

Exploring the Impact of Drug Properties on the Extent of Intestinal Lymphatic Transport - *In Vitro* and *In Vivo* Studies

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Received: 28 July 2014 / Accepted: 13 November 2014 / Published online: 27 November 2014
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ABSTRACT

Purpose Intestinal lymphatic transport of specific lipophilic drugs offers therapeutic advantages and maximises oral bioavailability. The aims of this study were; to compare intestinal lymphatic transport of a range of drugs and to investigate the influence of cyclosporine A on the mechanism/extent of lymphatic transport.

Methods Caco2 cells and an anaesthetised mesenteric lymphatic cannulated rat model were used for *in vitro* and *in vivo* studies. Lymphatic transport of three lipophilic drugs was directly compared in a long chain fatty acid formulation. In addition, the impact of cyclosporine A on triglyceride turnover was evaluated *in vivo* and *in vitro*.

Results The extent of intestinal lymphatic transport in rats was positively correlated with drug solubility in triglyceride and negatively correlated with drug aqueous solubility. Cyclosporine A displayed non-linear lymphatic transport kinetics and reduced intestinal lymph triglyceride. *In vitro* experiments indicated that the cellular processes affected were intracellular lipid processing and/or lipid secretion.

Conclusions The linear correlations obtained using a range of lipophilic drugs confirm that the simplified approach of determining aqueous or triglyceride drug solubility is useful in predicting the extent of lymphatic transport. *In vitro* experiments correlated with *in vivo* observations, demonstrating the usefulness of the Caco-2 model for mechanistic investigations.

KEY WORDS cyclosporine A · *in vitro-in vivo* correlations · lipid formulations · lymphatic transport

ABBREVIATIONS

BSA	Bovine serum albumin
DDT	Dichlorodiphenyltrichloroethane
FA	Fatty acids
FaSSIF	Fasted simulated state intestinal fluid
HPLC	High performance liquid chromatography
LCFA	Long chain fatty acids
LCT	Long chain triglyceride
LOQ	Limit of quantitation
PBS	Phosphate buffered saline
P-gp	P-glycoprotein
TG	Triglyceride
TLC	Thin layer chromatography
TRL	Triglyceride rich lipoproteins

INTRODUCTION

Oral delivery of poorly water soluble drugs is challenging due to limited and unpredictable absorption characteristics [1, 2]. Considerable attention has been focused on the use of lipid-based formulations to enhance oral absorption of such drugs [2]. The suggested mechanisms of absorption enhancement include; improved solubilisation [2, 3], increased membrane permeability, inhibition of pre-systemic drug metabolism and promotion of mesenteric lymphatic transport [4, 5]. Drugs that are absorbed from the intestine may access the systemic circulation *via* the hepato-portal blood pathway or *via* the mesenteric lymphatic pathway. Transporting drugs *via* the mesenteric lymphatic route offers key advantages such as the capacity to avoid first pass hepatic metabolism and, in the case of certain diseases, the potential to deliver therapeutic drugs, such as immuno-suppressants, anti-cancer drugs and anti-virals, directly to the pharmacological site of action [4, 6–8]. However, while the benefits of formulating drugs to enhance intestinal lymphatic transport are now well established, the

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factors governing intestinal lymphatic drug uptake are not completely understood.

Several factors can affect the extent of lymphatic drug transport - these include the physicochemical properties of drug, the formulation type and the composition of lipid excipients in the formulation [2, 6]. In the past, the key physicochemical properties which identified a drug as a possible candidate for lymphatic transport were a log $P > 5$ and a triglyceride (TG) solubility > 50 mg/ml [9]. These estimates were based on the assumption that the drug was transported in association with triglyceride rich lipoproteins (TRL), the knowledge that portal blood flow is 500-fold faster than intestinal lymph flow and the fact that the TRLs make up approximately 1% of the lymph [9]. This approach has successfully predicted the lymphatic transport of a number of compounds. For example, in the case of DDT (dichlorodiphenyltrichloroethane; log P 6.19, lipid solubility 80 mg/g) and halofantrine (log P 8.5, lipid solubility > 50 mg/ml), up to 15 and 20% respectively of the dose is transported in the lymph of rats following co-administration in lipid-based vehicles [10, 11]. In addition, hexachlorobenzene with a high log P (6.53) but low TG solubility (7.5 mg/g) was poorly transported in the lymph (3% of the dose) [10]. However, exceptions to this rule have also been reported. Low levels of lymphatic transport were reported in the cases of penclomedine (log P 5.48, TG solubility 174 mg/ml) [10] and Cl-176 (log P 5.83, TG solubility > 100 mg/ml) [12] (3% and $< 1\%$ of the administered dose, respectively). These results suggest that the initial parameters, log P and TG solubility, may not be the only physicochemical properties of the drug which exert influence on the extent of intestinal lymphatic transport. Mechanistic studies using a wider range of drugs and examining a greater number of physicochemical properties are required to fully elucidate this.

The composition of the lipid-based formulation is also known to exert a profound effect on the extent of lymphatic transport of drugs. In particular, it is now clear that vehicles rich in long chain fatty acids (LCFA) enhance lymphatic transport of certain drugs, compared to those based on short or medium chain fatty acids [13–15]. This is likely due to the greater effectiveness of the former to stimulate the production of TRLs. Other formulation-related factors may also play a role, such as the type of formulation (microemulsion *versus* micellar) [16] and the co-administration of bioactive excipients [8, 17, 18].

A range of *in vitro* and *in vivo* models have been utilised to study intestinal lymphatic drug transport. *In vivo* models include mesenteric lymph duct cannulation in anaesthetised and unanaesthetised rats [10, 12, 16–18]. Although rodents are more widely used, especially for early stage studies, a conscious dog model has been developed [19, 20], which facilitates more clinically relevant dosing studies. More recently, a lymph cannulated mouse model was established [21] which will facilitate future studies in various disease mouse models. That study also demonstrated that the extent of intestinal lymphatic transport of halofantrine is species specific (mouse (16%), rat (29%) and dogs

(57%)), which highlights another potential challenge to developing pre-clinical predictive models of lymphatic absorption of drugs. Furthermore, even within the same *in vivo* model, lymphatic transport can be affected by experimental factors such as the site of lymph collection (*i.e.* mesenteric *versus* thoracic) [22], the effect of general anaesthesia [23] and the period of fasting prior to dosing [24].

