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RESEARCH PAPER

Profiling the Role of Deacylation-Reacylation in the Lymphatic Transport of a Triglyceride-Mimetic Prodrug

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ABSTRACT

Purpose Recent studies have demonstrated the potential for a triglyceride (TG) mimetic prodrug to promote the delivery of mycophenolic acid (MPA) to the lymphatic system. Here, the metabolic pathways that facilitate the lymphatic transport of the TG prodrug (1,3-dipalmitoyl-2-mycophenoloyl glycerol, 2-MPA-TG) were examined to better inform the design of next generation prodrugs.

Methods *In vitro* hydrolysis experiments in simulated intestinal conditions and *in vivo* rat lymphatic transport experiments were conducted in the presence and absence of orlistat and A922500 (inhibitors of lipolysis and TG re-esterification, respectively), to evaluate the importance of 2-MPA-TG digestion and re-esterification of 2-MPA-MG (the 2-monoglyceride derivative) in promoting lymphatic transport.

Results 2-MPA-TG was rapidly hydrolysed to 2-MPA-MG on incubation with fresh bile and pancreatic fluid (BPF), but not in simulated gastric fluid, heat-inactivated BPF or BPF + orlistat. Orlistat markedly decreased lymphatic transport and systemic exposure of 2-MPA-TG derivatives suggesting that inhibition of pancreatic lipase hindered luminal digestion and absorption of the prodrug. A922500 also significantly decreased lymphatic transport of 2-MPA-TG but redirected MPA to the portal blood, suggesting that hindered re-acylation of 2-MPA-MG resulted in intracellular degradation.

Conclusion Incorporation into TG deacylation-reacylation pathways is a critical determinant of the utility of lymph directed TG-mimetic prodrugs.

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KEY WORDS DGAT · lipase · lymphatic transport · prodrug · triglyceride mimetic

ABBREVIATIONS

BPF	Bile and pancreatic fluid
DG	Diglyceride
DGAT	Diacylglycerol acyltransferease
DGTA	Diacylglycerol transacylase
FA	Fatty acid
ID	Intraduodenal
MG	Monoglyceride
MGAT	Monoacylglycerol acyltransferase
MPA	Mycophenolic acid
MW	Molecular weight
SGF	Simulated gastric fluid
TG	Triglyceride

INTRODUCTION

The lymphatic system consists of a specialised network of vessels, nodes and lymphoid tissues that are distributed throughout the body in close proximity to the vascular system. The lymphatics play a number of key roles in immune response, fluid balance, nutrient absorption, and tumour metastasis. Due to the unique anatomical and physiological characteristics of the lymphatic system, targeted drug delivery to the lymph has been suggested as a means to improve both pharmacokinetic and pharmacodynamic outcomes. For instance, lymphatic drug transport has the potential to enhance oral bioavailability through avoidance of first pass metabolism [1–3], to alter systemic drug disposition [4] and to enhance efficacy against lymph or lymphocyte mediated pathologies such as lymphatic tumor metastasis [5], autoimmune disease and transplant rejection [6, 7].

One means by which targeted delivery to the lymphatics may be achieved is to harness the endogenous pathways of dietary lipid transport *via* the intestinal lymphatics (see Fig. 1) [8, 9]. After ingestion, dietary triglyceride (TG) is hydrolysed by luminal lipases (largely derived from pancreatic secretions) to release diglyceride (DG), 2-monoglyceride (2-MG) and fatty acid (FA). 2-MG and FA are subsequently absorbed into enterocytes where 2-MG is re-esterified to DG and TG, by monoacylglycerol acyltransferases (MGATs) and diacylglycerol acyltransferases (DGATs), respectively [10]. Resynthesised TG is assembled into lipoproteins in the endoplasmic reticulum and the lipoproteins so formed are exocytosed from enterocytes into the intercellular space and from there cross the basement membrane to enter the lamina propria. Colloidal lipoproteins subsequently gain preferential access to the intestinal microlymphatics since diffusion across the vascular endothelium is more hindered than that across the lymphatic endothelium. Highly lipophilic drugs (or prodrugs) that are co-administered orally with lipids may associate with lipoproteins on passage across enterocytes, thereby achieving directed uptake into the intestinal lymphatic system in parallel with dietary TG [1, 11–13].

A recent study in our laboratory [14] compared different prodrug strategies to promote the delivery of an immunomodulator, mycophenolic acid (MPA), to target cells within the lymphatic system. In this study, a TG mimetic prodrug, 1,3-dipalmitoyl mycophenoloyl glyceride (2-MPA-TG, Fig. 2) was identified as an effective means by which the lymphatic transport of MPA could be promoted and increases in lymphatic transport of approximately 80-fold were apparent when compared to administration of parent MPA. Simpler prodrug candidates, such as alkyl ester and amide derivatives, were less effective and resulted in more moderate (up to 13-fold) increases in lymphatic transport. 2-MPA-TG was suggested to be particularly effective by virtue of biochemical integration into endogenous TG metabolic pathways (see Fig. 1).

The current studies sought to examine in detail the sequential site-specific biotransformation processes that were

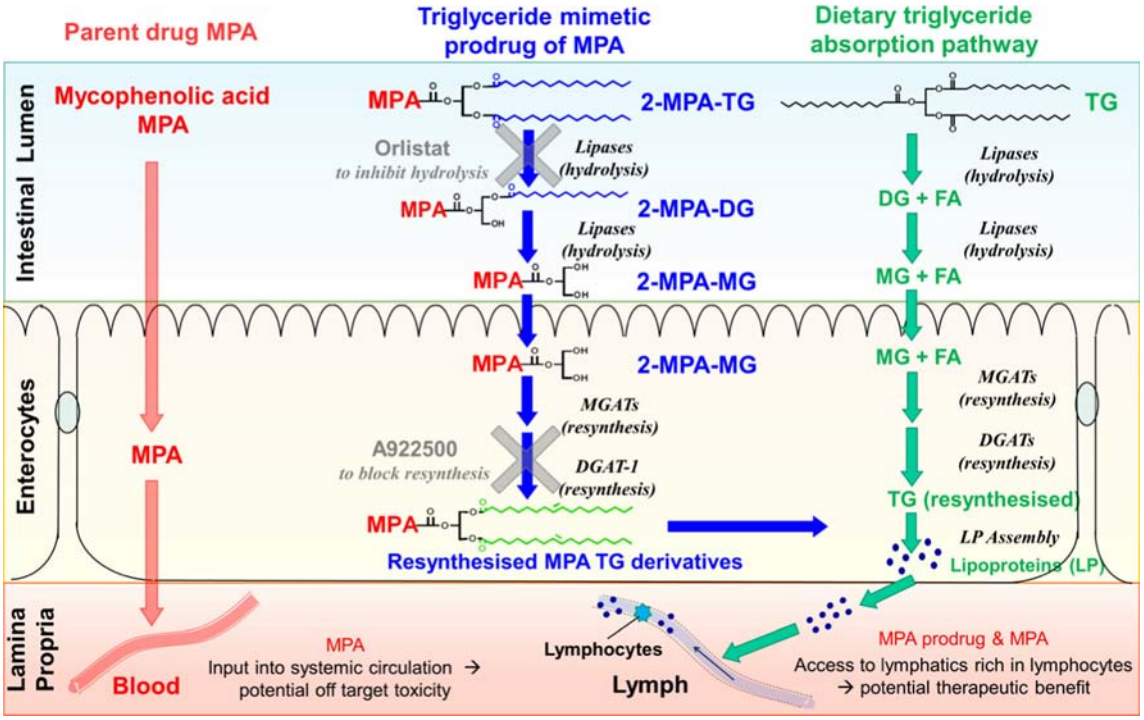

Fig. 1 Schematic diagram summarising dietary triglyceride (TG) transport routes into the intestinal lymphatics, the transport pathway of the parent drug MPA into portal blood, and the proposed metabolic pathways that result in the lymphatic transport of the triglyceride mimetic prodrug (2-MPA-TG). Dietary TG transport (*green pathway*): After ingestion, dietary TG is sequentially hydrolysed by digestive lipases in the gastrointestinal (GI) lumen to release diglyceride (DG), 2-monoglyceride (2-MG) and fatty acids (FA). 2-MG and FA are absorbed into enterocytes and re-synthesised to DG and then TG *via* consecutive catalysis by monoacylglycerol acyltransferases (MGATs) and diacylglycerol acyltransferases (DGATs). Reformed TG is assembled to form the core of lipoproteins (LP) and LP are subsequently exocytosed from the basolateral membrane of enterocytes into the intercellular space and from there cross the basement membrane to enter the lamina propria. From the lamina propria, access of LP to underlying blood capillaries is limited by the barrier properties and integrity of the vascular endothelium. Instead, LP preferentially gain access to the intestinal micro-lymphatics *via* passage through the gaps between adjacent lymphatic endothelial cells [15] and potentially *via* active transcellular transport pathways [16]. Parent drug MPA (red pathway): Orally dosed MPA is absorbed directly into the blood [14] (due to limited lipoprotein affinity) leading to non-specific exposure to both sites of activity (*e.g.* lymphocytes) and off-target toxicity. TG mimetic prodrug of MPA (*blue pathway*): 2-MPA-TG was designed to promote MPA lymphatic transport by mimicking dietary TG hydrolysis and resynthesis pathways in the GI lumen and enterocytes. Grey crosses: In the current study, orlistat and A922500 (inhibitors of pancreatic lipase and DGAT-1, respectively) were used as probes to evaluate the importance of digestion and re-esterification of 2-MPA-TG in promoting lymphatic transport.

