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Targeted delivery of a model immunomodulator to the lymphatic system: Comparison of alkyl ester versus triglyceride mimetic lipid prodrug strategies

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ABSTRACT

A lipophilic prodrug approach has been used to promote the delivery of a model immunomodulator, mycophenolic acid (MPA), to the lymphatic system after oral administration. Lymphatic transport was employed to facilitate enhanced drug uptake into lymphocytes, as recent studies demonstrate that targeted drug delivery to lymph resident lymphocytes may enhance immunomodulatory effects. Two classes of lymph-directing prodrugs were synthesised. Alkyl chain derivatives (octyl mycophenolate, MPA-C8E; octadecyl mycophenolate, MPA-C18E; and octadecyl mycophenolamide, MPA-C18AM), to promote passive partitioning into lipids in lymphatic transport pathways, and a triglyceride mimetic prodrug (1,3-dipalmitoyl-2-mycophenoloyl glycerol, 2-MPA-TG) to facilitate metabolic integration into triglyceride deacylation–reacylation pathways. Lymphatic transport, lymphocyte uptake and plasma pharmacokinetics were assessed in mesenteric lymph and carotid artery cannulated rats following intraduodenal infusion of lipid-based formulations containing MPA or MPA prodrugs. Patterns of prodrug hydrolysis in rat digestive fluid, and cellular re-esterification *in vivo*, were evaluated to examine the mechanisms responsible for lymphatic transport. Poor enzyme stability and low absorption appeared to limit lymphatic transport of the alkyl derivatives, although two of the three alkyl chain prodrugs — MPA-C18AM (6-fold) and MPA-C18E (13-fold) still increased lymphatic drug transport when compared to MPA. In contrast, 2-MPA-TG markedly increased lymphatic drug transport (80-fold) and drug concentrations in lymphocytes (103-fold), and this was achieved *via* biochemical incorporation into triglyceride deacylation–reacylation pathways. The prodrug was hydrolysed rapidly to 2-mycophenoloyl glycerol (2-MPA-MG) in the presence of rat digestive fluid, and 2-MPA-MG was subsequently re-esterified in the enterocyte with oleic acid (most likely originating from the co-administered formulation) prior to accessing the lymphatics and lymphocytes. Importantly, after administration of 2-MPA-TG, the concentrations of free MPA in the mesenteric lymph nodes were significantly enhanced (up to 28 fold) when compared to animals administered equimolar quantities of MPA, suggesting the efficient conversion of the esterified prodrug back to the pharmacologically active parent drug. The data suggest that triglyceride mimetic prodrugs have potential as a means of enhancing immunotherapy *via* drug targeting to lymphocytes and lymph nodes.

1. Introduction

Lymphocytes are the primary cellular component of the lymphatic system and play central roles in immune function including immune surveillance, allotransplant rejection, autoimmunity, and initiation of physiological responses to pathogen and carcinoma invasion. Lymphocytes are therefore the target site for many immunomodulators [1–4]. Most current immunomodulator treatments, however, have little intrinsic affinity for lymphocytes and therapy is often limited by off-target toxicity [5]. Lymphocytes are concentrated within the lymphatics and lymph nodes, and targeted delivery of drugs to the lymphatic system is expected to co-localise high concentrations of drugs and lymphocytes in a single compartment. Delivery of high concentrations of immunosuppressants to the lymphatics therefore has the potential to enhance local [6] and systemic [7] immunosuppression.

