106 ± 2.0	105 ± 0.8
Calcium (mmol/l)	5.6 ± 0.2	5.3 ± 0.15	5.3 ± 0.1	5.3 ± 0.4
Phosphorous (meq/l)	8.3 ± 0.8	8.1 ± 0.5	8.6 ± 0.2	8.1 ± 0.8
Magnesium (mmol/l)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
ALT (U/l)	32 ± 2	23 ± 6	37 ± 6*	31 ± 7
AST (U/l)	109 ± 14	121 ± 33	123 ± 18	118 ± 16
Alkaline phosphatase (U/l)	138 ± 43	87 ± 27	130 ± 13	92 ± 11
GGT (U/l)	1 ± 0	1 ± 1	0 ± 1	2 ± 1
Glucose (mmol/l)	7.4 ± 0.9	6.2 ± 0.5	6.7 ± 0.5	6.2 ± 0.8
BUN (mg/dl)	12 ± 2	14 ± 2	11 ± 2	12 ± 3
Creatinine (mmol/l)	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Cholesterol (mmol/l)	1.6 ± 0.4	1.4 ± 0.1	1.6 ± 0.2	1.5 ± 0.4
Triglycerides (mmol/l)	0.62 ± 0.22	0.28 ± 0.02	0.50 ± 0.16	0.28 ± 0.02
Total bilirubin (µmol/l)	1.71 ± 0	1.71 ± 0	1.71 ± 0	3.42 ± 1.71
Sorbitol dehydrogenase (U/l)	10 ± 3.0	19 ± 3.5	9 ± 2.3	15 ± 5.4
Albumin (g/l)	42 ± 2	44 ± 1	42 ± 1	44 ± 3
Total protein (g/l)	63 ± 3	63 ± 3	63 ± 2	63 ± 2
Globulin (g/l)	21 ± 1	19 ± 2	21 ± 2	20 ± 2

* $p \leq 0.05$ compared to controls.

^a Mean ± standard deviation.

Three 28-day repeat-dose toxicity studies were conducted using rats. The second of these studies included a 14-day recovery period. Animals in the first 2 studies were started at differing times varying as much as 3 weeks, even within the same group; however, statistical analysis showed that the initial body weights of the animals were similar in each study and the data could be taken into consideration. The third 28-day study was conducted under GLP and contained no technical issues that would affect interpretation of the data. In Study 1, total serum protein was significantly decreased in all dosed groups. The veterinary

pathologist attributed this finding to mild dehydration of the animals. This was not reported in Study 2 following the recovery period, although there was a significant decrease in albumin values from treated females. Neither effect was reported in Study 3. Differences in urinary pH reported in Study 1 and 2 were a function of diet and of no clinical significance. All other pre-necropsy findings were either limited to one sex, or were not dependent on dose. Pathologically, several animals in Study 1 and 2 showed changes relating to dosing injuries and subsequent systemic inflammation. In Study 3, the death of 2 rats was

Table 14
Mean^a organ weights (g) and ratio organ weight to body weight (%)—28-day Study 3