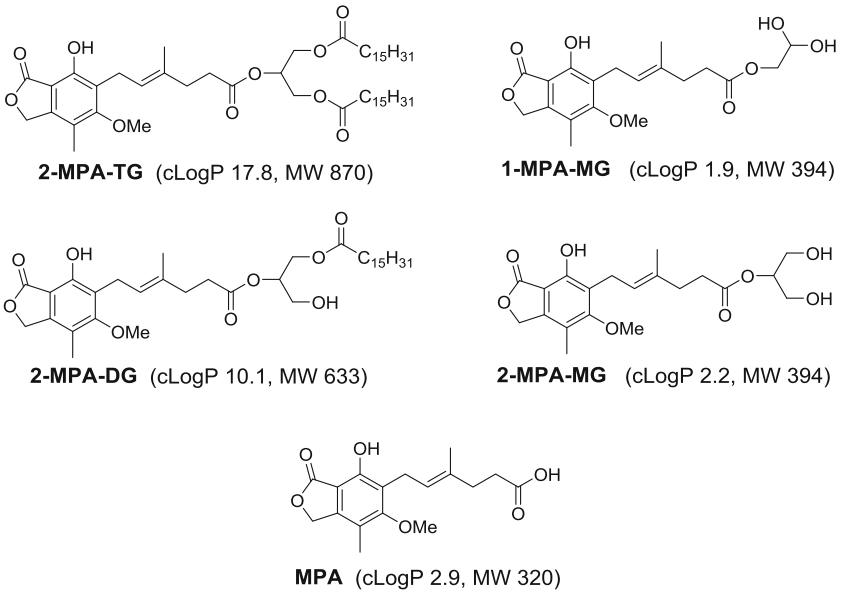

Fig. 2 Chemical structures of MPA, 2-MPA-TG, the hydrolysis products (2-MPA-DG and 2-MPA-MG) of 2-MPA-TG, and an analytical reference compound 1-MPA-MG with molecular weight (MW) and cLog P values calculated using ACD/Labs Release Software (version 9.12).

responsible for lymphatic transport of the biomimetic 2-MPA-TG prodrug, and in particular the enzymes involved in luminal deacylation and enterocyte-based reacylation, in order to better inform the design of next generation lymphotropic prodrugs.

To better understand the luminal events involved in the hydrolysis of 2-MPA-TG, *in vitro* studies were conducted under a variety of conditions, including incubation with simulated gastric fluid (SGF), bile and pancreatic fluid (BPF), fresh *versus* heat-inactivated BPF, and the lipase inhibitor orlistat. The importance of lipase-mediated hydrolysis in promoting intestinal absorption and lymphatic transport of 2-MPA-TG was also evaluated *in vivo via* examination of the lymphatic transport and bioavailability of 2-MPA-TG and its derivatives in triple-cannulated (mesenteric lymph-duct, carotid artery and duodenum) rats in the presence and absence of orlistat (illustrated by the upper cross in the (blue) prodrug pathway in Fig. 1). The necessity for enterocyte-based re-esterification of 2-MPA-MG (a hydrolysis product of 2-MPA-TG) for effective lymphatic transport of MPA, the enzymes involved in re-esterification and the potential for alternate metabolic routes for the monoglyceride equivalent 2-MPA-MG, were also explored in studies that examined lymphatic transport and bioavailability following administration of 2-MPA-TG in the presence and absence of A922500 (illustrated by the lower cross in Fig. 1), a selective inhibitor of DGAT-1 (which catalyses the final and rate-limiting step in enterocyte-based TG resynthesis). Lastly, liberation of the pharmacologically active MPA from its TG derivatives following access into the systemic circulation *via* the lymphatic route was investigated in lymph-duct intact rats. The data identify the critical metabolic steps required for lymphatic transport of 2-MPA-TG, including the enzymes responsible for hydrolysis (deacylation) and resynthesis (reacylation), and provide insight into the design criteria for future lymphotropic drug candidates.

MATERIALS AND METHODS

Chemicals and Prodrug Synthesis

Mycophenolic acid (MPA, >98%) was purchased from AK Scientific, Palo Alto, CA, USA. Orlistat, ketoprofen, oleic acid, formic acid, ammonium formate, ammonium acetate, and Tween 80 were purchased from Sigma-Aldrich, MO, USA. A922500 was purchased from Tocris Bioscience, Bristol, UK. Sodium hydroxide, hydrochloric acid and acetonitrile (ACN, for liquid chromatography) were purchased from Merck Pty Limited, Australia. Pentobarbitone was purchased from Virbac Animal Health, NSW, Australia. Ultrapure water was obtained from a Milli-Q^{TM} system (Millipore, MA, USA). All other chemicals were analytical grade or above.

The triglyceride mimetic prodrug 2-MPA-TG was synthesised as previously described [14]. A monoglyceride analogue of the prodrug 1-mycophenoloyl glycerol (1-MPA-MG, Fig. 2) was synthesised as a reference compound (see supplementary information).

Preparation of Lipid Formulations for *in Vitro* and *in Vivo* Experiments

Lipid-based formulations containing 2-MPA-TG were prepared as described previously [17]. Briefly, ~2 mg of 2-MPA-TG, 25 mg of Tween 80 and 40 mg of oleic acid were mixed in a glass vial as a lipid phase and incubated at 37°C for 12–18 h to equilibrate and to allow the prodrug to dissolve. An

aqueous phase consisting of 5.6 ml phosphate buffered saline (PBS, pH 7.4) was subsequently added to the glass vial and the formulation emulsified by ultrasonification with a Misonix XL 2020 ultrasonic processor (Misonix, Farmingdale, NY, USA) equipped with a 3.2-mm microprobe tip running at an amplitude of 240 μm and a frequency of 20 kHz for 2 min at room temperature. Prodrug concentrations in formulations were verified on the day of experiments using HPLC-MS. No prodrug degradation products (including free MPA or 2-MPA-MG) were present in the formulations.

Animal Care During *in Vivo* Experiments

All animal experiments were approved by the Monash Institute of Pharmaceutical Sciences 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. Male Sprague-Dawley rats (280–320 g) were maintained on a standard diet and then fasted overnight with free access to water prior to experiments. In all experiments rats were anaesthetised using a combination of ketamine, xylazine and acepromazine and placed on a heated pad at 37°C as described previously [18]. At the end of experiments rats were euthanised *via* intraperitoneal administration of 100 mg pentobarbitone.

Hydrolysis of 2-MPA-TG by Rat BPF or SGF

Rat bile and pancreatic fluid (BPF) was collected from anesthetised rats *via* insertion of a cannula (polyethylene tubing with 0.5 mm i.d. and 0.8 mm o.d.) into the common bile-pancreatic duct [19] as close as possible (0.3–0.5 cm) to its entry into the duodenum (*i.e.* below the point of entry of pancreatic secretions). This allowed simultaneous collection of bile and pancreatic fluid. BPF was collected continuously for 2 h and blank lipid formulation (prepared as described in 'Preparation of lipid formulations for in vitro and in vivo experiments' but without addition of drug) was infused into the duodenum at a rate of 2.8 ml/h during the collection period to mimic conditions during drug administration. Approximately 3 ml of BPF was collected during this time period and was used fresh (within 1 h) for *in vitro* prodrug hydrolysis experiments.

The hydrolysis experiments were conducted *via* incubation (37°C) of lipid formulations containing 2-MPA-TG with BPF or enzyme-free SGF (aqueous solution containing 0.07 M HCl and 0.2% NaCl, w/w [20]) under a range of conditions as described in Table I. Samples (10 μl) were taken at 2, 5, 10, 15, 30, 60, 90, 120, 180 min and added to 990 μl of ACN:water (4:1, v/v) to stop lipolysis, vortexed for 1 min and centrifuged at 4500 g for 5 min to precipitate any proteins prior to analysis. The supernatant (150 μl) was assayed by HPLC-MS (methods in supplementary information) for residual prodrug, and the products of prodrug hydrolysis including the DG derivative 2-MPA-DG, the MG derivative 2-MPA-MG, and free MPA (see Fig. 2).

