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Preclinical toxicity evaluation of erythrocyte-encapsulated thymidine phosphorylase in BALB/c mice and Beagle dogs: an enzyme replacement therapy for mitochondrial neurogastrointestinal encephalomyopathy

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Running title: Toxicity evaluation of EE-TP in mice and dogs

Abstract

Erythrocyte encapsulated thymidine phosphorylase (EE-TP) is currently under development as an enzyme replacement therapy for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal recessive disorder caused by a deficiency of thymidine phosphorylase. The rationale for the development of EE-TP is based on the pathologically elevated metabolites (thymidine and deoxyuridine) being able to freely diffuse across the erythrocyte membrane where the encapsulated enzyme catalyses their metabolism to the normal products. The systemic toxic potential of EE-TP was assessed when administered intermittently by intravenous bolus injection to BALB/c mice and Beagle dogs for 4 weeks. The studies consisted of one control group receiving sham-loaded erythrocytes twice weekly and two treated groups, one dosed once every two weeks, and the other dosed twice per week. The administration of EE-TP to BALB/c mice resulted in thrombi/emboli in the lungs and spleen enlargement. These findings were also seen in the control group and there was no relationship to the number of doses administered. In the dog, transient clinical signs were associated with EE-TP administration, suggestive of an immune based reaction. Specific anti-thymidine phosphorylase antibodies were detected in two dogs and in a greater proportion of mice treated once every two weeks. Non-specific antibodies were detected in all EE-TP treated animals. In conclusion these studies do not reveal serious toxicities that would preclude a clinical trial of EE-TP in patients with MNGIE, but caution should be taken for infusion-related reactions which may be related to the production of non-specific antibodies or a cell based immune response.

Key Words: enzyme replacement; erythrocyte carriers; mitochondrial neurogastrointestinal encephalomyopathy; thymidine phosphorylase; toxicity.

INTRODUCTION

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a rare autosomal recessive disease that is almost universally fatal. It causes relentless and progressive morbidity followed by premature death at an average age of 35 years (Garone *et al.*, 2011). MNGIE is caused by mutations in the nuclear *TYMP* gene that leads to a deficiency of thymidine phosphorylase (EC 2.4.2.4). Thymidine phosphorylase is a 47 kiloDalton subunit homodimer and catalyses the reversible phosphorylation of the pyrimidine nucleosides, thymidine and deoxyuridine to 2-deoxyribose 1-phosphate and their respective bases, thymine and uracil. The enzyme is part of the pyrimidine nucleoside salvage metabolic pathway and allows pyrimidine bases to be recycled for nucleotide biosynthesis, whilst the pentose 1-phosphates are converted to intermediates of the pentose phosphate shunt and glycolysis. A deficiency in thymidine phosphorylase activity results in elevated concentrations of thymidine and deoxyuridine in the plasma and cellular compartments which subsequently generate imbalances within the mitochondrial nucleotide pools, causing multiple deletions, point mutations and depletion of mitochondrial DNA (mtDNA), and ultimately mitochondrial dysfunction (Hirano *et al.*, 1994; Marti *et al.*, 2003; Spinazzola *et al.*, 2002; Valentino *et al.*, 2007). MNGIE is a multisystem disorder and is characterised clinically by leukoencephalopathy, ptosis and ophthalmoplegia, peripheral neuropathy and an enteric neuromyopathy manifesting as severe gastrointestinal dysmotility with cachexia. Allogeneic haematopoietic stem cell transplantation (HSCT) offers the possibility of a permanent correction of thymidine phosphorylase deficiency, but is still highly experimental, carrying a 44% mortality risk (Halter *et al.*, 2010). HSCT is limited by the availability of a matched donor, and patients are often in a poor clinical condition with an impaired capacity to tolerate transplant-related problems. The administration of HSCT to these patients presents pharmacological challenges in terms of administering drugs with possible mitochondrial toxicity, and the requirement for parenteral administration due to disturbed gastrointestinal function and impairment of absorption. A published consensus proposal for standardising an approach to allogeneic HSCT in MNGIE recommends restricting the recruitment of patients with an optimal donor to those without irreversible end-stage disease (Halter *et al.*, 2010). Patients who are oligosymptomatic are often

reluctant to undergo HSCT due to the high mortality risk. Many patients are therefore ineligible for this treatment option and clinical management is based on symptom relief and palliation. Thus there is a critical requirement to develop an alternative treatment.

Erythrocyte encapsulated thymidine phosphorylase (EE-TP) is under development as an enzyme replacement therapy for the treatment of MNGIE (Godfrin *et al.*, 2012; Godfrin and Bax, 2012; Moran *et al*. 2008). This therapeutic approach has the advantage of prolonging the circulatory half-life of the native enzyme to that of erythrocyte half-life (19 to 29 days) and potentially minimising the immunogenic reactions which are observed in enzyme replacement therapies administered by the conventional route (Bax *et al*., 1999, 2000; 2007, Godfrin *et al*., 2012). To formulate EE-TP in the clinical setting, a defined quantity of blood is removed from the patient and following separation of blood components, the erythrocytes are subjected to a reversible hypo-osmotic dialysis procedure in the presence of thymidine phosphorylase (EC 2.4.2.4) so that the enzyme becomes encapsulated. EE-TP is then administered back to the patient. The rationale for the development of EE-TP is based on thymidine and deoxyuridine being able to freely diffuse across cell membranes and exist in a state of equilibrium between the cellular and plasma compartments. It is proposed that regular intravenous administrations of EE-TP to patients with MNGIE will lead to a sustained reduction or elimination of plasma thymidine and deoxyuridine concentrations, resulting in a clearance from the cellular compartments and an amelioration of the intracellular nucleotide imbalances. EE-TP aims to arrest and reverse the progression of the clinical disease with consequent clinical improvement by reversal of the mitochondrial dysfunction in MNGIE.

The objective of the studies reported here was to evaluate the systemic toxic potential of EE-TP in BALB/c mice and Beagle dogs in support of clinical development of EE-TP. The mouse and dog were chosen as the first and second test species respectively because of their acceptance as predictors of toxic change in man and the requirement for a rodent and non-rodent species by regulatory agencies. The

BALB/c mouse was used because of previous experience with administration of EE-TP to that strain. The Beagle strain was employed because of the historical control data available and also because the dog also has a sufficient volume of blood to allow the formulation of EE-TP using autologous blood (Chalmers, 1985; Sprandel *et al.,* 1981). The studies were designed to meet the requirements of regulatory guidelines and were conducted in accordance with the requirements of current, internationally recognised Good Laboratory Practice Standards.

A standard toxicological evaluation was performed which included daily clinical signs, weekly body weight and food consumption, and end of study ophthalmic examinations, clinical pathology, organ weights, and complete gross necropsy on all animals and light microscopic examination of a range of tissues.

MATERIALS AND METHODS

Test and control materials

Recombinant *E.coli* thymidine phosphorylase was manufactured for this study by Sigma-Aldrich (Israel) and was supplied formulated in a potassium dihydrogen orthophosphate stabilization buffer with a specific activity of 178 to 211 IU/mg protein. The Master and working cell bank is stored in the Jerusalem Plasmid Bank. The specification and batch analysis release results for the recombinant enzyme employed in these studies are outlined in Table 1. The test material, EE-TP was formulated by encapsulation within dog or mouse erythrocytes as follows:

