Intestinal delivery in a long-chain fatty acid formulation enables lymphatic transport and systemic exposure of orlistat

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Abstract

Orlistat is a pancreatic lipase (PL) inhibitor that inhibits dietary lipid absorption and is used to treat obesity. The oral bioavailability of orlistat is considered zero after administration in standard formulations. This is advantageous in the treatment of obesity. However, if orlistat absorption could be improved it has the potential to treat diseases such as acute and critical illnesses where PL transport to the systemic circulation via gut lymph promotes organ failure. Orlistat is highly lipophilic and may associate with intestinal lipid absorption pathways into lymph. Here we investigate the potential to improve orlistat lymph and systemic uptake through intestinal administration in lipid formulations (LFs). The effect of lipid type, lipid dose, orlistat dose, and infusion time on lymph and systemic availability of orlistat was investigated. After administration in all LFs, orlistat concentrations in lymph were greater than in plasma, suggesting direct transport via lymph. Lymph and plasma orlistat derivative concentrations were ~8-fold greater after administration in a long-chain fatty acid (LC-FA) compared to a lipid-free, LC triglyceride (LC-TG) or medium-chain FA (MC-FA) formulation. Overall, administration of orlistat in a LC-FA formulation promotes lymph and systemic uptake which may enable treatment of diseases associated with elevated systemic PL activity.

Keywords: Lipid Formulation, Orlistat, Lipase inhibitor, Lymphatic, Fatty Acid, Drug Delivery

Abbreviations: acetonitrile (ACN), area under the curve (AUC), area under the first moment curve (AUMC), bioavailability (BA), chylomicron (CM), clearance (Cl), fatty acid (FA), intravenous (IV), lipid formulation (LF), long-chain fatty acid (LC-FA), long-chain triglyceride (LC-TG), medium-chain fatty acid (MC-FA), monoglyceride (MG), pancreatic lipase (PL), phosphate buffered saline (PBS), poorly water soluble drugs (PWSDs), triglyceride (TG)

1 Introduction

Orlistat is used clinically to treat obesity. It promotes weight loss by inhibiting gastric and pancreatic lipase (PL) activity locally in the intestinal lumen (Sternby et al., 2002) and thus the digestion and absorption of dietary lipids (Guerciolini, 1997). While the current clinical application of orlistat is for the treatment of obesity, it has the potential to provide therapy for other illnesses in which elevated PL activity in the lymph, blood or organs and tissues beyond the intestine contribute to disease progression, such as acute pancreatitis (Mittal et al., 2009; Navina et al., 2011; Patel et al., 2015) and trauma-haemorrhagic shock (Morishita et al., 2012; Qin et al., 2012). Moreover, orlistat has the potential to treat cancers via inhibition of fatty acid synthase that is overexpressed in tumours (Carvalho et al., 2008; Zhao et al., 2013). However, the absorption and bioavailability of orlistat is considered to be essentially zero after oral administration in current standard clinical formulations (that consist of solid powders in a hard gelatin capsule) in either the fed or fasted state (Zhi et al., 1995; Zhi et al., 1996). The bioavailability of orlistat is thought to be limited by low absorption and high first-pass metabolism (Zhi et al., 1999). For example, in healthy volunteers and human patients, the plasma concentrations of orlistat are negligible after oral administration in standard capsule formulations with more than 97% of the dose recovered in the faeces of healthy volunteers, and 83.1% in a non-metabolised form (Zhi et al., 1995; Zhi et al., 1999). The low absorption and systemic availability of orlistat after oral administration limits its potential to treat systemic conditions.

Orlistat (Fig 1 A) is a poorly water soluble and highly lipophilic drug with a cLogP of 8.1 (Wishart et al., 2006) and high long chain triglyceride (LC-TG) solubility (110 mg/ml) (Gade and Hurkadale, 2016). Previous studies have shown that co-administration of highly lipophilic drugs with a logP >5 and LC-TG solubility >50 mg/g (such as halofantrine (Trevaskis et al., 2013), CP524,515 and CP532,623 (Trevaskis et al., 2010), and Org 49209 (Caliph et al., 2014)) with lipid formulations (LFs) enhances their absorption and promotes their transport from the intestine via the lymphatic system (Han et al., 2015; Williams et al., 2013). This is because lipid co-administration leads to the formation of colloidal lipid structures within the intestinal lumen that enhance the solubilisation and absorption of highly lipophilic drugs. Additionally, lymphatic transport is increased because highly lipophilic drugs associate with intestinal lymph lipid transport pathways that are promoted by lipid co-administration (Trevaskis et al., 2008). For example, lipids such as TGs are hydrolysed to monoglycerides (MGs) and fatty acids (FAs) in the gastrointestinal lumen, absorbed into enterocytes, and resynthesized to triglycerides (TGs) that are assembled into chylomicrons (CMs). The CMs are exocytosed from enterocytes into the lamina propria which is supplied with blood capillaries and specialised lymphatic capillaries (lacteals) (Han et al., 2015; Mansbach and

Gorelick, 2007; Tso and Balint, 1986). The size of the CMs (200-1,000 nm) precludes access across the blood vasculature in which the adjacent endothelial cells are held together by tight junctions. CMs are therefore specifically transported from the intestine via the lymphatics as the lacteals are more permeable due to the presence of open button-like junctions between endothelial cells (Zhang et al., 2018).

We thus hypothesised that orlistat would be lymphatically transported if administered with an appropriate LF (i.e. dose and combination of lipids and lipophilic excipients). In this respect, long-chain (LC) lipids were expected to more effectively promote lymphatic transport of orlistat when compared to medium-chain (MC) length lipids (<12 carbons). This reflects the fact that MC lipids are mainly transported from the intestine directly via the blood circulation (Trevaskis et al., 2015b; Williams et al., 2013) whereas LC length lipids (>14 carbon) are assembled into intestinal CMs and stimulate intestinal lymphatic lipid transport. This is supported by previous studies showing significant increases in lymphatic drug transport on administration with LC lipids when compared to MC lipids (Trevaskis et al., 2013; Trevaskis et al., 2020). Since orlistat inhibits TG digestion and absorption, we also hypothesised that a LC-TG based formulation containing orlistat may less effectively facilitate lymphatic lipid and drug transport when compared to a LF containing pre-digested lipids (i.e. FAs) as the LC-TG would remain undigested and not absorbed.

Promoting lymphatic transport of orlistat through administration with an appropriately designed LF may additionally increase its systemic exposure and oral bioavailability by facilitating avoidance of first-pass metabolism (Shackleford et al., 2003; Trevaskis et al., 2009). After intestinal absorption, most drugs are transported from the intestine via the portal vein which flows to the liver leading to drug exposure to first-pass metabolism prior to entry to the systemic circulation. In contrast, for drugs that are incorporated into the intestinal lymphatic transport pathways, drug transport to the blood circulation is achieved via the lymphatic capillaries, vessels and nodes that converge at the thoracic lymph duct which empties directly into the systemic circulation at the subclavian vein thus bypassing the liver (Trevaskis et al., 2015b).

The current study therefore investigated the potential to enhance the absorption, lymphatic transport and systemic availability of intestinally administered orlistat via co-administration with LFs. The utility of different types of formulations: MC-FA, LC-FA, LC-TG and a lipid-free (control) formulation was compared. The impact of drug dose on orlistat absorption and lymphatic transport was also evaluated since orlistat may inhibit lipid digestion and absorption, and thus its own absorption and lymphatic transport at higher doses. In addition, the potential to prolong the time for which therapeutic orlistat concentrations are

achieved in lymph was investigated via continuous enteral infusion of orlistat. In particular, it was determined if the LFs were able to support the delivery of orlistat into lymph at concentrations that are expected to be therapeutically active (0.99 µg/ml orlistat in lymph according to (Qin et al., 2012)). Notably, continual enteral infusion is feasible in patients with acute and critical illnesses such as acute pancreatitis and trauma-haemorrhagic shock since patients are hospitalised and commonly administered enteral feeds (Banks and Freeman, 2006; Windsor et al., 1998).

2 Materials and methods

2.1 Chemicals

Orlistat was purchased from Sapphire Bioscience Pty. Ltd. (NSW, Australia). Oleic acid, octanoic acid, olive oil, Tween 80, formic acid and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich, MO. USA. Acetonitrile (ACN) for sample preparation and liquid chromatography was purchased from Merck Pty. Ltd. Ultrapure water was obtained from a Milli-Q^{TM} system (Millipore, MA, USA).

2.2 Experimental design

A range of LFs, as detailed in Table 1, were prepared to evaluate the effect of lipid type, lipid dose, and orlistat dose on the intestinal absorption, lymphatic transport and systemic availability of orlistat. Lymphatic transport was determined in a triple cannulated anaesthetised rat model with cannulas inserted into the mesenteric lymph duct, carotid artery and duodenum for lymph collection, blood collection, and formulation infusion, respectively. Systemic availability was determined in lymph-intact anaesthetised rats with cannulas inserted into the carotid artery and duodenum for blood collection and formulation infusion, respectively. To enable the calculation of absolute bioavailability an additional group was administered orlistat intravenously via a jugular vein cannula, and blood samples were collected from a carotid artery cannula over time. In this group the lymph duct was kept intact.

To determine the effect of lipid type on orlistat lymphatic transport and systemic availability, three different types of lipids were tested: oleic acid (i.e. LC-FA), octanoic acid (i.e. MC-FA), and olive oil (i.e. LC-TG). In addition, a lipid-free formulation was evaluated as a control formulation. After determining that co-administration with oleic acid (LC-FA) supported the highest absorption and lymphatic transport of orlistat, further investigations into the effect of lipid dose and orlistat dose focussed on the use of oleic acid based formulations. Three lipid doses were tested: 0 mg oleic acid, 40 mg oleic acid and 80 mg oleic acid with the drug dose kept consistent at 8 mg/kg. Three orlistat doses were subsequently tested (0.15 mg/kg, 8 mg/kg and 50 mg/kg), with the high dose (50 mg/kg) administered with 80 mg oleic acid and the lower doses administered with 40 mg oleic acid, since more oleic acid was required to

solubilise the high orlistat dose. A 50 mg/kg dose of orlistat has previously been shown effective in the treatment of acute pancreatitis, and hence was investigated here (Patel et al., 2015). The impact of infusion time on the lymphatic transport of orlistat was also investigated by comparing the administration of 1 mg/h orlistat and 20 mg/h oleic acid over 2 h versus 8 h.

Table 1

Experimental groups including formulations, administration details and studies conducted. All formulations were prepared in 5.6 ml 0.2% Tween 80 in PBS and infused intraduodenally at a rate of 2.8 ml/h for 2 h unless otherwise specified. Lymphatic uptake studies were conducted for all experimental groups except for the intravenously administered group in the last row. Bioavailability studies were only conducted in the groups indicated below.

Orlistat dose	**Lipid type & dose**
8 mg/kg	No lipid control
8 mg/kg	40 mg Octanoic Acid
8 mg/kg[a]	40 mg Olive Oil
8 mg/kg[a]	40 mg Oleic Acid
0.15 mg/kg	40 mg Oleic Acid
8 mg/kg	80 mg Oleic Acid
50 mg/kg	80 mg Oleic Acid
32 mg/kg[b]	160 mg Oleic Acid
0.4 mg/kg[c]	120 mg Soybean Oil

[a] Bioavailability studies were conducted

[b] Formulation was prepared in 22.4 ml PBS and the total volume and lipid/drug mass was infused intraduodenally over 8 h

[c] Formulation was prepared in 1 ml 2% glycerol, 1% egg phosphatidyl choline and infused intravenously at a rate of 0.2 ml/min for 5 min.

2.3 Preparation of orlistat lipid-based and control formulations

For the LFs for duodenal infusion, orlistat was added into a glass vial, mixed with lipid (oleic acid, olive oil or octanoic acid) and Tween 80 at the required concentrations for each formulation (as per Table 1) and was incubated at 37^{o}C for 2 h followed by 10-12 h incubation at room temperature. Subsequently, the required volume of phosphate buffered saline (PBS, pH 7.4) was added to the lipid phase (i.e. the orlistat, lipid and Tween 80 mixture). The formulations were emulsified with a Misonix XL 2020 ultrasonic processor (Misonix,

Farmingdale, NY, USA) fitted with a 3.2-mm microprobe tip. Ultrasonication was performed at 240 µm amplitude and 20 kHz frequency for 2 min at room temperature.

For the lipid-free control formulation, ~2 mg orlistat was dissolved in 112 mg of Tween 80. Subsequently, 5.6 ml of PBS (pH 7.4) was added. The formulation was the ultrasonicated as described for the LFs. For the IV formulation, 0.1 mg of orlistat was dissolved in 120 mg soybean oil in a glass vial. Subsequently, 1 ml of 2% glycerol and 1% egg phosphatidyl choline in water was added to the lipid phase and the formulation was ultrasonicated as described for the LFs. The drug concentration in all formulatons was verified by LC-MS/MS as described below. The particle size, polydispersity index and zeta potential (surface charge) of the formulations was determined by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) on a Malvern ZetaSizer NanoZA (Malvern Panalytical, UK). The data is summarised in Table 2.

Table 2

Particle size (expressed as mean diameter by intensity), polydispersity index and zeta potential of the formulations used for enteral administration in the studies. Data are mean ± SD for 3 formulations measured in triplicate.

Formulation	LC-FA	LC-TG	MC-FA	Lipid free
Partice Size (nm)	191 ± 1	267 ± 17	197 ± 53	12.2 ± 0.2
Polydispersity index	0.19 ± 0.01	0.22 ± 0.01	0.43 ± 0.11	0.12 ± 0.01
Zeta potential (mV)	-7.6 ± 0.2	-4.2 ± 0.7	-6.3 ± 0.4	-4.3 ± 0.3

2.4 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. Male Wistar rats ranging from 260-310 g were maintained on a standard diet and fasted overnight (14-16 h) with free access to water prior to commencement of the experiment. Anaesthesia was induced and maintained with 1.5-5% v/v isoflurane, according to response, delivered via a nose cone. To ensure body temperature was maintained, rats were kept on a 37°C heating pad during the surgical procedures and throughout the duration of the experiments.