	Group 1		Group 2		Group 3		Group 4	
	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 5)	Male (n = 5)	Female (n = 4)	Male (n = 5)	Female (n = 4)
Absolute (g)								
Adrenals	0.078 ± 0.012	0.081 ± 0.011	0.094 ± 0.013	0.08 ± 0.009	0.059 ± 0.010	0.089 ± 0.02	0.070 ± 0.016	0.105 ± 0.021
Kidneys	2.919 ± 0.28	1.961 ± 0.13	2.858 ± 0.24	1.727 ± 0.43	2.801 ± 0.14	2.019 ± 0.21	2.829 ± 0.36	2.038 ± 0.29
Liver	10.906 ± 0.74	7.603 ± 0.48	11.456 ± 0.76	7.019 ± 0.43	11.815 ± 0.33	8.083 ± 0.61	11.556 ± 1.81	8.380 ± 1.13
Testes ^b	5.278 ± 0.26	–	5.036 ± 0.35	–	5.248 ± 0.56	–	5.655 ± 1.93	–
Thymus	0.779 ± 0.24	0.627 ± 0.02	0.882 ± 0.21	0.589 ± 0.07	0.742 ± 0.15	0.545 ± 0.07	1.116 ± 0.67	0.656 ± 0.10
Spleen	0.941 ± 0.20	0.764 ± 0.14	0.834 ± 0.05	0.656 ± 0.14	0.794 ± 0.09	0.669 ± 0.06	0.749 ± 0.03	0.762 ± 0.07
Brain	2.203 ± 0.09	2.082 ± 0.07	2.231 ± 0.12	1.965 ± 0.13	2.487 ± 0.67 ^c	2.079 ± 0.08	2.111 ± 0.07	1.991 ± 0.12
Heart	1.446 ± 0.11	0.991 ± 0.04	1.492 ± 0.07	1.028 ± 0.08	1.426 ± 0.19	1.080 ± 0.13	1.468 ± 0.26	1.073 ± 0.14
Ratio organ weight to body weight (%)								
Adrenals	0.02 ± 0.002	0.030 ± 0.005	0.02 ± 0.004	0.032 ± 0.003	0.016 ± 0.003	0.033 ± 0.009	0.019 ± 0.0005	0.039 ± 0.007
Kidneys	0.761 ± 0.035	0.730 ± 0.075	0.725 ± 0.046	0.686 ± 0.177	0.741 ± 0.036	0.746 ± 0.06	0.766 ± 0.057	0.762 ± 0.068
Liver	2.848 ± 0.131	2.822 ± 0.155	2.913 ± 0.262	2.777 ± 0.118	3.129 ± 0.154	2.984 ± 0.054	3.115 ± 0.143	3.132 ± 0.232*
Testes ^b	1.384 ± 0.143	–	1.279 ± 0.085	–	1.396 ± 0.224	–	1.523 ± 0.425	–
Thymus	0.207 ± 0.077	0.233 ± 0.006	0.222 ± 0.044	0.233 ± 0.030	0.197 ± 0.043	0.201 ± 0.019	0.308 ± 0.208	0.248 ± 0.051
Spleen	0.246 ± 0.054	0.285 ± 0.059	0.212 ± 0.008	0.259 ± 0.051	0.210 ± 0.017	0.247 ± 0.013	0.205 ± 0.023	0.286 ± 0.019
Brain	0.577 ± 0.051	0.773 ± 0.029	0.567 ± 0.041	0.778 ± 0.05	0.675 ± 0.223 ^c	0.769 ± 0.022	0.577 ± 0.07	0.750 ± 0.078
Heart	0.379 ± 0.039	0.368 ± 0.021	0.379 ± 0.030	0.407 ± 0.031	0.376 ± 0.031	0.398 ± 0.025	0.398 ± 0.062	0.401 ± 0.034

* $p \leq 0.05$ compared to controls.

^a Mean ± standard deviation.

^b With epididymides.

^c $n = 4$.

attributed to gavage error. Any other reported lesions were common findings to the strain of rat, were observed sporadically at a low incidence, or no supporting histopathology was reported. In all three of these studies no consistent, dose-dependent adverse effects attributable to MTP treatment were reported.

While there is some evidence which shows that α -tocopheryl phosphate is cleaved by alkaline phosphatase to release α -tocopherol (Nakayama et al., 2003; Rezk et al., 2004; Negis et al., 2005), there is as yet no published data that allows the phosphorylated forms to be considered for classification as vitamin E. In order for it to be classified as such, its absorption, distribution, metabolism and excretion are also being assessed. Indeed, the IOM defines vitamin E activity to be limited to only the naturally occurring RRR-form, and the 3 synthetic 2R-stereoisomer forms (RSR-, RRS- and RSS-) of α -tocopherol. Other naturally occurring forms of vitamin E, these being the other 3 tocopherols and 4 tocotrienols, do not contribute toward meeting human vitamin E requirements, and also are not recognised well by α -tocopherol transfer protein in the liver. Evidence demonstrates that α -tocopheryl phosphate is natural and is de-phosphorylated to tocopherol, but the extent and rate of de-phosphorylation is only now being determined. Interestingly, alkaline phosphatase has also been shown to have phosphodiesterase activity (O'Brien and Herschlag, 2001), which may allow it to de-phosphorylate the α -di-tocopheryl phosphate. Therefore, information will continue to be gathered in order to determine if MTP will or can be classified as a new formulation of vitamin E. Information will also be gathered from longer-term exposure than from the 28 day repeat-dose

studies. The purpose of studying the safety of MTP was to provide data for its release onto the market as a nutritional supplement, including health beverages, and formulations for topical application.