Valuable mechanistic data has also been obtained from *in vitro* and *ex vivo* models. Gershkovich and Hoffman have shown that association of a drug with chylomicrons isolated from plasma correlated very closely with the degree of *in vivo* lymphatic transport reported in the literature [25]. This model may, therefore, be a useful tool for identification of drug candidates for intestinal lymphatic transport. The *in vitro* intestinal cell culture model, Caco2, is capable of secreting TRLs and provides an opportunity to perform mechanistic studies at the cellular level which are not possible in the more complex *in vivo* environment [17, 18].

A number of studies have explored correlations between physicochemical properties of drugs and *in vivo* intestinal lymphatic uptake, with the aim of developing more accurate screening tools to predict the lymphatic transport properties of new drug candidates [25, 26]. However, the reliability of these predictions is weakened by the fact that the *in vivo* data used for their development are derived from experiments where formulation type, anaesthetic status and site of lymphatic cannulation are not uniform. In particular, the differing lipid composition of formulations used in *in vivo* studies can lead to different rates and extents of triglyceride transport within intestinal lymph, thereby limiting direct comparisons between different studies. In an effort to overcome this, the current study directly compares the extent of intestinal lymphatic transport of three lipophilic drug compounds, with varying log P and lipid solubilities, by administering the drugs in the same lipid formulation, under the same experimental conditions, in the anaesthetised rat model. Further in-depth studies were carried out in the case of one of these drugs, cyclosporine A, to investigate the effects of dose on lymphatic TG and drug transport. Finally, *in vitro* mechanistic studies using the Caco-2 cell model were performed to help interpret the results obtained from the *in vivo* rat model. This type of combination *in vitro* / *in vivo* approach can provide useful information on the factors controlling intestinal lymphatic drug transport, help to identify potential drug candidates and assist in the design of effective lipid-based delivery systems to enhance lymphatic transport.

MATERIALS AND METHODS

Materials

Saquinavir base (Ro-31-8959/000) and internal standard (Ro 31-9564) were kindly donated by Roche Products UK (Herts,

UK). Labrasol (saturated polyglycolysed C8-C10 glycerides, 50–80% C8; 20–50% C10), Peceol and Plurol Oleique (polyglyceryl oleate) were kindly donated by Gattefosse (Saint-Priest, France) and cremophor EL (polyoxyl 35 castor oil) was donated by BASF (Ludwigshafen, Germany). The following reagents were obtained from Sigma-Aldrich (Ireland): Oleic acid (cis-9-Octadecenoic acid, Corn oil (composition), Tween 80, DDT (1,1-bis (4-chlorophenyl)-2,2,2-trichloroethane), triolein, foetal bovine serum, l-glutamine, phosphatidyl choline, cholesterol oleate, bovine albumin, hydrochloric acid, sodium hydroxide, pancreatin. The following reagents were obtained from Gibco: Dulbecco's modified Eagle's medium (DMEM), gentamicin, sodium pyruvate. Cyclosporine A, USP 26 standard, Batch 14305CC010 was donated by IVAX Pharmaceuticals, Czech Republic.

Formulations

LCFA Formulation

The long-chain fatty acid (LCFA) formulation employed in this study was an oleic acid microemulsion prepared as previously described [16]. The formulation consisted of: oleic acid (oil phase) (10% *w/w*, 6 mg per 3 ml dose), Plurol oleique® (surfactant) (12.92% *w/w*, 7.75 mg per 3 ml dose), and Labrasol® (surfactant) (77.08% *w/w*, 46.25 mg per 3 ml dose). Surfactant components were weighed into 10 ml vials and stirred (333 rpm) at 40°C. The oil phase was then added and stirred until the system was homogenous. The oil/surfactant blend was allowed to equilibrate for 24 h before further use and then diluted 1:50 with distilled deionised water, to form a stable microemulsion. Excess drug (DDT, saquinavir or cyclosporine A) was added to the microemulsion formulation, followed by sonication (5 min) and stirring overnight to obtain saturated solutions of each compound. Formulations were passed through a 0.45 µm filter prior to administration. Drug concentrations in all lipid formulations were verified on the day of dosing using a validated HPLC assay.

LCT Formulation

The long-chain triglyceride (LCT) formulation employed in this study was a corn oil microemulsion, which consisted of 30% *w/w* (18 mg per 3 ml dose) corn oil (oil phase) with the following surfactants: peceol (20% *w/w*, 12 mg per 3 ml dose), cremophor EL® (16.67% *w/w*, 10.02 mg per 3 ml dose) and Tween 80® (33.33% *w/w*, 19.99 mg per 3 ml). Surfactant components were weighed into 10 ml Regina® vials and stirred (333 rpm) at 40°C. The oil phase was then added and stirred until the system was homogenous. The oil/surfactant blend was allowed to equilibrate for 24 h before further use and then diluted 1:50 with distilled deionised water, to form the stable microemulsion. The required quantity of

cyclosporine A was added to microemulsion formulation and stirred for 2 h prior to intraduodenal dosing. Cyclosporine A concentration in the formulations was verified on the day of dosing using a validated HPLC assay.

Neoral®

Neoral® capsules (Novartis) were purchased for *in vitro* studies. Neoral® is a commercially available lipid formulation containing cyclosporine A (100 mg/ml). The Neoral® concentrate contains corn oil glycerides, ethanol, polyoxyl-40-hydrogenated castor oil, DL- α -tocopherol, glycerol and propylene glycol (Novartis Product Information, 2004). Prior to dosing the contents of a capsule were emptied and diluted 1:50 with distilled deionised water.

Solubility Measurements

For the determination of the solubility of drugs in triglyceride (Triolein), the standard shake flask method was utilised. Five ml of oil were placed in 10 ml glass ampoules to which excess drug (DDT, Saquinavir or cyclosporine A) had been added, sufficient to produce saturation. The flame-sealed vials were equilibrated at the required temperature in a water bath equipped with a shaker for up to 7 days. A vial was sampled from each replicate batch every 24 h. The samples were filtered through a 0.22 µm filter (Millipore) and diluted immediately with an appropriate quantity of mobile phase to prevent precipitation and subsequently analysed by HPLC. At least three determinations were made per sample, with a minimum of four experiments for each lipid solution. To confirm the recovery of drugs during the dilutions, samples of oils were spiked with a known concentration of drug, diluted as above and analysed by HPLC. Recovery of DDT was found to be 98–101%. The aqueous solubility of Saquinavir was similarly determined using this technique by adding excess Saquinavir to water in hermetically sealed vials and proceeding as above.