2.4.1 Lymphatic transport studies

The lymphatic transport of orlistat and triglyceride (TG), and plasma concentrations of orlistat, were assessed after duodenal administration of all formulations. In these experiments,

the mesenteric lymph duct, carotid artery and duodenum were cannulated as described previously (Edwards et al., 2001; Trevaskis et al., 2015a). After surgery, the rats were hydrated for at least 30 min via intraduodenal infusion of normal saline at 2.8 ml/h. The control or lipid-based orlistat formulations were then infused into the duodenum at 2.8 ml/h for 2 h for all experimental groups except for the one group that was infused at 2.8 ml/h for 8 h (Table 1). Following completion of formulation dosing, the intraduodenal infusion was switched back to 2.8 ml/h normal saline for the remainder of the experiment. Mesenteric lymph was collected continuously for 8-11 h following commencement of formulation dosing, into pre-weighed polyethylene tubes (Techno Plas Pty. Ltd., SA, Australia) containing 10-20 µl of 1000 IU/ml heparin (Clifford Hallam Healthcare Pty. Ltd., VIC, Australia). Lymph collection tubes were changed every hour and lymph flow was determined gravimetrically. Aliquots of 100 µl of lymph were stored at -20°C for later drug analysis by HPLC-MS/MS (as described below) and TG analysis using a commercial enzymatic kit assay (TR0100, Sigma-Aldrich, MO, USA). Any spare samples were transferred to -80°C for long term storage. 250 µl of blood from the carotid artery was also collected into polyethylene tubes together with 3 µl of 1000 IU/ml heparin at 10 time points: 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, and 8 h. An additional two time points for blood (9 h and 11 h) were collected for the experimental group administered the formulation for 8 h. Blood samples were centrifuged for 5 min at 2000 g to separate plasma. Plasma was stored as described for the lymph samples. Once the experiment was complete, rats were euthanized by a 1 ml injection of sodium pentobarbitone (100 mg/ml) via the carotid artery cannula.

2.4.2 Plasma pharmacokinetic and bioavailability studies

To enable determination of systemic exposure and absolute bioavailability (BA) following intestinal dosing of selected formulations (i.e. the 40 mg oleic acid and olive oil formulations containing 8 mg/kg orlistat which resulted in the highest and lowest lymphatic transport of orlistat across the formulations) a separate group of lymph-intact animals was cannulated at the duodenum (to enable formulation administration) and carotid artery (to enable blood sample collection). After surgery the rats were hydrated via intraduodenal infusion of 2.8 ml/h normal saline for at least 30 min, followed by 2 h intraduodenal infusion of the formulations at 2.8 ml/h. The hydration of the rats, collection of blood samples, separation of plasma, and euthanasia were the same as described for the lymphatic transport studies.

An intravenous pharmacokinetic study was also completed to enable the calculation of the absolute BA following intestinal administration. In this, the jugular vein and carotid artery of the rats were cannulated as described previously (Edwards et al., 2001; Trevaskis et al., 2015a). After surgery, the rats were rehydrated via the jugular vein infusion of normal saline for at least 30 min at 0.5 ml/h. Orlistat (0.4 mg/kg) dissolved in 120 mg soybean oil in 2%

glycerol, 1% egg phosphatidyl choline was administered as a short 1 ml IV infusion into the jugular vein cannula over 5 min. Following completion of formulation dosing, the jugular vein infusion was switched back to 0.5 ml/h normal saline for the remainder of the experiment. The collection of blood samples, separation of plasma, and euthanasia were the same as described for the lymphatic transport studies.

2.5 HPLC-MS/MS sample preparation and analysis

2.5.1 Sample preparation

Formulations containing 8-50mg/kg orlistat and 0.15 mg/kg orlistat were diluted 10,000-fold and 1,000-fold in ACN, respectively, prior to analysis. Lymph and plasma samples were processed using a protein precipitation method. Briefly, for samples with a concentration within 0.05-5 µg/ml, 900 µl of ACN was added to 100 µl of lymph or plasma. Samples were then vortexed for 1 minute, centrifuged at 10,000 g for 5 min, and 200 µl of supernatant was aliquoted into HPLC vials for analysis. For samples with a concentration within 5-50 µg/ml, 990 µl of ACN was added to 10 µl of lymph and vortexed for 1 minute. Then, 20 µl of the ACN-lymph mixture was aliquoted in a fresh tube and diluted with 180 µl of ACN. The diluted samples were then vortexed for 1 minute and centrifuged at 10,000 g for 5 min, and 200 µl of supernatant was aliquoted to HPLC vials for analysis.

2.5.2 HPLC-MS/MS analysis conditions

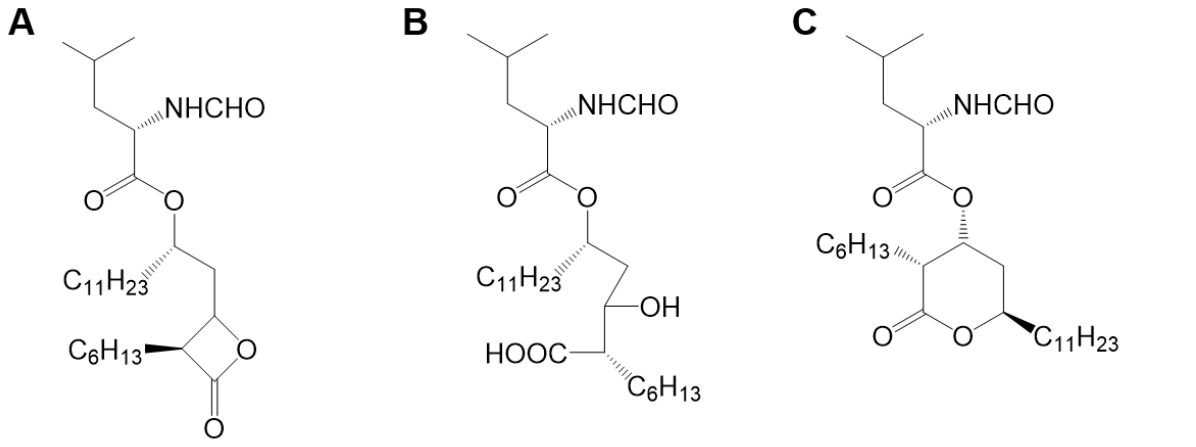
Concentrations of orlistat in lymph, plasma and formulations were analysed using a Shimadzu LCMS-8050 system (Shimadzu Scientific Instruments, Kyoto, Japan) consisting of a CBM-20A system controller, a DGU-20A5R degassing unit, two Nexera X2 LC-30 AD liquid chromatograph pumps, a Nexera X2 SIL-30AC autosampler, a CTO-20A column oven (held at 40^{o}C), and a LCMS-8050 triple quadrupole mass spectrometer with an atmospheric-pressure chemical ionization (APCI) interface. The desolvation line and heat block were kept at 250 ^{o}C and 400 ^{o}C, respectively. The interface and detector voltages were 4.5 and 2.18 kV respectively. The nebulizing gas and drying gas flow rates were 3 L/min and 10 L/min respectively. Chromatographic separation of the samples was achieved using a Phenomenex Kinetex C8 column (2.6µm, 100Å, 50 X 2.1 mm, NSW, Australia). The tray temperature in the autosampler was maintained at 15^{o}C. The mobile phase was a mixture of solvents A and B with a flow rate of 0.4 ml/min. Solvent A was 10:90 (% v/v) ACN-water with 0.1% formic acid and solvent B was 90:10 (% v/v) ACN-water with 0.1% formic acid. The injection volume was 10 µl and samples were run on the following gradient sequence: mobile phase B was first held at 75% for 0.5 min, then linearly increased to 100% over the next 0.3 min followed by a hold at 100% for 1.2 min, then a linear decrease to 75% over the next 0.1 min. Mobile phase B was kept at 75% for another 1.9 min prior to the injection of the next sample. The total run time was 4 minutes. The transition *m/z* of 496.40 ($[M+H]^+$) → 319.40 was used for the detection of

orlistat. The collision energy optimised for detecting orlistat was -14 eV. The retention time for orlistat in acetonitrile and in heat treated lymph/plasma (i.e. lymph and plasma that has been incubated at 80^{o}C and 60^{o}C respectively for 60 min to deactivate enzymes) was 1.7 min. When orlistat was present in untreated lymph/plasma two retention times were observed at 1.4 min and 1.7 min corresponding to the open and closed ring epimer of orlistat as described below and shown in Fig 1.

2.5.3 Calculation methods and validation of HPLC-MS/MS analysis methods for orlistat in formulations, lymph and plasma

For orlistat in ACN and diluted formulations the HPLC-MS/MS assay was validated on three separate days by running two standard curves and five replicates of quality control (QC) samples prepared at four different concentrations (i.e. 5, 10, 50, and 500 ng/ml). The assay was found to be accurate and precise for concentrations between 5-500 ng/ml (with <10% variation at all concentrations except at the lowest limit of quantitation for which it was <20%).

For orlistat in non-heat inactivated and heat inactivated lymph/plasma, the assay was validated on three separate days for the concentration range of 0.05-50 µg/ml by running two standard curves and five replicates of QC samples prepared at four different concentrations (i.e. 0.05, 0.1, 0.5, and 5 µg/ml). For lymph, five replicates of QC samples were also prepared at 50 µg/ml. As described above, two peaks were observed at retention times of 1.4 min and 1.7 min at *m/z* of 496.40 ($[M+H]^+$) → 319.40 in the LC-MS/MS profiles for orlistat in the lymph and plasma calibration standards, QCs, and *in-vivo* study samples. However, only one peak was observed (at RT of 1.7 min) when rat lymph or plasma were incubated at 80^{o}C or 60^{o}C, respectively, for 60 min prior to spiking the calibration standards and QCs. This heat pre-treatment would inactivate enzymes in the lymph and plasma. The second peak with same *m/z* ratio at 1.4 min in the non-treated lymph and plasma samples was therefore believed to result from enzyme mediated hydrolysis of the β lactone ring of orlistat to form the open ring epimer (Fig 1 B). Orlistat covalently binds to the active site of pancreatic lipase via its lactone ring and binding to the active enzyme results in ring opening of the lactone (Guerciolini, 1997). The closed and open ring epimers of orlistat are expected to be detected with the same *m/z* transition (but with different RTs), as the open ring epimer is chemically unstable (Stalder et al., 1992), resulting in acyl-group migration to form a six-membered lactone with the same mass as orlistat (Fig 1 C). Despite the presence of two peaks, it was possible to develop an accurate and precise method to quantify orlistat present in lymph and plasma based on the size and ratio of the two peaks as described below.



Chemical formula: $C_{29}H_{53}NO_5$
Exact mass: 495.39
Molecular weight: 495.75
CLogP: 8.61

Chemical formula: $C_{29}H_{55}NO_5$
Exact mass: 513.40
Molecular weight: 513.76
CLogP: 8.65

Chemical formula: $C_{29}H_{53}NO_5$
Exact mass: 495.39
Molecular weight: 495.75
CLogP: 9.10

Fig 1. Chemical structure of A) orlistat, B) orlistat open ring epimer and C) six-membered lactone epimer. The transition from orlistat (A) to the orlistat open ring epimer (B) is expected to occur when orlistat is in contact with enzymes such as pancreatic lipase (i.e. when in non-treated lymph or plasma). Orlistat and the open ring epimer were detected on the LC-MS/MS assay as peaks at retention time 1.7 min and 1.4 min, respectively. Orlistat and orlistat open ring epimer can be detected at the same *m/z* transition as the open ring epimer is unstable and can be converted to the six-membered lactone epimer (C) during LC-MS/MS analysis. CLogP values were determined by ChemDraw Professional 15.0 Suite software.

2.5 4 Back-calculation of orlistat concentration in lymph and plasma

Orlistat closed ring epimer (i.e. Fig 1 A) concentration in lymph and plasma samples was determined by comparing the area under the curve (AUC) of the LC-MS/MS peaks corresponding to the closed ring epimer (i.e. peaks with a retention time of 1.7 min) in the lymph/plasma samples to the AUCs from the heat inactivated lymph and plasma calibration standards.

Due to the lack of an authentic standard for the orlistat open ring epimer (i.e. Fig 1 B), a method was developed to back-calculate the total orlistat concentration in untreated lymph and plasma samples stemming from the collective concentrations of both the open and closed ring forms. Firstly, by comparing the AUC of the LC-MS/MS peaks from the heat inactivated and untreated calibration standards at the same nominal concentration, a correction factor k was calculated to correct for differences in peak area of the orlistat open ring epimer peak at 1.4 min and the orlistat closed ring epimer peak at 1.7 min at the same concentration, and therefore to allow determination of the concentration of the open ring epimer of orlistat by

comparing the AUC of the 1.4 min peak (ring opened form) against a standard curve of orlistat (i.e. the 1.7 min peak). k was calculated as:

$$k = \left(\frac{AUC_{\text{inactivated 1.7m orlistat peak}} - AUC_{\text{untreated 1.7m orilstat peak}}}{AUC_{\text{untreated 1.4m open ring peak}} \right)$$

Where $AUC_{\text{inactivated 1.7m orlistat peak}}$ is the AUC of the orlistat 1.7 min peak from heat inactivated biomatrix spiked with a known concentration of orlistat. When the same concentration of orlistat was spiked into non-heat treated biomatrix, $AUC_{\text{untreated 1.4m open ring peak}}$ and $AUC_{\text{untreated 1.7m orlistat peak}}$ represent the AUC of the peaks for the orlistat open ring epimer (at 1.4 min) and orlistat closed ring epimer (at 1.7 min) from untreated biomatrix spiked at the same concentration of orlistat as the heat inactivated biomatrix calibration standard. Multiple k values were obtained by spiking different concentrations of orlistat in both heat inactivated and untreated biomatrix. The k correction factors were very similar and thus the average ($k_{average}$) was used to calculate the equivalent orlistat AUC ($AUC_{equivalent}$) when both peaks were present in untreated samples using the following equation:

$$AUC_{equivalent} = AUC_{1.7m} + (AUC_{1.4m} \times k_{average})$$

Finally, the $AUC_{equivalent}$ in untreated samples was compared to a standard curve prepared from heat inactivated calibration standards to determine the equivalent total orlistat concentrations. The assay to determine total orlistat (i.e. concentration of both open and closed ring epimer) and closed ring orlistat was validated and found to be accurate and precise in the 0.05-50 µg/ml concentration range (accuracy 100 +/- 13%, and precision <15% variation except at the lowest limit of quantiation for which it was <20%).

