Lymphatic Transport and Lymphocyte Targeting of a Triglyceride Mimetic Prodrug is Enhanced in a Large Animal Model: Studies in Greyhound Dogs

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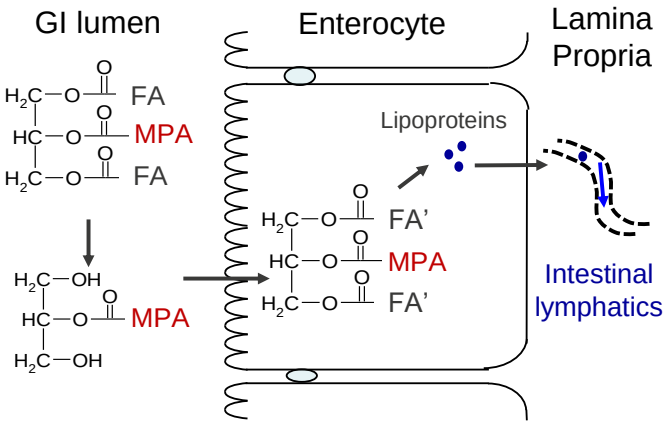
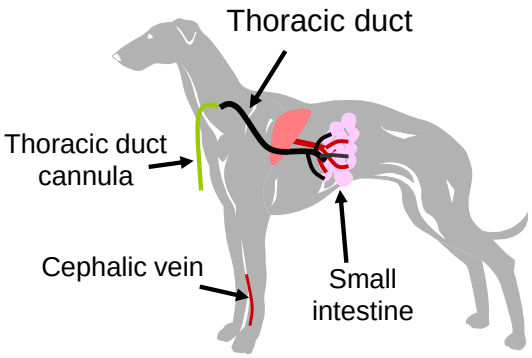




ABSTRACT

In previous studies, a triglyceride (TG) mimetic prodrug of the model immunomodulator mycophenolic acid (MPA), was shown to significantly enhance lymphatic transport of MPA-related species in the rat. The rat gastrointestinal tract, however, is somewhat different to that in higher order species such as dogs and humans and may underestimate lymphatic transport. Here the effectiveness of the prodrug strategy has been examined in conscious greyhound dogs, the GI physiology of which is more representative of that in humans. The bioavailability and lymphatic transport of free MPA and total MPA related materials were examined following oral administration of the parent drug (MPA) and the prodrug (2-MPA-TG) to both thoracic lymph-duct cannulated and intact (non-cannulated) greyhound dogs. The enrichment of free MPA in lymph nodes and lymph-derived lymphocytes was also determined to examine the efficiency of drug targeting to potential sites of action within the lymph. Via biochemical integration into a series of site-specific metabolic processes, the prodrug markedly increased (288-fold) lymphatic transport of total MPA related material (present as re-esterified 2-MPA-TG) when compared to the parent MPA and the extent of lymphatic transport was significantly greater in the dog (36.4 % of the dose recovered in lymph) when compared to the previous data in the rat (13.4 % of the dose). Conversion from 2-MPA-TG derivatives to parent MPA occurred in vivo, resulting in a marked increase in MPA concentrations in lymph nodes (5-6 fold) and lymph lymphocytes (21 fold), when compared to animals administered the parent drug. In conclusion, the data demonstrate that the TG prodrug of MPA facilitates efficient delivery of MPA to the lymphatic system in dogs and suggest that the TG prodrug strategy may more effectively facilitate targeted delivery and treatment in large animal models than in rats.

KEYWORDS

Lymphatic transport; prodrug; triglyceride mimetic; greyhound dogs; lymphocyte targeting

ABBREVIATIONS

AUC, area under the curve; DG, diglyceride; GI, gastrointestinal; MG, monoglyceride; FA, fatty acid; MW, molecular weight; MPA, mycophenolic acid; PC, phosphatidylcholine; TG, triglyceride.

INTRODUCTION

In light of an increasing awareness of the importance of the lymphatic system in disease progression and as a conduit for immune response, deliberate and directed delivery of therapeutic agents to the lymphatic system has received increasing attention in recent years[1]. This is particularly the case in therapeutic areas such as autoimmune disorders[2], vaccination[3], HIV infection[4], cancer[5], and metabolic syndrome[6] where disease development is closely linked to lymphatic anatomy and physiology. Directed delivery to the lymphatics from the interstitial space relies on specificity of drug transport across lymphatic endothelial cells in preference to vascular endothelia cells. For most small molecule drugs, however, this does not occur, regardless of the mechanisms of access to the interstitum (eg via parenteral administration or oral administration and absorption across the enterocyte). For these molecules, facile diffusion across vascular endothelial cells into blood capillaries results in very efficient transport into the blood since the rate of blood flow is typically orders of magnitude higher than that of lymph flow, and the transport sink is therefore much higher[1].

Targeted drug exposure to the lymphatic system, requires an approach that limits access to the vasculature and promotes transport into the lymphatic capillaries. In general this may be achieved by drug association with colloidal species, the size of which precludes facile diffusion across the continuous vascular endothelium, but allows access across the markedly discontinuous and more permeable lymphatic endothelium. These colloidal drug delivery systems may be either exogenously derived (e.g. parenterally administered polymeric or liposomal nanomaterials[7]) or endogenously sourced (e.g. via drug association in situ with albumin[3] or lipoproteins[8]). Our laboratory has focused recently on the use of intestinal lipoproteins as carriers for lymphatic drug delivery, and in particular on the design of triglyceride (TG) mimetic prodrugs, where the drug

molecule is conjugated to a glyceride backbone allowing biochemical incorporation into lipid processing and lipoprotein assembly pathways[9]. To this point our focus has been on glyceride mimetic prodrugs of the immunosuppressant mycophenolic acid (MPA, Figure 1). MPA acts via inhibition of lymphocyte proliferation, and enhanced delivery of MPA to the lymphatic system might therefore be expected to improve therapeutic efficacy. The TG mimetic prodrug of MPA (2-MPA-TG, Figure 1) was designed to integrate into the dietary TG metabolism pathway. Thus, 2-MPA-TG is first hydrolysed in the gastrointestinal (GI) lumen to form the equivalent monoglyceride (2-MPA-MG), resynthesised back to TG in the enterocyte and assembled into colloidal lipoprotein particles in the endoplasmic reticulum, prior to access into the intestinal lymphatics (Scheme 1A)[10].

The complexity of the biochemical processes involved in lipoprotein assembly and lymphatic access has dictated that most studies in the field rely on animal models to provide proof of concept. To study lymphatic drug transport in vivo, the most commonly employed model is the rat[2, 11, 12,13]. There are several advantages of using a small animal model such as the rat, including low cost, accessibility, ease of surgery and convenience of handling. In our recent studies, the use of the rat model has enabled relatively rapid screening of a number of prodrug candidates of MPA at moderate cost and complexity and has allowed an initial indication of the rank order of differences in lymphatic transport for different constructs. The rat model has also allowed the conduct of a range of mechanistic studies that have evaluated the mechanism of absorption and lymphatic transport of 2-MPA-TG[10, 14].