Lymphatic Transport and Bioavailability Studies in Rats

Cannulas were inserted into the duodenum (for formulation administration and rehydration), mesenteric lymph duct (for lymph collection) and carotid artery (for blood collection) of anaesthetised rats as previously described [17]. Post-surgery, rats were re-hydrated for 0.5 h *via* intraduodenal infusion of normal saline at 2.8 ml/h [17]. The lipid formulations were then infused into the duodenum at a rate of 2.8 ml/h for 2 h, after which the infusion was changed back to 2.8 ml/h normal saline for the remainder of the experiment [17]. Where indicated, the lipase inhibitor orlistat (0.9 mg in ethanol) or the DGAT-1 inhibitor A922500 (2 mg in DMSO) were added to the lipid formulations (followed by vortexing for 0.5 min) 5 min prior to infusion. The volume of solvent added when spiking the formulations was <1% of the total volume. Lymph was continuously collected for 8 h following initiation of formulation administration into pre-weighed eppendorfs containing 10 μl of 1000 IU/ml heparin, and the collection tubes were changed hourly. Two 20 μl aliquots of each hourly collected lymph sample were immediately transferred into eppendorf tubes and stored at −80°C prior to HPLC-MS analysis. The remaining samples were centrifuged at 2000 g for 5 min and the supernatant transferred to a new eppendorf and kept at 4°C for less than 24 h prior to assay of TG content using a commercial enzymatic analysis kit (TR0100, Sigma-Aldrich, MO, USA). Blood samples (250 μl) were also collected *via* the carotid artery cannula each hour for 8 h following the initiation of formulation infusion. Plasma was separated *via* centrifugation at 2000 g for 5 min prior to storage at −80°C until HPLC-MS analysis. To examine the profile of parent MPA release after access of prodrug derivatives into the systemic circulation *via* the lymphatics, plasma data was also obtained from a separate cohort of animals where the same dose of 2-MPA-TG (2 mg) was administered *via* ID infusion to mesenteric lymph-duct intact rats (*i.e.* where the lymph duct was not cannulated and where lymph drained directly into the systemic circulation).

Plasma Stability of 2-MPA-TG and 1-MPA-MG

The plasma stability of 2-MPA-TG and its intermediate hydrolytic product (2-MPA-MG) was estimated *via* stability studies of 2-MPA-TG and the synthesised isomer 1-MPA-MG in blank rat plasma. 1-MPA-MG was utilised as a surrogate for 2-MPA-MG since the 2-MPA-MG isomer is unstable and spontaneously isomerises to 1-MPA-MG. Briefly, 10 μl of 395 μg/ml 1-MPA-MG or 870 μg/ml 2-MPA-TG was dissolved in THF and spiked into 990 μl blank plasma taken

Table I Experimental conditions for *in vitro* hydrolysis by bile and pancreatic fluid (BPF) and simulated gastric fluid (SGF)

	BPF	BPF + 2 μM orlistat	BPF + 20 μM orlistat	BPF + 200 μM orlistat	BPF, heat-inactivated [a]	SGF [b]
Volume of BPF or SGF (ml)	0.375	0.375	0.375	0.375	0.375	0.9
Volume of formulation (ml)	0.625	0.625	0.625	0.625	0.625	0.1
Concentration of orlistat [c] (μM)	0	2	20	200	0	0

[a] Heat inactivation was conducted by heating freshly collected BPF at 80°C for 15 min. The pH (8.1–8.2) of BPF was unchanged after heat-inactivation

[b] A solution of HCl (0.07 M) and NaCl (0.2%, w/w) was used as a simulated gastric fluid (without pepsin) to mimic the low pH in the stomach (pH ~ 1.2) [20]

[c] Orlistat was pre-dissolved in 10 μl of 96% ethanol and mixed with 2-MPA-TG formulations prior to addition of BPF. Orlistat-free groups also contained 10 μl of 96% ethanol, accounting for 1% of total volume

from a pool of plasma collected from 10 male SD rats. Samples therefore contained 10 μM and 1% (v/v), respectively of 2-MPA-TG or 1-MPA-MG and THF and were incubated at 37°C for 3 h. At 2, 5, 10, 15, 30, 60, 90, 120 and 180 min after addition of 1-MPA-MG, 10 μl samples were taken and immediately added to 190 μl of 9:1 (v/v) acetonitrile-water to prevent further hydrolysis. At 5, 15, 30, 60, 120 and 180 min after addition of 2-MPA-TG, two replicate 10 μl samples were taken and immediately added to 190 μl of THF (for analysis of remaining 2-MPA-TG) and 190 μl of 9:1 (v/v) acetonitrile-water (for analysis of generated free MPA), respectively. After vortexing for 1 min, samples were centrifuged at 10,600 g for 5 min to precipitate any proteins and 150 μl of supernatant was used for HPLC-MS analysis of residual 2-MPA-TG, 1-MPA-MG and generated free MPA (see supplementary information).

Preparation of Lymph and Plasma Samples and HPLC-MS Analysis (see Supplementary Information)

Data Analysis

Lymphatic Transport Studies. Mass transport of TG and MPA prodrug derivatives into lymph during each 1 h collection period was calculated from the product of the volume of lymph collected and the measured concentrations of TG and MPA-related species in lymph, respectively. Lymphatic drug transport was expressed as both moles of free MPA and moles of all MPA-related derivatives. 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 prodrug administered. 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 supplementary information.

Pharmacokinetic Analysis. The areas under the plasma concentration-time profiles from zero to designated time intervals ($AUC_{0\text{-}t}$) were calculated using the linear trapezoidal method. The bioavailability of MPA (in lymph-duct intact animals) or the extent of absorption *via* the portal vein (in lymph-duct cannulated animals) was estimated *via* comparison of dose normalised $AUC_{0-5\ h}$ for MPA following intraduodenal administration of 2-MPA-TG and intravenous dosing of MPA. Truncated AUCs ($AUC_{0-5\ h}$) were used due to the presence of a second peak in the MPA plasma concentration *versus* time profile, likely reflecting enterohepatic recycling of MPA [14, 21, 22]. Bioavailability in lymph-duct cannulated animals was estimated *via* the addition of the portal vein absorption of MPA to the cumulative recovery of all MPA-related derivatives in the lymph.

Plasma Stability of 2-MPA-TG and 1-MPA-MG. The rates of degradation of 2-MPA-TG and 1-MPA-MG were estimated by comparison of changes to HPLC-MS peak areas of samples taken at different time points. The percent MPA generation was calculated as the ratio of the molar concentration of MPA produced during incubation relative to the molar concentration of MPA in the prodrug at the initiation of the incubation (*i.e.* 10 μM).

Statistical Methods

Statistical differences were determined by ANOVA followed by Tukey's test or Sidak 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

Hydrolysis of 2-MPA-TG in SGF and Rat BPF

The TG mimetic prodrug 2-MPA-TG was stable in SGF (data not shown), but was very rapidly hydrolysed in freshly

collected BPF (Fig. 3 A). 2-MPA-TG hydrolysis did not occur in heat treated BPF (data not shown), consistent with a role for heat-labile enzymes in prodrug breakdown. To confirm a role for pancreatic lipase in prodrug hydrolysis and to better illustrate the molecular conversions during hydrolysis, a series of experiments were subsequently undertaken in the presence of increasing concentrations of the lipase inhibitor orlistat. These data are described in Fig. 3.

The data in Fig. 3 reveal that orlistat effectively inhibited the hydrolysis of tri-, di-, and mono-glyceride equivalents of the prodrug in a concentration dependent manner, resulting in significant changes to the profiles of 2-MPA-TG disappearance (panel A), 2-MPA-DG generation and hydrolysis (panel B), 2-MPA-MG generation and hydrolysis (panel C) and MPA generation (panel D). Authentic standards for 2-MPA-MG and 2-MPA-DG were not synthesised as the 2-isomer spontaneously converts to the corresponding 1-isomer [23]. Absolute quantification of 2-MPA-MG or 2-MPA-DG was therefore not attempted. Instead, the profile for the species corresponding to 2-MPA-MG and 2-MPA-DG were plotted based on the peak areas obtained from HPLC-MS (gated to the respective molecular weights as described in supplementary information). To illustrate changes to the relative concentration of 2-MPA-MG, the highest peak area (measured at 5 min in the orlistat free group, Fig. 3 C) was set as 100%. In support of this suggestion, at this time almost all 2-MPA-TG and 2-MPA-DG had been hydrolysed and little MPA had been generated. To illustrate changes to 2-MPA-DG, the peak area obtained at 180 min in the 200 μM orlistat group (Fig. 3 B) was assigned a percentage value that was estimated by subtracting the % remaining of 2-MPA-TG and 2-MPA-MG from 100%.