The dog study followed the proposed clinical regime, in that autologous blood was removed from a subject for processing into test or control material and then administered back to the same subject; one day before each dosing occasion, 50 ml of blood was collected via a suitable vein from the appropriate dog into vacutainers with lithium heparin anticoagulant and transported to St. George's, University of London. EE-TP was formulated by encapsulation of thymidine phosphorylase within erythrocytes

using our established reversible hypo-osmotic dialysis process (Chalmers, 1985; Bax *et al*., 1999). Aseptic techniques and sterile materials were used throughout. Briefly, blood was centrifuged at 1,100 x *g* for 10 minutes, and the plasma and buffy coat removed and retained for later use. Erythrocytes were washed twice in cold (4^{o}C) phosphate buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl, 8.10 mM Na_2HPO_4, pH 7.4) The washed and packed erythrocytes were then mixed with cold PBS containing 200 IU/ml of thymidine phosphorylase to form a suspension with a haematocrit of 70%. The cell suspension was placed in a dialysis bag with a molecular weight cut-off of 12,000 Da and then dialysed against 40 volumes of hypo-osmotic buffer (5 mM KH_2PO_4, 5 mM K_2HPO_4, pH 7.4) at 4^{o}C with rotation at 8 rpm for 90 minutes. The lysed erythrocytes were resealed by dialysis against 40 volumes of PBS supplemented with 5mM $MgCl_2$, 5mM adenosine, and 5mM glucose (SPBS, pH 7.4) at 37^{o}C with rotation at 8 rpm for 60 minutes. The enzyme-loaded erythrocytes were then washed three times in SPBS, with centrifugation at 100 x *g* for 20 minutes. The cells displayed normocytic and normochromic morphology and were characterized for the following parameters: mean cell volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), extracellular haemoglobin concentration (Hb), encapsulated and extracellular thymidine phosphorylase activity (Table 2). The haematological parameters MCV, MCH and MCHC were determined using a Woodley MS4-5 haematology analyser. For the determination of extracellular Hb concentration and thymidine phosphorylase activity, extracellular fractions were obtained by adjusting the haematocrit of the washed and packed cells to 50% with PBS, followed by centrifugation at 1000 x *g* for 10 minutes. Hb was measured by spectrophotometry at 542nm using Drabkin's Reagent (Sigma-Aldrich, United Kingdom) and thymidine phosphorylase activity was determined as described below. The control material (sham-loaded erythrocytes) was formulated by subjecting erythrocytes to the same reversible hypo-osmotic dialysis process, but in the absence of thymidine phosphorylase. Cell recovery for EE-TP and sham-loaded cells was 62 ±1.2% and 59.2 ± 1.3 %, respectively. Plasma and white cells retained from the first centrifugation step and erythrocytes excess to requirements of the encapsulation procedure were added back to the test or control material to provide an infusion volume of 43-50 ml.

This step was included to mimic the regime used in the clinical setting to avoid depletion of important blood constituents and maintain blood volume.

In the mouse study, to avoid complications of over-sampling, allogeneic blood (rather than autologous blood) obtained from donor mice from the same strain was used to prepare the test and control material. A pre-determined volume of allogeneic blood in lithium heparin was supplied one day before dosing and processed into test and control material. The same reversible hypo-osmotic dialysis technique as described for the dog erythrocytes was used, accept that all washes (both pre and post dialysis) and iso-osmotic resealing were performed using SPBS containing 3mM glutathione, and the hypo-osmotic and iso-osmotic dialysis steps were conducted against 30 volumes of buffer (Murray *et al*., 2006). Cell recoveries were 36.7 ± 1.8 (n= 12) and 37.8 ± 2.1% (n= 9) respectively, for EE-TP and sham-loaded cells. The cells displayed normocytic and normochromic morphology and had the characteristics described in Table 2. After formulation, two volumes of test or control material were suspended in 1 volume of retained plasma.

For both species, within one hour of formulation, the test and control materials were dispatched at ambient temperature to Huntingdon Life Sciences for administration.

The stability of encapsulated thymidine phosphorylase activity was assessed over the proposed maximum time delay between formulation and infusion in the pre-clinical setting by analysis of thymidine phosphorylase activity after 0, 1, 4, 17 and 24 hours of storage at 4^{o}C and 22^{o}C. One hundred µl aliquots of EE-TP prepared from dog and mouse erythrocytes were stored in closed microtubes at the appropriate temperature and then frozen after the appropriate incubation time until analysis.

Thymidine phosphorylase analysis

Thymidine phosphorylase activity was determined by quantification of the rate of thymine formation using a validated high performance liquid chromatography (HPLC) method (manuscript in preparation). The method is linear over a thymine phosphorylase activity of 4.0 to 590 nmol/min/ml, and has a limit of detection and limit of quantification of 4.7 nmol/min/ml and 14.3 nmol/min/ml, respectively. Analyses were performed to verify the activity of cellular and extracellular thymidine phosphorylase activity in EE-TP and to confirm the absence of enzyme activity in the control material. Lysed (thawed from -80^{o}C) erythrocytes and extracellular fractions were diluted 1:1420 and 1:10, respectively with TRIS buffer (125 mM, pH 7.4). Twenty five µl of the diluted erythrocyte lysate or extracellular fraction was then added to 100 µl phosphate buffer (100 mM, pH 6.5) and 25 µl thymidine standard (10 mM), mixed and incubated at 37°C for 10 minutes. The reaction was terminated with 25 µl 40% trichloroacetic acid (TCA). Samples were centrifuged at 12,000 rpm for 2 minutes and the supernatant washed twice with water-saturated di-ethyl ether for 2 minutes on a shaker to remove TCA. A sample volume of 10 µl was injected into the HPLC. A pre-packed Spherisorb 5 ODS column (125 x 4.6 mm i.d.) was used in an isocratic system at a flow rate of 1.0 ml/min with a run time of 8 minutes. The buffer consisted of ammonium acetate (40 mM) with the ion-pairing agent tetrabutylammonium sulphate (5 mM), adjusted to pH 2.70 with HCl. The HPLC trace was recorded at 254 nm and 0.1 AUFS. Metabolites were identified by comparing spectra with pure standards.

Animal husbandry

This aspect of the studies was performed at Huntingdon Life Sciences, UK. All in-life experimental procedures were performed in compliance with the Animals (Scientific Procedures) Act 1986. Animal housing complied with the United Kingdom Home Office Code of Practice for the Housing and Care of Animals used on Scientific Procedures. The study designs were reviewed and approved by the holders of the Home Office Project Licences at Huntingdon Life Sciences.

Mice. BALB/c mice were purchased from a commercial breeder and were acclimatized for 18 days. At the start of treatment the mice were 10 weeks old, with bodyweights of 21.5 to 26.7 g for males, and 18.7 to 22.6 g for females. The animals were housed up to three per cage for females and singularly for males. The temperature and relative humidity were maintained within the range of 19 to 23^{o}C and 40 to 70%, respectively. Artificial lighting was controlled to give a 12 hour light/dark cycle. Food (Rat and Mouse No. 1 Maintenance Diet) and water were given *ad libitium*.

Dogs. Pure-bred Beagle dogs of known lineage were obtained from a commercial breeder and were inoculated against canine distemper virus, canine hepatistis virus, canine parainfluenza virus, canine parvovirus, *Leptospira canicola*, *Leptospira icterohaemorrhagiae* (by subcutaneous injection of DHPPi and Leptospira) and *Bordetella bronchiseptica* vaccine (Intrac® given intranasally). On arrival, animals also received a veterinary examination and received a course of oral treatment with the anthelmintic 'Drontal Plus®' (praziquantel, pyrantel embonate and febantel. The dogs were allowed to acclimatise to housing conditions for at least four weeks before the start of treatment. At the start of treatment the dogs were approximately 24 to 27 weeks of age and weighed 9.6 to 11.8 kg for males and 7.07 to 10.0 kg for females. The animals were housed in trios of the same sex and dose group, except for the period immediately before each administration. Each individual pen was equipped with under-floor heating and graded whitewood sawdust was used as litter and changed daily. Room temperature was maintained in the range of 15 to 24^{o}C and air extraction was via a balanced system designed to provide approximately 12 air changes per hour. Lighting was controlled to give a 12 hour light/dark cycle. Each dog was offered 400 g of a standard dry pelleted diet (Teklad 2021 Dog Maintenance Diet) daily. Food was offered midmorning and each dog was allowed access to it for at least one hour, after which time any uneaten food was removed and subsequently weighed and discarded. Water was given *ad libitium*.

Administration of test and control materials

For both species an intermittent intravenous bolus injection route of administration was chosen to simulate the conditions of clinical administration. The mice received an intravenous bolus injection at a volume-dose of 4 ml/kg/occasion, using a graduated syringe and needle inserted into the tail vein. In the dogs, venous access was gained via cephalic or saphenous veins, alternated at each administration. A catheter primed with a small volume of saline was then connected to a syringe driver (Harvard Apparatus PHD2000 infusion pump) and the entire volume of blood was administered back to the dog from which it came from at an infusion rate of 10 ml/minute (except for Group 3 males on Day 15, where an infusion rate of 5 ml/minute was used).