Animal Studies

All animal experiments were performed in accordance with EU directive 86/609 (as implemented in Ireland by Statutory Instrument 17/9) in association with BioResources unit, Trinity College, Dublin, which is registered with the Department of Health and Children in Ireland. Male Wistar rats, (280–320 g) were fasted for 24 h with free access to water. The animals were anaesthetized for the duration of the experiment using 50 mg/kg sodium pentobarbital given by intraperitoneal injection. The duodenum and mesenteric lymph duct were cannulated as previously described [8, 14, 27]. 3 ml of the test formulations was administered by intraduodenal infusion over 2 h (*i.e.* 1.5 ml/h). Normal saline was then infused at a rate of

1.5 ml/hr for the remaining 6 h of the experiment to rehydrate the animal. Lymph was collected hourly for 8 h (*i.e.* 2 h dosing and 6 h post-dosing periods), in pre weighed glass tubes containing anticoagulant (heparin). Lymph triglyceride (expressed as mg equivalents of C18 LCT) was determined using a Triglyceride Enzymatique PAP 150 assay (bioMeirieux) as described previously [16].

Analytical Procedures for Animal Studies

DDT

Lymph concentrations of DDT were determined using a previously validated HPLC method for DDT analysis [27]. The stationary phase was a reversed phase MOS Hypersil C8 column (15×4.6 cm id, particle size 5 µm), equipped with a guard column of the same material. The mobile phase was 75:25 acetonitrile:water, with a flow rate of 2 ml/min and UV detection at $\lambda=238$ nm. Retention times for DDT and internal standard DDI were 2.6 and 3.5 min respectively. 0.15 ml lymph samples were then extracted with diethyl ether (5 ml). The organic phase was decanted and evaporated to dryness. The residue was dissolved in HPLC mobile phase (0.12 ml), vortexed for 1 min, and 20 µl was injected onto the column. The analysis of DDT in lymph exhibited excellent linearity ($r^2\geq 0.99$) over the concentration range of 3.34–167 µg/ml. The LOQ was 3 µg/ml. The extraction recovery of DDT in lymph was $91.66\pm 7.46\%$ and recovery of the internal standard was $>90\%$.

Cyclosporine A

Lymph concentrations of cyclosporine A were determined using a validated method for cyclosporine analysis. The stationary phase was a reversed phase C18 column (25×4.6 cm id, particle size 5 µm). The mobile phase was 69:31 acetonitrile:water, with a flow rate of 1.4 ml/min and UV detection at $\lambda=202$ nm. Retention times for cyclosporine A and internal standard cyclosporine D were 19 mins and 26 mins respectively. 1.4 ml of distilled water, 400 µl of 1 M sodium hydroxide and 200 µl of 0.1 M HCl was added to 0.2 ml lymph samples. The samples were then extracted with diethyl ether (5 ml). The organic phase was decanted and evaporated to dryness. The residue was dissolved in HPLC mobile phase (0.12 ml), vortexed for 1 min and 90 µl was injected onto the column. The analysis of cyclosporine in lymph exhibited excellent linearity ($r^2\geq 0.99$) over the concentration range of 3.5–85 µg/ml. The LOQ was 2.99 µg/ml. The extraction recovery of both cyclosporine A and the internal standard cyclosporine D was in excess of 90%.

Saquinavir

Lymph concentrations of saquinavir were determined using a previously validated method for saquinavir analysis [8, 16]. The stationary phase was a 5 µm Megellen C8 column (250×4.6 mm, Phenomenex), equipped with a guard column of the same material. The mobile phase consisted of a 63:37 mix of acetonitrile:ammonium acetate (10 mM), with a flow rate of 1 ml/min and UV detection at $\lambda=238$ nm. The retention times for saquinavir and internal standard (Ro 31-9564) were 9 and 21 min, respectively. Twenty µl of a 5 µg/ml solution of internal standard, Ro 31-9564, in mobile phase was added to 200 µl of lymph sample. The lymph samples were then made basic by the addition of 50 µl of 10 M K_2CO_3 and extracted with diethyl ether (4 ml). Following centrifugation of the mixture for 5 min at 3000 rev/min, the tubes were placed into a dry ice/acetone bath. The organic phase was decanted and evaporated to dryness under a stream of nitrogen. The residue was dissolved in HPLC mobile phase (0.15 ml), vortexed for 1 min and 50 µl was injected onto the column. The analysis of saquinavir in lymph exhibited excellent linearity ($r^2\geq 0.99$) over the concentration range of 40–800 ng/ml. The LOQ was 40 ng/ml. The extraction recovery of saquinavir from lymph was $>93\%$, whereas the recovery of internal standard was $>97\%$.

In all cases, the extent of lymphatic transport was calculated using the concentration of drug found in each lymph sample multiplied by the volume of the lymph produced per hour and expressed as a cumulative percentage of the dose.

Cell Culture Studies

Caco-2 cells (passage 30), a human colonic adenocarcinoma cell line, were obtained from the European Collection of Animal Cell Cultures, UK. Cells were cultured using DMEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 µg/ml gentamicin and 1 mM sodium pyruvate. For experiments, cells were seeded onto semi-permeable filters (Costar® Transwell™ polycarbonate filters, 0.4 µm pore, 24 mm diameter, 4.71 cm² surface area) from a re-suspended cell pellet at a density of 63,000 cells/cm² and grown for 19–23 days to allow differentiation.

Fully differentiated monolayers were washed three times with serum-free culture medium and then incubated (1.5 ml in apical chamber and 3 ml in basolateral chamber) with serum-free culture medium for 3 h. TEER measurements were taken. Medium was removed and the test or control vehicle (labelled with trace [¹⁴C]) added to the apical chamber (1.5 ml). 3 ml serum-free culture medium was placed in the basolateral chamber. Monolayers were incubated for 20 h under standard conditions (37°C, 5% CO₂, 90% relative humidity). After incubation, TEER values were re-read to check monolayer integrity.