The rates of prodrug hydrolysis in blank BPF and in BPF with only 2 μM orlistat were similar and very rapid, with only a trace amount of 2-MPA-TG detected after 2 min. This coincided with the transient appearance of 2-MPA-DG and rapid production of 2-MPA-MG. At later time points the concentrations of 2-MPA-MG declined (Fig. 3 C) and were accompanied by the release of free MPA (Fig. 3 D). In contrast, 2-MPA-TG hydrolysis was slowed by the presence of

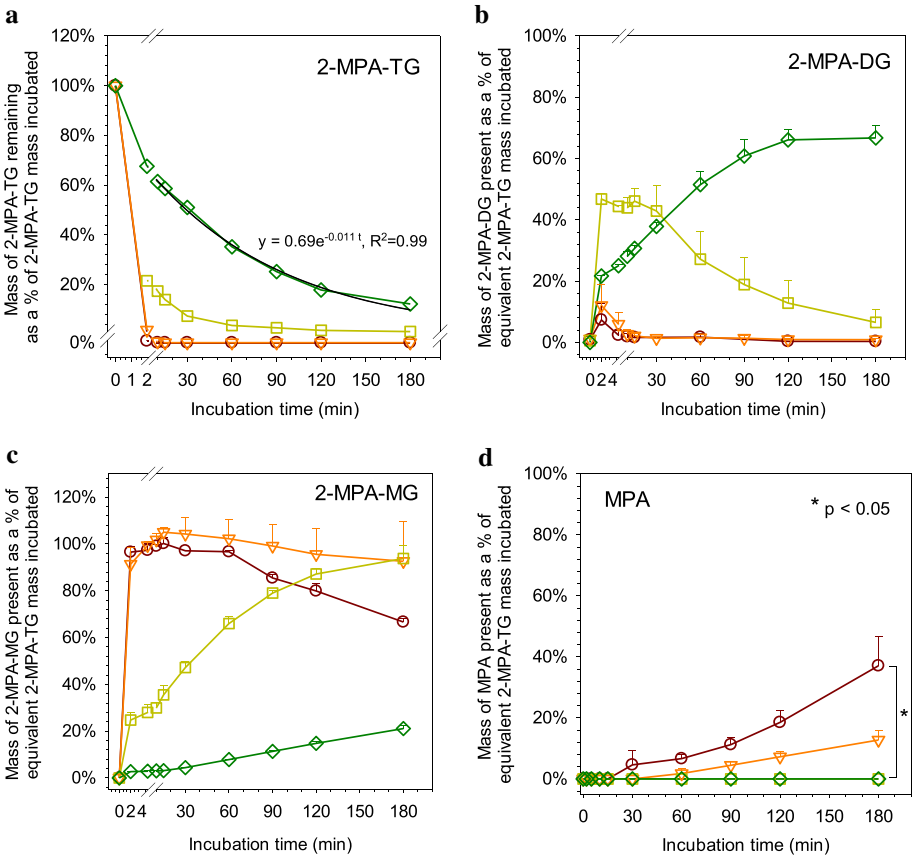

Fig. 3 Percent mass changes of 2-MPA-TG (panel (**a**)), 2-MPA-DG (panel (**b**)), 2-MPA-MG (panel (**c**)) and MPA (panel (**d**)) on *in vitro* incubation of 2-MPA-TG with rat BPF in the presence of different orlistat concentrations (0 μM-*circles*, 2 μM-*triangles*, 20 μM-*squares*, 200 μM-*diamonds*). Orlistat inhibited hydrolysis of 2-MPA-TG, 2-MPA-DG and 2-MPA-MG in a concentration dependent manner, resulting in different rates of 2-MPA-TG disappearance (panel (**a**)), different profiles of appearance and disappearance of 2-MPA-DG (panel (**b**)) and 2-MPA-MG (panel (**c**)) and subsequent hydrolysis to MPA (panel (**d**)). An exponential regression line (with $R^2 = 0.99$) is plotted in panel (**a**) to describe the first order degradation of 2-MPA-TG in the presence of 200 μM orlistat during the 2–180 min time interval, giving a first-order rate constant $k = 0.011\ \text{min}^{-1}$. Regression *lines* to describe the degradation of 2-MPA-TG in the presence of lower concentrations of orlistat were not plotted due to the very high rate of hydrolysis. Data are shown as mean ± range, $n = 2$.

20 μM orlistat and significantly slowed by 200 μM orlistat. In both cases, concentrations of 2-MPA-DG persisted for longer periods, 2-MPA-MG was more stable and MPA was not detected. In the presence of 200 μM orlistat, hydrolysis of 2-MPA-TG was incomplete after 3 h. 12% remained as 2-MPA-TG, approximately 67% converted to 2-MPA-DG and 21% to 2-MPA-MG. Free MPA was not detected. In the absence of orlistat all enzymatic processes were more rapid (and in the case of initial fatty acid lipolysis too rapid to quantify) and MPA production was evident (Fig. 3 D).

Under conditions of partial lipase inhibition (*e.g.* in the presence of 20 μM orlistat) the molecular conversions during prodrug hydrolysis were most readily illustrated (Fig. 4). Thus, in the very early stage of digestion (0–2 min), rapid disappearance of 2-MPA-TG was evident. This coincided with rapid production of the DG equivalent (2-MPA-DG). Subsequently (2–180 min), 2-MPA-TG hydrolysis continued but at a slower rate. The concentrations of 2-MPA-DG also declined, suggesting functionality as an intermediate. Simultaneously the concentrations of a species with a molecular weight consistent with 2-MPA-MG increased. No free MPA was detected under these conditions.

Lymphatic Transport and Bioavailability Studies

The transport of total MPA-related derivatives into lymph (Fig. 5 A and Table II) after intraduodenal infusion of the 2-MPA-TG prodrug has been shown previously to be significantly greater (13.4%, $p<0.05$) than that of MPA after administration of MPA alone (0.17%) [14]. After administration of MPA alone, only free MPA was detected in lymph. In contrast, after 2-MPA-TG dosing, the majority of the prodrug derivatives in lymph were detected following base catalysed hydrolysis and likely represent glyceride derivatives of the prodrug that were produced by re-esterification of the MG-derivative of the prodrug with enterocyte-sourced fatty acids [14].

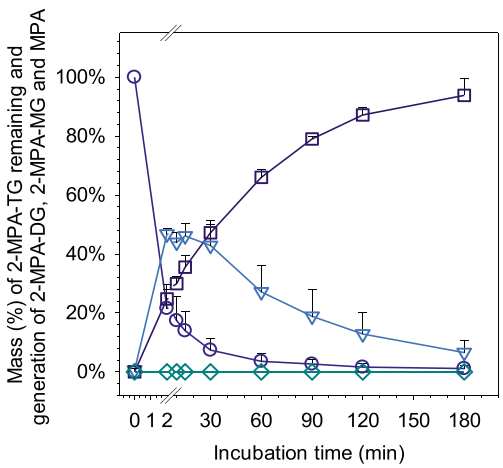

Fig. 4 *In vitro* digestion of 2-MPA-TG in rat bile and pancreatic fluid (BPF) in the presence of 20 μM of orlistat. The reduced hydrolysis rate due to lipase inhibition facilitated display of the sequential lipolysis profiles of the triglyceride mimetic prodrug 2-MPA-TG. Data are shown for 2-MPA-TG (*circles*), 2-MPA-DG (*triangles*), 2-MPA-MG (*squares*) and free MPA (*diamonds*) as mean ± range ($n=2$).

In the current study, co-administration of either a pancreatic lipase inhibitor - orlistat (0.9 mg) or a DGAT-1 inhibitor - A922500 (2 mg) with the 2-MPA-TG prodrug formulations significantly reduced the lymphatic transport of MPA and total MPA derivatives (Fig. 5 A, B and Table II). Lymphatic transport of total MPA derivatives was 10-fold lower (1.3 vs 13.4%, $p<0.05$) in orlistat-dosed rats, and concentrations of free MPA in the lymph were below the LOQ (0.1 μg/ml). A922500 almost abolished (0.32 vs 13.4%, $p<0.05$) the transport of total MPA derivatives into lymph and approximately half (0.15%) of the (albeit low levels) of lymphatically transported MPA-related materials were present as free MPA.

Previous studies [14] have shown that the only species present in the systemic circulation after intraduodenal administration of MPA or 2-MPA-TG to lymph-cannulated rats is free MPA (intact prodrug was undetectable, and re-assay of plasma after liberation of MPA using the hydrolysis method gave almost identical levels of MPA, Fig. 5 D). In the current study, neither free MPA or MPA derivatives (as assayed using the hydrolysis method) were detectable in plasma (or were present in only trace amounts) following ID infusion of 2-MPA-TG together with orlistat to lymph duct-cannulated rats (Fig. 5 D and Table II). In contrast, after administration of 2-MPA-TG with A922500, the main species present in the systemic circulation was free MPA and significant quantities of MPA derivatives were not detected after hydrolysis of plasma (Fig. 5 D). Plasma concentrations of MPA peaked at 2 h, consistent with the end of formulation infusion, and consistent with the T_{max} obtained following ID infusion of free MPA in the previous study [14]. The estimated absorption of 2-MPA-TG *via* the portal blood after co-administration with A922500 was 35.5% (*via* comparison of the plasma MPA $AUC_{0-5\ h}$ with that obtained following IV infusion of MPA (Table II). The data were therefore similar to that obtained previously following administration of MPA alone (39%).