The study consisted of one control (Group 1) and two treated groups (Groups 2 and 3) for each species and which were treated as outlined in Table 3. Groups 1 and 3 were treated twice per week. Group 2 was treated once every two weeks, according to the proposed clinical regime, with a proposed maximal dose level of 200 IU/kg. The twice weekly administration to Group 3 was intended to achieve an exposure approximately four times higher than the proposed clinical dose to achieve a satisfactory safety margin for clinical trials.

Serial observations

Animals were inspected visually at least twice daily for evidence of ill-health or reaction to treatment. On dosing days detailed observations were recorded at the following times in relation to dose administration: predose, immediately after infusion, between 0.5 and 2 hours after completion of dosing, and as late as possible in the working day. The bodyweight of each animal was recorded weekly (mouse) and twice per week (dog) during the acclimatization period, on the day treatment commenced (Day 1), twice weekly throughout the treatment period, and before necropsy. The weight of food supplied to each cage of mice and each individual dog, the weight that remained, and an estimate of any spilled was recorded during the acclimatization period and throughout the study. For the mouse, the weekly consumption per animal (g/animal/week) was calculated for each cage.

Ophthalmoscopic examinations were conducted predose and prior to each animals scheduled euthanization. Prior to each examination, the pupils of each animal were dilated using tropicamide ophthalmic solution (Mydriacyl). The adnexae, conjunctiva, cornea, sclera, anterior chamber, iris (pupil dilated), lens, vitreous and fundus were examined.

In the dog, electrocardiograph tracings were recorded from all animals on one occasion during the pretreatment period for the three standard limb leads (I, II, II) and the three augmented limb leads (aVR, aVL and aVF). Further tracings were obtained during Week 3, two and 24 hours after infusion for Group 2, and during Week 4, two and 24 hours after infusion for Groups 1 and 3. The traces were examined visually for any abnormalities of the electrical complexes and the heart rate was recorded.

Clinical pathology

Clinical pathology samples were collected for the evaluation of haematology and clinical chemistry. In the dog these were obtained via the jugular vein before treatment commenced and during Week 4 before dosing. Additional samples were taken for haematology on Day 14 from all dogs, and on Day 21 from male dogs only. Mouse blood samples were taken at termination only; the animals were held under light general anaesthesia induced by isoflurane and blood samples were withdrawn from the retro-orbital sinus. Haematology parameters were measured in the first five mice per sex per group, and clinical chemistry parameters measured in the second five mice per sex per group.

Haematology parameters were analysed in blood collected into tubes containing EDTA as an anticoagulant using a Bayer Advia 120 haematology analyser and included: haematocrit (Hct), Hb, erythrocyte count (RBC), reticulocyte count, MCH, MCHC, MCV, total white cell count (WBC), differential WBC count (neutrophils, lymphocytes, eosinophils, basophils, monocytes, large unstained cells) and platelet count. In the mouse, bone marrow samples were obtained from the tibia and femur

during necropsy for examination of bone marrow haematology. Prepared smears were air drier, fixed in methanol and stained using a romanowsky procedure.

Clinical chemistry parameters were examined in plasma (separated from blood which had been collected into lithium heparin) using a Roche PP Modular Analyser and included: alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, total bilirubin, urea, creatinine, glucose, total cholesterol, triglycerides, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein and albumin. In the dog electrophoretic protein factions Albumin, ∝1 globulin, ∝2 globulin, β globulin, and γ globulin were processed using a Helena SPIFE 3000 with agarose gel and scanning with a densitometer. Albumin/globulin ratio was calculated from total protein concentration and analysed albumin concentration.

Coagulation parameters prothrombin time and activated partial thrombopastin time were measured for the dog in blood collected into citrate using an ACL 9000 Analyser.

Overnight urine produced was collected from all dogs before treatment commenced and during Week 4. Dogs were placed in individual metabolism cages without food or water and urine was collected for approximately 16.5 hours. Samples were examined for appearance, volume, pH, specific gravity, protein, glucose, ketones and bile pigments. A microscopic examination of the urine sediment was performed by centrifugation of an aliquot of urine and staining the resulting deposit with Kova stain before spreading on a microscope slide.

Anti-thymidine phosphorylase antibodies

Blood samples for analysis of anti-thymidine phosphorylase antibodies were collected from all mice and dogs into standard serum tubes during pretreatment, on Day 17 (male dogs only) and at termination.

After separation by centrifugation at 2000 *g* at 4°C for 10 minutes the serum was collected into aliquots and frozen at -70°C until analysis. Samples were analysed for anti-thymidine phosphorylase antibodies using validated GLP electrochemiluminescence detection methods (Authorised Method Numbers IM/2011/0193 and IM/2011/0194, manuscript submitted).

Necropsy and histology

All animals were euthanized either 1 day (dogs) or 2 days (mice) following the administration of the last dose; mice were sacrificed by carbon dioxide asphyxiation, and dogs by an overdose of sodium pentobarbitone solution (200 mg/ml) by intravenous injection and subsequent exsanguination. A full macroscopic examination of the tissues was performed. Any abnormality in the appearance or size of any organ and tissue was recorded and the required tissue samples preserved in the appropriate fixative. Testes were fixed in modified Davidson's fluid and eyes were fixed in Davidson's fluid prior to transfer to 70% industrial methylated spirit. All other tissues were preserved in 10% neutral buffered formalin. Tissues to be examined were dehydrated, embedded in paraffin wax, sectioned at approximately 4 to 5 micron thickness and stained with haematoxylin and eosin. The following tissues were examined microscopically: adrenals, brain, femur with joint, heart, kidneys, liver, lungs, spinal cord, sternum, stomach, thyroid and uterus. For bilateral organs, sections of both organs were prepared. Findings were either reported as present or assigned a severity grade. In the latter case one of the following five grades was used- minimal, slight, moderate, marked or severe.

Statistical analyses

Data are expressed as mean ± SD or as mean ± SEM. For the mouse studies, statistical analyses were carried out separately for males and females. Data relating to food consumption were analysed on a cage basis for females, and individually for males. For all other parameters, the analyses were carried out using the individual animal as the experimental unit. Comparisons were Group 1 versa 2, and Group 1 versa 3. The sequence of statistical tests employed for bodyweight, food consumption, organ weight

and clinical pathology data was firstly a parametric analysis if Bartlett's test for variance homogeneity (Bartlett, 1937) was not significant at the 1% level; groups were compared using t-tests, and secondly a non-parametric analysis if Bartlett's test was still significant at the 1% level following both logarithmic and square-root transformations; groups were compared using Wilcoxon rank sum tests (Wilcoxon, 1945). For clinical pathology data, if 75% of the data (across all groups) were the same value, for example c, Fisher's Exact tests (Fisher, 1973) were performed. Treatment groups were compared using pairwise comparisons of each dose group against the control both for values $<$c versus values $\geq$c, and for values $\leq$c versus values $>$c, as applicable. For organ weight data, analysis of covariance was performed using terminal bodyweight as the covariate (Angervall and Carlstrom, 1963). The treatment comparisons were made on adjusted group means to allow for differences in bodyweight which might influence organ weight. Significant differences between control and treated groups are expressed as $*p < 0.05$ and $**p < 0.01$. Due to the small numbers on the dog study, statistical analyses were not performed.

RESULTS

EE-TP stability and dose

Storage of EE-TP for up to 24 hours at temperatures of 4^{o}C and 22^{o}C had no significant effect on erythrocyte associated thymidine phosphorylase activity (Figure 1). Low concentrations of extracellular Hb and low extracellular activities of thymidine phosphorylase demonstrated that the EE-TP was stable 24 hours after formulation (Table 2). The maximum time delay between EE-TP formulation and infusion in these studies was 5 hours. Table 3 shows the doses administered.

Clinical observations

Mouse. One male receiving EE-TP once every 2 weeks (Group 2) died immediately following the final dose on Day 29. The post mortem examination showed no gross findings, however, the histopathological examination revealed a marked presence of thrombi/emboli in the lungs and this was

considered to be the reason for death. From Week 4 an ungroomed appearance was observed for the males in all groups (including control), accompanied by piloerection for both test substance treated groups. A hunched posture was noted for males receiving the twice weekly administration. Dark tails, dark patches on tails and/or scabbing were observed for all groups (including control) and were considered to reflect the intravenous route of administration.