5.6 Data analysis for lymphatic transport, pharmacokinetic and bioavailability studies

For the lymphatic transport studies, mass transport of orlistat in lymph was calculated by multiplying the volume of lymph collected by the measured concentration of orlistat in lymph. Drug concentrations in lymph and plasma were dose normalised to 8 mg/kg (unless stated otherwise). Lymph:plasma concentration ratios were calculated by dividing the average orlistat lymph concentration for each hourly collection period by the orlistat plasma concentration measured at the end of the hourly collection period. For instance, the lymph:plasma concentration ratio for the 2 h time period was calculated as follows:

$$\frac{\text{Concentration measured in lymph collected from 1-2 h}}{\text{Concentration measured in plasma collected at 2 h}}$$

The plasma concentration was set at the lower limit of quantification (i.e. 0.05 µg/ml) for timepoints where the plasma concentration was below 0.05 µg/ml when determining the lymph:plasma concentration ratio.

For the plasma pharmacokinetic studies, the absolute bioavailability following intestinal delivery of orlistat was calculated from:

$$\text{Absolute bioavailability} = \frac{AUC_{0\text{-}8h}\ \text{intestinally administered}}{AUC_{0\text{-}8h}\ \text{IV administered}} \times \frac{\text{Orlistat Dose}_{\ IV}}{\text{Orlistat Dose}_{\ intestinal}}$$

Where $AUC_{0\text{-}8h}$ intestinally administered and IV administered are the area under the plasma concentration time curves from time 0 to 8 h after intestinal and IV administration, respectively. Plasma AUCs were calculated using the linear trapezoidal method.

The percentage of the total systemic exposure contributed by lymphatic transport for each group was estimated as follows:

$$\text{Contribution of lymphatic transport to systemic exposure} = \left(1 - \frac{AUC_{0\text{-}8h}\ \text{lymph cannulated}}{AUC_{0\text{-}8h}\ \text{lymph intact}}\right) x\ 100$$

Where $AUC_{0\text{-}8h}$ lymph cannulated and lymph intact are the area under the plasma concentration time curves from time 0 to 8 h after intestinal administration in lymph cannulated and lymph intact rats, respectively. AUCs were calculated using the linear trapezoidal method.

For the IV administered group, non-compartmental pharmacokinetic parameters were calculated using WinNolin® Software (WinNolin® professional version 5.2.1, Pharsight Corporation, CA, USA). The terminal elimination rate constant (k) was determined by regression analysis of the elimination phase. Plasma half-life ($t_{1/2}$) was calculated from ln(2)/k. The AUC from time 0 to infinity was calculated from the plasma concentration versus time profile using the linear trapezoidal method extrapolated to infinity by dividing the last measured concentration by the elimination rate constant k. Drug clearance (Cl) was calculated by dividing the administered dose by the AUC. The area under the first moment curve (AUMC) was calculated from the area under the product of the concentration and time curve extrapolated to infinity. The steady state volume of distribution was determined from the product of Cl and AUMC/AUC.

5.7 Statistics

GraphPad Prism for Windows V7.01.180 (GraphPad Software Inc. Ca, USA) was used to perform statistical analyses. One-way ANOVA followed by Tukey's multiple comparisons test (for comparisons between three or more groups) or an unpaired t test (for comparisons between two groups) was used to determine significant differences with a level of $p = 0.05$ set as significant (unless otherwise noted).

3 Results

3.1 The effect of lipid type on the lymphatic transport of orlistat and triglyceride

The cumulative lymphatic transport of orlistat was significantly greater when it was administered in the LC-FA (i.e. oleic acid) based formulation (at 2.6% of the dose for total orlistat and 0.6% of dose for closed-ring orlistat over 8 h) when compared to the lipid free (i.e. control), LC-TG (i.e. olive oil) and MC-FA (i.e. octanoic acid) based formulations for which lymphatic transport was relatively low at <0.9% of dose over 8 h for total orlistat (i.e. open plus closed ring forms) (Fig 2 A-B and Table 3). The LC-TG and MC-FA based formulations therefore did not promote lymphatic transport of orlistat relative to the lipid-free formulation. The cumulative lymphatic transport of TG after administration of the different lipid types was also significantly greater for the LC-FA formulation when compared to the LC-TG, MC-FA, and lipid-free formulations (Fig 3 A-B). A broad correlation between the cumulative % of the orlistat dose transported in lymph (in both closed and open ring forms) and the cumulative mass of TG transported in lymph in individual rats was evident, with an r^2 value of 0.61 (Fig 3 C).

The peak concentration (C_{max}) of orlistat in lymph generally occurred at 2-3 h post-dose for all formulations after which time the lymph concentrations of orlistat declined. In all groups, lymph concentrations were low beyond 5 h post-dosing. The orlistat C_{max} in lymph was significantly higher when it was administered in the LC-FA formulation when compared to the LC-TG, MC-FA and lipid free formulations, as was expected from the higher cumulative lymphatic transport in this group (Fig 2 A-D).

In the plasma of lymph cannulated animals, orlistat was only measurable in open ring form. The closed ring form was present in some samples but below the limit of quantitation. In contrast to the lymph profiles, orlistat plasma concentrations were substantially higher after administration in the lipid free formulation when compared to the LC-TG, MC-FA and LC-FA formulations (Fig 2 E). However, it must be noted that the lymph is diverted out of the body in these animals such that the plasma concentrations do not represent the total exposure to the drug and only represent the proportion of the dose absorbed directly into blood from the intestine. Across all formulations, plasma C_{max} generally occurred between 2-3 h post-dosing followed by a decline beyond 6 h post-dosing. The plasma concentrations of orlistat were lower (2-56 fold) than in lymph across all time points and in all groups. The lymph:plasma concentration ratio of total orlistat at 3 h post-dose was significantly higher following administration of the LC-FA formulation when compared to the other formulations (Fig 2 F).

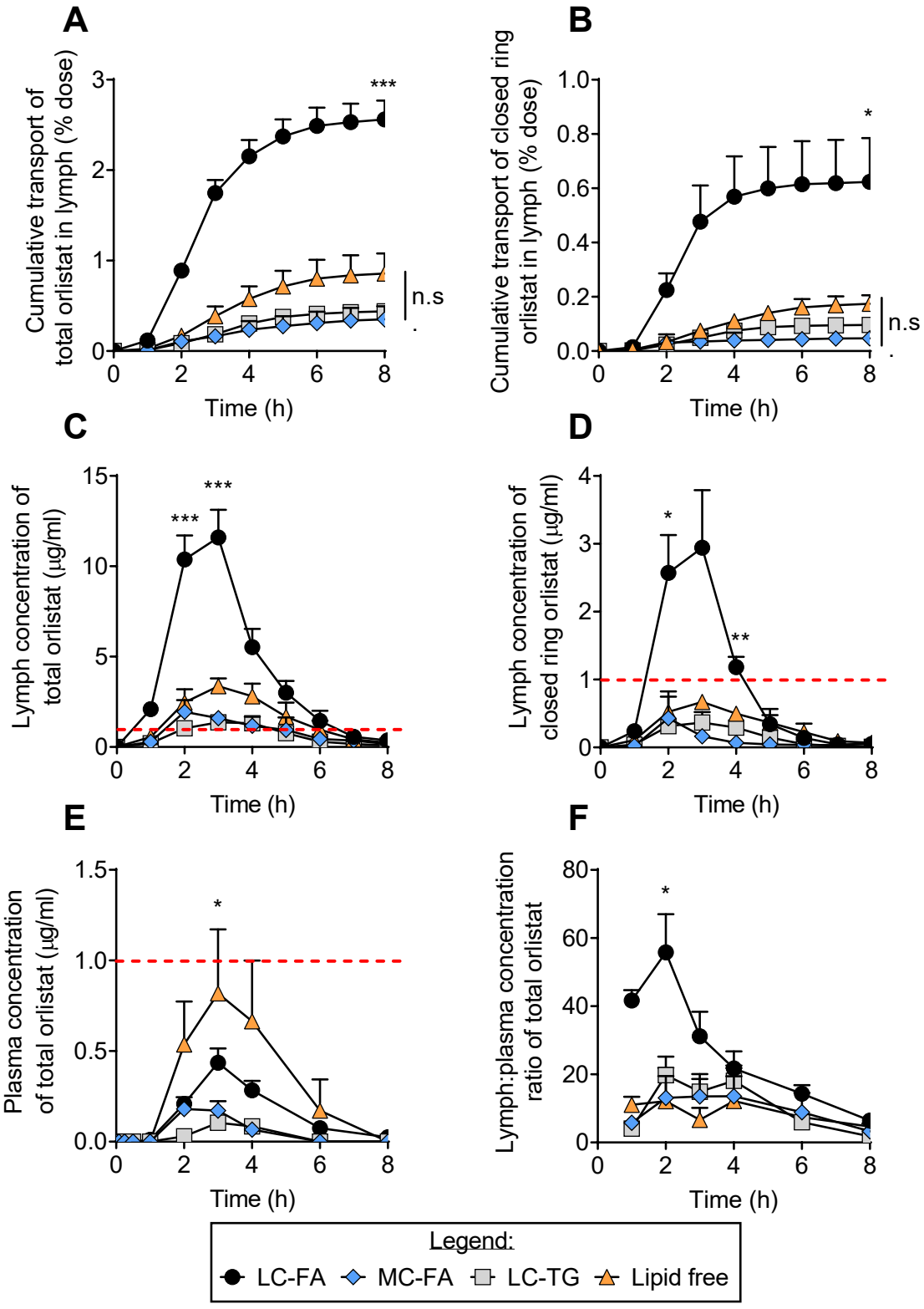


Fig 2. Impact of formulation lipid type on mesenteric lymph transport and plasma pharmacokinetics of orlistat in lymph cannulated rats. A) Cumulative lymphatic transport of total orlistat (open and closed ring forms) over time, B) Cumulative lymphatic transport of closed ring orlistat over time, C): Dose-normalised lymph concentration of total orlistat (open

and closed ring forms) over time, D) Dose-normalised lymph concentration of closed ring orlistat over time, E) Dose-normalised plasma concentration of total orlistat (mostly in the open ring form) over time, and F) The ratio of lymph to plasma concentrations of total orlistat over time, in mesenteric lymph duct cannulated, anesthetised rats following intraduodenal infusion of formulations from 0 to 2 h. All formulations contained 2 mg of orlistat. For the lipid-based formulations, orlistat was dispersed in 40 mg oleic acid (LC-FA), octanoic acid (MC-FA) or olive oil (LC-TG) with 25 mg Tween 80 and 5.6 ml PBS. For the control lipid-free formulation, orlistat was dispersed in 112 mg Tween 80 and 5.6 ml PBS. In panel C-E orlistat concentrations are dose-normalised to 8 mg/kg. The red dashed line indicates the expected minimum therapeutic concentration of orlistat (i.e. 0.99 µg/mL). Data is presented as mean ± SEM for oleic acid LC-FA (circle, n=5), octanoic acid MC-FA (diamond, n=4), olive oil LC-TG (square, n=4) and control lipid-free (triangle, n=3) formulations. For panel E significance applies to lipid free versus MC-FA and lipid free versus LC-TG only. Significant difference to other groups determined from one-way ANOVA: ***p≤0.001, *p≤0.05

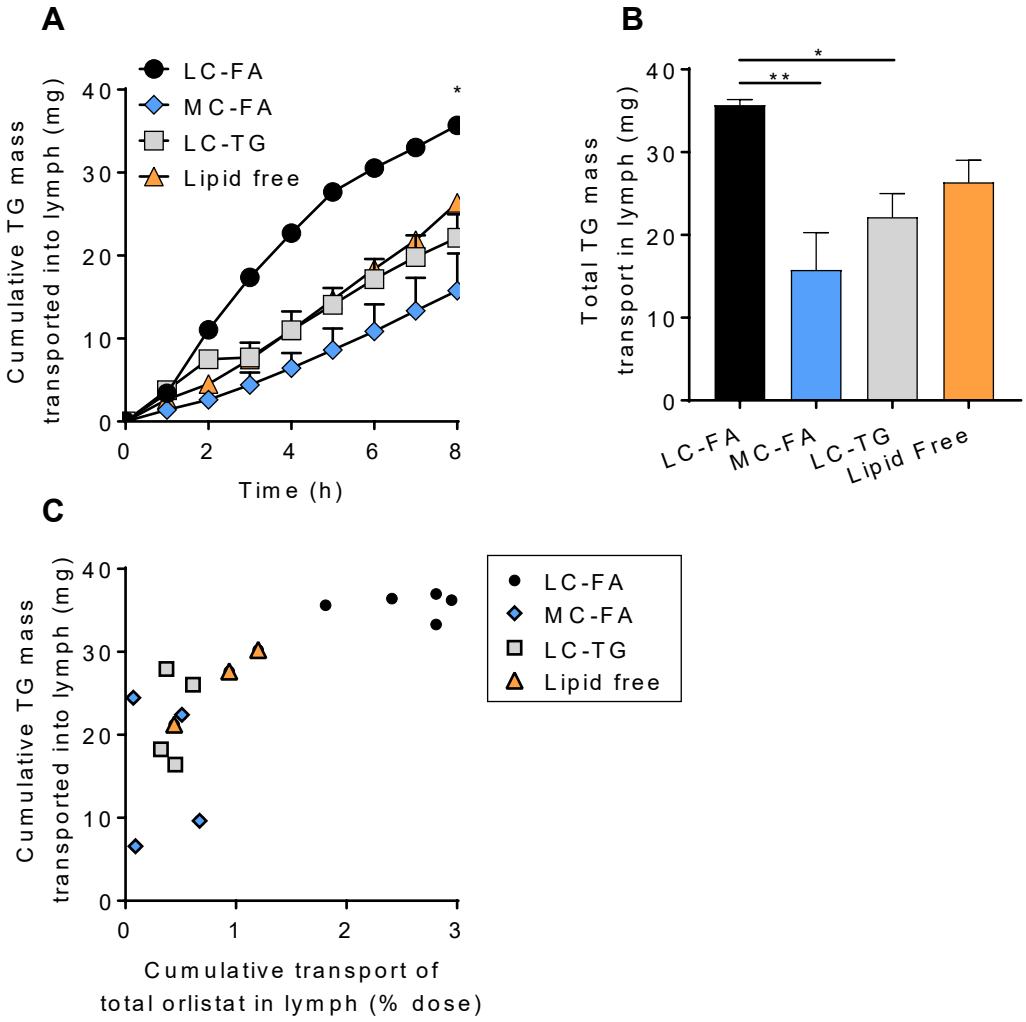


Fig 3. Impact of formulation lipid type on mesenteric lymph transport of triglyceride (TG). A) Cumulative lymphatic transport of TG over time, B) Total mass of TG transported in mesenteric lymph over 8 h, and C) Cumulative TG mass transported into lymph over 8 h *versus* cumulative lymphatic transport of total orlistat over 8 h (with each symbol representing an individual rat) following intraduodenal infusion of formulations to anesthetised, mesenteric lymph duct cannulated rats. All formulations contained 2 mg of orlistat and were administered from 0-2 h. For the lipid-based formulations, orlistat was dispersed in 40 mg oleic acid (LC-FA), octanoic acid (MC-FA) or olive oil (LC-TG) with 25 mg Tween 80 and 5.6 ml PBS. For the control lipid-free formulation, orlistat was dispersed in 112 mg Tween 80 and 5.6 ml PBS. Data is presented as mean ± SEM for oleic acid LC-FA (circle, black, n=5), octanoic acid MC-FA (diamond, blue, n=4), olive oil LC-TG (square, grey, n=4), and control lipid-free (triangle, orange, n=3) formulations. Significant difference to other groups determined from one-way ANOVA: ** $p \leq 0.01$, * $p \leq 0.05$