Whilst the rat model provides robust data in a time- and cost-effective manner, it has limitations when seeking to accurately extrapolate lymphatic transport results to larger animal models and ultimately to the clinic. For example, a recent study[15] compared lymphatic lipid and drug transport

in mice, rats and dogs. The study reported similar extents of lymphatic lipid transport across species but uncovered significantly different profiles for lymphatic transport of halofantrine (a highly lipophilic drug). In particular, lymphatic drug transport was seemingly lower in the smaller species. The lower extent of lymphatic drug transport in rodents when compared to higher order species may result from differences in GI physiology, particularly bile flow and GI volume. Bile flow is continuous in rats and is therefore independent of food intake, making it difficult to obtain a fed-state GI environment that reflects the human situation. The inherently small GI volume of rats also precludes the use of full-size, clinically relevant dosage forms and in anaesthetised animals prevents oral administration; as such formulations must be infused intraduodenally. Furthermore, in cases where TG mimetic prodrugs are used to promote lymphatic transport, the biotransformation pathways (as described in Scheme 1A) that range from intestinal digestion to absorption, re-esterification in the enterocyte and secretion into lymph, are highly enzyme dependent and to this point have not been examined in species other than the rat. It is therefore important to verify that in large animals (where GI physiology is closer to that in humans) the chain of biotransformation events designed for the prodrugs are similar (and as effective) as they are in small animals.

The current study therefore aimed to confirm the utility of the TG prodrug strategy in a conscious greyhound dog model (Scheme 1 B)[13, 16, 17]. This is the first study to describe the processing of glyceride mimetic prodrugs in a higher order species. Lymphatic transport was examined following oral administration of MPA and 2-MPA-TG to fed greyhound dogs and free MPA enrichment in lymph-derived lymphocytes and lymph nodes determined. A marked increase in free MPA exposure to lymph lymphocytes and mesenteric lymph nodes was observed suggesting that the triglyceride mimetic prodrug was effective in larger animal models.

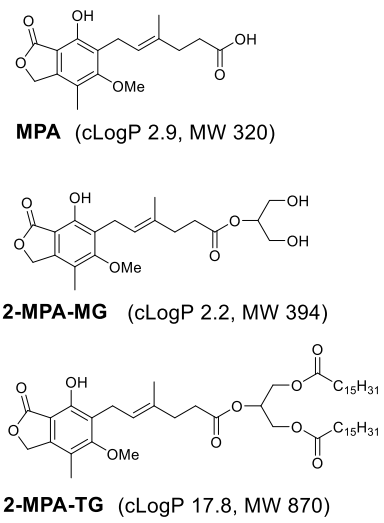
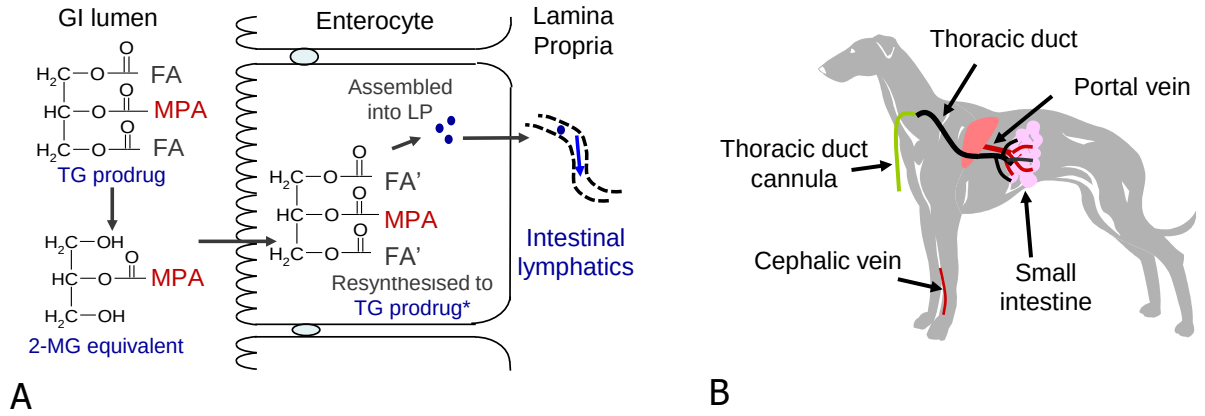


Figure 1. Chemical structures of MPA, 2-MPA-TG and the hydrolysis product (2-MPA-MG) of 2-MPA-TG with molecular weight (MW) and cLog P values calculated using ACD/Labs Release Software (version 9.12).



Scheme 1. Panel A shows the metabolic pathways that result in the lymphatic transport of the triglyceride mimetic prodrug (2-MPA-TG). After ingestion, the prodrug is sequentially hydrolysed by digestive lipases in the gastrointestinal (GI) lumen to release the 2-monoglyceride (2-MG) equivalent and FA. The 2-MG derivative and FA are absorbed into enterocytes and re-synthesised to TG derivatives. Reformed TG prodrug molecules are then assembled into lipoproteins (LP) and LP are subsequently exocytosed from the basolateral membrane of enterocytes. Following entry into the underlying lamina propria, LP (loaded with prodrug derivatives) preferentially gain access to the intestinal micro-lymphatics. Panel B shows the greyhound dog model used in the current study, where the thoracic lymph duct and the cephalic veins are cannulated for the collection of lymph and blood samples, respectively[17].

EXPERIMENTAL SECTION

Chemicals and prodrug

Mycophenolic acid (MPA, > 98%) was purchased from AK Scientific, Palo Alto, CA, USA. The TG mimetic prodrug 2-MPA-TG was synthesised as previously described[9]. Lipoid E PC S (lecithin from egg, approximately 99% phosphatidylcholine (PC)) was from Lipoid GmbH, Ludwigshafen, Germany. Maisine 35-1 was obtained from Gattefosse, France. Ketoprofen (internal standard), disodium EDTA, ammonium formate, ammonium acetate, formic acid, soybean oil, Cremophor EL, phosphate buffered saline (PBS, pH 7.4), microcrystalline cellulose (Fluka Avicel® PH-101), sodium taurodeoxycholate 97% (NaTDC) and porcine pancreatin (8xUSP specification activity) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Chloroform, methanol, ethanol, acetonitrile (ACN), sodium hydroxide and hydrochloric acid were purchased from Merck Pty Limited, Australia. Sodium starch glycolate (Explotab®) was obtained from Mendell, NY, USA. Ultrapure water was prepared by a Milli-Q™ system (Millipore, MA, USA). All other chemicals were analytical grade or above.

Formulation preparation

2-MPA-TG was prepared in a long-chain lipid based self emulsifying drug delivery system (SEDDS) according to previously established methods[17]. The SEDDS formulation consisted of 30.5% w/w soybean oil, 30.5% w/w Maisine 35-1, 31.6% w/w Cremophor EL and 7.4% w/w ethanol. Formulations were filled into soft gelatin capsules using a 3 mL syringe and 21 G needle and the capsules sealed using a warm spatula. For administration of 2-MPA-TG, dogs were dosed with two × 0.5 g (size 0) capsules containing a total dose of 50 mg 2-MPA-TG dissolved in 950 mg of the SEDDS formulation. MPA was also prepared as a lipid-free powder formulation for oral

administration. MPA (50 mg) was mixed with 200 mg of Avicel PH101 and 200 mg of Explotab and filled into a 0.5 g (size 0) hard gelatin capsule.