Preparation of Vehicles for Cell Culture Studies

Sixteen μl of ethanolic [^{14}C] oleic acid was pipetted into 10 ml vials and left to dry under a stream of nitrogen. 40 μl of the required vehicle (LCT formulation or Neoral®) was then added and the vials were incubated in a shaking water bath at 37°C for 1 h to allow for equilibration. Vehicle samples were then digested by addition of 2 ml bile salt solution containing pancreatin (0.02 mg/ml of an 8x concentrate), 6 mM sodium taurocholate and 1.5 mM lecithin in a Tris-maleate buffer (10 mM Tris-base and 150 mM sodium chloride calcium chloride adjusted to pH 7.4 with sodium hydroxide) and incubated in a shaking water bath at 37°C for a further 2 h. Each system was then diluted 1 in 4 with serum-free cell DMEM containing BSA (final concentration of BSA was 0.125 mM). Control buffer (containing pancreatin) was treated in the same manner. The final vehicle was sterilised by filtration through a sterile 0.2 μm pore filter.

Analytical Procedures for Cell Culture Studies

Preparation of Samples for Analysis

Apical contents were collected and a 100 μl aliquot analysed directly by liquid scintillation counting to determine the end concentration of label (unabsorbed label). Basolateral contents were collected and stored at 2–8°C for lipid extraction. For intracellular determination, monolayers were washed twice in 3% *w/w* BSA in phosphate buffered saline (pH 7.4) (PBS) to remove bound fatty acid and then twice with PBS alone. Cells were scraped into ice cold PBS using a rubber cell scraper and lysed by repeated passage through a 21-gauge needle and addition of Triton X (to 0.5% *w/w*). EDTA (25 μl of a 100 mM aqueous solution), NaN_3 (25 μl of a 100 mM aqueous solution) and PMSF (10 μl of a 2 mM ethanolic solution) were added as preservatives and the volume was made up to 2 ml with PBS, pH 7.4. Cell lysates were stored at 2–8°C for up to 3 days before further analysis. One aliquot was removed for lipid analysis and the remainder used for protein determination using the Bio-Rad assay kit.

Lipid Extraction

Lipid extraction was carried out according to the method of Folch *et al.* [17, 18, 28]. Briefly, 5 ml of an extraction mixture of chloroform: methanol (2:1) with 0.01% *v/v* BHT (as antioxidant), was added to 0.5 ml of basolateral sample or 200 μl of cell lysate in a borosilicate glass tube and vortexed for 1 min. The tubes were centrifuged at 4000 rpm for 10 min to separate aqueous and organic phases. The bottom organic layer (containing lipids) was removed with a glass Pasteur pipette and transferred to a fresh borosilicate glass tube. Chloroform (2 ml) was added to the aqueous phase to remove

any residual lipid, followed by vortexing and centrifugation as before. The pooled organic sample was dried under nitrogen and stored at –20°C until further analysis.

Separation of Lipid Classes

The various lipid classes were separated by thin layer chromatography (TLC) as described [29]. Silica 60 LKD 10 lane TLC plates (Whatman) were activated by heating an oven at 100°C for 1 h, then allowed to cool. Lipid extracts were reconstituted in 40 μl of chloroform with vortexing. Reconstituted extracts were spotted onto individual lanes of the plate in 5 μl aliquots, drying between each addition. Basolateral and cellular lipid extracts isolated from a single Transwell™ filter were spotted onto the plate in neighbouring lanes. Triglyceride, phospholipid and cholesterol ester standards were also spotted onto individual lanes of each plate. Standards were prepared as follows and stored under nitrogen at –80°C for up to 2 months: Triglyceride- 10 μl solution of pure corn oil in chloroform (15 μl spotted onto plate); Phospholipid- 2 mg/ml solution of phosphatidyl choline in chloroform (20 μl spotted onto plate); Cholesterol ester- 3 mg/ml solution of cholesterol oleate in chloroform (15 μl spotted onto plate).

TLC plates were allowed to dry at room temperature and then partially developed in methanol by allowing the solvent to run just beyond the loading band, then air-dried and fully developed in a TLC tank containing 80 ml of developing solvent (petroleum ether: diethyl ether: glacial acetic acid (80:20:1)). After developing for 40 min the plates were air-dried and then transferred to a glass tank containing iodine vapour (generated from iodine granules). Lipid spots were stained brown. The lipid classes were identified by comparison with the standards. Cellular lipids were visualised directly by this technique, but basolateral samples were not visible (lipid content was too low). The exact location of lipid spots for individual basolateral samples were identified by comparison with the neighbouring cellular extract (isolated from the same Transwell™ filter). This accounted for any minor differences in retention value of lipid classes caused by administration of different vehicles. Lipid spots were scraped into scintillation vials. 4 ml of scintillation fluid was added and the vial vortexed for 1 min to solubilise lipid. Content of radiolabelled lipid was determined by liquid scintillation counting and was corrected for cell protein content (Bio-Rad DC protein assay kit). Secreted lipid and lipoprotein data was expressed as percent dose per mg protein.

Statistics

Statistical analyses were performed by one way analysis of variance (Figs. 2 and 5) or two way analysis of variance (Fig. 4) followed by Bonferroni post hoc test using Graph Pad.

RESULTS

Lymphatic Transport of Lipophilic Drugs from LCFA Formulation

Intestinal lymphatic transport was evaluated in anaesthetised rats dosed intraduodenally with three lipophilic drugs, DDT, saquinavir and cyclosporine A, each of which was formulated in the same LCFA formulation. Table I presents a summary of the physicochemical properties of these drugs and lymphatic transport kinetics observed. The data in Table I show that the highest percentage transport in the lymph was achieved for DDT as expected, given that it is the most lipophilic of the three drugs (based on log P and LCT solubility). Peak lymph transport and cumulative lymph transport after 8 h were also highest for DDT. In contrast, the percentage transported in the lymph for saquinavir and cyclosporine A was 0.05 and 2.3% respectively, which can in part be related to the low LCT solubility of the former and low log P of the latter.

In order to explore the relationships between the various physicochemical properties of the three drugs and their extent of lymphatic transport, log P, LCT solubility and aqueous solubility were plotted against the percentage of the dose transported in the intestinal lymph (Fig. 1). The relationship between lymph transport and log P and TG solubility has been well documented in the literature [10]. Typically, high log P >5 and TG solubility >50 mg/ml are predictive of extensive lymph transport [10], although there are exceptions to this, as highlighted in the introduction. The correlation between percentage lymphatic transport and log P was found to be weak (Fig. 1a) ($R^2=0.6412$). Weak predictive correlations based on log P have similarly been reported in other studies [25]. In contrast, a very strong correlation between percentage lymphatic transport and LCT solubility was noted for the compounds studied (Fig. 1b) ($R^2=0.9899$).