3.2 The effect of lipid dose on the lymphatic transport of orlistat and triglyceride

The LC-FA based formulation therefore supported increased lymphatic transport of orlistat when compared to the other LFs and lipid free formulation (Fig 2 A). Next, the impact of increasing LC-FA (i.e. oleic acid) dose from 40 mg to 80 mg while keeping the drug dose constant was tested to determine if this could promote a further increase in lymphatic drug and TG transport. Doubling the LC-FA dose from 40 mg to 80 mg significantly enhanced the lymphatic transport of total orlistat (open and closed ring forms) from 2.6 to 3.6 % of the dose and enhanced the lymphatic transport of the more active closed ring form of orlistat from 0.6% to 1.6% of the dose. Both LC-FA formulations promoted lymphatic transport of orlistat relative to the lipid-free control formulation (Fig 4 A-B, and Table 3). The lymph C_{max} for total orlistat and closed ring orlistat was also significantly higher after co-administration with 80 mg when compared to 40 mg of LC-FA (Fig 4 C-D). Both LC-FA formulations resulted in higher concentrations of orlistat in lymph when compared to plasma (Fig 4 E). In contrast to the differences seen in lymph concentrations, plasma concentrations of total orlistat in the lymph cannulated animals (which consisted almost entirely of the open ring form) were similar after co-administration with either 40 mg or 80 mg LC-FA (Fig 4 E). The lymph to plasma concentration ratio of total orlistat was thus greater following administration with 80 mg compared to 40 mg of LC-FA for up to 4 h post-dose (Fig 4 F). Interestingly, in contrast to the lymphatic transport of orlistat, the cumulative lymphatic transport of TG was similar for both lipid doses (i.e. 40 mg and 80 mg LC-FA) (Fig 5 A-B).

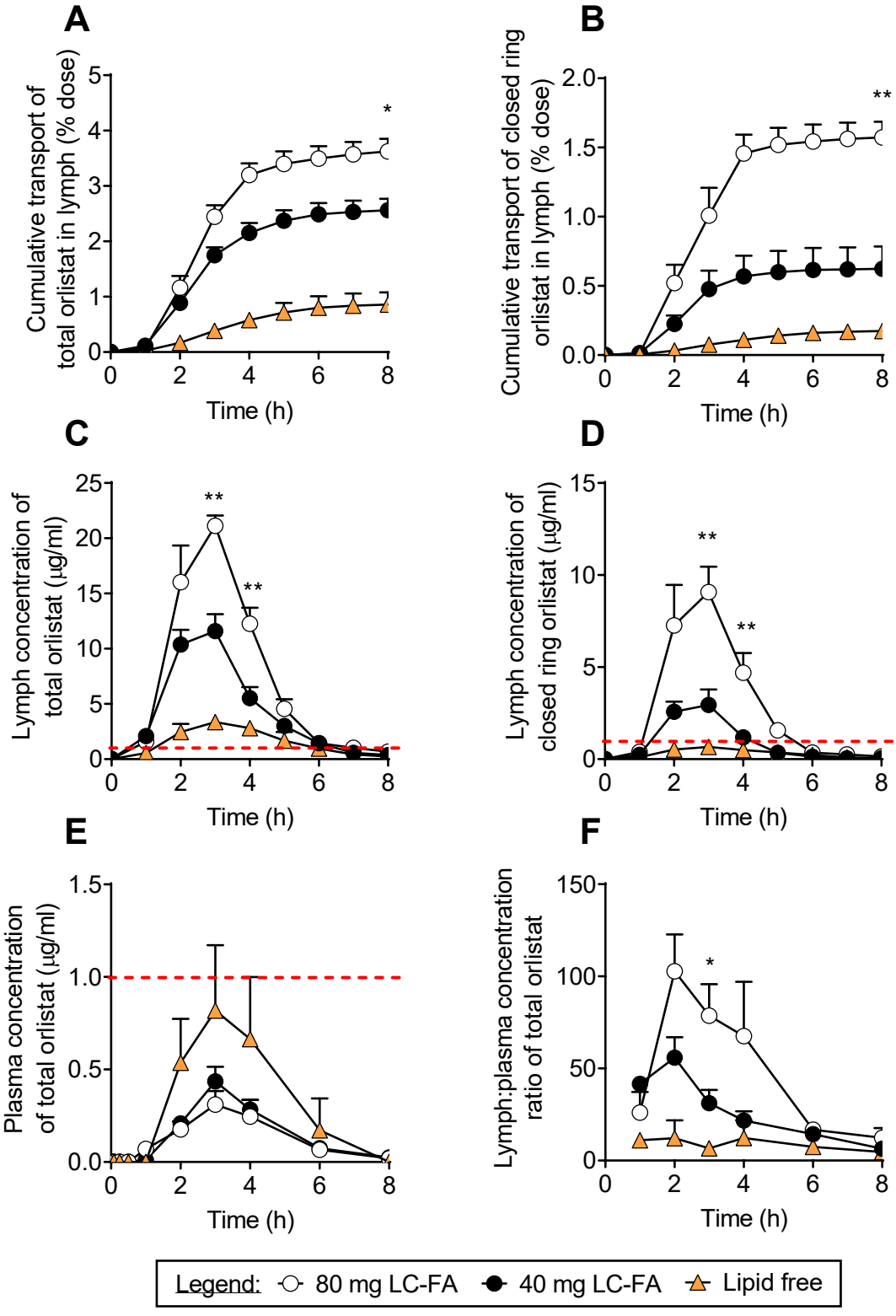


Fig 4. Impact of lipid dose on mesenteric lymph transport and plasma pharmacokinetics of orlistat in lymph cannulated rats. A) Cumulative lymphatic transport of total orlistat (open and closed ring forms) over time, B) Cumulative lymphatic transport of closed ring orlistat over time, C) Dose-normalised lymph concentration of total orlistat (open and closed ring forms) over

time, D) Dose-normalised lymph concentration of closed ring orlistat over time, E) Dose-normalised plasma concentration of total orlistat (mostly in the open ring form) over time, and F) The ratio of lymph to plasma concentrations of total orlistat over time in mesenteric lymph duct cannulated, anesthetised rats following intraduodenal infusion of formulations from 0 to 2 h. All formulations contained 2 mg of orlistat dispersed in 40 mg, 80 mg, or 0 mg (i.e. control) of long chain fatty acid (LC-FA, i.e. oleic acid). For the lipid-based formulations, 25 mg Tween 80 and 5.6 ml PBS was added with orlistat and the oleic acid. For the control, orlistat was dispersed in 112 mg of Tween 80 and 5.6 ml PBS. In panel C-E orlistat concentrations are dose-normalised to 8 mg/kg. The red dashed line indicates the expected minimum therapeutic concentration of orlistat (i.e. 0.99 µg/mL). Data is presented as mean ± SEM for 80 mg LC-FA (open circle, n=4), 40 mg LC-FA (circle, n=5), and control (triangle, n=3). Significant difference to other groups from one-way ANOVA: ** p≤0.01, *p≤0.05

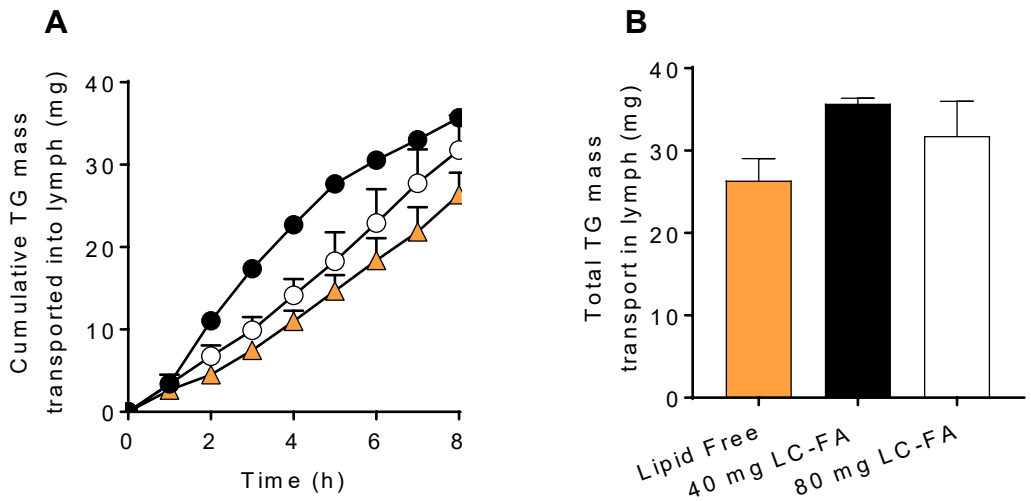


Fig 5. Impact of lipid dose on mesenteric lymph transport of triglyceride (TG). A) Cumulative lymphatic transport of TG over time and B) Total mass of TG transported in mesenteric lymph over 8 h in mesenteric lymph duct cannulated, anesthetised rats following intraduodenal infusion of formulations from 0 to 2 h. All formulations contained 2 mg of orlistat dispersed in 40 mg, 80 mg, or 0 mg (i.e. control) of long chain fatty acid (LC-FA, i.e. oleic acid). For the lipid-based formulations, 25 mg Tween 80 and 5.6 ml PBS was added with orlistat and the oleic acid. For the control, orlistat was dispersed in 112 mg of Tween 80 and 5.6 ml PBS. Data is presented as mean ± SEM for 80 mg LC-FA (open circle, n=4), 40 mg LC-FA (circle, n=5), and control (triangle, n=3). Data were not significant between groups from one-way ANOVA.

3.3 The effect of orlistat dose on the lymphatic transport of orlistat and triglyceride

The effect of orlistat on lymphatic TG and thus drug transport is likely to be orlistat dose-dependent. As such we determined the impact of orlistat dose on the absorption and lymphatic transport of TG and orlistat. Three different orlistat doses were evaluated: 0.15, 8 and 50 mg/kg. The 0.15 and 8 mg/kg drug doses were administered with 40 mg LC-FA whereas the 50 mg/kg drug dose was administered with 80 mg LC-FA as this dose of lipid was required to solubilise the drug. As expected, the lymphatic transport of orlistat varied with orlistat dose. Interestingly, either increasing the orlistat dose or decreasing the orlistat dose negatively influenced the lymphatic transport of orlistat relative to administration of the 8 mg/kg dose. The cumulative lymphatic transport of total orlistat (i.e. in open and closed ring forms) was 2.6% of dose for the 8 mg/kg orlistat dose and <0.8% of dose for the 50 mg/kg and 0.15 mg/kg orlistat doses (Fig 6 A). Similarly, the cumulative transport of the more active closed ring form of orlistat was greater for the 8 mg/kg orlistat dose compared to the 50 mg/kg and 0.15 mg/kg orlistat doses (Fig 6 B).

In contrast, the cumulative lymphatic transport of TG was greater after administration of the lower orlistat dose (i.e. 0.15 mg/kg orlistat versus 8 mg/kg or 50 mg/kg orlistat) (Fig 7A). Interestingly, the total TG mass transported in lymph was similar after administration of the 8 mg/kg or 50 mg/kg orlistat formulation despite the lipid dose being doubled from 40 mg to 80 mg in the 50 mg/kg group (Fig 7). However, the TG mass transport in the group administered 50 mg/kg orlistat with 80 mg oleic acid was similar to the TG mass transport seen previously when 8 mg/kg orlistat was administered with 80 mg oleic acid suggesting that at both the 8 and 50 mg/kg orlistat dose, the orlistat inhibits lymphatic TG transport.

As might be expected, the total concentration of orlistat (open and closed ring forms) in lymph was significantly higher from 1-4 h post-dose after administration of the higher drug dose (i.e. 50 mg/kg orlistat) (Fig 6 C). This occurred despite a significantly higher percent of the orlistat dose being recovered in lymph at the same time points when the 8 mg/kg orlistat formulation was administered (Supplementary Fig 1). When the 0.15 mg/kg orlistat formulation was administered, total orlistat (i.e. open plus closed ring forms) lymph concentrations were only above the limit of quantification (LOQ) of the assay (0.05 µg/ml) from 1-3 h post dose (Fig 6 C and Supplementary Fig 2). Similarly, after administration of the low dose (0.15 mg/kg) orlistat formulation, the concentrations of the closed ring form of orlistat in lymph and total orlistat in plasma were below the LOQ for the assay. For the other groups, total orlistat concentrations in plasma (which mostly comprised the open ring form) were lower than in lymph, and the plasma C_{max} generally occurred 2-3 h post-dosing. As expected, the total orlistat plasma concentration between 2-4 h post-dosing was significantly higher after administration of the 50 mg/kg versus the 8 mg/kg orlistat formulation (Fig 6 E). The

lymph:plasma concentration ratio were high (>20 from 1-4 h post-dose) and did not significantly differ between the 8 mg/kg and 50 mg/kg orlistat dose groups (Fig 6 F).