The lymphatic system is the main conduit for the transport of dietary lipids (such as triglyceride, TG) and lipid soluble vitamins from the intestine to the systemic circulation [8,9]. After ingestion, dietary TG is hydrolysed in the GI lumen, absorbed into enterocytes, resynthesised to TG, assembled into lipoproteins, and ultimately taken up into the

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intestinal micro-lymphatics (see Fig. 1). Within the lymphatics, lipids in the form of lipoproteins, drain through a series of capillaries, nodes and ducts, finally emptying into the systemic circulation at the junction of the left subclavian vein and internal jugular vein. Highly lipophilic compounds (usually log P > 5, long-chain TG solubility >50 mg/g) associate with lipoproteins on passage across enterocytes and in doing so achieve directed uptake into the intestinal lymphatic system in parallel with dietary (or formulation-derived) TG (see Fig. 1) [9]. Drug transport into the intestinal lymphatics is therefore enhanced by the coadministration of lipids with intrinsically highly lipophilic drugs (e.g. CP524,515 [10] and CRA13 [11]) or *via* the administration of lipophilic prodrugs (e.g. prodrugs of testosterone [12,13], chlorambucil [14], L-dopa [15], LK-903 [16], valproic acid [17], nicotinic-acid and naproxen [18]).

Most currently used immunomodulators are not sufficiently lipophilic to have intrinsic affinity for developing lymph lipoproteins and are therefore not lymphatically transported. For example, cyclosporine A and sirolimus are poorly transported *via* the lymphatics (1.3% and <1% dose [7]) following either oral or intraduodenal administration. The current study therefore aimed to target the delivery of a widely used immunosuppressant, mycophenolic acid (MPA, cLog P 2.9, cLog $D_{7.4}$ 0.26), to lymphocytes in the intestinal lymphatics using lipophilic prodrug approaches. MPA was employed as a model compound as it exerts its immunosuppressant effects *via* the inhibition of a cytoplasmic enzyme (inosine-5-monophosphate dehydrogenase, IMPDH) that is involved in purine biosynthesis and is required for DNA replication in, and proliferation of, lymphocytes [21]. Two classes of prodrugs (Fig. 2) were explored for their potential to promote MPA delivery to lymphocytes in the lymphatics. The first comprised three highly lipophilic alkyl derivatives of MPA: a C8 ester, a C18 ester, and a C18 amide with cLog P values of 7.1, 12.4 and 11.2 respectively. The alkyl derivative prodrugs were expected to promote lymphatic transport by passive partitioning into developing lipoproteins in enterocytes due to their physicochemical affinity for lipophilic environments. The second class of prodrug was a TG mimetic prodrug (1,3-dipalmitoyl-2-mycophenoloyl glycerol, 2-MPA-TG, cLog P 17.8). The TG mimetic was designed to incorporate into the processes of TG hydrolysis in the GI lumen (to generate the monoglyceride derivative of 2-MPA-TG) followed by re-esterification in the enterocyte (to reform TG derivatives of MPA), and finally to access the lymphatics in association with intestinal lipoproteins.