In vitro digestion TG prodrug in simulated intestinal fluid

In previous studies in our laboratory[10], rat bile and pancreatic secretions collected via a cannula in the common bile duct was used ex vivo to profile the luminal (intestine) hydrolysis of the TG mimetic prodrug (i.e. from the TG to the MG derivative). Collection of dog bile and pancreatic fluid was impractical in the current studies and therefore simulated digestive fluid containing porcine pancreatic lipases was used to predict the likely catabolism of 2-MPA-TG in the intestinal lumen in dogs. This medium has similar lipase activity when compared to the dog and human GI environment, and has been commonly used as a substitute to study the digestion/performance of lipid formulations[18]. Digestion experiment was performed as described previously[19] with slight modifications. Briefly, 2-MPA-TG (20 µg/ml) was firstly dispersed in 900 µl of digestion buffer (50 mM TRIS maleate, 150 mM NaCl, 5 mM $CaCl_2.2H_2O$, pH 6.5) containing 5 mM NaTDC and 1.25 mM PC. Experiments were performed at 37 ^oC. Digestion was initiated by addition of 100 µl of pancreatin extract dispersed in digestion buffer providing 1,000 TBU of lipase activity. At 0 (prior to addition of lipase), 5, 10, 15, 30, 60, 90, 120, 180 min, aliquots of 20 µl sample were added to 980 µl of ACN to stop lipolysis, vortexed for 0.5 min and centrifuged at 4500 g for 5 min to precipitate proteins prior to analysis. The supernatant was analysed by HPLC-MS for the prodrug and potential products of prodrug hydrolysis including free MPA and the 2-MG derivative, 2-MPA-MG (Fig. 1).

Animal studies

All animal experiments were approved by the local animal ethics committee and were conducted in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines. Adult male greyhound dogs (32-38 kg) were used in all studies.

Six dogs were employed for lymphatic transport studies. The thoracic lymph duct was cannulated under surgical anaesthesia as previously described[13]. Following surgery, dogs were allowed to recover unrestrained in a closed run overnight (12–16 h) and returned to normal ambulatory movement before commencement of the study. In the initial recovery period fluids were administered IV to ensure adequate hydration and to prevent hypoproteinemia. Water was also available *ad libitum* throughout the experiment period. Prior to drug administration, a 20 G intravenous catheter was inserted into the cephalic vein to enable serial blood sampling and the catheter kept patent by periodic flushing with heparinised saline (1 IU/ml). To limit possible dehydration due to the continuous collection of thoracic lymph, 25 ml normal saline was also administered hourly by IV bolus during the sampling period. The dogs were fed a standard can of commercial dog food (680 g) containing 5% fat and 7.5% protein 30–40 min prior to drug administration. 2-MPA-TG (50 mg) or MPA (50 mg) formulations were administered to the fed greyhound dogs by placing the capsule(s) as far posterior to the pharynx as possible, closing the mouth and rubbing the throat to stimulate swallowing. Subsequently 50 mL of water was administered orally. Lymph was collected continuously into pre-weighed 50 ml collection tubes containing 75 mg di-sodium EDTA for the duration of the 10 h post-dosing period. Individual lymph samples for each half hourly or hourly collection period were combined and the mass of lymph collected determined gravimetrically. Several 20 and 200 µl aliquots of each lymph sample were transferred into individual 1.5 ml eppendorf tubes and stored at -80 °C until analysis of drug concentrations. The remaining lymph from each collection period (half hourly or hourly) was

transferred into 10 ml tubes that were centrifuged at 2000 g for 10 min to obtain lymphocyte pellets that were stored at -80 °C until analysis of drug concentrations. Systemic blood samples (3 ml) were taken via the indwelling cephalic vein catheter and placed in individual heparinised tubes (13×75mm BD Vacutainer®, 68 IU). Blood samples were collected at pre-dose (−5min) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, and 10 h following drug administration. Plasma was separated by centrifugation and stored at −80°C prior to analysis by LC-MS. At the end of the sampling period, dogs were humanely killed via an intravenous dose of 200 mg/kg phenobarbitone (Jurox, Silverwater, Australia).

In a separate cohort of 3 dogs no lymph duct cannulation surgery was conducted to enable a typical pharmacokinetic study (collection of blood). The dogs received either an IV infusion (over 5 min) of MPA (10 mg MPA dissolved in 10 ml of 95:5 (v/v) PBS: ethanol or an oral administration of the MPA or 2-MPA-TG formulation described above. A wash out period of 1 week was allowed between treatments and the formulations were randomised across the animals. At the end of the pharmacokinetic study, a further dog was added to provide a cohort of 4 animals to examine drug accumulation in lymph nodes. Two dogs were administered orally with the MPA formulation and two dogs were administered 2-MPA-TG formulation as described above. At 2h post dose, dogs were killed by IV injection of pentobarbitone, and the mesenteric lymph nodes (draining the gastrointestinal lymphatic tissue) and axillary lymph nodes (draining the peripheral lymphatic tissue) were collected via blunt dissection and stored at -80 °C prior to assay.

Sample preparation

The concentrations of free MPA and total MPA related material in dog lymph and plasma were measured using similar methods to those described previously for rat samples[9], with slight modification. Briefly, lymph samples were prepared for HPLC-MS analysis of free MPA

concentrations via addition of 1000 µl acetonitrile to a 200 µl lymph sample followed by vortexing for 0.5 min and then centrifugation at 4500 g for 5 mins. The supernatant (150 µl) was used for HPLC-MS analysis. To quantify total MPA related material in the lymph (i.e. free MPA plus MPA glycerides) following prodrug administration, hydrolysis of potential glyceride derivatives of MPA in lymph was achieved via addition of 140 µl 0.5 M NaOH in 1:1 (v/v) ethanol:water to 20 µl of lymph and heating at 60 ^{o}C for 8 min. Subsequently, 70 µl of 1 M HCl in water was added to each sample to stop the hydrolysis and to neutralise samples. After vortexing for 0.5 min, a 100 µl aliquot of the sample was diluted with 4900 µl mobile phase (described below in HPLC-MS analysis), vortexed for a further 1 min, centrifuged at 4500 g for 5 min and 150 µl of the supernatant was analysed for total MPA derivatives by HPLC-MS. The

In order to prepare samples for HPLC-MS/MS analysis of free MPA concentrations in dog plasma, a 200 µl aliquot of plasma was spiked with 10 µl of internal standard solution (10 µg/ml ketoprofen in ACN) and vortexed for 20s. After addition of 250 µl of saturated $(NH_4)_2SO_4$, samples were vortexed for 10s, followed by addition of 500 µl ACN (containing 0.1% (v/v) formic acid and 2 mM ammonium acetate). The mixture was then vortexed for 1 min and centrifuged at 14,000 g for 10 min, and 150 µl of the supernatant was subsequently transferred to vials for HPLC-MS/MS analysis. For the assay of the concentrations of total MPA related materials in plasma, an aliquot of 200 µl plasma was spiked with 10 µl of internal standard solution (10 µg/ml ketoprofen in ACN) and hydrolysed via incubation with 200 µl of 0.5 M NaOH at 80 ^{o}C for 10 min. Subsequently, 100 µl of 1 M HCl in water was added to each sample to stop the hydrolysis, prior to sample dilution with 900 µl mobile phase (described below in HPLC-MS analysis). Samples were then vortexed for a further 1 min, centrifuged at 4500 g for 5 min and 150 µl of the supernatant was analysed for total MPA derivatives by HPLC-MS.

For measurement of free MPA concentrations in lymphocytes, lymphocytes that had been pelleted from 10 ml of lymph were resuspended in 30 µl of saline and spiked with 10 µl of ACN (to match the preparation of the standards, into which 10 µl of MPA solution in ACN was spiked) and 10 µl of ketoprofen solution (2.5 µg/ml in ACN, as internal standard) and subsequently vortexed for 1 min. ACN (500 µl, containing 0.1% (v:v) formic acid and 2 mM ammonium acetate) was then added to lyse cells and precipitate proteins and samples were vortexed for 1 min prior to centrifugation at 4500 g for 10 min. The supernatant (150 µl) was subsequently transferred to vials for LC-MS/MS analysis.