Cumulative TG recovery in lymph following intraduodenal infusion of MPA or 2-MPA-TG in lipid formulations containing 40 mg oleic acid and 25 mg Tween 80 is shown in Fig. 5 C and Table II. The overall lymphatic transport of TG was significantly lower ($p<0.05$) in the presence of orlistat (32±4 mg) when compared to control (56±11 mg). In contrast, cumulative TG recovery in rats co-administered A922500 (48±11 mg) was not statistically different to that in the control group but was delayed during the 1 h to 3 h period. Thus, the TG mass in lymph samples collected from 1 to 2 h (7.3±1.8 mg vs 13.5±3.8 mg) and 2–3 h (5.7±3.7 mg vs 15.1±2.5 mg) was statistically significantly lower following administration of the 2-MPA-TG lipid

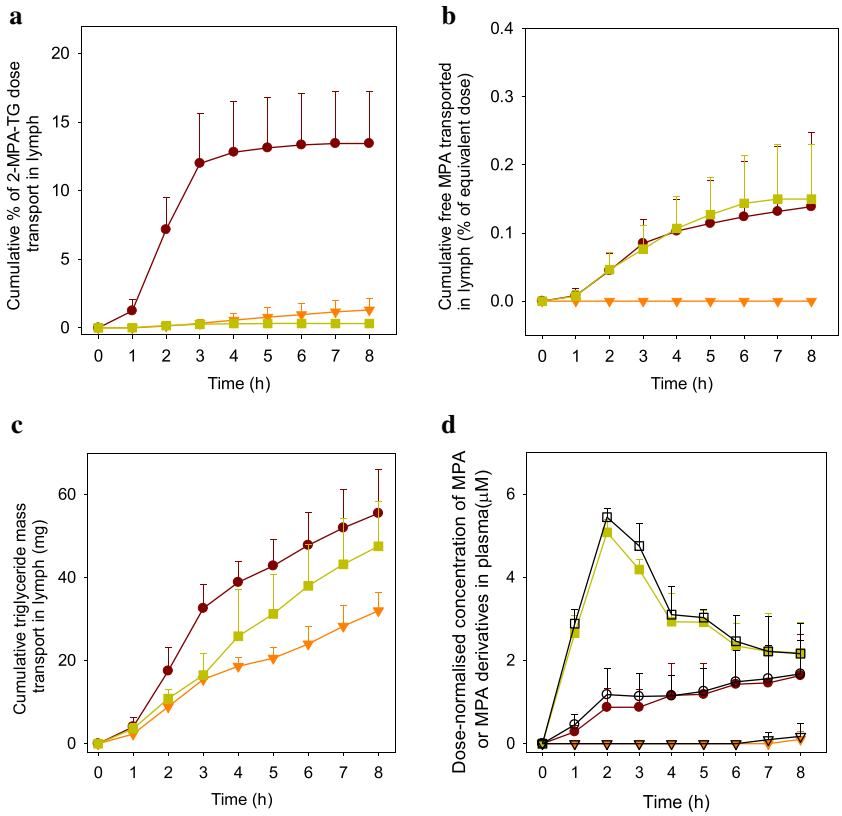

Fig. 5 Cumulative lymphatic transport of total MPA-related derivatives (% of prodrug dose, panel (**a**)), free MPA (% of MPA equivalent dose, panel (**b**)) triglyceride (mg, panel **c**), and dose-normalised plasma concentrations (panel (**d**), where doses are normalised to a 3 mg/kg equivalent dose of MPA) of free MPA (*closed symbols*) and total MPA derivatives (*open symbols*, assayed after liberation of MPA by hydrolysis) *versus* time following intraduodenal infusion (over 2 h) of MPA and 2-MPA-TG formulations to anaesthetised, mesenteric lymph-duct cannulated rats. Formulations contained 2 mg of MPA or 2-MPA-TG dispersed in 40 mg oleic acid, 25 mg Tween 80 and 5.6 ml PBS, in the absence or presence of pancreatic lipase inhibitor orlistat (0.9 mg) or DGAT-1 inhibitor A922500 (2 mg). Data are presented as Mean ± SD for 2-MPA-TG (*circles*, $n = 5$, closed circles are data reproduced from a previous study [14] for comparison), 2-MPA-TG with orlistat (*triangles*, $n = 3$) and 2-MPA-TG with A922500 (*squares*, $n = 3$).

formulation in the presence of A922500 when compared to control.

In order to probe the release of MPA from prodrug derivatives following access into the systemic circulation *via* the lymphatic system (and therefore to provide an indication of the potential of the prodrug to liberate MPA *in vivo*), a separate cohort of lymph-duct intact rats were dosed with 2-MPA-TG and plasma levels of MPA and total MPA derivatives were compared with the data obtained in the lymph-duct cannulated group (Fig. 6). As reported above, in the lymph-diverted group, free MPA was the only species present in the systemic circulation. In contrast, in lymph-intact animals both free MPA and other MPA containing species were present in the plasma. This was evidenced by comparison of the plasma MPA levels obtained in the absence and presence of plasma sample pre-processing using alkaline hydrolysis. In general, hydrolysis of plasma samples resulted in the release of higher quantities of MPA, consistent with liberation from a circulating MPA glyceride. This was most obvious at the end of formulation infusion (2 h), when the concentration of total MPA equivalents (4.50±0.97 μM) was significantly higher ($p<0.05$) than that of free MPA (2.28±0.64 μM).

Plasma Stability of 2-MPA-TG and 1-MPA-MG

The degradation of 2-MPA-TG and 1-MPA-MG in blank rat plasma was investigated to provide an estimate of the likely stability of the TG prodrug and its intermediate hydrolytic product (2-MPA-MG) in plasma and to thereby provide further clarity as to the mechanisms underlying absorption and transport of 2-MPA-TG *via* the lymphatic system and portal vein. 1-MPA-MG was employed instead of 2-MPA-MG due to the difficulty in synthesising and isolating 2-MPA-MG. Upon incubation with blank rat plasma, 2-MPA-TG was slowly hydrolysed with 76% of the prodrug remaining after 3 h, and approximately 11% of the mass of 2-MPA-TG converted to free MPA over 3 h (Fig. 7 A). The release of 2-MPA-DG and 2-MPA-MG from 2-MPA-TG was detected in trace amounts but was not quantified due to lack of authentic standards. However, the total mass of these two species is likely

Table II Summary of lymphatic transport of MPA, total MPA derivatives and triglyceride, and estimates of MPA and prodrug absorption into portal blood following intraduodenal infusion (over 2 h) of MPA or 2-MPA-TG (in the presence or absence of 0.9 mg of the lipase inhibitor orlistat or 2 mg of the DGAT-1 inhibitor A922500) to anaesthetised, mesenteric lymph-duct cannulated rats. The last column summarises the plasma PK data obtained in mesenteric lymph-duct intact rats administered 2-MPA-TG. Doses are normalized to a 3 mg/kg equivalent MPA dose and data are presented as mean ± SD

	2-MPA-TG control [a] ($n=5$)	2-MPA-TG + Orlistat ($n=3$)	2-MPA-TG + A922500 ($n=3$)	MPA [a]	2-MPA-TG Lymph-duct intact ($n=4$)
Transport of total MPA derivatives in lymph (% of dose)	13.4 ± 3.8% [b]	1.3 ± 0.8%	0.32 ± 0.14%	0.17 ± 0.12% ($n=5$)	N/A
Transport of free MPA in lymph (% of equivalent dose)	0.14 ± 0.11%	0	0.15 ± 0.08%	0.17 ± 0.12% ($n=5$)	N/A
Transport of triglyceride in lymph (mg)	56 ± 11	32 ± 4 [c]	48 ± 11	48 ± 14 ($n=5$)	N/A
Dose-normalised plasma $AUC_{0\text{-}5\ h}$ of MPA ($\mu g \times h/ml$)	1.21 ± 0.65 [d]	0 [d]	5.23 ± 0.38	5.70 ± 1.33 ($n=6$)	2.40 ± 0.60 [e]
Estimated portal blood absorption*	8.3 ± 4.4% [d]	0 [d]	35.5 ± 2.6%	38.8 ± 9.0% ($n=6$)	N/A
Estimated absolute bioavailability**	21.7%	1.3%	35.8%	39.0%	16.3%