There was no apparent effect of treatment on bodyweight, body weight gain or food consumption. Findings from the ophthalmoscopic examinations performed pretreatment and in Week 4 were within normal limits for animals of this age and strain. There was no evidence of a treatment-related effect on any ocular structures.

Dog. There were no unscheduled deaths during the study. Animals dosed once every two weeks (Group 2, dosed on Days 1, 15 and 29) showed no clinical signs on the first administration. However on the second administration (Day 15) transient post dose underactivity was noted in three males and two females. On the third administration (Day 29) transient post dose underactivity was noted for all three males and three females. On Day 29 additional transient clinical signs included: unusual respiration (panting) in one male and one female, loose or liquid faeces in one male and one female, and vomiting in one female. Pale gums were noted occasionally throughout the study for some animals.

Males dosed twice per week (Group 3, dosed on Days 1, 4, 8, 11, 15, 18, 22, 25 and 29) showed no clinical signs on dosing Days 1 and 4. From Day 5 onwards pale gums were noted for all males. However on Day 11 after dosing, one male vomited, a second dog was noted with loose faeces and the third dog was transiently underactive with loose faeces and vomiting. During dosing on Day 15 all three male dogs were underactive during dosing and this continued to be noted for one dog until the end of the working day. Loose faeces were noted for this dog during dosing, and body tremors and vomiting after dosing. As a result of these findings, a decision was made to pre-medicate the male dogs from Day

18 onwards with antihistamine, corticosteroid anti-inflammatory and anti-emetic treatments approximately one hour prior to dosing. As a result, the levels of clinical signs were significantly reduced at dosing Days 18, 22 and 25, with only post dose underactivity noted in two of the dogs. However, on Day 29 the level of underactivity increased for two dogs both during dosing and post dosing.

Females dosed twice per week (Group 3) showed the same range of clinical signs as those seen within the males but in general these signs appeared later in the study. On Day 15 one female was noted as unsteady at the end of dosing and on Day 18 another female was noted as underactive during dosing. Due to these finding both these females received predose medication in future treatments; despite this both animals were noted as underactive following dosing on Day 25, and one was again underactive on Day 29. Pale gums were noted, but at a lower incidence than seen within the males of this group, but was also observed in one control dog.

There was a higher incidence of bruising and scabbing at the injection sites of males that had received twice weekly treatments in comparison with control. There were no apparent increases in incidence within the females or within dogs that were treated every two weeks.

Group mean bodyweight gain in males and females receiving twice weekly treatments (Group 3) was less than that of the control (Group 1). The mean bodyweight gain in females receiving treatment every second week (Group 2) was also lower than that of the controls (Figure 2). However this result was due to one female, which was the smallest animal in the study. This difference from the control is therefore not considered to be related to treatment. There was no apparent effect of treatment on food consumption, ophthalmoscopic and electrocardiographic examinations.

Clinical Chemistry and Haematology

Mouse. Table 4 shows the results of selected clinical chemistry parameters for mice after receiving treatment for 4 weeks. Higher than control concentrations of cholesterol (1.08 - 1.15 fold higher) and triglyceride (1.62-2.84 fold higher) were recorded for both male groups treated with EE-TP. In the absence of similar differences in the females or corroborative pathology, these higher concentrations were considered not to be of any toxicological importance. The plasma urea concentration was lower for females treated once every 2 weeks and both sexes treated twice per week compared to the control. This difference reflects the control group urea values being slightly higher than the expected background range (5-95 percentile: females 5.44 to 7.08 mM) and consequently is not considered to be related to EE-TP administration. All other inter-group differences after four weeks of treatment were minor or were confined to one sex and were attributed to normal biological variation. Such changes included the variations observed for creatinine and calcium in males and alkaline phosphatase in females.

No changes were observed in the haematological parameters investigated which were considered to be an effect of EE-TP administration. Anisocytosis was observed for the majority of animals (including control) and was more marked in the males. Higher than control mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were observed for both groups of treated females and a lower than control haematocrit was observed for females treated once every 2 weeks (Table 5). In the absence of a dose relationship or a similar finding in the males this difference from control was considered unlikely to be an effect of treatment. No abnormal microscopic findings were observed in the bone marrow.

Dog. No treatment-related changes were observed in the clinical chemistry parameters investigated. All inter-group differences were minor and reflected trends that were present pretreatment or lacked dosage-relationship and are therefore attributed to normal biological variation. No changes were observed in the urinalysis parameters which were considered to be effect of treatment. Urinary chloride

and sodium were increased for two males receiving twice weekly treatments when compared with control and pre-treatment values. However, no similar findings were noted in the females (Table 6).

Haematology investigations performed on Days 14 and 21 (males only) and in Week 4 revealed reductions in haematocrit, haemoglobin concentration and erythrocyte count, with an associated increase in reticulocytes within all groups including the controls, in comparison with pre-treatment. There was some variation in the magnitude of response between animals within the same treatment group, the magnitude of these changes were greater midway through the study (Day 14) and lessened, but still not returning to pre-treatment levels by the end of the study; animals treated once every two weeks were less affected than those treated twice weekly; females were less affected than males. All other inter-group differences were minor, reflected trends that were present pre-treatment or lacked dosage-relationship and were therefore attributed to normal biological variation (Table 7). A reduction in platelet count, when compared with the controls, was observed on Day 14 and/or Day 21 (males only) for males and females receiving treatments every two weeks and for males and females receiving twice weekly treatments. During Week 4 similar reductions were observed within these groups, however the magnitude of changes was less, and approximately half the animals treated once every two weeks and one of the animals treated twice per week returned to values similar to pre-treatment (Table 7).

Anti-thymidine phosphorylase antibodies

Samples taken on Day 29 for all mice and dogs treated with EE-TP were positive for the presence of non-specific antibodies. A higher titre was noted for mice treated twice weekly (Group 3). No antibodies were detected in the control group animals. For the dog a confirmatory assay showed the presence of anti-thymidine phosphorylase-specific antibodies for one male receiving EE-TP once every two weeks (Group 2), and one male receiving EE-TP twice per week (Group 3). For the mouse the presence of anti-thymidine phosphorylase-specific antibodies was detected for 7 of 9 males and 7 of 10

females receiving EE-TP once every two weeks (Group 2) and 4 of 10 males and 1 of 10 females receiving EE-TP twice per week (Group 3).

Organ Weights and Anatomic Pathology

Mouse. The bodyweight adjusted spleen weights for both sexes treated twice per week were significantly higher than the control group ($p < 0.05$ for male, and $p < 0.01$ for female). Additionally the absolute spleen weights were higher than the expected background range (5 - 95 percentile: males 0.089 - 0.103 g, females 0.085 - 0.102 g) for all animals in the study (male and female, both treatment groups and control). All other differences from control, including those achieving statistical significance were only observed in a single sex, showed no dose relationship or were considered to be minor. Such differences from control included low brain (treated once every two weeks, $p<0.05$) and thymus (treated once every two weeks, $p<0.05$; treated twice weekly $p<0.01$) weights in males.

Macroscopic examination performed after 4 weeks of treatment revealed enlargement of the spleen in a majority of the animals in all groups including the control. Dark areas on the parenteral site (tail vein) were observed in some animals in all groups and were procedural in origin. The incidence and distribution of all other findings were consistent with the common background of BALB/c mice. Changes related to treatment with EE-TP as observed by light microscopy were noted in the lungs. Thrombi/emboli in the lungs were recorded in all groups, including the control group, accompanied by perivascular inflammatory cells in EE-TP treated animals (Figure 3). There was an increased incidence and severity noted for the treated groups compared with control, however, there was no relationship to the number of doses administered (Table 8). At the parenteral injection site perivascular inflammation/haemorrhage was observed in all groups. This finding correlated with the gross pathology observation of dark tails at necropsy and was related to the intravenous administration procedure and not to EE-TP. No microscopic lesions were observed in the spleen.

Dog. For dogs receiving twice weekly treatments (Group 3) the thymus weights of all three females and two of the three males were lower than the control weights. The brain weights in all 3 males receiving twice weekly treatments (Group 3) were increased when compared with the controls. However in the absence of similar findings within the females or any associated microscopic findings in the brain, these weight differences are considered not to be related to treatment. Increased liver weights were apparent for all animals receiving twice weekly treatments (Group 3) and for males receiving treatment once every two weeks (Group 2). However, there was no microscopic correlate and therefore these differences are considered not to be related to treatment. An increased group mean spleen weight was also observed for two of the three females receiving twice weekly treatments. No similar change was noted within the males and no microscopic correlate was observed and therefore these differences are considered not to be related to treatment. All other inter-group differences were minor or lacked dosage-relationship and were therefore attributed to normal biological variation (Table 9).