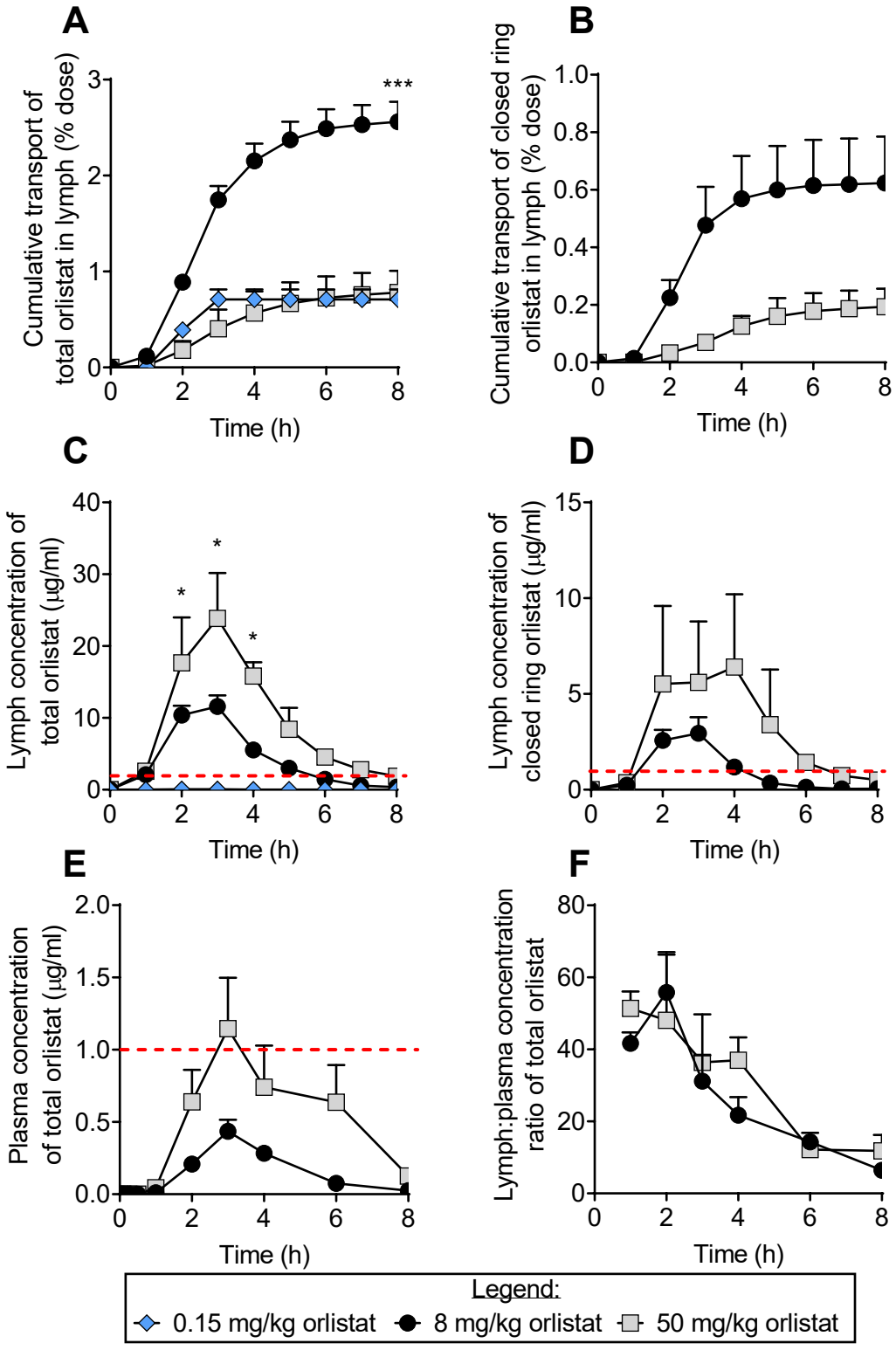


Fig 6. Impact of orlistat dose on mesenteric lymph transport and plasma pharmacokinetics of orlistat in lymph cannulated rats. A) Cumulative lymphatic transport of total orlistat (open and

closed ring forms) over time, B) Cumulative lymphatic transport of closed ring orlistat over time, C) Dose-normalised lymph concentration of total orlistat (open and closed ring forms) over time, D) Dose-normalised lymph concentration of closed ring orlistat over time, E) Dose-normalised plasma concentration of total orlistat (mostly in the open ring form) over time, and F) The ratio of lymph to plasma concentrations of total orlistat over time in mesenteric lymph duct cannulated, anesthetised rats following intraduodenal infusion of formulations from 0 to 2 h. Orlistat was dispersed in long chain fatty acid (LC-FA, i.e. oleic acid), 25 mg Tween 80 and 5.6 ml PBS. For the 8 mg/kg and 0.15 mg/kg orlistat formulations, 2 mg and 0.035 mg of orlistat were incorporated into the formulation, respectively, with 40 mg LC-FA. For the 50 mg/kg orlistat formulation, 13.5 mg orlistat and 80 mg LC-FA were incorporated into the formulation as the drug mass was not soluble at the lower oleic acid dose. The red dashed line indicates the expected minimum therapeutic concentration of orlistat (i.e. 0.99 µg/mL). Data is presented as mean ± SEM for the 0.15 mg/kg orlistat formulation (blue diamond, n=4); the 8 mg/kg orlistat formulation (black circle, n=5); and the 50 mg/kg orlistat formulation (grey square, n=3). Orlistat closed ring and orlistat total plasma concentrations measured for the 0.15 mg/kg group were all below the lower limit of quantification (i.e. 0.05 µg/ml) of the HPLC-MS/MS assay. Significant difference to other groups determined by one-way ANOVA for data with three groups or unpaired t test for data with two groups: ***p≤0.001, *p≤0.05

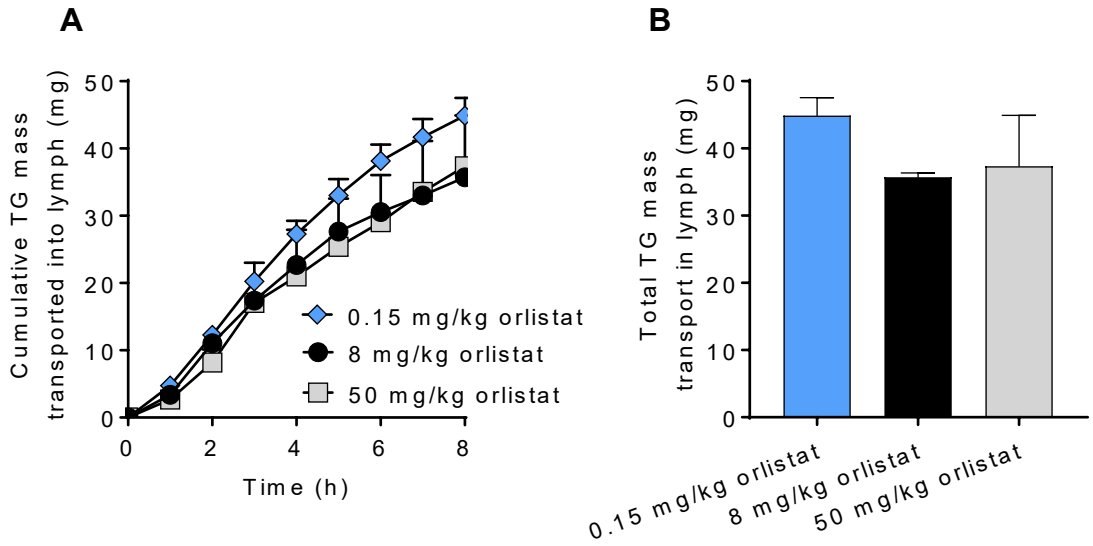


Fig 7. Impact of orlistat dose on mesenteric lymph transport of triglyceride (TG). A) Cumulative lymphatic transport of TG over time, and B) Total mass of TG transported in mesenteric lymph over 8 h, following intraduodenal infusion of formulations to anesthetised, mesenteric lymph duct cannulated rats. Orlistat was dispersed in long chain fatty acid (LC-FA, i.e. oleic acid), 25 mg Tween 80 and 5.6 ml PBS. For the 8 mg/kg and 0.15 mg/kg orlistat formulations, 2 mg and

0.035 mg of orlistat were incorporated into the formulation with 40 mg LC-FA. For the 50 mg/kg orlistat formulation, 13.5 mg orlistat and 80 mg LC-FA were incorporated into the formulation as the drug mass was not soluble at the lower oleic acid dose. Data is presented as mean ± SEM for the 0.15 mg/kg orlistat formulation (blue diamond, blue, n=3), 8 mg/kg orlistat formulation (black circle, black, n=5), and 50 mg/kg orlistat formulation (grey square, grey, n=3). Data were not statistically different across groups between groups from one-way ANOVA.

3.4 The effect of infusion time on the lymphatic transport of orlistat and triglyceride

The impact of infusion time on lymphatic transport was also tested with the aim to further increase the total mass of orlistat transported in lymph. Three experimental groups were compared: (1) 2 h infusion (4 mg/kg/h orlistat in 20 mg/h LC-FA), (2) 8 h infusion (4 mg/kg/h orlistat in 20 mg/h LC-FA), and (3) 2 h infusion high drug dose (HD) (25 mg/kg/h orlistat in 40 mg/h LC-FA). The cumulative percentage of the dose of orlistat transported into lymph in both open and closed ring form was similar in the groups administered orlistat with 20 mg/h LC-FA over 2 h or 8 h but significantly lower in the group administered the high drug and LC-FA dose over 2 h (Fig 8, Panel A and B, and Table 3).

The lymph concentrations of total orlistat did not significantly differ at 0-2 h after commencing dosing of the 8 h and 2 h infusion because the orlistat dose and formulation were the same in the first 2 h (i.e. 4 mg/kg/h orlistat in 20 mg/h LC-FA). In the 8 h infusion group a steady state and peak rate of orlistat transport into lymph was reached between 4-9 h after commencing the 8 h infusion. By 7-8 h after commencing dosing the lymph concentration of orlistat was substantially greater in the 8 h infusion group when compared to the 2 h infusion groups as expected since the drug continued to be dosed. In contrast, for the group administered the higher drug dose over 2 h the total and closed ring orlistat concentrations in lymph peaked at 3 h then declined, and the peak concentrations were slightly higher than the groups administered the lower drug dose, though this was not significant (Fig 8, Panel C-D). The mass transport of total orlistat in lymph was significantly greater for the 8 h infusion group administered the lower drug dose (i.e. 32 mg/kg over 8 h) when compared to the 2 h infusion group administered the higher drug dose (i.e. 50 mg/kg orlistat over 2 h), suggesting that a prolonged infusion of a moderate rather than high drug dose is preferred to enhance total orlistat exposure in the lymph (Supplementary Figure 4.3). Interestingly, the cumulative lymphatic transport of TG was not statistically different across the three groups at 8 h post-dosing (Fig 9, Panel A). A steady-state rate of TG transport into lymph was evident in the 8 h infusion group apart from the final collection timepoints at 10-11 h (Fig 9, Panel B).

Total orlistat concentrations (which mostly consisted the open ring form) were lower in plasma when compared to lymph for the 2 h and 8 h infusion groups (Fig 8, Panel E). The orlistat plasma C_{max} occurred at 3 h post-dose for the 2 h infusion group and 8-9 h post-dose for the 8 h infusion group followed by a decline in plasma concentrations due to ceasing formulation infusion. At 8 h post dose, the orlistat plasma concentration was not statistically different between the 2 h and 8 h infusion groups (Fig 8, Panel E). The lymph:plasma concentration ratios (which were relatively high and ranged from 48-57 at the C_{max}) did not significantly differ for the three experimental groups (Fig 8, Panel F).

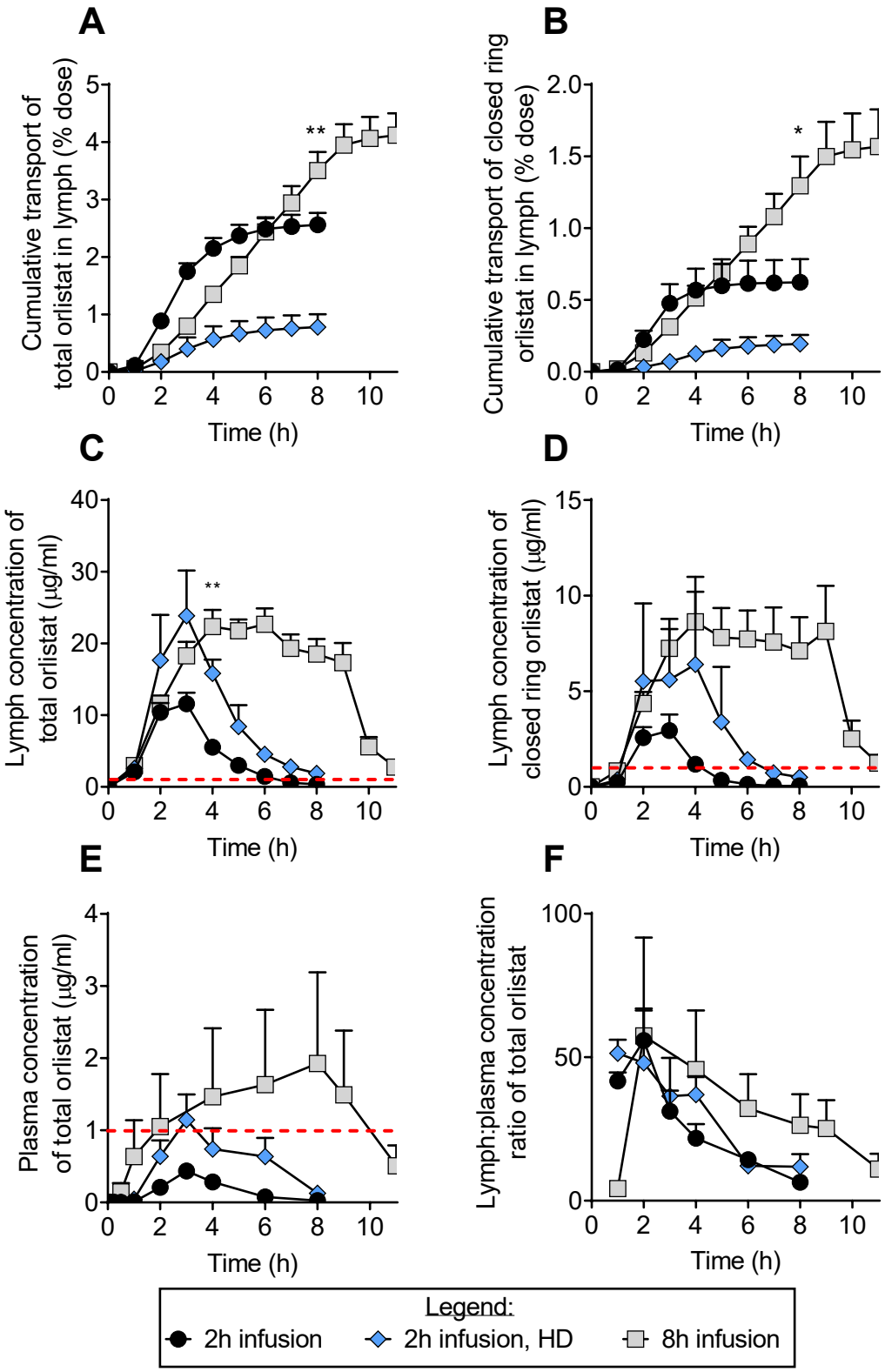


Fig 8. Impact of orlistat formulation infusion time on mesenteric lymph transport and plasma pharmacokinetics of orlistat in lymph cannulated rats. A) Cumulative lymphatic transport of total orlistat (open and closed ring forms) over time, B) Cumulative lymphatic transport of