Aqueous solubility was also investigated as a predictor of lymphatic transport of lipophilic drugs. Interestingly, Fig. 1c shows a very strong negative correlation between lymph transport and log aqueous solubility ($R^2=0.9962$). This data, using the three drugs in this study, suggests that predictions of intestinal lymphatic transport based on aqueous drug solubility and LCT solubility are superior to predictions based on log P.

Effects of Lipophilic Drugs on Lymph Triglyceride Output

Figure 2 shows the lymph TG output, after intraduodenal administration of three lipophilic drugs (DDT, saquinavir and cyclosporine A) to rats, in an LCFA formulation (as used in Table I), compared to a saline-treated group. Interestingly, although the three drugs were administered in an equivalent lipid formulation, the rate of triglyceride turnover within intestinal lymph was highest for the DDT group compared to the cyclosporine A and saquinavir groups (Fig. 2a). Relative to a saline treated control group, the cumulative extent of lymph TG recovered after 8 h was significantly higher in the case of the DDT-treated group (Fig. 2b, $*p<0.05$). However, in cyclosporine A and saquinavir treated groups, while higher TG turnovers relative to the saline control treated animals were observed, the cumulative extent of TG transport after 8 h was not statistically significant different. Although no control data for TG transport following administration of a drug free formulation is available, previous studies in our laboratory have demonstrated that the presence of DDT does not alter lymph TG output when administered in lipid-based formulations. As all three drugs were administered in the same formulation vehicle, this could suggest that the drug compounds, saquinavir and cyclosporine A, negatively impact lymph TG turnover.

Table I Comparison of physicochemical properties, peak lymph transport ($\mu\text{g/ml}$), cumulative lymphatic transport (μg) and percentage of the dose transported in the mesenteric lymph for DDT, saquinavir and cyclosporine A after administration of a LCFA formulation to rats. Mean \pm SEM, $n \geq 3$

Drug	Log P	TG solubility (mg/g) ^D	Aqueous solubility (ng/ml) ^D	Dose (mg)	Peak lymph transport ($\mu\text{g/ml}$)	Cumulative lymphatic transport (μg) ^A	% Dose transported in lymph ^A
DDT	6.19	176.57 \pm 8.45 ^B	3.1 ^E	2.24	178.88 \pm 15.65	340.54 \pm 44.99	15.2 \pm 2
Saquinavir	4.50	1.87 \pm 0.48	57,000 \pm 12,100	5	1.45 \pm 1.12	2.59 \pm 1.48	0.05 \pm 0.05
Cyclosporine A	2.92	44.9 \pm 15.1 ^B	7300 \pm 1300 ^C	3.21	12.36 \pm 13.67	73.81 \pm 38.38	2.3 \pm 0.05

^A after 8 h

^B Gershkovich & Hoffman 2005 [25]

^C Ismailos et al. 1991 [37]

^D Solubility at 37°C

^E Patton et al. 1984 [38] (Solubility at 23°C)

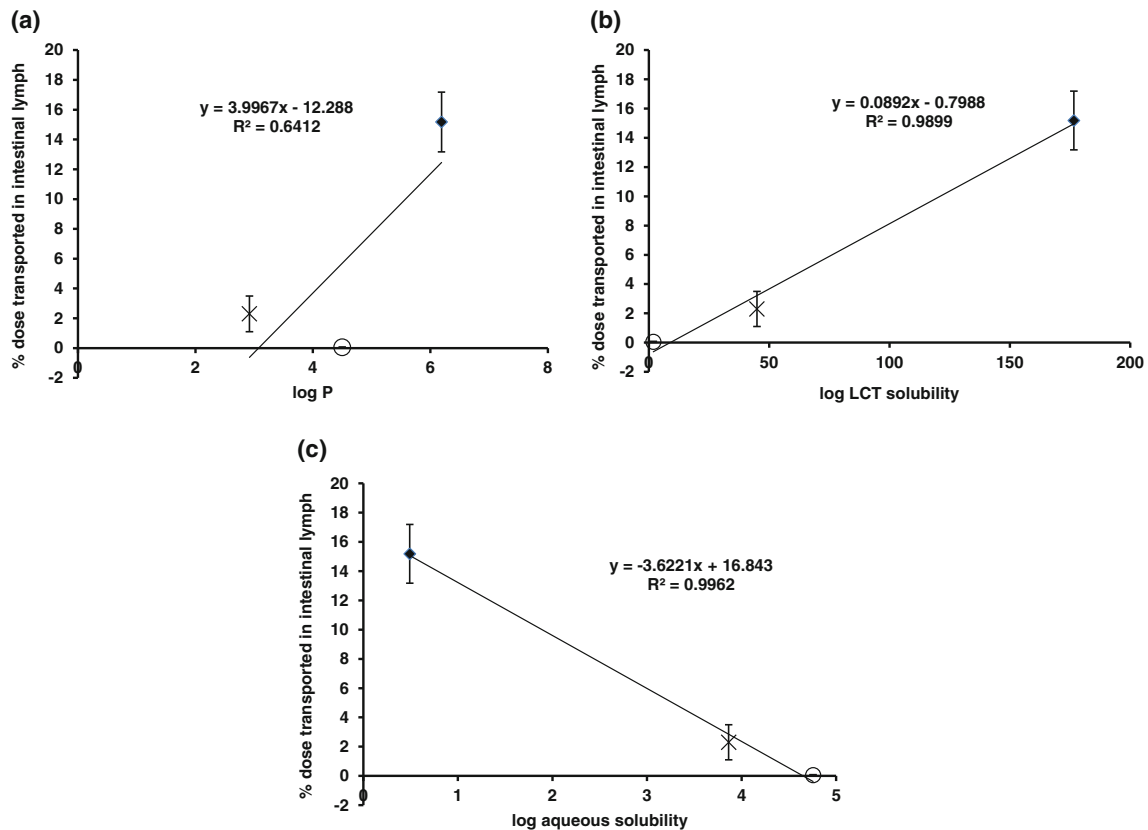


Fig. 1 Relationship between the percent of the dose transported in intestinal lymph and physicochemical properties of the drugs. DDT (◆), saquinavir (○) and cyclosporine A (×) were formulated in a LCFA formulation and administered intraduodenally to rats. The percentage of the dose transported in the lymph was determined after 8 h and was plotted against (a) log P, (b) LCT solubility and (c) log aqueous solubility.