Macroscopic examination performed after 4 weeks of treatment revealed smaller thymuses in all three females and two of the three males receiving twice weekly treatments (Group 3). Perivascular red areas observed at some parenteral sites were procedural in origin. The incidence and distribution of all the other findings were consistent with the common background. Changes related to treatment with EE-TP seen by light microscopy were in the thymus of animals given the test substance twice weekly. The thymus had slight to marked atrophy in these animals (Figure 4).

DISCUSSION

MNGIE is a rare inherited disease for which at present there are no regulatory approved therapies. The disease is relentlessly progressive and degenerative, with patients dying at an average age of 35 years (Garone *et al*., 2011). EE-TP is under pre-clinical and clinical development as an orphan designated enzyme replacement therapy for patients with MNGIE (EC register number EU/3/11/856). Therapy is proposed for patients in whom there is no matched donor, where the risk of mortality from allogeneic

HSCT would be too high, or as a rescue or maintenance therapy prior to the availability of a suitable HSCT donor. The objective of this GLP study was to assess the systemic toxic potential of EE-TP when administered intermittently by intravenous bolus injection to mice and dogs for 4 weeks. The animals employed in this study were normal and thus the administration of EE-TP has the potential to cause imbalances in the pyrimidine metabolism resulting in toxic or adverse effects. In the normal state plasma thymidine and deoxyuridine concentrations are undetectable due to circulating platelets and lymphocytes (which contain thymidine phosphorylase) regulating the homeostasis of these metabolites; it was considered that toxic effects due to pyrimidine metabolism imbalances would be insignificant during these 4 week studies (Shaw *et al*; 1988).

EE-TP was formulated using a reversible hypo-osmotic dialysis, permitting the administration of 330 to 364 IU/kg/occasion in the mouse and 194 to 200 IU/kg/occasion in the dog. The proposed anticipated clinical dose of thymidine phosphorylase is 200 IU/kg/two weeks, and thus exposures 6.6 fold and 4 fold higher than this dose were administered respectively in the mice and dogs receiving EE-TP twice per week, achieving satisfactory safety margins for clinical trials. The stability data demonstrated that the time delay between EE-TP formulation and infusion had no effect of the encapsulated thymidine phosphorylase activity and thus the dose administered. Extracellular thymidine phosphorylase activity at six hours post formulation represented 0.2% and 0.4% of the total erythrocyte encapsulated enzyme for the mouse and dog erythrocyte, respectively demonstrating the stability of the erythrocyte carrier during the period between EE-TP formulation and infusion. In the clinical setting it is anticipated that EE-TP infusion will take place within 24 hours of formulation.

In the 4 week mouse study, an increased incidence and severity of thrombi/emboli in the lungs was recorded in EE-TP treated animals, compared with the controls. This was associated with the presence of perivascular inflammatory cells which is a normal response to the presence of thrombi/emboli. Thrombi in the lungs resulted in the death of one test substance treated mouse and may also be linked to

the clinical signs noted towards the end of the treatment period (ungroomed appearance, piloerection, and hunched posture). The mouse studies employed allogeneic erythrocytes and as this finding was not observed in the dog studies which used autologous erythrocytes, this effect may be related to the intravenous administration of foreign erythrocytes and/or the action of antibodies on the administered erythrocytes. The perivascular recruitment of inflammatory cells may also be a consequence of lung inflammation caused by the local release of thymidine phosphorylase, and would explain the greater incidence and severity of thrombi and emboli in the EE-TP treated animals. There is no evidence of this effect in the lungs of the patient who has received twice monthly infusions of erythrocyte encapsulated adenosine deaminase over a period of 16 years (Bax *et al*., 2007).

The splenic enlargement and high spleen weights observed in both EE-TP treated and control mice may reflect a pooling of erythrocytes due to administration to animals with a full complement of erythrocytes, or alternatively a sequestration of infused erythrocytes by the spleen for conditioning and later release back into the circulation. The spleen acts as a reservoir for blood and is the most discriminating organ for monitoring the integrity of erythrocytes and removes senescent or abnormal erythrocytes from the circulation (Harris *et al*., 1957). The absence of microscopic lesions in the spleen would indicate that spleen enlargement was not caused by an excessive destruction of erythrocytes by erythrophagocytosis. Our *in vivo* studies of human chromium (^{51}Cr)-labeled erythrocyte carriers demonstrated a sequestration of between 51 and 97% of the cells within the first 144 hours of infusion, as measured by surface counting, and this was followed thereafter by an almost total release of cells back into the circulation suggesting that the erythrocytes were initially retained by the spleen for repair and this mechanism could explain the splenic enlargement observed here (Bax *et al*., 1999).

Anisocytosis was observed in the majority of treated and control mice and this was probably induced by the dialysis procedure, which in the mouse erythrocyte results in a cell volume reduction of 29 to 39%.

This compares to a reduction of 6 to 18% in the human and dog erythrocyte volumes and a subsequent *in vivo* normalisation of cell size.

Administration of EE-TP to mice once every two weeks or twice weekly resulted in an antibody response, with a greater incidence of anti-thymidine phosphorylase antibodies observed in the group treated once every 2 weeks. These results are consistent with our previous studies in the BALB/c mouse where the administration of antigen-loaded erythrocytes was shown to elicit humoral immune responses (Murray *et al*., 2006). The source of thymidine phosphorylase employed in these current studies was a recombinant *E.coli* protein sharing a 40% amino acid sequence homology with the human sequence (Barton *et al*., 1992). Although encapsulation within erythrocytes would be predicted to reduce immunogenicity of the native enzyme, an intravascular release of thymidine phosphorylase from damaged or fragile erythrocytes is likely to elicit an immunogenic reaction. This is of significant interest with regard to the clinical application of EE-TP; despite raising an anti-thymidine phosphorylase antibody response, the erythrocyte carrier can simultaneously protect the encapsulated thymidine phosphorylase from circulating antibodies. In the clinical setting, immunogenic reactions have been reported to occur with most therapeutic proteins, with the incidence ranging between less than 10% of patients, to nearly 100% (Schellekens, 2004). The monitoring of antibodies responses during pre-clinical and clinical safety testing of therapeutic proteins and, in some instances, after marketing approval is thus an important issue.

In the dog, although treatment twice weekly was tolerated for 4 weeks, the observed level of clinical signs during or shortly after dosing from Day 11 onwards were significant, and increased with each subsequent dosing occasion. In the clinical setting, nausea and erythema of the face, neck and in the arm proximal to infusion have previously been observed in patients receiving EE-TP under compassionate use, and these were successfully prevented using antihistamine, corticosteroid anti-inflammatory and anti-emetic drugs prior to treatment. This approach was undertaken for dogs receiving twice weekly treatments from Day 18

onwards for a majority of the animals. As a result of these predose medications the incidence and degree of clinical signs was initially notably reduced, however with each subsequent administration the signs increased again. Dogs treated every second week showed a similar pattern of clinical signs on the second and third administration (Day 15 and 29). The delayed appearance of these clinical signs, their transient nature associated with dosing and their subsequent successful treatment with anti-inflammatory drugs, suggested an immune based response to the administration of EE-TP. As there was no similar finding within the control dogs, it is concluded that thymidine phosphorylase was responsible. Non-specific antibodies were detected at the end of the study in all dogs given EE-TP and specific anti-thymidine phosphorylase antibodies were detected in only two dogs. It would therefore appear that the clinical signs were associated with the non-specific antibodies or with a cell based immune response, and not with a specific anti-thymidine phosphorylase response. The thymidine phosphorylase preparation contained low levels of process related endotoxins, and although a majority of these would have been removed by the dialysis process employed in the formulation of EE-TP, there is the possibility that the formation of non-specific antibodies was caused by the presence of residual endotoxins. The Food and Drug Administration (USA) recommends a maximum endotoxin limit of 5 EU/Kg body weight for parentally administered drugs; the endotoxins levels in the thymidine phosphorylase preparation prior to EE-TP formulation were well below this limit (Brito and Singh, 2011). The absence of specific anti-thymidine phosphorylase antibodies in a majority of the dogs would suggest that removal of EE-TP from the circulation and subsequent catabolism by lysosmal enzymes (the normal route for the degradation of senescent erythrocytes) does not lead to antigen presentation, unlike in the mouse (Murray *et al.*, 2006). It also indicates that there was minimal intravascular haemolysis of the enzyme-loaded erythrocytes, which if occurred would have most likely resulted in the production of specific antibodies against the released thymidine phosphorylase.