For analysis of free MPA concentrations in lymph nodes following administration of MPA or 2-MPA-TG, lymph nodes were first cut into small pieces and approximately 100 mg placed into an Eppendorf tube followed by addition of 10 µl of 50 µg/ml ketoprofen (in 1:1 (v/v) ACN:water) as an internal standard and 100 µl of 1% formic acid aqueous solution. Using an Agile portable tissue grinder (ACT-AG 3080, ACTGene, Inc., Piscataway, NJ, USA), samples were homogenised at 3000 rpm for 1 min, followed by the addition of 1 ml ACN into the eppendorf tube and a further 30s of homogenization. After 1 min of vortex and centrifugation at 5000 rpm for 5 min, 150 µl of supernatant was transferred to vials for HPLC-MS/ MS analysis. Validation experiments were conducted to ensure that sample processing did not lead to liberation of MPA. In these experiments, approximately 100 mg samples of blank lymph nodes were spiked with known concentrations (3-1500 ng) of intact prodrug and then processed as described above. Liberated MPA concentrations were below the limit of quantification of the assay confirming lack of liberation of free MPA during sample processing.

HPLC-MS and HPLC-MS/MS analysis

HPLC-MS analysis of in vitro digestion and lymph samples was conducted using a Shimadzu LCMS 2010 system (Shimadzu Scientific Instruments, Kyoto, Japan) as described previously[9]. HPLC-MS/MS analysis of plasma, lymphocyte and lymph node samples was performed on a Shimadzu LC-MS 8030 system (Shimadzu Scientific Instruments, Kyoto, Japan) consisting of a CBM-20A system controller, a DGU-20A5 solvent degasser, two LC-30AD pumps, an SIL-30AC MP autosampler, a CT-20A column oven (held at 40°C), and a triple quadrupole mass spectrometer with an electrospray ionization interface (ESI). The desolvation line (DL) and the heat block were maintained at 250°C and 400°C, respectively. Interface and detector voltages were 4.5 kV and 1.82 kV, respectively. The nebulising gas flow rate and drying gas flow rate were 3 l/min and 15 l/min, respectively. The prepared plasma (15 µl), lymphocyte (10 µl) or lymph node (10 µl) samples were injected onto a C18 Ascentis Express column (50 mm × 2.1 mm; 2.7 µm, Supelco, United States). The tray temperature in the autosampler was maintained at 15°C. The mobile phase was a mixture of solvent A and B with a flow rate of 0.3 ml/min. Solvent A was milli Q water with 0.1% formic acid and 2 mM ammonium acetate. Solvent B was methanol with 0.1% formic acid and 2 mM ammonium acetate. The gradient was initiated with 30% solvent B from time zero to 0.40 min, then linearly increased to 95% over 0.40 min, and remained at 95% for 0.70 min, before returning to 30% over 0.70 min and equilibration prior to the next injection. The total run time was 3.5 minutes. The transitions m/z 338.15 → 207.05 and m/z 272.10 → 77.10 were used for detection of MPA and ketoprofen, respectively. The collision energies optimised for the method were 25.6 and 49 eV, respectively for MPA and ketoprofen. The assay was validated by replicate analysis of QC samples at low medium and high nominal concentrations.

Data analysis

Lymphatic transport studies: The mass transport of free MPA and 2-MPA-TG derivatives in lymph during each sample collection period was calculated from the product of the volume of lymph collected and the measured concentrations of the analyte in lymph, respectively. Lymphatic drug transport was expressed as both moles of free MPA and moles of MPA derived from all MPA-related species. The cumulative percentage of free MPA transported into lymph was calculated as the mole ratio (as a percentage) of MPA in lymph relative to the equivalent number of moles of MPA or prodrug administered. For the prodrug, the cumulative percentage of total 2-MPA-TG in lymph over time was calculated as the mole ratio of all MPA-related species in lymph relative to the equivalent moles of prodrug administered, where total MPA-related species in lymph were quantified after alkaline hydrolysis of lymph as described in the section 'Sample preparation' above.

Pharmacokinetic analysis: The areas under the plasma MPA concentration-time profiles from zero to designated time intervals (AUC_{0-t}) were calculated using the linear trapezoidal method. Following IV administration, the plasma concentrations declined log-linearly for the first 2 h, but beyond 3 h an increase in plasma concentrations was evident. This likely reflects enterohepatic recycling of MPA, which was also seen in rats[9]. Thus, the $AUC_{0-\infty}$ following IV administration was extrapolated from 2 h onwards, i.e. $AUC_{0-\infty}=AUC_{0-t}+C_t/k$, where the elimination rate constant k was obtained from the regression of the plasma concentration-time curve before 2 h (t=1.5 h, in the cases where the plasma concentration was below LOQ at 2 h). In the cases of oral dosing of MPA or 2-MPA-TG, the limited number of data points before a second peak in the MPA plasma concentration versus time profile precluded accurate calculation of the elimination rate constant k for MPA. Thus, AUC_{0-3h} instead of $AUC_{0-\infty}$ was used in these studies as a surrogate for the analysis of MPA plasma exposure. In lymph duct cannulated animals, the extent of absorption into the

portal blood was estimated via comparison of dose normalised $AUC_{0\text{-}3h}$ following oral dosing of MPA or 2-MPA-TG (only MPA but not prodrug derivatives were detected in plasma) with the $AUC_{0\text{-}\infty}$ obtained following IV administration of free MPA. Bioavailability was estimated by adding the % drug absorption into the blood to the cumulative % transported into the lymph. In the case of lymph duct intact animals (i.e. non-lymph cannulated animals), bioavailability was calculated by comparison of dose normalised free MPA $AUC_{0\text{-}3h}$ following oral dosing of MPA or 2-MPA-TG with the $AUC_{0\text{-}\infty}$ obtained following IV infusion of MPA.

Statistical methods: Statistical differences were determined by student t test for comparisons between two groups or by ANOVA followed by Tukey's test for multiple comparisons at a significance level of p=0.05, using GraphPad Prism for Windows V6.0.0 (GraphPad Software Inc, CA, USA).

RESULTS

In vitro digestion of 2-MPA-TG in simulated intestinal fluid

In the presence of pancreatic enzymes, hydrolysis of the fatty acid chains from 2-MPA-TG was sufficiently rapid that the original prodrug was undetectable after 5 min (data not shown). This coincided with rapid production of the MG form of the prodrug, 2-MPA-MG (Figure 2). Subsequently, the concentration of 2-MPA-MG decreased over time and approximately 20% remained after 3 h incubation. Using the data post-peak concentration of 2-MPA-MG (from 5 min onwards), concentrations declined following first-order kinetics with a degradation rate constant k of 0.0102 ± 0.0003 min^{-1} and thus a half-life $t_{1/2}$ of 68 min ($t_{1/2} = 0.693/k$). The decrease in 2-MPA-MG concentration was accompanied by a corresponding increase in free MPA in the incubation media.