closed ring orlistat over time, C) Dose-normalised lymph concentration of total orlistat (open and closed ring forms) over time, D) Dose-normalised lymph concentration of closed ring orlistat over time, E) Dose-normalised plasma concentration of total orlistat (mostly in the open ring form) over time, and F) The ratio of lymph to plasma concentrations of total orlistat over time in mesenteric lymph duct cannulated rats following intraduodenal infusion of formulations. Low dose formulations consisted of 4 mg/kg/h orlistat, 12.5 mg/h Tween 80, and 20 mg/h LC-FA (i.e. oleic acid) administered over 2 or 8 h for the short (2 h) versus long (8 h) infusion group. High orlistat dose (HD) infusion consisted of 25 mg/kg/h orlistat, 12.5 mg/h Tween 80, and 40 mg/h LC-FA administered over 2 h. In panel C-E orlistat concentrations are dose normalised to 8, 32 and 50 mg/kg for the 2 h infusion, 8 h infusion, and 2 h HD infusion groups, respectively. The red dashed line indicates the expected minimum therapeutic concentration of orlistat (i.e. 0.99 µg/mL). Data is presented as mean ± SEM for 2 h infusion (circle, black, n=5), 2 h HD infusion (diamond, blue, n=3), and 8 h infusion (square, grey, n=4). For Panel A-C, significance specifically applies to 8 h infusion versus 2 h infusion only. Significant difference to other groups determined from one-way ANOVA: ** p≤0.01, *p≤0.05

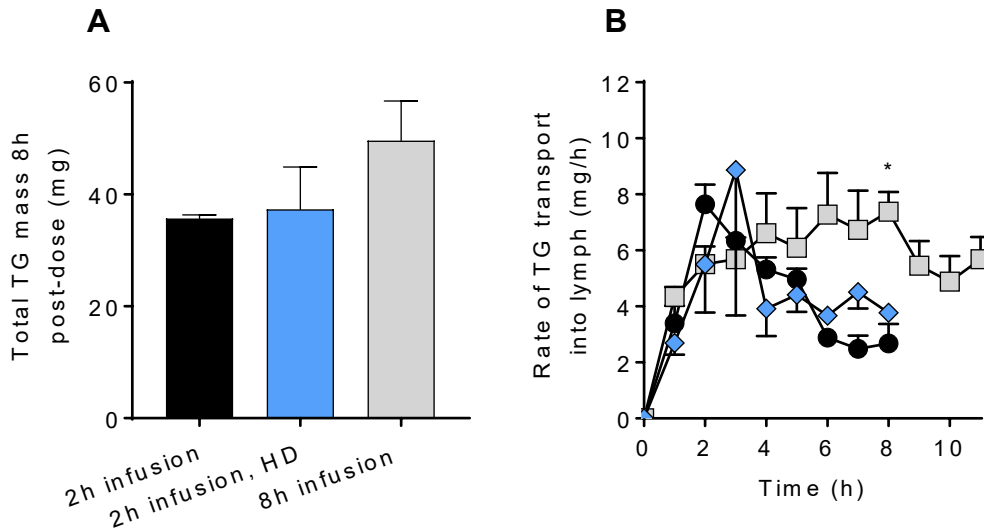


Fig 9. Impact of orlistat formulation infusion time on mesenteric lymph transport of triglyceride (TG). A) Total mass of TG transported in mesenteric lymph over 8 h, and B) Rate of TG transport into lymph over time following intraduodenal infusion of formulations to anesthetised, mesenteric lymph duct cannulated rats. Low dose formulations consisted of 4 mg/kg/h orlistat, 12.5 mg/h Tween 80, and 20 mg/h LC-FA (i.e. oleic acid) administered over 2 or 8 h for the short (2 h) versus long (8 h) infusion group. High orlistat dose (HD) infusion consisted of 25 mg/kg/h orlistat, 12.5 mg/h Tween 80, and 40 mg/h LC-FA administered over 2 h. Data is presented as mean ± SEM for 2 h infusion (circle, black, n=5), 2 h HD infusion (diamond, blue,

n=3), and 8 h infusion (square, grey, n=4). For Panel A-C, significance specifically applies to 8 h infusion versus 2 h infusion only. Significant difference to other groups determined from one-way ANOVA: *p≤0.05

Table 3 Summary of mesenteric lymphatic transport and plasma Cmax for orlistat in lymph cannulated rats administered the different formulations.

Formulation dosed	Cumulative % total orlistat dose in lymph over 8 h	Cumulative % closed form orlistat dose in lymph over 8 h	Lymph Cmax of total orlistat (µg/ml)	Lymph Cmax of closed form orlistat (µg/ml)	Plasma Cmax of total orlistat (µg/ml)	Cumulative TG mass transport in lymph (mg) over 8 h
8 mg/kg orlistat in no lipid (control)	0.86 ± 0.38%	0.17 ± 0.03%	3.36 ± 0.43	0.67 ±0.09	0.82 ± 0.35	26.36 ± 2.67
8 mg/kg orlistat in 40 mg MC-FA	0.33 ± 0.15%	0.05 ± 0.03%	1.60 ± 0.39	0.16 ± 0.07	0.17 ± 0.05	15.78 ± 4.49
8 mg/kg orlistat in 40 mg LC-TG	0.44 ± 0.06%	0.10 ± 0.02%	1.36 ± 0.36	0.37 ± 0.16	0.10 ± 0.02	22.16 ± 2.84
8 mg/kg orlistat in 40 mg LC-FA	2.56 ± 0.21% [b]	0.62 ± 0.16%	10.9 ± 1.09	2.94 ± 0.85	0.44 ± 0.08	35.7 ± 0.64
8 mg/kg orlistat in 80 mg LC-FA	3.62 ± 0.23%	1.57 ± 0.11% [c]	21.13 ± 0.93	9.08 ± 1.38	0.31 ± 0.07	31.77 ± 4.22
0.15 mg/kg orlistat in 40 mg LC-FA	0.59 ± 0.21%	0.24 ± 0.09%	0.06 ± 0.01	Below LOQ [a]	Below LOQ [a]	46.93 ± 2.76
50 mg/kg orlistat in 80 mg LC-FA	0.76 ± 0.23%	0.19 ± 0.06%	23.85 ± 6.31[d]	5.61 ± 3.18	0.97 ± 0.43	37.34 ± 7.58
32 mg/kg orlistat in 160 mg LC-FA (8 h infusion)	4.12 ± 0.38%	1.29 ± 0.20%	22.37 ± 2.30	8.63 ± 2.35	1.93 ± 1.26	49.62 ± 7.06

n=3-6 rats per group, data is presented as mean ± SEM. Statistical significance between groups was determined by one-way ANOVA.

[a] All lymph/plasma samples were below LOQ (i.e. <0.05 µg/ml) for the HPLC-MS/MS assay.

[b] Significantly greater when compared to all other experimental groups for the same parameter except for the groups administered 8 mg/kg orlistat in 80 mg LC-FA (2 h infusion) and 30 mg/kg orlistat in 160 mg LC-FA (8 h infusion) ($p \leq 0.001$)

[c] Significantly greater when compared to all other experimental groups for the same parameter except for the group administered 32 mg/kg orlistat in 160 mg LC-FA (8 h infusion) ($p \leq 0.001$)

[d] Significantly greater when compared to all other experimental groups for the same parameter except for the groups administered 8 mg/kg orlistat in 80 mg LC-FA (2 h infusion) and 30 mg/kg orlistat in 160 mg LC-FA (8 h infusion) ($p \leq 0.01$)

3.5 The bioavailability and systemic exposure of orlistat

To further quantify the contribution of lymphatic transport to the systemic availability of orlistat after administration in the LFs, the area under the plasma concentration versus time profiles (AUC) of total orlistat were compared in lymph intact and lymph cannulated rats administered the LC-FA and LC-TG based formulations (as these promoted the highest and lowest extent of lymphatic transport, respectively). The absolute bioavailability of total orlistat in the groups administered these formulations was also determined by comparing the plasma AUC of total orlistat to rats administered orlistat 0.4 mg/kg IV (Supplementary Fig 5). In both the groups administered orlistat intestinally and IV, plasma orlistat was almost entirely present in the open ring form suggesting that orlistat was rapidly hydrolysed in the systemic circulation. The closed ring form was detected in some samples but was below the limit of quantitation of the assay (0.05 µg/ml). Plasma concentrations over time and the bioavailability attributed by the orlistat open ring form were substantially higher for the LC-FA formulation (bioavailability of 3.6%) when compared to the LC-TG formulation (bioavailability of 0.7%) (Fig 10 and Table 4). In all rats administered orlistat into the intestine, the plasma C_{max} of orlistat occurred at 3-4 h post-dose followed by a decline beyond 6 h. In the rats administered the LC-TG based formulation there was no significant difference in total orlistat plasma concentrations between the lymph intact and lymph cannulated groups. In contrast after dosing the LC-FA formulation, the orlistat plasma concentrations were significantly greater in lymph intact versus lymph cannulated rats at 2 and 3 h after commencing dosing (Fig 10). By determining the proportional reduction in the plasma AUC in lymph intact versus lymph cannulated/diverted rats, ~33 and 14 % of systemic exposure of orlistat was estimated to be contributed by the lymphatically transported drug for the LC-FA and LC-TG formulations, respectively. Therefore, a substantially greater percentage of systemic exposure appeared to be contributed by lymphatic transport for the LC-FA based formulation when compared to the LC-TG formulation. This is consistent with the orlistat lymphatic transport data (Table 3).

From the ratio of the percent orlistat dose transported in lymph (in both open and closed ring forms) and the calculated bioavailability of total orlistat in lymph intact rats, 71% and 64% of the systemic exposure was estimated to be contributed by lymphatically transported drug for the LC-FA and LC-TG formulations, respectively (Table 4). An explanation for the differences in the calculated contribution of lymphatic transport from recovery in mesenteric lymph versus the reduction in plasma AUC in lymph cannulated vs lymph intact groups is not apparent at this time. It may be related to differences in drug absorption, distribution and clearance across the groups. Alternatively, the estimated bioavailability may not be completely accurate as the plasma AUC were truncated to 8 h. Also the bioavailability calculation assumes that the clearance and disposition in the intestinally and IV administered

groups are similar. Previous studies have shown that the clearance and disposition of lipophilic drugs administered IV is formulation dependent and differs upon drug entry into the circulation in lymph versus blood (Caliph et al., 2012). The formulation used for IV dosing in the current study was lipid based and the systemic clearance, volume of distribution and apparent elimination half-life were 119.3 ± 13.4 ml/h*kg, 0.1 ± 0.0 L/kg, and 0.5 ± 0.0 h, respectively.

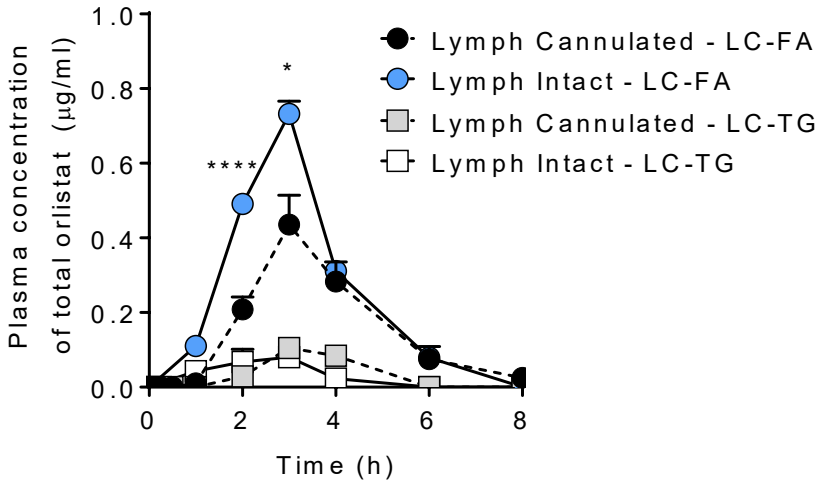


Fig 10. Effect of lipid type on systemic exposure of orlistat. Dose-normalised plasma concentrations of total orlistat (which was predominantly in the open ring form) over time in anesthetised, carotid artery cannulated and mesenteric lymph duct cannulated (dotted line) or lymph-intact (solid line) rats following intraduodenal infusion of formulations over 2 h. Formulations contained 8 mg/kg orlistat dispersed in 40 mg LC-FA (i.e. oleic acid) or LC-TG (i.e. olive oil) with 25 mg Tween 80 and 5.6 ml PBS. Data is presented as mean ± SEM for LC-FA (circle, black, n=5 for lymph cannulated and circle, blue, n=4 for lymph intact) and LC-TG (square, grey n=4 for lymph cannulated and square, white, n=3 for lymph intact). Significant difference to other groups determined from one-way ANOVA: ***p≤0.001, *p≤0.05

Table 4 Summary of the orlistat plasma and lymph pharmacokinetic data in lymph cannulated (LC) vs lymph intact (LI) rats administered 8 mg/kg orlistat in 40 mg LC-FA or LC-TG. Data is presented as mean ± SEM for n=3-5 rats. Note that orlistat in plasma was almost entirely in open ring form in all animals.