Haematology investigations performed during and at the end of the treatment period revealed in all dog groups an apparent haemorrhagic-type anaemia (reductions in haematocrit, haemoglobin concentration

and erythrocyte count), with a subsequent increase in reticulocytes. This change is most likely to be due to the hypo-osmotic dialysis procedure used to prepare the sham- and thymidine phosphorylase-loaded erythrocytes; there is a cell loss of approximately 38% during the process and a reduction in the cellular haemoglobin concentration. This would explain why dogs treated once every two weeks were less affected than those treated twice weekly. The haematology results from the end of the treatment period indicated that although these changes were still present, the parameters were returning to normal levels. The increase in reticulocyte numbers demonstrated that the bone marrow was responding to the anemia, and for this reason these changes are considered not to be adverse. Pale gums noted during for treated animals may be a result of these haematology changes. The magnitude in the reduction of platelet counts observed in EE-TP treated dogs appeared to be less at the end of the treatment period, also suggesting an adaptive response and for this reason these changes are considered not to be adverse.

Microscopic changes related to test article were seen as slight to markedly atrophic in the thymus of dogs receiving EE-TP every two weeks (Group 3). This finding correlated with the small size of the thymus observed during macroscopic examination as well as weight changes in the females. The thymus is known to be sensitive to acute stress-induced atrophy and these changes are likely to be related to the stress produced by the clinical reaction which in turn may underlie the lower bodyweight gain by this group (Pearse, 2006).

In conclusion the results of these preclinical studies do not reveal any potential serious toxicities that would preclude the use of EE-TP in a phase II clinical trial in patients with MNGIE, but caution should be taken for infusion-related reactions which may be related to the production of non-specific antibodies or a cell based immune response.

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FIGURE LEGENDS

FIG. 1. Thymidine phosphorylase activity encapsulated in (a) mouse and (b) dog erythrocytes as a function of storage time at 4 and 22^{o}C. Results are expressed as mean ± SEM of 4 experiments.

FIG 2. Bodyweights for male and female dogs throughout the acclimatization period and during four weeks of treatment. Results are expressed as mean for each group of 3 dogs.

FIG. 3. Lung sections from (a) control mouse receiving sham-loaded erythrocytes showing normal histology, (b) control mouse receiving sham-loaded erythrocytes showing embolus occluding pulmonary arteriole, (c) mouse treated twice per week with EE-TP showing emboli and perivascular inflammatory cells and d) mouse treated once every two weeks showing emboli and perivascular inflammatory cells. 10X original magnification for all micrographs, stained with haematoxylin and eosin.

FIG. 4. Thymus sections from a) control dog receiving sham-loaded erythrocytes showing normal histology, b) dog treated twice per week with EE-TP showing moderate atrophy, and c) dog treated twice per week with EE-TP showing marked atrophy. 4X original magnification for all micrographs, stained with haematoxylin and eosin.

TABLES

TABLE 1

Specification and batch analysis release results for recombinant thymidine phosphorylase

Attribute	Acceptance criteria	Batch analysis results
Appearance	Clear to slightly hazy colourless to yellow liquid	Slightly hazy faint yellow liquid
MW size	Main band consistent with reference standard 45 kD	45 kD
	Purity ≥ 95%	≥ 99%
Endotoxin	NMT 4,000 EU/ml	90- 150 EU/ml
Identity	Matches N terminal amino acid sequence (NCBI BLAST) of MFLAQEIIRK	Identical to NCBI BLAST (100% alignment)
Residual host cell DNA	NMT 10ppb	0.0002 to 0.001 ppb
Bioburden	NMT 10 CFU/ml	0 CFU/ml
Kanamycin	NMT 10ppm	0.23 ppm

TABLE 2

Haematological characteristics (Mean ± SEM) of test and control materials

Parameter		Mouse		Dog	
		EE-TP (n= 9)	Sham-loaded (n=9)	EE-TP (n=72)	Sham-loaded (n=54)
MCV (fl)		37.9 ± 0.4	37.1 ± 1.0	59.2 ± 0.4	61.2 ± 0.4
MCH (pg)		10.2 ± 0.4	10.1 ± 0.4	18.1 ± 0.2	18.8 ± 0.2
MCHC (g/dl)		27.2 ± 1.0	26.8 ± 1.2	30.6 ± 0.2	30.9 ± 0.2
Extracellular Hb (g/l) post formulation (hour):	0	0.2 ± 0.0*	0.2 ± 0.1*	0.1 ± 0.1*	0.1 ± 0.1*
	6	0.2 ± 0.1*	0.2 ± 0.1*	0.1 ± 0.1*	0.1 ± 0.1*
	24	0.6 ± 0.2*	0.6 ± 0.2*	0.6 ± 0.3*	0.6 ± 0.2*
Encapsulated thymidine phosphorylase (IU/ml)		123.7 ± 8.5	NA	147.9 ± 5.9	NA
Extracellular thymidine phosphorylase (IU/ml) post formulation (hour):					
	0	0.2 ± 0.1*	NA	0.3 ± 0.0*	NA
	6	0.3 ± 0.1*	NA	0.6 ± 0.0*	NA
	24	1.7 ± 0.1*	NA	2.4 ± 0.1*	NA

NA, Not applicable

* n = 4

TABLE 3

Treatment groups in 4 Week mouse and dog studies

Species	Group	Treatment	Mean Dose (IU/kg/occasion)	Days of dosing	No. of animals	
					Male	Female
Mouse	1	Sham loaded erythrocytes	0	1,4,8,11,15,18, 22, 25, and 29	10	10
	2	EE-TP	364 ± 48	1, 15, 29	10	10
	3	EE-TP	330 ± 23	1,4,8,11,15,18, 22, 25, and 29	10	10
Dog	1	Sham loaded erythrocytes	0	1,4,8,11,15,18, 22, 25, and 29	3	3
	2	EE-TP	194 ± 15	1, 15, 29	3	3
	3	EE-TP	200 ± 7	1,4,8,11,15,18, 22, 25, and 29	3	3

TABLE 4

Selected clinical chemistry parameters (Mean ± SD) for mice treated for 4 Weeks

Parameter/Sex	Treatment		
	Control Twice weekly	Once every two weeks	Twice weekly
	Group 1 (n=5)	Group 2 (n=5)	Group 3 (n=5)
Cholesterol (mM)			
Males	2.73 ± 0.22	3.15 ± 0.30*	2.97 ± 0.29
Females	2.31 ± 0.04	2.24 ± 0.23	2.09 ± 0.08*
Triglyceride (mM)			
Males	0.92 ± 0.12	2.62 ± 0.30**	1.49 ± 0.39**
Females	1.62 ± 0.77	2.13 ± 0.26	1.43 ± 0.25
Urea (mM)			
Males	9.69 ± 1.20	8.34 ± 1.51	7.95 ± 0.45*
Females	8.36 ± 0.82	6.84 ± 0.58**	6.20 ± 0.84**
Creatinine (µM)			
Males	6.0 ± 1.5	5.0 ± 1.6	4.0 ± 1.9*
Females	7.0 ± 1.7	8.0 ± 2.2	7.0 ± 1.8
Calcium (mM)			
Males	2.21 ± 0.06	2.14± 0.11	2.36 ± 0.15**
Females	2.21 ± 0.20	2.20 ± 0.29	2.32 ± 0.10
Alkaline phosphatase (U/l)			
Males	127 ± 11.3	131 ± 11.2	118 ± 25.9

Females	159 ± 10.1	153 ± 7.8	141 ± 17.7*

Groups compared using student's t-test

* $p<0.05$ (for comparisons with Group 1)