Effect of Cyclosporine A dose on TG in Intestinal Lymph and Lymphatic Cyclosporine A Transport

In order to explore further the potential influence of a drug on TG levels in lymph, a dose escalation study was carried out using cyclosporine A. Cyclosporine A was formulated in a LCT formulation and intraduodenally administered at three doses, 1.6, 2.2 and 5.1 mg (Fig. 3). A LCT formulation, comprising a higher amount of LCT (30 versus ~10% LCFA in the previous experiments), was chosen for this study to explore potential cyclosporine A effects at higher levels of LCT administration. In addition, the LCT formulation was designed using corn oil as the LCT oil phase as the current clinically utilised formulation is a corn oil based LCT formulation (*i.e.* Neoral®).

Firstly, in order to elucidate further the negative effect of cyclosporine A on lymph TG output, the cumulative lymph TG as a function of time for three doses of cyclosporine A, was compared to saline and to vehicle treated animals (Fig. 3a). Lymph TG increased over time after administration of the vehicle (LCT formulation without cyclosporine A), the maximal extent of TG after 8 h was 40.9 mg. By comparison, a reduction in lymph TG was observed when cyclosporine A was administered in the same LCT formulation. In particular, at the two highest doses, 5.1 and 2.2 mg, cumulative lymph

TG was significantly lower compared to vehicle from 4 and 5 h respectively ($*p < 0.05$). The rank order for cumulative triglyceride output was: LCT formulation (no cyclosporine A) > 1.55 > 2.2 > 5.1 mg cyclosporine A > saline. This is in agreement with the decrease in lymph TG output observed for cyclosporine A in Fig. 2b.

Subsequently, cumulative cyclosporine A concentrations in intestinal lymph were measured as a function of time for each dose (Fig. 3b). Cyclosporine A levels in intestinal lymph were lowest with the lowest dose, 1.5 mg and increased significantly for the 2.2 mg dose ($*p < 0.05$). However at the higher dose of 5.1 mg, no further increases in cyclosporine A levels were observed (Fig. 3b) relative to the 2.2 mg dose. These findings suggest that the transport kinetics for cyclosporine A in the intestinal lymphatic system were non-linear as lymph levels of the drug did not increase with increasing dose. It would appear that the effect of cyclosporine A on reducing lymph TG turnover may at least partly explain this non-linear transport.

In Vitro Investigations of the Effect of Cyclosporine A on Cellular Lipid Processing

Given the observation of non-linear lymphatic transport characteristics at increasing doses of cyclosporine A, as well as the

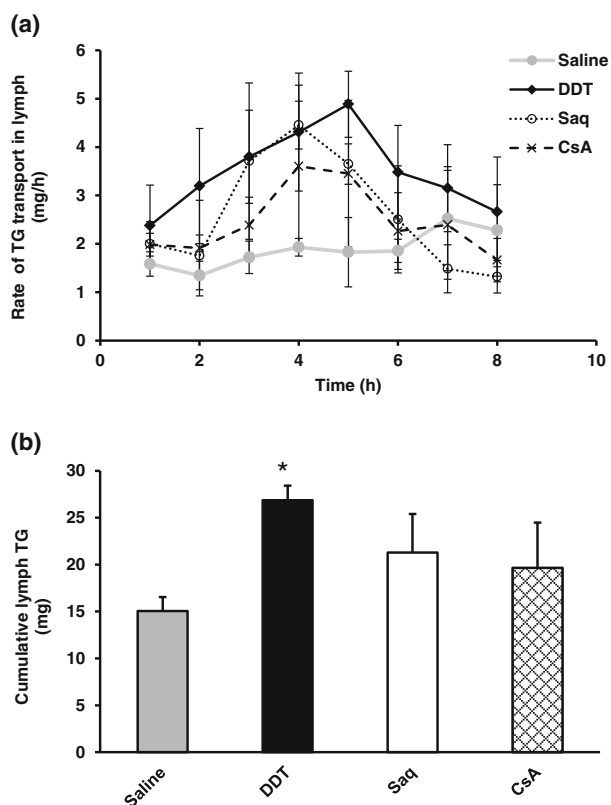


Fig. 2 Effect of lipophilic drugs on lymph TG transport. **(a)** Rate of lymph transport of TG (mg/h) over time and **(b)** Cumulative intestinal lymphatic TG output (mg) at 8 h, following intraduodenal administration of DDT, saquinavir (Saq) and cyclosporine A (CsA) in a LCFa formulation. Saline-treated animals were included as a control. Mean \pm SEM, $n \geq 3$.

potential negative consequences of a reduced TG turnover following clinical use of cyclosporine A formulations, it was interesting to investigate this drug further in a cell culture model, to gain more in-depth insights into the effects of cyclosporine A on lymph TG at the cellular level. Furthermore, we wanted to investigate whether these potential effects would be evident with the commercially available cyclosporine A LCT formulation (Neoral®). In order to assess the equivalence of the cyclosporine A-containing LCT formulation developed in house and Neoral®, TG and cyclosporine levels in intestinal lymph were compared following intra-duodenal dosing to anaesthetised rats. As is evident in Fig. 4a and b, there were no significant differences in the extent of lymph TG and cyclosporine outputs between the two formulations. Hence the LCT formulation developed in house can be considered equivalent to Neoral® in terms of intestinal output of TG. Subsequently, to compare the equivalence of the LCT formulation and Neoral® in terms of FA content, the two formulations were pre-digested by incubation with pancreatin-containing fasted state simulated intestinal fluid (FaSSIF). Free fatty acid release was similar from both formulations (2.6 ± 0.08 nM for in-house LCT formulation; 2.1 ± 0.2 nM for Neoral®).