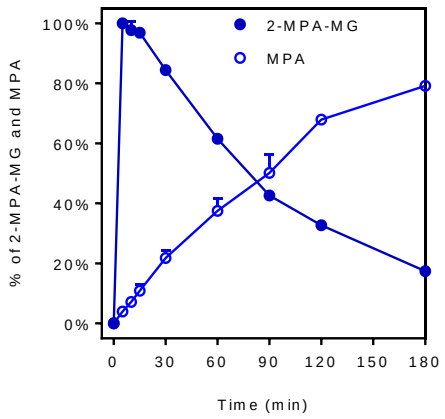


Figure 2. In vitro digestion of 2-MPA-TG in simulated digestive fluid containing porcine pancreatic lipases. Hydrolysis of the TG prodrug resulted in almost immediate production of the monoglyceride form of the prodrug, 2-MPA-MG (closed circles). The concentration of 2-MPA-MG subsequently declined and was accompanied by the release of free MPA (open circles). Data are shown as Mean ± SEM, n=3.

Lymphatic transport

During the lymph collection period, in two of the four dogs receiving 2-MPA-TG, the thoracic lymph-duct cannula was accidentally pulled out by the dogs at 1.5 h and 5.5 h post prodrug dosing. This precluded complete lymph collection for 10 h post dose as scheduled. However, the majority of MPA related materials in lymph were recovered in the first 1.5 h in the other two dogs. The data from the two dogs where the cannulas were removed was therefore included (but assuming no further transport from the last measured time point), realising that the mean data may slightly underestimate total lymphatic transport. Lymphatic transport of total MPA related derivatives was significantly greater following administration of 2-MPA-TG when compared to MPA (Table 1 and Figure 3A). Administration of the TG mimetic prodrug resulted in a 288-fold increase (36.4 % of dose, $p<0.05$) in MPA related derivatives (presumably primarily as re-esterified 2-MPA-TG) when compared to administration of parent MPA (0.13% of dose). The recovery of free MPA in the lymph following prodrug administration was low (0.13% of an equivalent molar dose) and similar

to the value obtained following parent drug (Table 1 and Figure 3B). Lymph flow rates were variable across all animals (Figure 3 legend), but previous studies suggest that changes in lymph flow reflect different levels of hydration and movement and simply dilute the lipoproteins employed to transport lymphatically transported drugs. As such variations in lymph flow are not well correlated with differences in lymphatic drug transport[20].

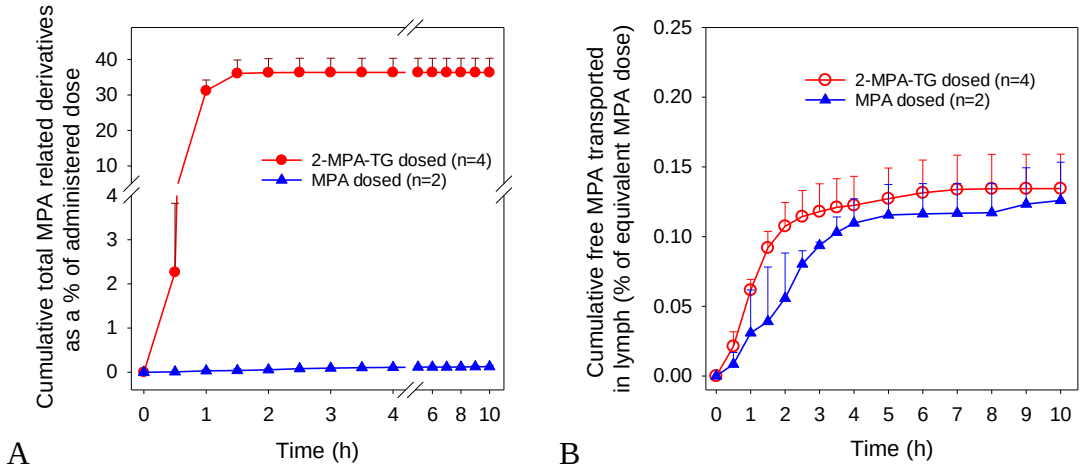


Figure 3. Cumulative lymphatic transport of total MPA related derivatives (% of dose of 2-MPA-TG or MPA, panel A) and free MPA (% of equivalent dose, panel B) versus time in thoracic lymph duct cannulated greyhound dogs following oral administration in the fed state. Each dog received either 50 mg of 2-MPA-TG formulated in a long-chain lipid based SEDDS, or 50 mg of MPA mixed with 200 mg Explotab® and 200 mg Avicel®. Data are presented as mean ± SEM for 2-MPA-TG (circles, n=4) and mean ± range for MPA (triangles, n=2) dosed groups, respectively. For comparison purpose, the data for free MPA in Panel B is a reproduction of that in Panel A. Lymph flow rates were 170, 579, 405, and 1559 ml over 1.5, 5, 10 and 10 h for 2-MPA-TG dosed animals and 625 and 1310 ml over 10 h for MPA dosed animals.

Drug Enrichment in lymphocyte pellets and lymph nodes

Figure 4A shows the dose-normalised concentrations of free MPA in lymphocytes obtained from thoracic lymph after oral administration of 2-MPA-TG and MPA. Administration of the TG mimetic prodrug resulted in markedly higher concentrations of free MPA (most obviously in the first 2 hour post-dose period, consistent with the period of maximal lymphatic transport). The peak concentrations for free MPA in lymphocyte pellets was 2710 ± 1123 ng/g after 2-MPA-TG

administration, 21 fold higher ($p<0.05$) than that after MPA dosing (127 ± 62 ng/g). Figure 4B shows the dose-normalised concentrations of free MPA in mesenteric and axillary lymph nodes obtained after administration of 2-MPA-TG or MPA. Due to ethics restrictions, only a limited number of animals were employed and only 2 replicates were available at a single time point (2 h). This prevents rigorous statistical analysis. Nonetheless the individual data (Figure 4B) show a trend towards higher (5–6 fold) concentrations of free MPA in both the gastrointestinal (mesenteric node) and the peripheral (axillary node) lymphoid organs after administration of the prodrug and slightly higher (1.5-1.9 fold) levels in the mesenteric lymph nodes when compared to peripheral lymph nodes.