** $p<0.01$ (for comparisons with Group 1)

TABLE 5

Selected haematology parameters (Mean ± SD) for mice treated for 4 Weeks

	Treatment		
Parameter/Sex	Control Twice weekly	Once every two weeks	Twice weekly
	Group 1 (n=5)	Group 2 (n=5)	Group 3 (n=5)
RBC ($x10^{12}$/l)			
Males	10.03 ± 0.41	9.80 ± 0.55	9.83 ± 0.53
Females	9.79 ± 0.29	9.72 ± 0.73	9.22 ± 0.32
Hb (g/dl)			
Males	15.2 ± 0.66	14.7 ± 0.99	14.9 ± 0.79
Females	15.1 ± 0.28	15.5 ± 0.99	14.6 ± 0.52
Hct (l/l)			
Males	0.48 ± 0.03	0.48 ± 0.02	0.47 ± 0.02
Females	0.48 ± 0.01	0.48 ± 0.03	0.45 ± 0.01*
MCH (pg)			
Males	15.2 ± 0.06	15.1 ± 0.96	15.1 ± 0.23
Females	15.4 ± 0.36	16.0 ± 0.17**	15.8 ± 0.16*
MCHC (g/dl)			
Males	32.0 ± 0.82	30.9 ± 2.19	31.8 ± 0.77
Females	31.5 ± 0.77	32.3 ± 0.51*	32.6 ± 0.38*

Groups compared using student's t-test

* $p<0.05$ (for comparisons with Group 1)

**$p<0.01$ (for comparisons with Group 1)

TABLE 6

Urinalysis Parameters (Mean ± SD) for Dog predose and after treatment for 4 Weeks

		Treatment		
Parameter/Sex	Day	Control Twice weekly Group 1 (n=3)	Once every two weeks Group 2 (n=3)	Twice weekly Group 3 (n=3)
pH				
Males	Predose	5.1 ± 0.17	5.2 ± 0.06	5.2 ± 0.21
	28	5.0 ± 0.25	5.1 ± 0.10	6.2 ± 0.46
Females	Predose	5.3 ± 0.12	5.3 ± 0.12	5.3 ± 0.06
	28	5.0 ± 0.12	5.1 ± 0.12	5.5 ± 0.38
Specific gravity (g/l)				
Males	Predose	1034 ± 7.0	1031 ± 4.6	1035 ± 3.5
	28	1028 ± 12.3	1030 ± 2.6	1019 ± 4.5
Females	Predose	1027 ± 5.1	1027 ± 9.0	1034 ± 3.6
	28	1025 ± 2.3	1029 ± 8.1	1025 ± 4.6
Protein (g/l)				
Males	Predose	0.10 ± 0.02	0.10 ± 0.02	0.14 ± 0.09
	28	0.09 ± 0.06	0.09 ± 0.02	0.04 ± 0.02
Females	Predose	0.10 ± 0.01	0.08 ± 0.04	0.11 ± 0.02
	28	0.10 ± 0.05	0.09 ± 0.05	0.05 ± 0.02
Chloride (mmol)				
Males	Predose	41.67 ± 6.19	27.33 ± 9.21	28.46 ± 11.10
	28	34.47 ± 6.51	36.86 ± 15.65	58.99 ± 14.83
Females	Predose	41.07 ± 5.48	27.37 ± 7.59	37.95 ± 3.34
	28	32.58 ± 5.41	19.52 ± 6.61	35.99 ± 17.04
Sodium (mmol)				
Males	Predose	13.44 ± 1.93	5.03 ± 2.35	6.41 ± 6.05
	28	10.68 ± 4.57	11.47 ± 8.82	52.90 ± 2.92
Females	Predose	6.28 ± 1.52	4.93 ± 1.97	8.38 ± 4.78
	28	4.31 ± 0.86	3.75 ± 3.09	21.34 ± 23.84
Potassium (mmol)				
Males	Predose	33.67 ± 3.84	21.53 ± 4.95	22.89 ± 8.75
	28	26.28 ± 4.75	27.78 ± 8.31	24.67 ± 4.03
Females	Predose	28.35 ± 3.71	22.04 ± 7.01	28.96 ± 1.66
	28	23.67 ± 5.25	14.72 ± 2.34	17.49 ± 2.71

TABLE 7

Selected haematology parameters (Mean ± SD) for dogs predose, during and after treatment for 4 Weeks

		Treatment		
Parameter/Sex	Day	Control Twice weekly Group 1	Once every two weeks Group 2	Twice weekly Group 3
RBC ($x10^{12}$/l)				
Males	Predose	6.20 ± 0.39	5.92 ± 0.32	5.68 ± 0.09
	14	5.50 ± 0.18	5.51± 0.19	4.45 ± 0.38
	21	5.40 ± 0.25	5.39 ± 0.39	4.67 ± 0.22
Females	28	5.70 ± 0.27	6.10 ± 0.47	5.10 ± 0.46
	Predose	5.85 ± 0.36	6.04 ± 0.17	6.19 ± 0.66
	14	5.17 ± 0.51	5.83 ± 0.37	5.76 ± 0.29
	28	5.15 ± 0.32	6.06 ± 0.23	5.76 ± 0.61
Hb (g/dl)				
Males	Predose	14.1 ± 0.75	13.0 ± 0.31	13.0 ± 0.61
	14	12.8 ± 0.26	12.4 ± 0.25	10.8 ± 1.10
	21	12.3 ± 0.42	12.2 ± 1.25	11.2 ± 1.00
	28	12.5 ± 0.31	13.3 ± 1.05	11.7 ± 1.53
Females	Predose	14.1 ± 1.10	14.0 ± 0.30	14.2 ± 1.45
	14	12.2 ± 1.39	13.5 ± 0.95	13.0 ± 0.35
	28	12.2 ± 0.62	13.3 ± 0.50	12.6 ± 1.48
Hct (l/l)				
Males	Predose	0.43 ± 0.03	0.40 ± 0.01	0.40 ± 0.02
	14	0.37 ± 0.01	0.36 ± 0.01	0.31 ± 0.03
	21	0.38 ± 0.02	0.37 ± 0.04	0.34 ± 0.03
	28	0.40 ± 0.02	0.42 ± 0.03	0.37 ± 0.43
Females	Predose	0.42 ± 0.03	0.42 ± 0.01	0.42 ± 0.04
	14	0.37 ± 0.04	0.40 ± 0.02	0.40 ± 0.01
	28	0.39 ± 0.02	0.42 ± 0.02	0.40 ± 0.05
Reticulocyte (%)				
Males	Predose	1.30 ± 0.21	0.80 ± 0.27	0.90 ± 0.40
	14	2.00 ± 0.20	1.00 ± 0.22	2.80 ± 0.51
	21	1.80 ± 0.22	1.30 ± 0.28	2.80 ± 1.14
Females	28	1.40 ± 0.32	1.40 ± 0.34	2.40 ± 0.95
	Predose	1.50 ± 0.60	1.40 ± 0.16	1.50 ± 0.19
	14	2.20 ± 0.39	1.40 ± 0.24	2.30 ± 0.23
	28	2.00 ± 0.26	1.60 ± 0.56	2.80 ± 1.39
Platelet count (x 10^9/l)				
Males	Predose	352 ± 30.2	376 ± 50.3	390 ± 11.5
	14	353 ± 34.4	280 ± 63.1	122 ± 40.1
	21	361 ± 27.0	207 ± 19.3	233 ± 14.8
	28	401 ± 19.6	310 ± 21.1	286 ± 41.0
Females	Predose	349 ± 18.5	354 ± 84.9	370 ± 30.0
	14	368 ± 21.1	239 ± 66.4	290 ± 26.9
	28	359 ± 31.5	300 ± 57.5	227 ± 115.0

TABLE 8

Summary of treatment related findings in the lungs of mice after treatment for 4 weeks

Sex /Group		Male 1	Male 2	Male 3	Female 1	Female 2	Female 3
Dose frequency		Twice/week	Once/two weeks	Twice/week	Twice/week	Once/two weeks	Twice/week
Thrombus/Embolus							
	Minimal	1	3	2	1	3	5
	Slight	0	2	0	0	2	2
	Moderate	0	0	0	0	1	2
	Marked	0	0	0	0	1	0
	Total	**1**	**5**	**2**	**1**	**7**	**9**
Perivascular Inflammatory cells							
	Minimal	0	2	1	0	3	6
	Slight	0	1	0	0	2	1
	Moderate	0	0	0	0	1	1
	Total	**0**	**3**	**1**	**0**	**6**	**8**
Number of animals examined		10	9	10	10	10	10