Taken together, these results indicate a degree of safety of MTP, and work will continue to determine if its safety profile is similar to α -tocopherol and its more common derivatives.

Conflict of interest statement

EO is employed by Phosphagenics Ltd., which also funded the salaries of RL, RG and SG.

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References

- Ames, B.N., McCann, J., Yamasaki, E., 1975. Methods for detecting carcinogens and mutagens with the Salmonella/microsome-mutagenicity test. *Mutat. Res.* 31, 347–364.
- Cosmetic Ingredient Review Expert Panel (CIREP), 2002. Final report on the safety assessment of tocopherol, tocopheryl acetate, tocopheryl linoleate, tocopheryl linoleate/oleate, tocopheryl nicotinate, tocopheryl succinate, dioleoyl tocopheryl methylsilanol, potassium ascorbyl tocopheryl phosphate, and tocophersolan. *Int. J. Toxicol.* 21 (Suppl. 3), 51–116.
- Gianello, R., Libinaki, R., Azzi, A., Gavin, P.D., Negis, Y., Zingg, J.-M., Holt, P., Keah, H.-H., Griffey, A., Smallridge, A., West, S., Ogru, E., 2005. α -Tocopheryl phosphate: a novel, natural form of vitamin E. *Free Radical Biol. Med.* 39, 970–976.

- Hathcock, J.N., Azzi, A., Blumberg, J., Bray, T., Dickinson, A., Frei, B., Jialal, I., Johnston, C.S., Kelly, F.J., Kraemer, K., Packer, L., Parthasarathy, S., Sies, H., Traber, M., 2005. Vitamins E and C are safe across a broad range of intakes. *Am. J. Clin. Nutr.* 81, 736–745.
- IOM, 2000. Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. A report of the panel on dietary antioxidants and related compounds, subcommittees on upper reference levels of nutrients and interpretation and uses of dietary reference intakes, and the standing committee on the scientific evaluation of dietary reference intakes. Food and Nutrition Board, Institute of Medicine. National Academy Press, Washington, DC.
- ISCN, 1995. An International System for Human Cytogenetic Nomenclature. Felix Mitelman (ed.), S. Karger, Switzerland.
- Miller, E.R., Pastor-Barriuso, R., Dalal, D., Riemersma, R.A., Appel, L.J., Guallar, E., 2005. Meta-Analysis: High-dosage vitamin E supplementation may increase all-cause mortality. *Ann. Int. Med.* 142, 37–46.
- Munteanu, A., Zingg, J.M., Ogru, E., Libinaki, R., Gianello, R., West, S., Negis, Y., Azzi, A., 2004. Modulation of cell proliferation and gene expression by α -tocopheryl phosphates: relevance to atherosclerosis and inflammation. *BBRC* 318, 311–316.
- Nakayama, S., Katoh, E.M., Tsuzuki, T., Kobayashi, S., 2003. Protective effect of α -tocopherol-6-O-phosphate against ultraviolet B-induced damage in cultured mouse skin. *J. Invest. Dermatol.* 121, 406–411.
- Negis, Y., Zingg, J.-M., Ogru, E., Gianello, R., Libinaki, R., Azzi, A., 2005. On the existence of cellular tocopheryl phosphate, its synthesis, degradation and cellular roles: a hypothesis. *IUBMB Life* 57, 23–25.
- O'Brien, P.J., Herschlag, D., 2001. Functional Interrelationships in the alkaline phosphatase superfamily: phosphodiesterase activity of *Escherichia coli* alkaline phosphatase. *Biochemistry* 40, 5691–5699.
- Ogru, E., Gianello, R., Libinaki, R., Smallridge, A., Bak, R., Geytenbeek, S., Kannar, D., West, S., 2003. Vitamin E phosphate: an endogenous form of vitamin E. Medimond S.r.l., Bologna, Italy.
- Rezk, B.M., Haenen, G.R.M.M., van der Vijgh, W.J.F., Bast, A., 2004. The extraordinary antioxidant activity of vitamin E phosphate. *Biochim. Biophys. Acta* 1683, 16–21.