N/A Data not obtained in lymph-duct intact rats

* Percentage of drug dose absorbed into portal blood in lymph-duct cannulated animals was estimated as the ratio of dose normalised (to 3 mg/kg) plasma $AUC_{0-5\ h}$ of free MPA (no other MPA derivatives were detected in plasma) following intraduodenal dosing of MPA or 2-MPA-TG *versus* the plasma $AUC_{0-5\ h}$ following IV dosing of MPA (plasma $AUC_{0-5\ h}$ was 14.7 $\mu g \times h/ml$) (data reported previously [14]). Absolute bioavailability could not be calculated due to second peaks in the plasma concentration profiles as described in the methods section

** For lymph-duct cannulated animals, absolute bioavailability was estimated by the sum of lymphatic transport of total MPA-related derivatives (Row 1) and estimated portal blood absorption (Row 5). For lymph-duct intact animals, absolute bioavailability of MPA was estimated as the ratio of dose normalised (to 3 mg/kg) plasma $AUC_{0-5\ h}$ of free MPA following intraduodenal dosing of 2-MPA-TG *versus* the plasma $AUC_{0-5\ h}$ following IV dosing of MPA (14.7 $\mu g \times h/ml$)

[a] Data reproduced from a previous study [14]

[b] Significantly greater in rats administered 2-MPA-TG when compared to MPA and 2-MPA-TG co-administered with orlistat and A922500 ($p < 0.05$)

[c] Significantly lower in rats co-administered orlistat and 2-MPA-TG when compared to rats administered 2-MPA-TG alone ($p < 0.05$)

[d] Significantly lower in rats administered 2-MPA-TG alone or co-administered with orlistat when compared to MPA, or 2-MPA-TG co-administered with A922500 ($p < 0.05$)

[e] Significantly lower in lymph-duct intact rats administered 2-MPA-TG when compared to that in lymph-duct cannulated rats administered MPA alone or 2-MPA-TG co-administered with A922500 ($p < 0.05$)

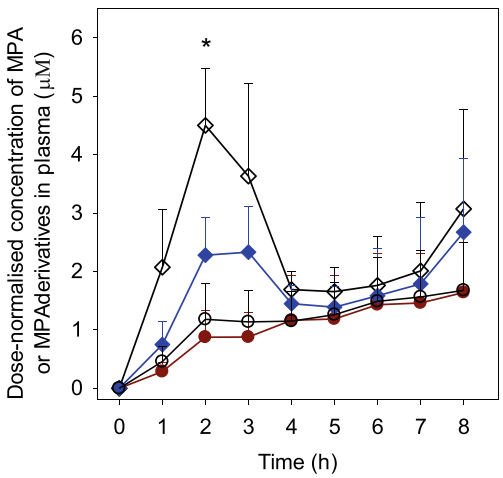

Fig. 6 Dose-normalised (normalised to a 3 mg/kg equivalent dose of MPA) plasma concentrations of free MPA (*closed symbols*) and total MPA derivatives (*open symbols*, assayed after liberation of MPA by hydrolysis) *versus* time following intraduodenal infusion (over 2 h) of 2-MPA-TG formulations to anaesthetised, mesenteric lymph-duct intact (*diamonds*, $n=4$) or cannulated (*circles*, $n=5$, closed circles were data represented from a previous study [14] for comparison) rats. Data are presented as mean ± SD. * The concentrations of total MPA derivatives in plasma were statistically significantly greater than that of free MPA in mesenteric lymph-duct intact rats at the end of prodrug infusion period (2 h).

to be less than 13% based on the difference between the mass of 2-MPA-TG remaining and the mass of MPA produced. In contrast, hydrolysis of 1-MPA-MG proceeded rapidly *via* first-order kinetics with corresponding generation of free MPA (Fig. 7 B). The degradation rate constant (k) and half-life ($t_{1/2}$, calculated as 0.693/k) calculated from the regression line ($R^2=0.96$) for 2-MPA-TG degradation were 0.0015 min^{-1} and 460 min, respectively. The k and $t_{1/2}$ calculated from the regression line ($R^2=0.99$) for 1-MPA-MG degradation were 0.084 min^{-1} and 8.3 min, respectively.

DISCUSSION

TG mimetic prodrugs have been described previously to improve oral bioavailability [24, 25], to enhance activity [13, 26] and to reduce off-target toxicity [27]. In most cases lymphatic transport has been suggested as the means by which delivery benefits accrue, although the extent of lymphatic transport has not always been measured directly [26–28]. The details of the metabolic pathways responsible for prodrug biotransformation and lymphatic transport have also not been

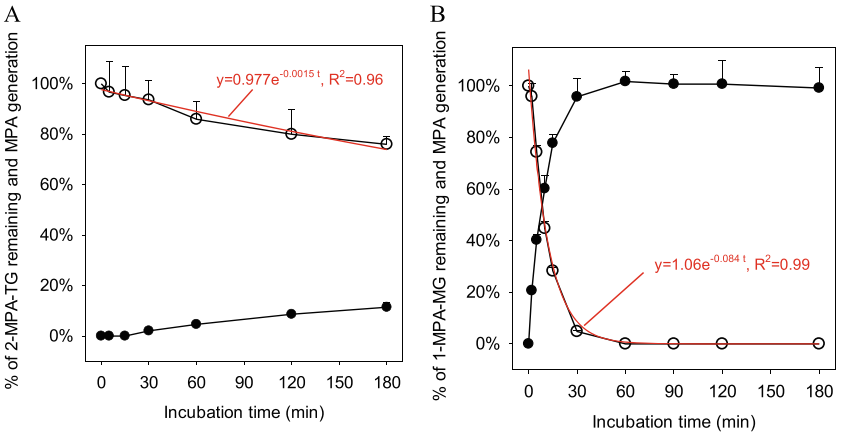

Fig. 7 Stability of 2-MPA-TG and the MG analogue 1-MPA-MG in blank rat plasma. Panel (**a**). Hydrolysis of 2-MPA-TG (*open circles*) occurred slowly and approximately 11% of the mass of 2-MPA-TG was converted to free MPA (*closed circles*) over 180 mins. A trace amount of 2-MPA-DG and 2-MPA-MG resulting from hydrolysis of 2-MPA-TG were detected but not quantified due to lack of authentic standards (data not shown). Panel (**b**). In contrast, hydrolysis of 1-MPA-MG (*open circles*) proceeded rapidly *via* first-order kinetics with corresponding generation of free MPA (*closed circles*). The elimination rate constants, *k*, calculated from the regression lines for 2-MPA-TG and 1-MPA-MG degradation were 0.0015 ± 0.0004 and 0.084 ± 0.003 min^{-1}, respectively. Data are shown as mean ± SD, $n = 3$.

described in detail previously or have only been partially described [28]. The current studies were conducted to provide a better indication of the metabolic pathways that drive lymphatic access for TG mimetic prodrugs.

Luminal Hydrolysis of 2-MPA-TG

In simulated gastric fluid (SGF), 2-MPA-TG was stable for the 3 h incubation period (data not shown). This suggests that the acidic pH of the stomach is unlikely to hydrolyse the prodrug. The prodrug is also unlikely to be absorbed from the stomach either intact, or in the form of hydrolysed derivatives, as the absorptive surface area of the stomach is small, and pre-duodenal lipases have relatively low activity [29], when compared to pancreatic lipases. The prodrug is thus likely to pass intact into the small intestine for digestion and absorption. In the event of more significant gastric digestion, dosage forms could be enteric coated.

The lipolysis of 2-MPA-TG was very rapid in freshly collected rat BPF (Fig. 3) consistent with our recent study [14] and others in which the digestion of TG mimetic prodrugs has been assessed in the presence of pancreatic lipases [28, 30]. The fact that no hydrolysis was evident in heat-inactivated BPF (data not shown) suggested that hydrolysis of the prodrug was enzyme mediated. To probe the enzymes involved, a pancreatic lipase inhibitor, orlistat was co-incubated with 2-MPA-TG in BPF. Orlistat suppressed the lipolysis of 2-MPA-TG in a concentration-dependent manner (Fig. 3), consistent with previous studies demonstrating concentration-dependent inhibition of dietary TG digestion by orlistat *via* covalent interaction with pancreatic lipase [31, 32]. Though significant, the inhibition of hydrolysis of 2-MPA-TG was incomplete even at the highest orlistat concentration (200 μM), again consistent with previous studies with TG emulsions [31]. The data suggest that pancreatic lipase is a major contributor to lipolysis of 2-MPA-TG but does not exclude the potential involvement of other hydrolytic enzymes, for example, carboxyl ester hydrolase [33] and pancreatic lipase-related protein 2 [34].