Experiment group	**Plasma C_{max} (µg/ml)**	**Plasma AUC (µg*h/ml)**	**Absolute bioavailability (%)** [a]	**Lymphatic transport (% dose over 8 h)**	**% contribution of lymphatic transport to systemic availability** [b]
LC-FA – LC	0.44 ± 0.08 [c]	1.33 ± 0.34	2.41 ± 0.61% [d]	2.56 ± 0.21%	Calculation 1: 71 ± 6% Calculation 2: 51 ± 2% Calculation 3: 33%
LC-FA – LI	0.73 ± 0.03	1.99 ± 0.06	3.60 ± 0.11% [e]		
LC-TG – LC	0.10 ± 0.01	0.33 ± 0.13	0.59 ± 0.23%	0.44 ± 0.06%	Calculation 1: 64 ± 9% Calculation 2: 42 ± 3% Calculation 3: 14%
LC-TG – LI	0.24 ± 0.25	0.38 ± 0.01	0.69 ± 0.02%		

[a] To determine bioavailability a group was IV administered 0.4 mg/kg orlistat in 120 mg soybean oil. Plasma AUC of total orlistat was 3.45 ±0.42 µg*h/ml.

[b] Percent contribution of lymphatic transport to systemic availability was estimated via three calculation methods: (1) the ratio of % orlistat dose transported into lymph in lymph cannulated rats and absolute bioavailability in lymph intact rats; (2) the ratio of cumulative % dose transported into lymph in lymph cannulated rats and the sum of % dose transported in lymph and bioavailability calculated in lymph cannulated rats; and (3) the proportional reduction in mean plasma AUC in lymph cannulated versus lymph intact rats.

[c] Significantly less when compared to the same data for lymph intact rats ($p \leq 0.05$)

[d] Significantly greater when compared to the LC-TG lymph cannulated group ($p \leq 0.05$)

[e] Significantly greater when compared to LC-TG based formulation dosed groups for the same parameter ($p \leq 0.01$)

4 Discussion

The utility of LFs to assist the absorption of poorly water soluble drugs (PWSDs) has been recognised and studied for several decades, with several lipophilic drugs currently marketed in LFs (Porter et al., 2008). LFs promote the absorption of PWSDs by presenting the drug in a pre-solubilised form (thus avoiding dissolution limited absorption) and enhancing drug solubilisation within the gastrointestinal lumen. The latter occurs via the formation of colloidal lipid structures in the intestinal lumen that have enhanced solubilisation capacity for lipophilic drugs when compared to fasted intestinal contents. For a subset of very highly lipophilic drugs, LFs also increase drug bioavailability by promoting lymphatic drug transport, an absorption route that avoids hepatic first pass metabolism (Williams et al., 2013). Orlistat, a highly lipophilic PWSD with a clogP of 8.1 (Wishart et al., 2006), is poorly bioavailable when administered in standard formulations such as dry powders in capsules (2007; 2009; Zhi et al., 1995; Zhi et al., 1999). This is believed to result from both poor absorption and high first pass metabolism of orlistat. As orlistat is a highly lipophilic PWSD we explored the potential to enhance the absorption, lymphatic transport and systemic availability of orlistat via co-administration with LFs. The impact of orlistat dose and co-administration with different lipid types and doses on orlistat absorption and lymphatic transport, as well as the mechanisms of orlistat absorption were studied. The ultimate aim was to identify an enteral formulation to enhance the lymphatic and systemic availability of orlistat for the treatment of systemic conditions associated with elevated pancreatic lipase activity in lymph such as acute pancreatitis and trauma-haemorrhagic shock.

Co-administration with the LC-FA, oleic acid, significantly enhanced total orlistat transport in the lymph when compared to administration with lipid-free or MC-FA formulations (Table 3). This is consistent with published data showing that lymphatic drug transport is significantly higher on co-administration with LC lipids when compared to MC lipids (Caliph et al., 2000; Han et al., 2014; Khoo et al., 2003; Trevaskis et al., 2013). Co-administration with LC lipids is suggested to enhance lymphatic lipid and therefore drug transport because LC lipids are assembled into intestinal lipoproteins that are transported from the intestine via the lymphatic system. In contrast, MC lipids are absorbed from the intestine directly into the mesenteric blood capillaries that flow into the portal vein and therefore do not substantially promote lymphatic lipid and drug transport (Trevaskis et al., 2015b). In support of this concept the transport of lipid (TG) in lymph was significantly greater in the groups dosed with LC-FA versus MC-FA in the current study (Fig 3 A-B). In addition, the MC-FA may have supported lower drug absorption and therefore lower subsequent lymphatic drug transport since MC lipids sometimes display lower drug solubilisation capacity upon dilution in the gastrointestinal tract when compared to LC lipids (Williams et al., 2013). In support of this, the total orlistat

concentrations in both mesenteric lymph (Fig 2 C-D) and plasma (Fig 2 E) were substantially higher after co-administration with LC-FA versus MC-FA suggesting higher overall absorption of orlistat after dosing with the LC-FA.

While LC-TGs are normally expected to promote lymphatic drug transport, in this study, co-administration with olive oil (i.e. a LC-TG) did not significantly increase orlistat recovery in lymph when compared to the MC-FA and lipid-free formulation (Fig 2 A-D and Table 3). This result is most likely attributed to the pharmacological action of orlistat i.e. inhibition of PL mediated digestion of the TG in the formulation and thus TG absorption and transport into lymph (Trevaskis et al., 2015b). This may have reduced the absorption of orlistat as the orlistat would remain associated with undigested olive oil droplets in the gastrointestinal lumen.

Furthermore, the digestion of the olive oil in this formulation may have been lower than expected as the studies were conducted in anaesthetised animals where intestinal transit and digestion can be reduced. Indeed, a previous study by Porter et al. showed that the absorption and lymphatic transport of halofantrine (also a highly lipophilic poorly water-soluble drug) is impaired when it is administered to anaesthetised when compared to conscious rats in a TG based emulsion. The difference in absorption and lymphatic transport between conscious and anaesthetised rats could, however be reduced by administration of halofantrine in a pre-dispersed (micellar) and digested (FA and MG based) LF (Porter et al., 1996). In acute and critical illnesses such as severe acute pancreatitis and trauma-haemorrhagic shock, patients are generally unconscious and sedated such that administration of orlistat to treat conditions will require that the drug is administered via an enteral feeding tube. Therefore, intestinal infusion in a LC-FA formulation will provide an additional advantage in this setting.

Although oleic acid is in a digested form, the lymphatic transport of TG after administration of orlistat in the oleic acid based formulation was significantly lower than the lymphatic TG transport reported previously in our lab following administration of the same LF with other drugs (Supplementary Fig 4) (Han et al., 2015). Additionally, the data is consistent with a previous study where co-administration with 2.8-3.2 mg/kg orlistat was found to reduce lymphatic transport of lipid and a TG mimetic prodrug (Han et al., 2015). In that study it was proposed that orlistat may inhibit other stages of the lipid absorption and metabolic pathway in addition to lipid digestion in the intestinal lumen. Orlistat may thus have partly inhibited its own lymphatic uptake by reducing lymphatic TG transport. In support of this, other drugs with similar lipophilicity to orlistat, such as halofantrine, display greater lymphatic uptake than orlistat (Caliph et al., 2000). We can suggest three potential mechanisms by which orlistat may inhibit lymphatic transport of lipids. i) Orlistat may inhibit the release of cholecystokinin from the duodenum, thereby altering small intestine motility and lipid/drug absorption (Mathus -

Vliegen et al., 2004). ii) Binding of orlistat to PL may directly inhibit the absorption of FAs. It has been reported previously that binding of PL at the apical border of enterocytes facilitates FA absorption (Bosner et al., 1989). iii) Orlistat may inhibit intracellular lipases such as pancreatic triacylglycerol lipase that are involved in the mobilization of endogenous TG pool in the enterocytes (Mahan et al., 2001; Porter et al., 2007).

The lymphatic transport of orlistat increased significantly when the LC-FA dose was doubled from 40 mg to 80 mg (i.e. 160 mg/kg to 320 mg/kg oleic acid dose) (Fig 4 A-D). In a previous study in our lab the lymphatic transport of the lipophilic drug halofantrine was found to increase with lipid dose but reach a plateau at ~200 mg/kg lipid dose in mice, rats and dogs (Trevaskis et al., 2013). We thus believe it is likely that co-administration with lipid doses higher than 80 mg (320 mg/kg) of oleic acid will not further increase the lymphatic transport of orlistat.

The intestinal absorption and lymphatic transport of orlistat was expected to be orlistat dose-dependent since orlistat is a PWSD so likely to display dose-dependent solubilisation and absorption. In addition, the pharmacological action of orlistat (i.e. lipase inhibition) results in a negative effect on dietary lipid digestion and absorption and increasing orlistat dose may reduce lymphatic TG and therefore drug transport. In support of this, our lab previously found that co-administration with ~3 mg/kg orlistat significantly reduced lymphatic transport of TG and a lipophilic prodrug (Han et al., 2015) whereas administration with 0.15 mg/kg orlistat did not (Supplementary Figure 4.2, unpublished). It was therefore hypothesized that a greater percentage of the orlistat dose would be absorbed after administration of 0.15 mg/kg vs 8 mg/kg orlistat since no inhibition of luminal hydrolysis of TG and DG would occur at the lower dose. Interestingly, this was not the case and in fact, the lymphatic transport of total orlistat (as a % dose) was significantly lower after administration of the lower drug dose (Fig 6 A and Table 3). The reduced lymphatic transport may have resulted from orlistat binding to lipases in the gastrointestinal lumen (Lewis and Liu, 2012). In addition, the metabolism of orlistat may be dose dependent such that any orlistat that is absorbed after administration of the 0.15 mg/kg dose may be rapidly metabolised prior to systemic entry. In support of this, a previous study in obese patients found that ~42% of the mass of orlistat that is absorbed from the intestinal tract is rapidly converted into two major metabolites (Zhi et al., 1996).

The lymphatic transport of orlistat was also reduced when the orlistat dose was increased from 8 mg/kg to 50 mg/kg (Fig 6 A-B, and Table 3). This was likely due to dose dependent solubilisation of orlistat where a lower proportion of the drug dose was solubilised at the higher dose leading to reduced drug absorption. In addition, it is also possible the increased orlistat dose resulted in greater inhibition of lymphatic lipid and thereby drug

transport. In support of this, the cumulative TG mass transport in lymph for the high orlistat dose group (i.e. 50 mg/kg) was similar to the intermediate orlistat dose group (i.e. 8 mg/kg) despite that a higher dose of lipid (i.e. 80 mg versus 40 mg of oleic acid) was administered (Fig 7B). The middle 8 mg/kg dose of orlistat thus appeared to be an optimal dose for orlistat absorption and lymphatic transport.

In an attempt to further increase the mass transport of orlistat in lymph, 4 mg/kg/h orlistat and 20 mg/h LC-FA were infused for 8 h rather than 2 h. This did indeed increase drug concentrations and mass transport in lymph suggesting that greater masses of orlistat can be transported in lymph via infusion of a lower drug concentration over a longer infusion time.

Within the lymph samples across all groups and all time points 10-37 % of the orlistat was present in the closed ring form and the remaining was in open ring form. The proportion of orlistat in the closed ring form was highest from 0-2 h post-dose as would be expected shortly after drug administration. The closed ring form of orlistat is considered ~1000-fold more active than the open ring form (Ballinger and Peikin, 2002; Zhi et al., 1996; Zhi et al., 1999) although it is uncertain as to whether the ring opening of orlistat in the lymph (and plasma) in this study was due to intended pharmacological activity (i.e. binding to and inhibiting lipases) or by unintended hydrolysis by non-specific esterases. Nonetheless, the lymph concentration of orlistat present in closed ring form was consistently above the therapeutic concentration of orlistat (i.e. 0.99 µg/ml) after administration with the LC-FA formulation, but not the lipid free formulation confirming that the LC-FA formulation supports delivery of pharmacologically active concentrations of orlistat in lymph. Within the plasma samples, orlistat was almost entirely present in open ring form. Orlistat was thus rapidly hydrolysed to the open ring form in the systemic circulation due to either passage through the liver, or the presence of more lipases and/or esterases in plasma when compared to lymph (Mittal et al., 2009). In support of this, more orlistat was converted to the open ring form on contact with plasma than lymph during HPLC-MS assay development and validation.

The concentrations of closed ring orlistat in mesenteric lymph after administration of all formulations were above the IC_{50} of orlistat in buffer (i.e. 0.01-0.40 µg/ml (Johnston and Goldberg, 2006). However, the IC_{50} may differ in lymph and *in vivo* because of drug association with lipids and lipoproteins, and other physiological variables (Bläckberg et al., 1981; Borgström, 1975; Rathelot et al., 1976). Indeed, a previous study reported that 0.99 µg/ml orlistat is required for effective inhibition of PL in mesenteric lymph (Qin et al., 2012). Moreover, it is unclear whether the open ring form of orlistat in lymph and plasma is the result of PL inhibition or esterase mediated hydrolysis. Thus, in future studies we will determine if orlistat is therapeutically active in lymph and plasma, and able to effectively treat conditions

where elevated pancreatic lipase in lymph and/or blood drive disease progression such as acute pancreatitis (Mittal et al., 2009; Navina et al., 2011; Patel et al., 2015) and trauma haemorrhagic shock (Morishita et al., 2012; Qin et al., 2012). Overall, the LC-FA formulation supported the highest concentrations of orlistat in lymph which were well above the anticipated therapeutic concentration.

5 Conclusion

The studies conducted here confirm the potential to improve the absorption and lymphatic transport of orlistat through co-administration with appropriately designed LFs. A detailed analysis of the effect of lipid type (LC-FA, MC-FA or LC-TG), lipid dose, orlistat dose, and infusion time on orlistat absorption and lymphatic transport was completed. Overall, co-administration with a LC-FA based formulation provided the highest extent of lymphatic drug and lipid transport when compared to the MC-FA, LC-TG and lipid-free formulations. Increasing the LC-FA dose from 40 to 80 mg significantly improved lymphatic drug transport. An increase or decrease in orlistat dose from 8 mg/kg orlistat appeared to negatively influence lymphatic drug transport, but not lymphatic lipid transport. Extending the infusion time of orlistat to 8 h resulted in greater total mass recovery of orlistat in lymph and the maintenance of high drug concentraiotns in lymph and plasma for longer periods. However, the highest peak lymph concentration of orlistat was obtained when the highest orlistat dose was administered with 80 mg LC-FA over 2 h infusion. Whether higher peak concentrations or longer periods above a threshold concentration are more useful therapeutically is unknown at this time. Mesenteric lymph concentrations of closed ring orlistat were higher than the IC_{50} of orlistat for all formulations. However, the LC-FA based formulation had the highest measured lymph concentrations. A LC-FA formulation strategy therefore shows significant promise in being able to deliver therapeutically active concentrations of orlistat to lymph and plasma to provide effective treatment of conditions associated with elevated pancreatic lipases in lymph or systemically such as acute and critical illnesses.