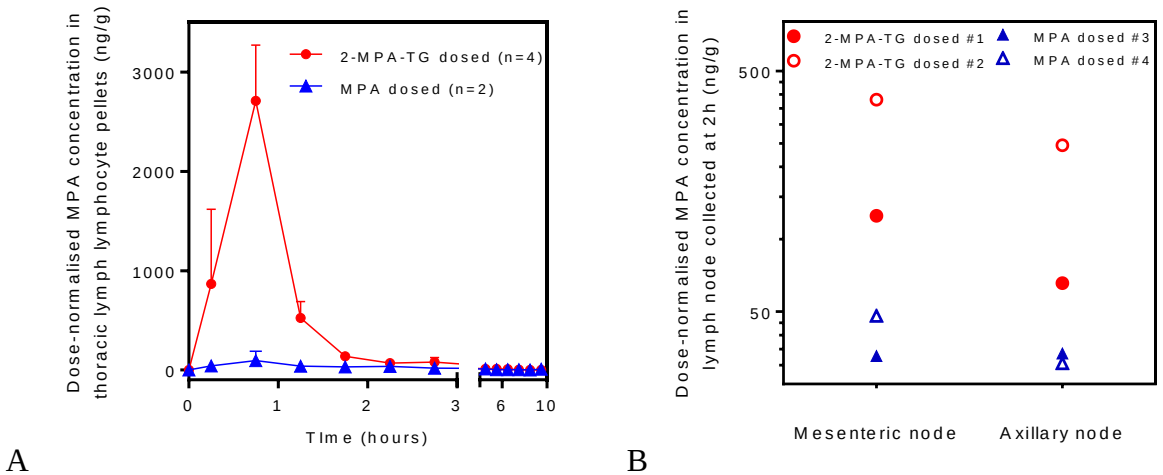


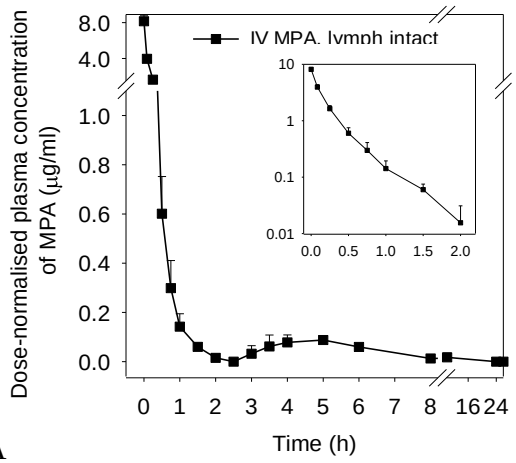
Figure 4. Dose-normalised free MPA concentrations in lymphocytes pellets separated from thoracic lymph samples (Panel A) or in mesenteric and axillary lymph nodes (Panel B) following oral administration of 50 mg of 2-MPA-TG or MPA to thoracic lymph-duct cannulated (Panel A) or intact (Panel B) greyhound dogs. Doses are normalised to a 1 mg/kg equivalent dose of MPA. Data in Panel A are presented as mean ± SEM (circles, n=4) for 2-MPA-TG dosed group and mean ± range (triangles, n=2) for MPA administered group. In Panel B, open and closed circles represent lymph node samples collected from two individual dogs at 2 h post dosing of 2-MPA-TG, and open and closed triangles represent lymph node samples collected from two individual dogs at 2h following MPA administration.

Bioavailability studies

Figure 5 illustrates the plasma concentrations of free MPA and total MPA related material versus time profiles following IV (Panel A) administration of MPA or following oral dosing of MPA or 2-MPA-TG to thoracic lymph duct cannulated (Panel B) or intact (Panel C) dogs. Following IV administration of MPA, the plasma concentrations declined log-linearly for the first 2 h, but an increase in plasma concentrations was evident beyond 3 h. This suggested enterohepatic recycling of MPA, as has been reported previously[9, 21, 22]. Second peaks were also seen in the profiles after oral administration of MPA or the prodrug. The second peak in the plasma profiles and the limited sample points available precluded accurate calculation of an elimination rate constant (and therefore absolute bioavailability calculation using $AUC_{0-\infty}$). An indication of the bioavailability of MPA (in lymph duct intact animals) or the extent of MPA and prodrug absorption into portal blood (in lymph duct cannulated animals) was thus obtained by comparing the normalised AUC_{0-3h} in the oral group with the normalised, extrapolated $AUC_{0-\infty}$ obtained from the IV group (summarised in Table 1).

Total and free levels of plasma MPA in lymph cannulated animals administered the prodrug (Figure 5B), were lower when compared to animals administered MPA, since a significant proportion of the dose was collected in the lymph in animals administered prodrug but not in animals administered MPA. In contrast, in non-lymph cannulated animals (Figure 5C) plasma levels of free MPA were similar after administration of both prodrug and MPA, suggesting conversion to free MPA from re-esterified glyceride derivatives following entry of lymph into the systemic circulation. Peak plasma levels of free MPA, however, were lower and later after administration of prodrug, consistent with a lag time due to the kinetics of conversion of MPA-TG to MPA. Analysis of plasma for total MPA-related materials in non-lymph cannulated animals

after administration of prodrug (Figure 5C), revealed higher plasma levels, consistent with the presence of MPA-glycerides in the plasma. However direct comparison of free MPA plasma levels and plasma levels of MPA associated with glycerides such as re-esterified 2-MPA-TG is not possible since each species will have different systemic pharmacokinetics (e.g. volume of distribution and clearance). The differences between free and total MPA concentrations in plasma were most obvious 0.5-1.5 h post prodrug dosing, consistent with the peak time for lymphatic transport of 2-MPA-TG. The higher exposure of total MPA after administration of the prodrug therefore likely reflects altered plasma pharmacokinetics of 2-MPA-TG and the lag time associated with systemic conversion to free MPA.



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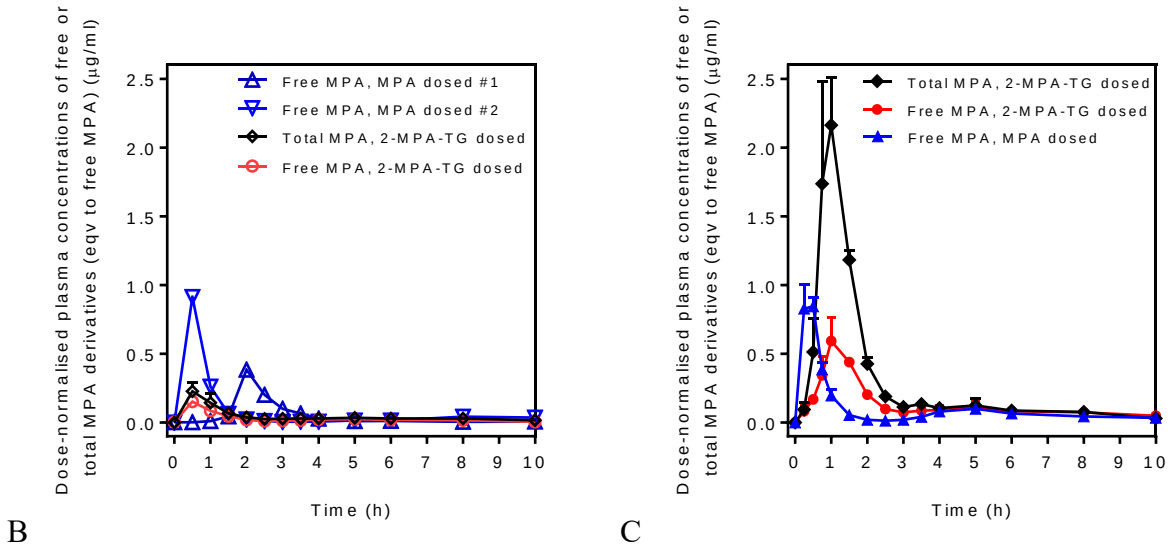

Figure 5. Dose-normalised plasma concentrations of free MPA and total MPA related materials (expressed in free MPA equivalent concentrations) following IV infusion of MPA (Panel A, the insert shows the plasma concentrations (µg/ml) versus time (h, 0-2h) on a log scale) or oral administration of 2-MPA-TG or MPA to thoracic lymph duct cannulated (Panel B) or intact (Panel C) dogs in the fed state. The IV formulation contained 10 mg MPA dissolved in 10 ml of vehicle (95:5 (v/v) - PBS:ethanol), the oral formulations contained 50 mg 2-MPA-TG in a long-chain lipid based SEDDS or 50 mg MPA in a solid capsule. Doses are normalized to a 1 mg/kg equivalent dose of MPA, and data are presented as mean ± SEM (n=3-4) except for the group of two lymph duct cannulated dogs that received the oral MPA formulation, where data were plotted individually (open triangle and open inverted triangles in Panel B).

Table 1. Summary of lymphatic transport of free MPA and total MPA derivatives and AUC of MPA concentration in plasma following oral administration of MPA or 2-MPA-TG to thoracic lymph-duct cannulated dogs, and summary of plasma MPA AUC following IV or oral administration of MPA or 2-MPA-TG to lymph-duct intact dogs. Doses are normalized to a 1 mg/kg equivalent MPA dose and data are presented as mean ± SD for 2-MPA-TG (n=4) and mean ± range for MPA (n=2) dosed dogs.