Suppression of digestion using orlistat allowed better visualisation of the intermediate lipolysis product, 2-MPA-DG, during hydrolysis of 2-MPA-TG to 2-MPA-MG. This was not easily seen during lipolysis in freshly collected BPF due to the very rapid degradation of 2-MPA-TG to 2-MPA-DG and then to 2-MPA-MG. At the highest concentration of orlistat employed (200 μM), the hydrolysis of both 2-MPA-TG and 2-MPA-DG was significantly inhibited ($p < 0.05$), with markedly delayed production of 2-MPA-DG and 2-MPA-MG respectively.

Although lipolysis was not completely inhibited *in vitro* in the presence of 200 μM orlistat, ID administration of 0.9 mg orlistat (a dose estimated to give rise to luminal concentrations of 200 μM orlistat given that the ID formulation was administered at a rate of 5.6 ml over 2 h and the flow rate of BPF is approximately 1.5 ml/h) with 2-MPA-TG led to a significant reduction in lymphatic transport of MPA and MPA-related species *in vivo*. The lymphatic transport of total prodrug derivatives decreased ten-fold following co-administration with orlistat to only 1.3% of the administered 2-MPA-TG dose (Fig. 5 A). The systemic (plasma) exposure of MPA derivatives was also negligible (Fig. 5 D). The data suggest that inhibition of hydrolysis of 2-MPA-TG precluded conversion to 2-MPA-MG, and that the intact triglyceride mimetic was unable to be efficiently absorbed (presumably due to constraints of molecular size, Fig. 8). The small lymphatically transported fraction appeared to be almost entirely in the form of glyceride derivatives of 2-MPA-MG as no free MPA (or intact prodrug) was detected in lymph. This likely resulted from absorption and

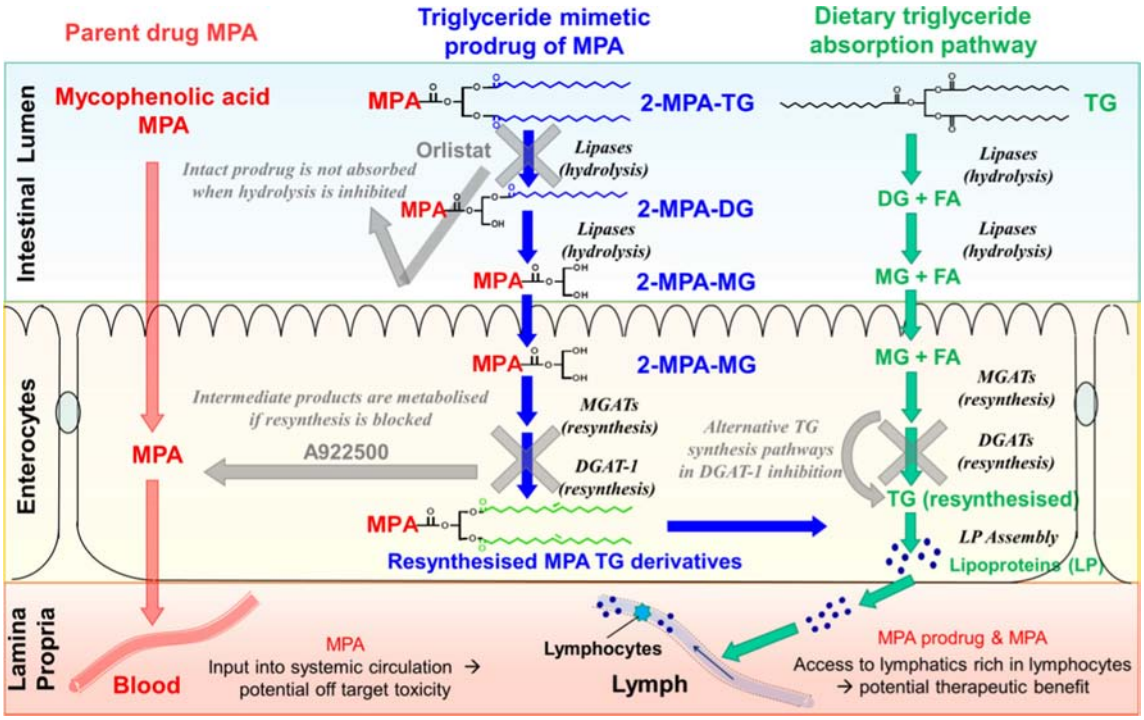

Fig. 8 Dietary TG transport routes into the intestinal lymphatics and the proposed metabolic pathways that result in access of 2-MPA-TG derivatives to blood and lymph. 2-MPA-TG was designed to promote MPA lymphatic transport by mimicking dietary TG hydrolysis and resynthesis pathways in the GI lumen and enterocytes. The current study demonstrates that pancreatic lipase and DGAT-1 are crucial for efficient lymphatic transport following administration of 2-MPA-TG. Grey crosses and arrows: The inhibition of hydrolysis by the lipase inhibitor (orlistat) results in impaired absorption of the prodrug and inhibition of resynthesis by A922500 results in redirection of drug transport to the portal blood. In contrast, the re-synthesis of dietary TG appears to be rescued by alternative metabolic pathways when DGAT-1 is inhibited by A922500.

re-esterification of the small mass of 2-MPA-MG that was formed in the GI lumen in the absence of complete inhibition of 2-MPA-TG lipolysis.

Interestingly, and somewhat unexpectedly, the recovery of TG in the mesenteric lymph following administration of the oleic acid containing formulation (Table II) with orlistat was also significantly ($p < 0.05$) reduced (32 mg) when compared to the orlistat-free 2-MPA-TG group (56 mg). This was not expected as the main lipid source in the formulation was oleic acid, not TG, and therefore did not require lipase-mediated digestion prior to absorption. The reduction in lipid transport may have occurred as a result of three possible scenarios. 1) Direct inhibition of fatty acid uptake into enterocytes by orlistat. Binding of pancreatic lipases to the brush broader membrane of enterocytes has been previously reported to facilitate cellular fatty acid uptake [35]. Covalent binding between orlistat and pancreatic lipases may therefore interfere with fatty acid absorption. 2) Inhibition of hydrolysis of phospholipids secreted in bile. A previous study [36] has described inhibition of phospholipase A2 by orlistat, (although the phospholipase used in the previous study was not sourced from pancreatic secretions), resulting in reduced absorption and reduced availability of endogenous fatty acids and lysophospholipids for lipoprotein assembly. 3) Inhibition of intracellular lipases, including enterocyte-based pancreatic triglyceride lipase [37]. These enzymes mobilise endogenous lipid droplets in the enterocyte as a source of lipids for lymphatic transport. Although orlistat is poorly absorbed into the systemic circulation [38], in the current study the local concentrations of orlistat in enterocytes may have been sufficient to inhibit intracellular lipase activity and thus reduce lipid transport *via* the lymphatic route.

Enterocyte Based Re-Esterification and Metabolism of 2-MPA-TG

Following luminal lipolysis of TG and absorption of MG and fatty acids into enterocytes, dietary MG are firstly resynthesised to DG by fatty acyl:CoA and MGATs, and subsequently to TG *via* the action of DGATs. This occurs on the endoplasmic reticulum membrane prior to assembly of TG into lipoproteins for lymphatic transport [10, 39]. In the current studies, the DGAT-1 inhibitor A922500 [40] was used as a tool to inhibit the last step of TG re-esterification (*i.e.* from DG forms of MPA to TG forms of MPA) to evaluate the necessity of resynthesis to TG in enabling lymphatic transport as well as to investigate potential links between metabolism/transport of TG and TG mimetic prodrugs.

Co-administration of the DGAT-1 inhibitor A922500 [40] with 2-MPA-TG effectively abolished lymphatic transport of MPA derivatives (Fig. 5 A) suggesting that complete resynthesis of intermediate MPA derivatives to TG forms is critical for efficient lymphatic transport. In contrast, although co-administration of A922500 suppressed the lymphatic transport of MPA and MPA-related species, plasma levels of free MPA were significantly enhanced when compared to control animals (Fig. 5 D). Importantly, no other MPA-related species were present in plasma suggesting pre-systemic conversion to free MPA. The high systemic exposure of free MPA after administration of 2-MPA-TG in the presence of A922500 therefore suggests that DG forms of the prodrug (that are blocked from re-esterification to TG and incorporation into lymph transport pathways) were metabolised in the enterocyte, releasing free MPA which was then able to access the portal blood (Fig. 8). This hypothesis is also consistent with the relatively low stability of a 1-MPA-MG derivative ($t_{1/2}$< 10 min) in rat plasma (Fig. 7 B), when compared to 2-MPA-TG (Fig. 7 A). Hydrolytic activity in enterocytes is expected to be at least as high as that in plasma due to the presence of carboxylesterases [41], monoglyceride lipases [42] and pancreatic lipases [37]. Unfortunately, the stability of 2-MPA-MG or its DG forms in plasma or enterocytes could not be probed directly as these isomers are inherently unstable (reverting to the 1-MG form) and could not be isolated. Nonetheless the data with the 1-MG isomer provide some indication that 2-MPA-MG and its DG forms may be relatively unstable in both plasma and enterocytes in the absence of incorporation into TG resynthesis pathways. In support of this suggestion, the bioavailability of MPA following ID infusion of 2-MPA-TG + A922500 or MPA was similar (35.8 vs 39.0%, Table II), suggesting that absorption of 2-MPA-MG was similarly efficient to that of parent MPA (which is also consistent with similarities in molecular properties *e.g.* MW 394 vs 320, cLogP 2.2 vs 2.9 respectively), and that incompletely re-esterified MPA derivatives (*i.e.* DG forms) are readily hydrolysed in the presence of A922500 resulting in the liberation of free MPA.