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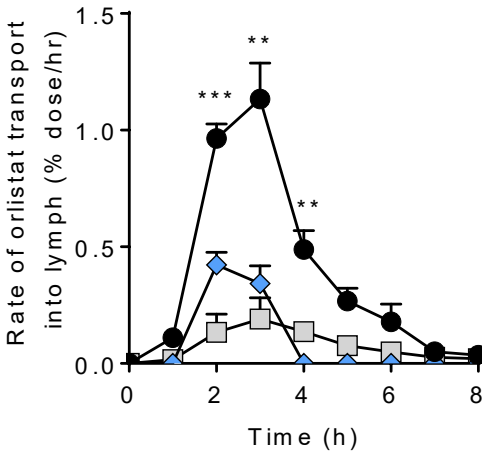
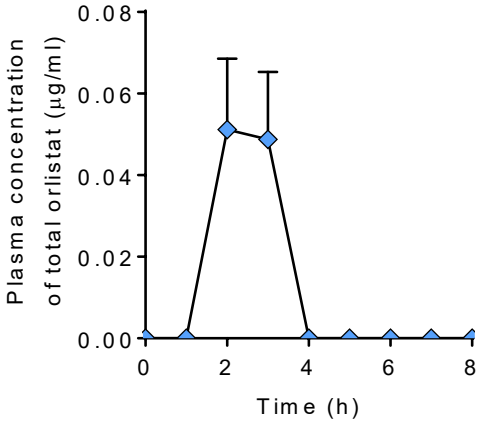
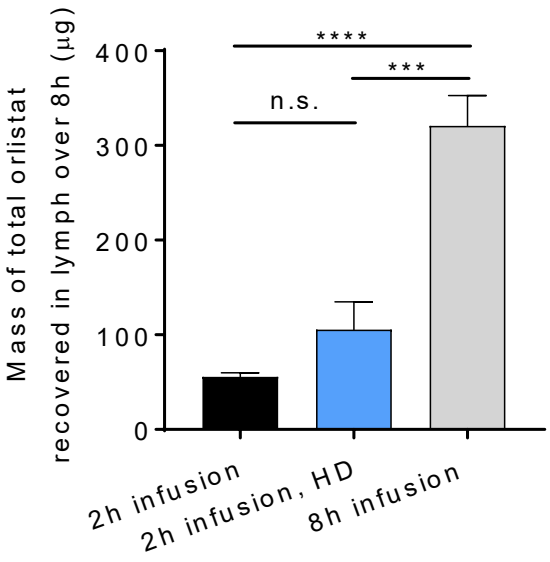
Supplementary information



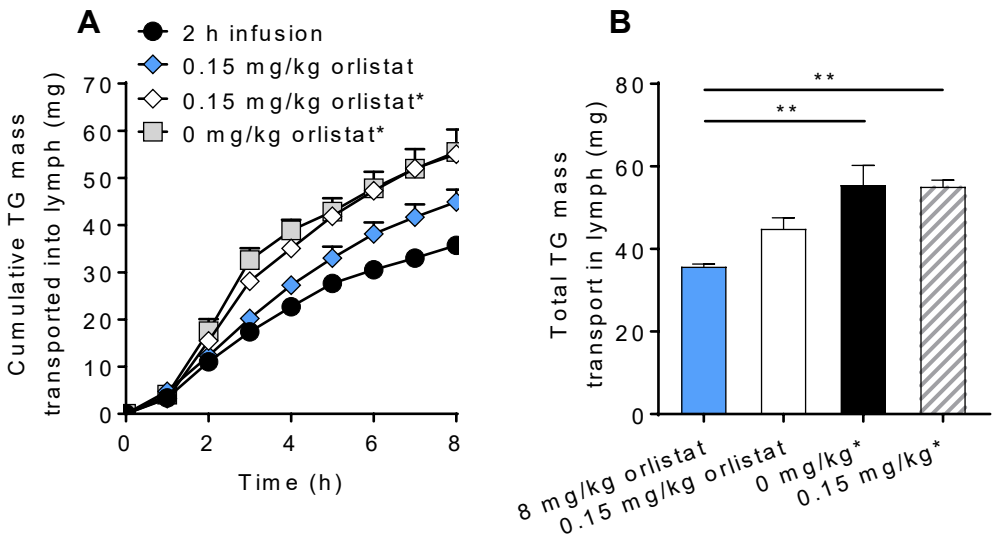
Supplementary Fig 1. Rate of orlistat transport into lymph (% dose/h) over time in mesenteric lymph duct cannulated, anesthetised rats following intraduodenal infusion of formulations from 0 to 2 h. Orlistat was dispersed in long chain fatty acid (LC-FA, i.e. oleic acid), 25 mg Tween 80 and 5.6 ml PBS. For the 8 mg/kg and 0.15 mg/kg orlistat formulations, 2 mg and 0.035 mg of orlistat were incorporated into the formulation, respectively, with 40 mg LC-FA. For the 50 mg/kg orlistat formulation, 13.5 mg orlistat and 80 mg LC-FA were incorporated into the formulation as the drug mass was not soluble at the lower oleic acid dose. Data is presented as mean ± SEM for the 0.15 mg/kg orlistat formulation (blue diamond, n=4); the 8 mg/kg orlistat formulation (black circle, n=5); and the 50 mg/kg orlistat formulation (grey square, n=3). Significant difference to other groups determined by one-way ANOVA: ***p≤0.001, **p≤0.01



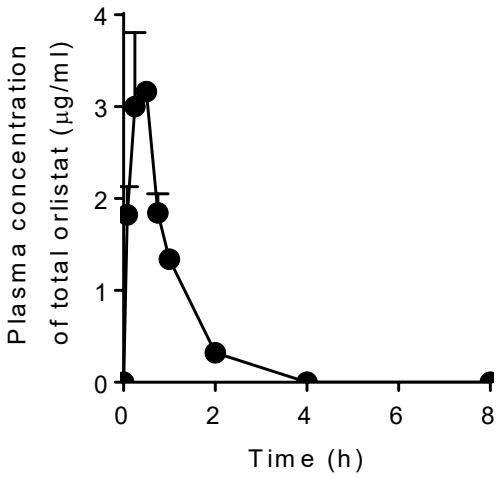
Supplementary Fig 2. Concentration of orlistat in lymph over time in mesenteric lymph duct cannulated, anesthetised rats following intraduodenal infusion of the 0.15 mg/kg orlistat formulation (i.e. 0.035 mg orlistat dispersed in 40 mg oleic acid, 25 mg Tween 80, and 5.6 ml PBS) from 0 to 2 h. Data is presented as mean ± SEM.



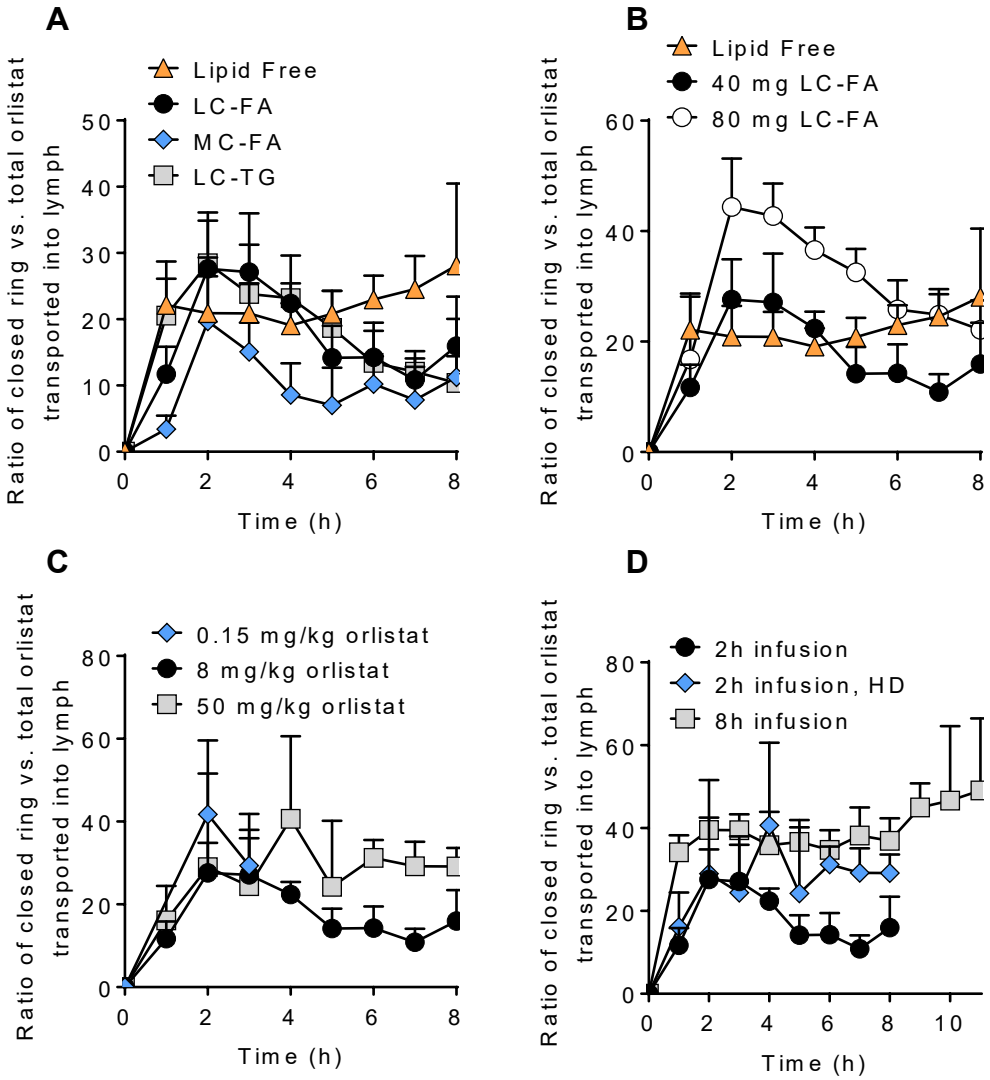
Supplementary Fig 3. Total mass of TG transported in mesenteric lymph over 8 h in mesenteric lymph duct cannulated rats following intraduodenal infusion of formulations. Low dose formulations consisted of 4 mg/kg/h orlistat, 12.5 mg/h Tween 80, and 20 mg/h LC-FA (i.e. oleic acid) administered over 2 or 8 h for the short (2 h) versus long (8 h) infusion group. High orlistat dose (HD) infusion consisted of 25 mg/kg/h orlistat, 12.5 mg/h Tween 80, and 40 mg/h LC-FA administered over 2 h. In panel C-E orlistat concentrations are dose normalised to 8, 32 and 50 mg/kg for the 2 h infusion, 8 h infusion, and 2 h HD infusion groups, respectively. Data is presented as mean ± SEM for 2 h infusion (circle, black, n=5), 2 h HD infusion (diamond, blue, n=3), and 8 h infusion (square, grey, n=4). For Panel A-C, significance specifically applies to 8 h infusion versus 2 h infusion only. Significance by one-way ANOVA: *** $p \leq 0.001$, ****$p < 0.0001$



Supplementary Fig 4. A comparison of the impact of orlistat dose on mesenteric lymph transport of triglyceride (TG) measured from this study versus Han et al. (Han et al., 2015). Starred groups are from Han et al. A) Cumulative lymphatic transport of TG over time, and B) Total mass of TG transported in mesenteric lymph over 8 h, following intraduodenal infusion of formulations to anesthetised, mesenteric lymph duct cannulated rats. Groups from this study were administered 0.15 or 8 mg/kg orlistat dispersed in 40 mg oleic acid, 25 mg Tween 80 and 5.6 ml PBS. Groups from Han et al. were administered 2 mg of mycophenolic acid TG prodrug in 40 mg oleic acid, 25 mg Tween 80 and 5.6 ml PBS. In the Han study, orlistat (0.9 mg in ethanol) was added to the lipid formulation 5 min prior to dosing; the volume of solvent spiked was <1% of the total formulation prepped. The 0.15 mg/kg orlistat data from Han et al., is not published. Data is presented as mean ± SEM for 8 mg/kg orlistat (black circle, n=5), 0.15 mg/kg orlistat (blue diamond, blue, n=3), 0 mg/kg orlistat* (grey square, n=5), and 0.15 mg/kg orlistat* (white diamond, n=4). Data were not significant between groups from one-way ANOVA.



Supplementary Fig 5. Dose-normalised plasma concentrations of total orlistat (which was predominantly in the open ring form) over time in anesthetised, carotid artery and jugular vein cannulated lymph-intact rats following intravenous administration. Orlistat IV formulation contained 0.4 mg/kg orlistat dispersed in 1% egg phosphatidylcholine, 2% glycerol in PBS and was administered at a rate of at a rate of 0.2 ml/min for 5 min**.** Data is presented as mean ± SEM, n=3.



Supplementary Fig 6. The ratio of closed ring versus total orlistat in mesenteric lymph from, A) Experimental groups dosed different lipid types, B) Experimental groups dosed different lipid dose, C) Experimental groups dosed different orlistat dose, and D) experimental groups with different formulation infusion time. All groups are conducted with mesenteric lymph duct cannulated, anesthetised rats following intraduodenal infusion of formulations from 0 to 2 h, except for the 8 h infusion group which is intraduodenally administered formulation from 0 to 8 h. Rate of infusion was 2.8 ml/h.

References

Han, S., Hu, L., Quach, T., Simpson, J., Trevaskis, N., Porter, C.H., 2015. Profiling the Role of Deacylation-Reacylation in the Lymphatic Transport of a Triglyceride-Mimetic Prodrug. Pharm Res 32, 1830-1844.