	Oral administration to lymph cannulated dogs		Oral administration to lymph intact dogs		IV administration to lymph intact dogs
	2-MPA-TG (n=4)	MPA (n=2)	2-MPA-TG (n=3)	MPA (n=3)	IV MPA (n=3)
Transport of total MPA derivatives in lymph (% of dose)	36.4 ± 8.0 [a]	0.13 ± 0.03	NA	NA	NA
Transport of free MPA in lymph (% of equivalent dose)	0.13 ± 0.05	0.13 ± 0.03	NA	NA	NA
$AUC_{0\text{-}3h}$ of free MPA in plasma (μg×h/ml)*	0.16 ± 0.06 [b]	0.53 ± 0.11	0.76 ± 0.22	0.64 ± 0.05	1.87 ± 0.20 [a] ($AUC_{0\text{-}\infty}$)
Bioavailability of MPA**	45 %	28 %	40 %	34 %	100 %

* The value for the IV group was $AUC_{0\text{-}\infty}$. The AUC between 0-4 h instead of 0-3 h was used for one animal in the lymph cannulated oral MPA group due to a delay in the plasma concentration peak.

** For the lymph duct cannulated group, the bioavailability was estimated by the sum of the lymphatically transported fraction and the extent absorbed via the portal blood (calculated by comparison of the AUC following oral dosing with the AUC after IV administration). For example, the bioavailability following oral administration of 2-MPA-TG to lymph cannulated dogs was obtained by 36.4 % + 8.7 % = 45.1% %, where 36.4 % is the percentage of lymphatic transport and 8.7 % reflects the ratio of the AUC following oral administration (0.16 μg×h/ml) to the AUC following IV dosing (1.87 μg×h/ml).

[a] significantly greater when compared to all other groups in the same row ($p<0.05$)

[b] significantly smaller when compared to all other groups in the same row ($p<0.05$)

DISCUSSION

Rodents, most commonly rats, have been used routinely as animal models to study lymphatic drug transport in vivo. There are, however, several differences in GI physiology between the rat and higher order species such as dogs and humans, and therefore cross species comparative studies are required to provide greater confidence in extrapolation to humans. The current study has employed a conscious greyhound dog model to confirm earlier findings in rats and to provide more confidence in the likely utility of a lymph-directing prodrug strategy in clinical studies. To our knowledge, this is first study using a large animal model to quantify lymphatic transport of a TG mimetic prodrug (where the prodrug must integrate into lipid digestion and resynthetic pathways).

Lymphatic drug transport in greyhound dogs

The lymphatic transport of MPA following oral administration of parent MPA to dogs was low (0.13% dose). This likely reflects similar partitioning of MPA between blood and lymph capillaries (since it has little specific affinity for lymph lipoproteins), and significantly higher mass transport in blood due to higher blood flow. The data obtained is comparable to previous studies in rats (0.17% dose)[9]. Similar to the previous data in rats, the TG mimetic prodrug enhanced the lymphatic recovery of total MPA related materials, although the degree of enhancement in dogs was far greater than previous studies in rats. Lymphatic transport of MPA-related material was increased over 280-fold in the dog model (albeit as the resynthesized glyceride) when compared to parent MPA. It is likely that 2-MPA-TG was processed in a similar fashion to that previous reported in rats (Scheme 1A)[9, 10]. That is, hydrolysis of 2-MPA-TG by digestive lipases in the GI lumen to release the 2-MG analogue and FA; absorption of 2-MPA-MG, FA and the digestion products of formulation and dietary lipids; re-synthesis to DG and ultimately TG derivatives of MPA from 2-MPA-MG via monoacylglycerol acyltransferases and diacylglycerol acyltransferases; assembly of

re-esterified 2-MPA-TG into intestinal lymph LP and finally LP exocytosis from the enterocyte and preferential uptake into intestinal micro-lymphatics.

Direct confirmation of all steps in the prodrug metabolic pathway was not practical in the current studies due to the difficulties in obtaining multiple biological samples from dogs (e.g. bile and pancreatic secretions). A number of lines of indirect evidence, however, are consistent with this proposed pathway. Firstly, incubation of 2-MPA-TG with a surrogate digestive medium (containing porcine pancreatic extract) revealed rapid generation of the 2-MPA-MG derivative (Figure 2), suggesting that the TG prodrug is hydrolysed efficiently to form the MG equivalent in the GI lumen of the greyhound dogs. This ensures the generation of the necessary intermediate for subsequent absorption (into enterocytes) and resynthesis (back to TG derivatives) prior to lymphatic transport [10]. HPLC-MS analysis of the dog lymph samples collected following prodrug administration revealed that the MPA related products in lymph consisted mainly of resynthesised TG derivatives (as described in a previous study[9] in rats) with molecular weights that were different to the original 2-MPA-TG (where the fatty acid chains in the 1 and 3 position of the glycerol backbone were palmitic acid (MW 870)). This suggests that 2-MPA-MG was re-esterified with FA other than the FA originally conjugated in the 1' and 3' position in the prodrug (multiple different FA are present in administered food and the lipid formulation).

After rapid generation of 2-MPA-MG from the TG prodrug (Figure 2), 2-MPA-MG was further hydrolysed to release free MPA. Degradation in the porcine lipase medium (20 % 2-MPA-MG remained after 3 h) was more rapid than that reported previously after incubation with rat bile and pancreatic fluid (where 70% of 2-MPA-MG remained after 3 h)[9]. This is consistent with the expectation that lipase enzyme levels in higher order species such as dogs and humans (and the pig, realising the use of porcine lipase here), particularly in the fed state, is higher than that in

rats[23]. The exact mechanism for the hydrolysis of 2-MPA-MG is not clear at this point. It may reflect direct cleavage of the 2' ester bond between MPA and the glycerol moiety, even though the 2' position is thought to be relatively insensitive to (i.e. protected from) pancreatic lipase hydrolysis. Alternatively, migration of MPA from the 2' to the 1' position is possible (as in the case for FA[24]) followed by hydrolysis in the 1' position (which is highly susceptible to lipase digestion)[25]. Degradation of the monoglyceride form of the prodrug in the intestine would be expected to reduce lymphatic transport of MPA since the monoglyceride is a key substrate for post-absorptive re-esterification. The data in the current studies, however, suggest that lymphatic transport was both rapid (90% of the lymphatically transported fraction was completed within 1 h (Figure 3A)) and significant (~40% of the dose), and therefore luminal instability of 2-MPA-MG does not appear to have significantly reduced utility here. Nonetheless structural modification of the prodrug to promote re-esterification or reduce intestinal instability may promote lymphatic transport further.