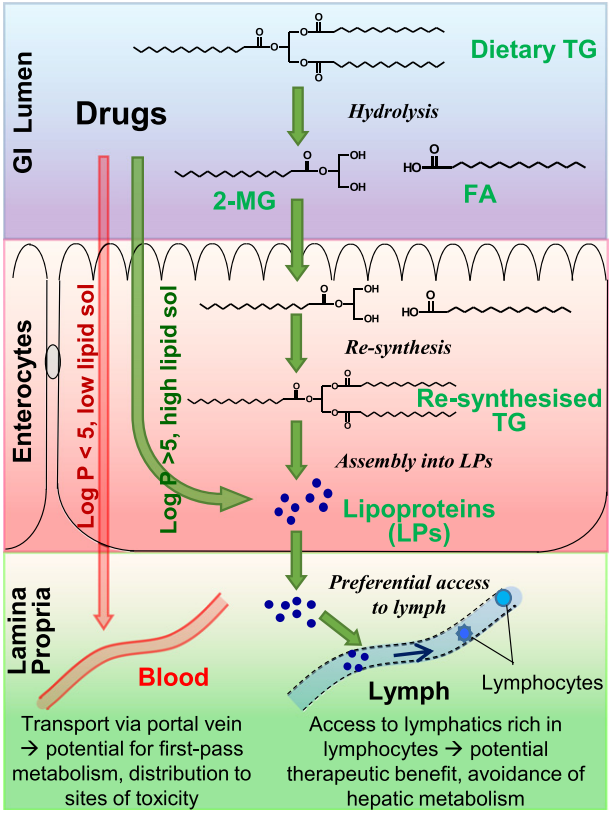

Fig. 1. Triglyceride (TG) transport into the intestinal lymphatics and drug transport into the lymph *via* association with lymph lipoproteins. After ingestion, dietary TG is hydrolysed by digestive lipases in the gastrointestinal (GI) lumen to release 2-monoglyceride (2-MG) and fatty acids (FA). 2-MG and FA are absorbed into enterocytes, re-synthesised to TG and assembled to form the core of lipoproteins (LPs). LPs are subsequently exocytosed from the basolateral aspect of enterocytes into the underlying lamina propria. From the lamina propria, access of LPs to the underlying blood capillaries is limited by the barrier properties of the vascular endothelium. Instead, LPs preferentially access the intestinal micro-lymphatics *via* passage through larger inter-endothelial gaps [19] and potentially *via* active transcellular transport pathways [20]. Highly lipophilic compounds may partition into LPs on passage across enterocytes to gain access to the intestinal lymphatic system in parallel with dietary TG [9].

2. Materials and methods

2.1. Chemicals

The mycophenolic acid (MPA) prodrugs — MPA-C8E, MPA-C18E, MPA-C18AM and 2-MPA-TG were synthesised from MPA (>98%, AK Scientific, Palo Alto, CA, USA) as described in the Supplementary information. Ketoprofen (internal standard), oleic acid and Tween 80 were purchased from Sigma-Aldrich, MO, USA. Acetonitrile for liquid chromatography was purchased from Merck Pty Limited, VIC, Australia. Ultrapure water was obtained from a Milli-Q™ system (Millipore, MA, USA). All other chemicals were analytical grade or above.

2.2. Preparation of lipid formulations for in vitro and in vivo experiments

Lipid-based formulations of MPA or MPA prodrugs were assembled as described previously [22]. Briefly, ~2 mg of MPA-C8E, MPA-C18E, 2-MPG-TG, or 1 mg of MPA-C18AM (the dose of MPA-C18AM was limited by low drug solubility in the formulation), 40 mg oleic acid and 25 mg Tween 80 were mixed in a glass vial and incubated at 37 °C for 12–18 h to equilibrate. An aqueous phase consisting of 5.6 ml phosphate buffered saline (PBS, pH 7.4) was subsequently added to the lipid phase (MPA was dissolved in PBS for the preparation of MPA containing formulations) and the formulation emulsified by ultrasonication 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. Drug/prodrug concentrations in all formulations were verified using HPLC–MS. No prodrug degradation products (including free MPA or 2-MPA-MG) were found in the formulations on the day of experiments.

2.3. Lymphatic transport, lymph node exposure, and bioavailability 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. Male Sprague–Dawley rats (280–320 g) were maintained on a standard diet and fasted overnight with free access to water prior to experiments. Anaesthetised rats (using ketamine, xylazine and acepromazine [23]) were placed on a heated pad at 37 °C and cannulas were inserted into the duodenum (for formulation administration and rehydration), mesenteric lymph duct (for lymph collection) and carotid artery (for blood collection) as previously described [22]. Post-surgery, rats were re-hydrated for 0.5 h *via* intraduodenal infusion of normal saline at 2.8 ml/h [22]. The lipid formulations were infused into the duodenum at 2.8 ml/h for 2 h, after which the infusion was changed to 2.8 ml/h normal saline for the remainder of the experiment. Lymph was continuously collected for 8 h into pre-weighed Eppendorf tubes containing 10 μl of 1000 IU/ml heparin. The collection tubes were changed hourly and lymph flow was measured gravimetrically. Aliquots