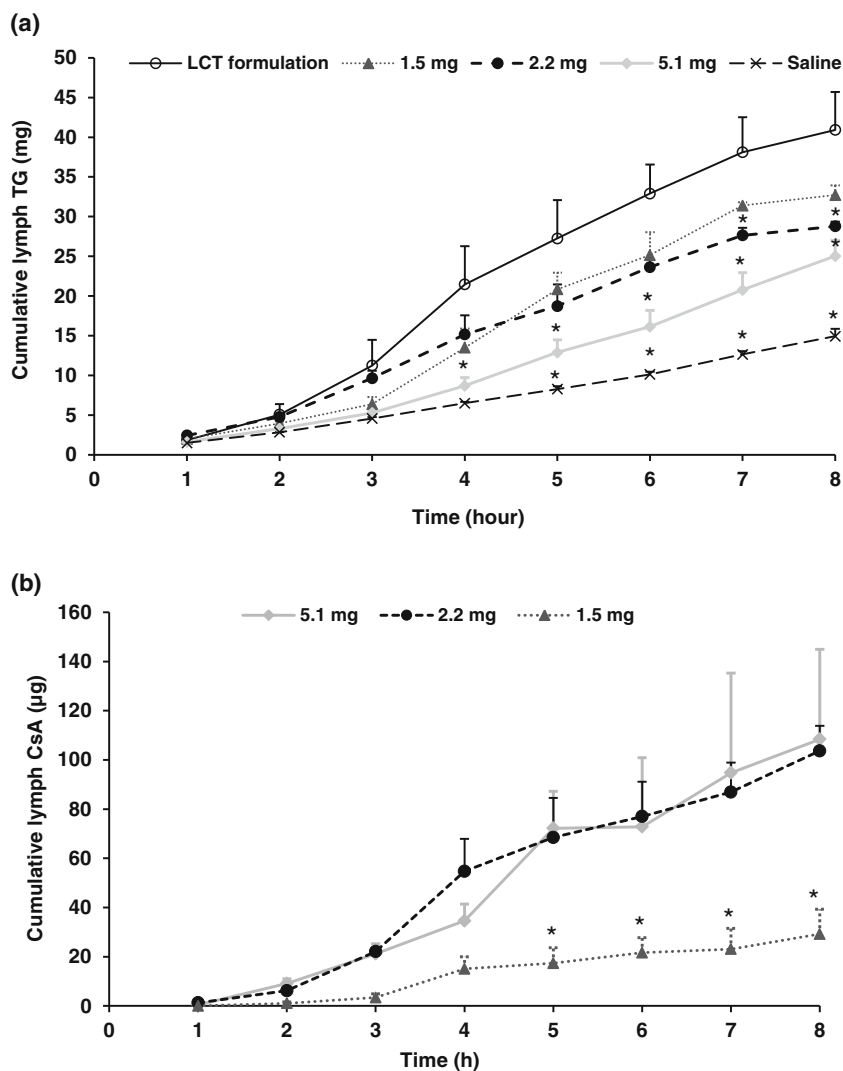
The effect of Neoral® versus a cyclosporine A-free LCT formulation was then explored in a Caco-2 model. These investigations focused on the effect of cyclosporine A on lipid uptake at the apical side, intracellular lipid processing and lipid secretion at the basolateral side. The test formulations were administered to the apical compartment and after 20 h, apical, intracellular and basolateral lipids and lipoprotein were analysed. Pancreatin-treated FaSSIF was included as a control.

Processing of lipoprotein begins with fatty acid uptake. After the incubation period, a sample of the apical compartment contents was taken and the proportion of ^{14}C -labelled oleic acid remaining was quantified, allowing an estimation of fatty acid uptake to be made. Figure 5a shows that 27 and 29% lipid remained in the apical compartment after administration of LCT formulation (cyclosporine A free) and Neoral® (with cyclosporine A) respectively. This data suggests that presence of cyclosporine A in the formulation does not impact on uptake of triglyceride fatty acids.

The next stage in lipoprotein metabolism is intracellular lipid accumulation. Intracellular triglyceride, phospholipid and cholesterol ester were analysed (Fig. 5b). Intracellular triglyceride levels were significantly reduced in the Neoral® group (29%, $*p < 0.05$), compared to the LCT formulation group (62%). In addition, intracellular accumulation of both phospholipid and cholesterol ester were significantly lower in the Neoral® group (4.25 and 0.46% respectively, $*p < 0.05$) compared to the LCT formulation group. In fact, cholesterol ester accumulation was four-fold lower when cells were treated with Neoral® compared to the cyclosporine A-free formulation. This current data suggests that cyclosporine A dramatically affects intracellular lipid processing and accumulation, in particular with regards to triglyceride and cholesterol ester. Additional data using the LCT formulation containing cyclosporine would provide further evidence to this effect and eliminate any variation which could arise due to the differences in the LCT and Neoral formulations.

The final stage in lipid metabolism to be explored was the secretion of triglyceride, phospholipid and cholesterol ester from the basolateral side of the cells. Reflecting the intracellular findings, Fig. 5c shows that Neoral® dramatically and significantly reduced secretion of triglyceride compared to the LCT formulation group ($*p < 0.05$). Decreased chylomicron secretion has previously been reported in Caco-2 cells after administration of verapamil (a known P-gp inhibitor) [18, 30]. Cyclosporine A is another known P-gp inhibitor which may at least partly explain the observed effects on TG secretion shown in Fig. 5c. Although lowest intracellularly, cholesterol ester secretion was highest in the cyclosporine A treated cells, but this was not significantly different from the other groups

Fig. 3 Effect of cyclosporine A dose on (a) intestinal lymph triglyceride output and (b) intestinal lymph cyclosporine A transport. Three doses of cyclosporine A were intraduodenally administered to rats in a LCT formulation and cumulative TG (mg) (a) or cumulative cyclosporine A (μg) (b) recovered in the mesenteric lymph was plotted against time (h). 1.5 (black dotted line and black triangle), 2.2 (black dashed line and black circle) and 5.1 (grey solid line and grey diamond) mg cyclosporine A. Vehicle (black line and empty circle) and saline (dashed line and cross) were included as controls. Mean \pm SEM, $n \geq 3$. (a) $*p < 0.05$ compared to vehicle (LCT formulation without cyclosporine A), (b) $*p < 0.05$ compared to the two highest doses of cyclosporine A.



due to high levels of variability in the data. This observation is broadly consistent with reports that long-term administration of cyclosporine A elevates plasma total cholesterol in humans [31].

Overall, the data in Fig. 5 suggests that the presence of cyclosporine A reduces the triglyceride secretion efficiency, while increasing cholesterol ester secretion and also altering intracellular accumulation of phospholipid and cholesterol ester.

DISCUSSION

Numerous studies have been conducted with a view to predicting intestinal lymphatic transport of lipophilic drugs based on their physicochemical properties [10, 16, 25]. However, direct comparison of these studies is seriously limited by the differences in animal models (rats, dogs, mice),

experimental conditions (anaesthetised *versus* unanaesthetised) and differences in the composition of the lipid formulations used in each of the studies. Hence, studies that have aimed to develop predictive models for lymphatic drug transport based on diverse data derived from studies involving different animal models, under different experimental conditions with different formulations, may be questionable. In the current study, we attempted to overcome these limitations by using the same vehicle (LCFA formulation) to deliver three drugs under identical experimental conditions.