The absolute lymphatic recovery (36.4%) of 2-MPA-TG in greyhound dogs following oral administration was markedly greater than previous studies in rats (13.4%) after intraduodenal administration. This is consistent with previous observations of the lymphatic transport of highly lipophilic drugs such as halofantrine[15] and CP524,515[11, 16] where lymphatic transport was significantly greater in dogs when compared to rats. The mechanism(s) of lymphatic access of inherently lipophilic drugs, however, is different to that of the current prodrug. In the case of halofantrine and CP524,515, lymphatic access is expected to occur via partitioning of drug into lipoproteins on passage across the enterocyte. This process is driven by differential affinity of the drug for the lipid core of lipoprotein versus the largely aqueous cytosol. In contrast, 2-MPA-TG is expected to mimic, and incorporate into, the TG metabolic pathway. The increase in lymphatic

transport of total MPA related materials after administration of 2-MPA-TG to dogs, when compared to previous data in rats, may therefore have resulted from (1) more effective absorption of the monoglyceride derivative (2-MPA-MG) from the GI lumen and/or (2) more efficient prodrug association with enterocyte resident lipoproteins in the dog model. The latter appears more likely to be the main reason for the increase in lymphatic transport in dogs since absorption of 2-MPA-MG from the GI lumen was ~35% of the infused dose in rats[10], and bioavailability was only slightly higher (~ 45 %, Table 1) in dogs. As such a much higher proportion (36.4 % out of 45 %) of the absorbed MPA derivatives were incorporated into lymph lipoproteins in the dog, when compared to the rat (13.4 % out of 35 %). An explanation for these differences is unclear at this stage, but may reflect differences in the re-esterification of 2-MPA-MG to TG derivatives, the association of the resynthesised derivatives with lipoproteins, the relative doses of co-administered lipids, the storage/mobilisation of lipoproteins in enterocytes, blood versus lymph flow, or enterocyte based first pass metabolism.

Conversion to free MPA from MPA-glycerides in lymphocytes and lymph nodes

As shown in Figure 3B, free MPA concentrations in lymph fluid collected following prodrug administration were low. This was not unexpected. The release of free MPA from the re-esterified prodrug likely requires sequential hydrolysis by lipases and esterases. A preliminary study in our laboratory has shown that the combination of lipoprotein lipase (LPL) and rat lymph or plasma (both of which might be expected to contain a range of esterases) is able to release free MPA from 2-MPA-TG. In contrast, lymph or plasma alone, or LPL alone is not sufficient to trigger release (data not shown). Such combined activity, however is not expected to be high in the lymph fluid, especially realizing the relatively rapid transit time through the mesenteric lymph. In contrast, much higher free MPA levels were observed in lymphocyte pellets and lymph nodes following

prodrug administration (when compared to administration of MPA alone), consistent with higher residence times and higher levels of lipases and esterases in lymphocytes or intra-/peri-lymph node substructures. In the plasma, levels of MPA were similar after administration of prodrug or MPA suggesting systemic release of MPA. Enhanced release in plasma rather than lymph fluid most likely reflects differences in residence time during circulation in the systemic circulation when compared to a single pass through the lymphatics. Release in the lymphatics, therefore, is likely to occur after sequestration into lymph nodes or uptake into lymphocytes where residence times are extended.

In previous rat studies[9, 10], quantification of released MPA in lymph lymphocytes was not possible, due the extremely small quantities of cells that could be recovered from the small volumes of lymph collected in rats (~ 1 ml/h). In the dog model, however, much larger volumes of lymph (>50 ml/h) could be collected and free MPA concentrations in lymph lymphocytes were therefore measured to provide a direct indication of the conversion of 2-MPA-TG to MPA in lymphocytes (Figure 4A). Administration of the prodrug resulted in a marked increase (21 fold; 2710 vs 127 ng/g) in MPA exposure in lymphocytes, when compared to data obtained after administration of parent drug (MPA). This is comparable to the data obtained in previous rat studies where the MPA concentrations in mesenteric lymph nodes following 2-MPA-TG dosing were up to 28 fold higher than that after MPA administration[9]. The current studies did not examine the mechanisms by which MPA was released in dog lymphocytes, although similar mechanisms in dogs and rats might be expected. LPL and lipoprotein receptors on the surface of lymphocytes[26] enable the uptake of lipoprotein associated TG and liberation of FA[27]. Similar mechanisms might be expected to facilitate the conversion of lipoprotein associated 2-MPA-TG to free MPA on the

lymphocyte surface. Alternatively, following lipoprotein uptake, drug release may occur via hydrolysis by lysosomal lipase[28] or non-specific esterases[29].

To assess the conversion from prodrug derivatives to free MPA in a more physiologically relevant environment (e.g. secondary lymphoid tissues where MPA inhibits lymphocyte proliferation), mesenteric and axillary lymph node samples were taken and analysed for drug concentrations (Figure 4B). Due to ethical constrains, a limited number of dogs were employed for this terminal study (n=2 for each group) and data was only obtained at a single time point (2 h). Nonetheless, the data suggest that compared to oral administration of MPA, administration of the prodrug enhanced free MPA exposure to both gut-associated (as represented by the mesenteric node) and peripheral (as represented by the axillary node) lymphoid tissues, with, on average, somewhat higher levels in the mesenteric lymph node. The levels obtained were consistent with the levels required to inhibit lymphocyte proliferation[30]. The presence of free MPA in lymph nodes following prodrug administration confirms the conversion of TG prodrug derivatives to free MPA. Two possible mechanisms of drug release are evident. In the first instance, MPA in lymph nodes may result from distribution of MPA into the node from the systemic circulation. Administration of 2-MPA-TG to lymph duct intact dogs resulted in normalised plasma AUCs of MPA that were comparable to those obtained after administration of MPA alone (Figure 5C and Table 1) although at the chosen sampling point, systemic plasma levels of MPA after administration of 2-MPA-TG were higher than after administration of MPA, due to a delay in release of MPA from the prodrug (Fig 5C). Concentrations of MPA in lymph nodes might therefore, at least in part, reflect systemic MPA levels. In light of the much higher levels of MPA in lymphocytes, however, it is also possible that the increase in MPA concentration in the nodes after administration of the prodrug may have resulted from hydrolysis of MPA from MPA glycerides in lymph via LPL expressed on the surface

of lymphocytes, or via intracellular lipases or esterases (as described above). Adipose tissue surrounds lymph nodes and has been shown to hydrolyse TG to provide fatty acids for utilisation by lymphocytes in the lymph node [31]. MPA release may also, therefore, have been catalysed by lipases associated with peri-nodal adipose tissue. Increases in lymph node MPA were apparent for both mesenteric lymph nodes and axillary lymph nodes, but were slightly higher for mesenteric lymph nodes. This is consistent with the fact that mesenteric lymph nodes are exposed directly to high concentrations of MPA glycerides in mesenteric lymph, whereas free MPA in axillary lymph nodes is likely to be sourced from MPA glycerides in lymph lipoproteins or lymph lymphocytes after entry into the systemic circulation.

CONCLUSIONS

The current studies examined the ability of a triglyceride mimetic prodrug, 2-MPA-TG, to promote lymphatic transport and lymphocyte targeting of MPA in a conscious greyhound dog model. The dog model was employed since the GI physiology of the dog is more representative of that in humans than that of small animal models, such as the rat used in previous studies. Following oral administration of 2-MPA-TG to fed greyhound dogs, a marked increase in lymphatic transport of total MPA related derivatives (288 fold) was evident when compared to that obtained after MPA dosing. Conversion to the pharmacologically active parent (MPA) was also observed, resulting in significantly higher concentrations of free MPA in lymph-sourced lymphocytes (21 fold) or lymph nodes (5-6 fold) following administration of the prodrug when compared to MPA. Importantly, higher drug concentrations at the target sites were achieved without increasing systemic exposure. Thus, the data suggest that the 2-MPA-TG prodrug is at least as effective in increasing lymphatic

transport of MPA in large animal models when compared to small animal models, increasing confidence that this could be replicated in clinical studies.

ACKNOWLEDGMENT

This work was financially supported by the National Health and Medical Research Council of Australia and Australian Research Council. The authors also thank Dr Tri-Hung Nguyen, Dr Yan Yan Yeap, Ms Orlagh Feeney and Mr Jianqing Hu for assistance in animal studies.

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