Interestingly, the absolute bioavailability of MPA in lymph cannulated animals following ID infusion of 2-MPA-TG (21.7% - calculated from the % MPA absorption into the blood plus the % dose recovered in lymph)) was lower than that after co-administration of 2-MPA-TG + A922500 (35.8%) or dosing of MPA alone (39.0%). This seems unlikely to have resulted from reduced absorption of 2-MPA-TG from the GI lumen (since luminal conditions were essentially the same), and also does not appear to result from incomplete liberation of MPA from any MPA-TG that might be in the systemic circulation in lymph cannulated animals (since MPA plasma levels were the same after alkaline hydrolysis of plasma to liberate any esterified MPA, Fig. 5 D). Instead it may reflect sequestration of the re-esterified prodrug in lipid stores in enterocytes prior to complete release into the blood or lymph over an extended period. This temporary storage effect has been previously documented for dietary TGs [43, 44].

Specific Metabolic Sensitivity of TG and TG Mimetic Prodrug to DGAT Inhibition

Although lymphatic drug transport was essentially abolished by co-administration with A922500, lymphatic transport of TG was only delayed and the mass of TG transported was not significantly different over the 8 h collection period. This result for TG transport is consistent with previous publications [45, 46] showing that TG absorption and chylomicron synthesis is not decreased quantitatively in DGAT-1 deficient mice but may be delayed. In these previous studies, alternate enzymes (DGAT-2 and diacylglycerol transacylase, DGTA) were suggested to be responsible for intestinal TG synthesis in the absence of DGAT-1 activity [39, 46] (Fig. 8). In the current study, these alternate pathways appear to have facilitated TG lipid transport but were seemingly unable to rescue lymphatic drug transport. The data suggest that in contrast to DG, DGAT-2 or DGTA are unable to re-esterify the modified DG forms of MPA.

The difference in metabolic pathways for natural DGs and DG analogues of MPA suggests that whilst DG prodrug analogues are able to mimic endogenous DGs with respect to recognition by DGAT-1, they appear to be relatively poor substrates for DGAT-2 and DGTA. This in turn appears to have resulted in the release of MPA due to exposure to hydrolytic enzymes in enterocytes such as carboxylesterases [41], monoglyceride lipases [42] and pancreatic lipases [37]. Blocking DGAT-1 and therefore instituting the requirement for access to DGAT-2 is also likely to increase the potential for prodrug hydrolysis since DGAT-1 is active on the luminal side of the endoplasmic reticulum [39, 47] (and therefore readily available) whereas transport to the cytosolic side of the endoplasmic reticulum is required to access DGAT-2 [39, 48], dictating greater time for access to competitive enzyme systems. Finally, TG synthesis *via* the DGTA pathway requires catalysis of two *sn*-1,2 or *sn*-2–3 DG molecules to form TG plus 2-MG *via* molecular transfer of a fatty acid in the 1- or 3- position [49]). Within this pathway, donation of a fatty acid from the MPA-DG analogue is required and generates 2-MPA-MG. In the absence of rapid resynthesis, this MG derivative may be susceptible to hydrolysis back to MPA (Fig. 7).

Reversion to MPA from TG Derivatives Following Access into the Systemic Circulation

Typically, prodrugs need to revert to the parent drug in order to exert their pharmacological effect. This is expected to be

the case for the MPA prodrug described here as preliminary studies have shown that unlike MPA, the TG mimetic prodrug has little effect on lymphocyte proliferation *in vitro* (data not shown). The ability to release MPA from TG derivatives *in vivo* is therefore an important indicator for the potential of this prodrug strategy to improve therapeutic efficacy. In a previous study [14] we reported that administration of 2-MPA-TG resulted in a ten-fold increase in free MPA exposure in mesenteric lymph nodes (when compared to administration of an equimolar quantity of free MPA) suggesting that MPA was liberated within lymph nodes. However differences in lymphatic transport of total MPA-related material between administration of MPA-TG and MPA were far higher (~80 fold), suggesting that prodrug conversion may be incomplete. In the current study we therefore further explored MPA liberation within the systemic circulation (rather than simply within the lymphatics). This was undertaken to give a better indication of the potential for *in vivo* liberation of MPA and has implications for the use of TG mimetic prodrugs for applications beyond targeting lymph-resident activity sites (such as the use to overcome first pass metabolism).

The exposure of MPA and MPA derivatives in the systemic circulation following administration of 2-MPA-TG to mesenteric lymph-duct cannulated vs intact rats was compared to profile the liberation of MPA from prodrug derivatives that entered the blood circulation *via* the lymph. As shown previously [14] the only species present in the systemic circulation after intraduodenal administration of 2-MPA-TG to lymph-duct cannulated rats was free MPA, since re-assay of plasma after liberation of MPA using the alkaline hydrolysis method gave almost identical levels of MPA (Fig. 6). In lymph-diverted rats, the appearance of MPA in plasma was therefore expected to be almost exclusively due to the portal blood absorption of MPA. Based on comparison of $AUC_{0-5\ h}$ as described previously, the extent of portal blood absorption was estimated to be 8.3% of dose, and this appeared to represent the proportion of MPA that had been generated in the GI lumen or in enterocytes, most likely due to instability of the intermediate 2-MPA-MG. In contrast, entry of prodrug derivatives into the systemic circulation *via* the lymph gave rise to an increase in plasma MPA levels (mainly in the first 3 h) in lymph-intact animals (Fig. 6), when compared to data in lymph cannulated animals. This suggests that the lymph-carried prodrug derivatives that entered the plasma were able to release MPA *in vivo*, presumably mediated by lipoprotein lipase and monoglyceride lipase [50] that are responsible for dietary TG metabolism. Interestingly, however, re-assay of plasma samples after alkaline hydrolysis in lymph intact animals resulted in an approximate doubling of plasma MPA suggesting that the reversion to MPA from prodrug derivatives occurred slowly (Fig. 6). Furthermore, by subtracting the % MPA absorption into the blood in lymph cannulated rats (8.3%, Table II) from the bioavailability of MPA in lymph intact rats (16.3%, Table II), an estimate of the increase in MPA bioavailability due to entry into the systemic circulation *via* the lymph can be attained (8.0%). This increase only accounts for 60% of the extent of lymphatic transport of total MPA derivatives (13.4±3.8%) and suggests that the release of MPA from prodrug derivatives post entry into the systemic circulation was incomplete (realising that this estimation requires the assumption that the clearance of IV dosed MPA was the same as that of MPA generated from prodrug derivatives). The slow and potentially incomplete parent drug release from 2-MPA-TG indicates that optimisation of the *in vivo* reversion properties of glyceride based lymph directed prodrugs may further benefit the utility of this prodrug strategy.

CONCLUSIONS

The studies described here confirm the importance of two steps in the TG metabolic pathway that are critical to efficient intestinal lymphatic transport of a TG mimetic prodrug (Fig. 8). Firstly, luminal hydrolysis is required to generate the MG equivalent of the prodrug, and this is required for absorption. Evidence to support this suggestion is provided by data showing that inhibition of lipolysis using orlistat resulted in significantly lower lymphatic recovery and negligible absorption into the systemic circulation. Subsequently, complete re-esterification of the absorbed MG derivative 2-MPA-MG to the 2-MPA-TG derivative is required to allow lymphatic transport since inhibition of DGAT-1 almost completely blocked lymphatic drug transport and instead appeared to promote hydrolysis of the prodrug intermediate and redirection of MPA into the portal blood. Finally, analysis of prodrug reversion post lymphatic transport suggests that liberation of MPA in the systemic circulation occurs, but is incomplete, suggesting future directions for prodrug optimisation. The data therefore suggest that ideal TG mimetic prodrugs should seek to enable luminal hydrolysis (at the 1- and 3- positions on the glycerol backbone), to maximise stability of the MG derivative in GI lumen and enterocyte, to retain substrate specificity for MGAT and DGAT resynthetic enzymes in the enterocyte, and to facilitate parent drug release in the vicinity of targets.

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