TABLE 9

Weights of selected organs (Mean ± SD) for dogs receiving treatment for 4 Weeks

	Treatment		
Parameter/Sex	Control Twice weekly	Once every two weeks	Twice weekly
	Group 1	Group 2	Group 3
Thymus (g)			
Males	11.91 ± 2.67	18.43 ± 13.40	4.12 ± 2.56
Females	19.56 ± 12.15	10.97 ± 5.4	2.35 ± 0.91
Brain (g)			
Males	83.0 ± 5.2	82.5 ± 2.4	94.5 ± 2.8
Females	78.3 ± 7.2	76.6 ± 9.9	73.9 ± 5.3
Liver (g)			
Males	365 ± 7	415 ± 36	471 ± 21
Females	324 ± 33	311 ± 32	379 ± 29
Spleen (g)			
Males	94.1 ± 34.1	113.6 ± 17.0	111.8 ± 29.9
Females	74.4 ± 19.0	62.1 ± 5.1	98.3 ± 33.3
Heart (g)			
Males	83.1 ± 11.0	84.4 ± 9.2	84.1 ± 8.5
Females	79.1 ± 9.1	71.5 ± 10.5	79.5 ± 8.1
Kidneys (g)			
Males	54.3 ± 3.6	60.6 ± 4.4	59.1 ± 2.5

Females	47.9 ± 3.4	49.9 ± 5.8	51.3 ± 4.1

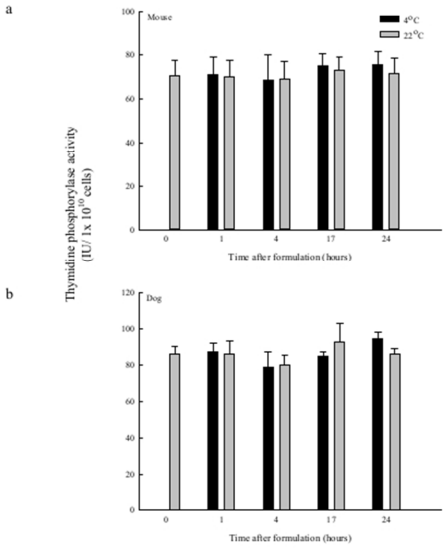

FIG. 1. Thymidine phosphorylase activity encapsulated in (a) mouse and (b) dog erythrocytes as a function of storage time at 4 and 22ºC. Results are expressed as mean ± SEM of 4 experiments.
179x232mm (120 x 120 DPI)

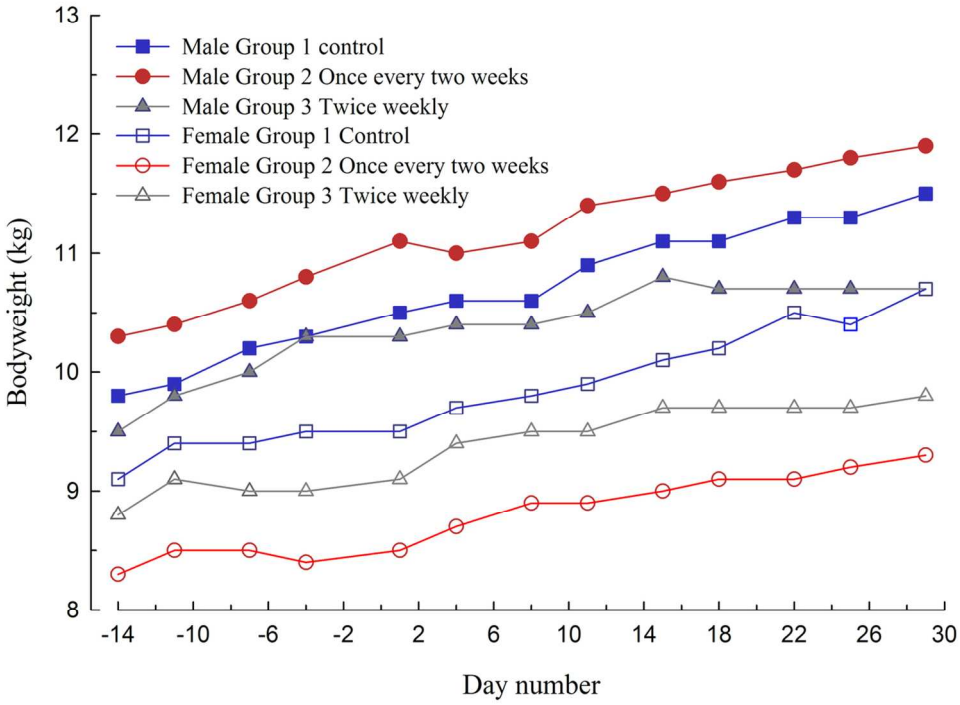

FIG 2. Bodyweights for male and female dogs throughout the acclimatization period and during four weeks of treatment. Results are expressed as mean for each group of 3 dogs.
118x92mm (300 x 300 DPI)

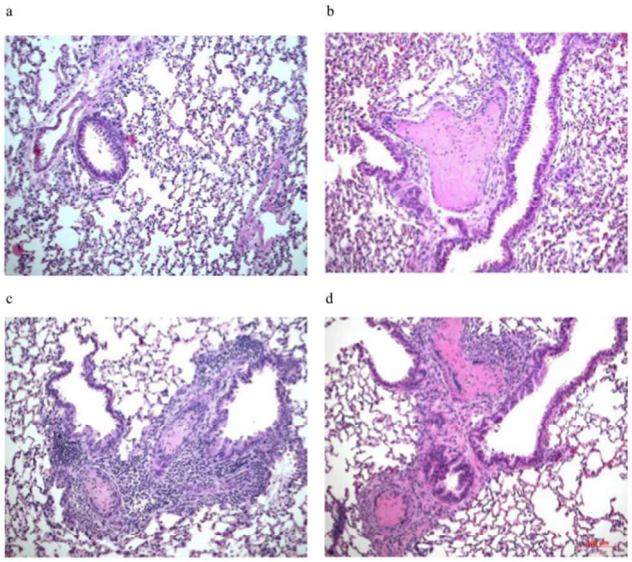

FIG. 3. Lung sections from (a) control mouse receiving sham-loaded erythrocytes showing normal histology, (b) control mouse receiving sham-loaded erythrocytes showing embolus occluding pulmonary arteriole, (c) mouse treated twice per week with EE-TP showing emboli and perivascular inflammatory cells and d) mouse treated once every two weeks showing emboli and perivascular inflammatory cells. 10X original magnification for all micrographs, stained with haematoxylin and eosin.
179x232mm (120 x 120 DPI)

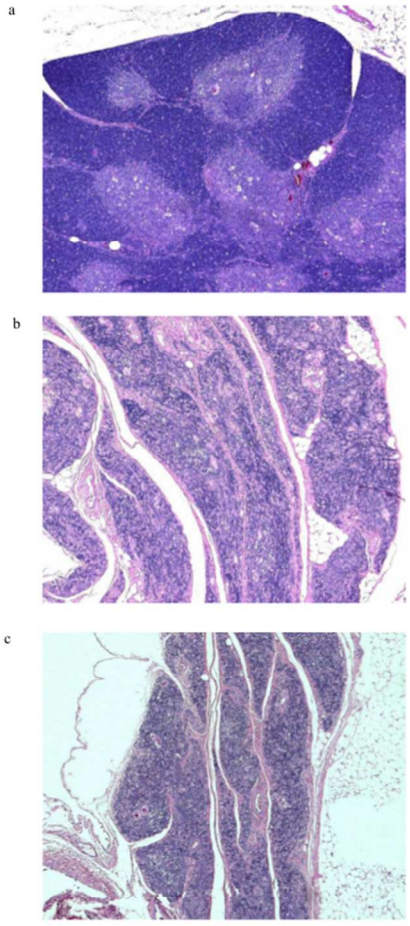

FIG. 4. Thymus sections from a) control dog receiving sham-loaded erythrocytes showing normal histology, b) dog treated twice per week with EE-TP showing moderate atrophy, and c) dog treated twice per week with EE-TP showing marked atrophy. 4X original magnification for all micrographs, stained with haematoxylin and eosin.
179x232mm (120 x 120 DPI)