Although traditionally relied on, a number of studies have shown log P to be a relatively poor predictor of lymphatic transport. Penclomidine was shown to be poorly transported in the lymph despite its log $P > 5.48$ and high TG solubility [10]. An *in silico* computational method (based on a variety of molecular predictors) was found to be superior to log P for predicting intestinal lymph transport of lipophilic molecules [26]. Gershkovich and colleagues reported a weak correlation between lymphatic bioavailability and both log P and TG

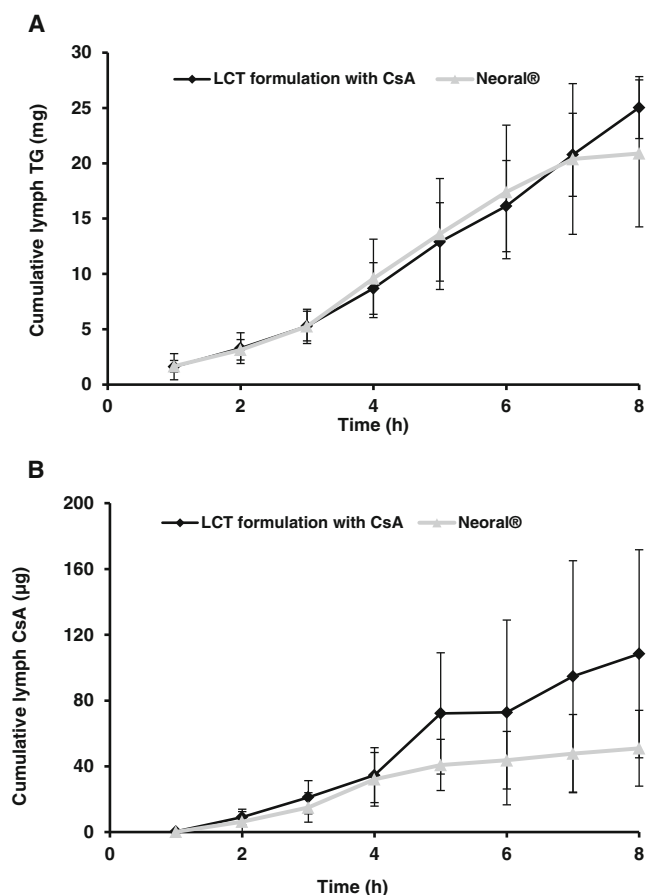


Fig. 4 Comparison of lymph triglyceride and cyclosporine A output after administration of in-house and commercially available cyclosporine A formulations. Cyclosporine A (CsA) was intraduodenally administered to rats in the in-house LCT formulation (5.08 mg CsA) and Neoral® (6 mg CsA), **(a)** the cumulative TG (mg) and, **(b)** the cumulative cyclosporine A (μg) recovered in the mesenteric lymph (Mean \pm SEM, $n \geq 3$) plotted against time (h).

solubility following examination of a range of lipophilic drugs including cyclosporine A, DDT, halofantrine and others [25]. The data shown in Fig. 1a regarding log P is in agreement with these reports. However in our study, LCT solubility was strongly correlated with intestinal lymphatic transport, in contrast to the work by Gershkovich [25]. This potentially reflects the more diverse set of lipophilic drugs investigated in the Gershkovich study, but also highlights a potential risk of developing predictions where aspects of the data set are taken from previously published studies. For example, in our study the extent of intestinal lymphatic transport of cyclosporine A was determined to be 2.3%, whereas Gershkovich *et al.* relied on an estimate 0.47% based on a study involving oral dosing of cyclosporine A dissolved in olive oil in conscious thoracic duct cannulated rats. While methodological differences between both studies may explain the approximate 5 fold difference in overall level of transport, this observation serves to highlight potential weaknesses in the predictions developed.

We also observed a strong inverse correlation between lymph transport and log aqueous solubility (Fig. 1c). This is

an interesting observation as traditionally it was considered that the key rate-limiting step influencing intestinal lymphatic transport was the lipophilicity of the drug, which, at a mechanistic level, was considered to be related to the ability of highly lipophilic drugs to be associated with (or partition into) TRLs. However, it may also be considered that the key determining factor may be low aqueous solubility of the drug. While it is important to acknowledge that low aqueous solubility will invariably be highly correlated with high lipophilicity, nonetheless in our study, aqueous solubility appears to be superior to the Log P based predictions and equivalent to predictions based on TG solubility. In terms of a mechanistic interpretation of the impact of aqueous solubility on the route of uptake (*i.e.* lymphatic versus portal uptake), it is generally considered that drug uptake *via* the portal route is favoured for drugs with high aqueous solubility. Portal blood flow acts as an absorption sink, competing with the intestinal lymph for the absorption of drugs. Therefore low aqueous solubility may be rate limiting for uptake into the portal blood, due to reduced diffusion out of enterocyte cells into the portal blood. This has an indirect effect of increasing drug content within enterocyte cells, with the consequence of increased association of the drug with the TRL and, therefore, driving uptake into the lymphatic system.

In summary, with a relatively simplified approach of determining drug solubility in aqueous or TG solutions, reliable preliminary predictions of the extent of intestinal transport may be achieved. This has the advantage of avoiding the need for more complicated *in silico* modelling or complex *ex vivo* analytical procedures. However, a broader set of drugs, evaluated under equivalent experimental/formulation conditions will be required to confirm the validity of this simplified predictive approach.

It has been widely reported that lipid vehicles, in particular LCFA based vehicles, stimulate lymph TG output, which in turn promotes the lymphatic transport of lipophilic drugs [13, 21]. However, in the current study, lymph TG output was reduced in the cases of saquinavir and cyclosporine A compared to DDT, when all three drugs were administered in the same LCFA formulation, suggesting that the drugs themselves can exert a profound influence on lymph TG output and can, in fact, override the formulation effects (Fig. 2). This is in agreement with a previous study which showed that saquinavir formulations reduced the total amount of TG in the lymph [8]. In addition, chylomicron flow has also been shown to decrease following administration of agents including colchicine [32].

This inhibitory effect of cyclosporine A was not limited to the LCFA formulation, but was also evident in the in-house and commercial LCT formulations. Lymph TG levels were higher following administration of a cyclosporine A-free LCT formulation (40.9 ± 8.3 mg after 8 h, Fig. 3a) compared to both the in-house LCT:cyclosporine A formulation ($25.0 \pm$

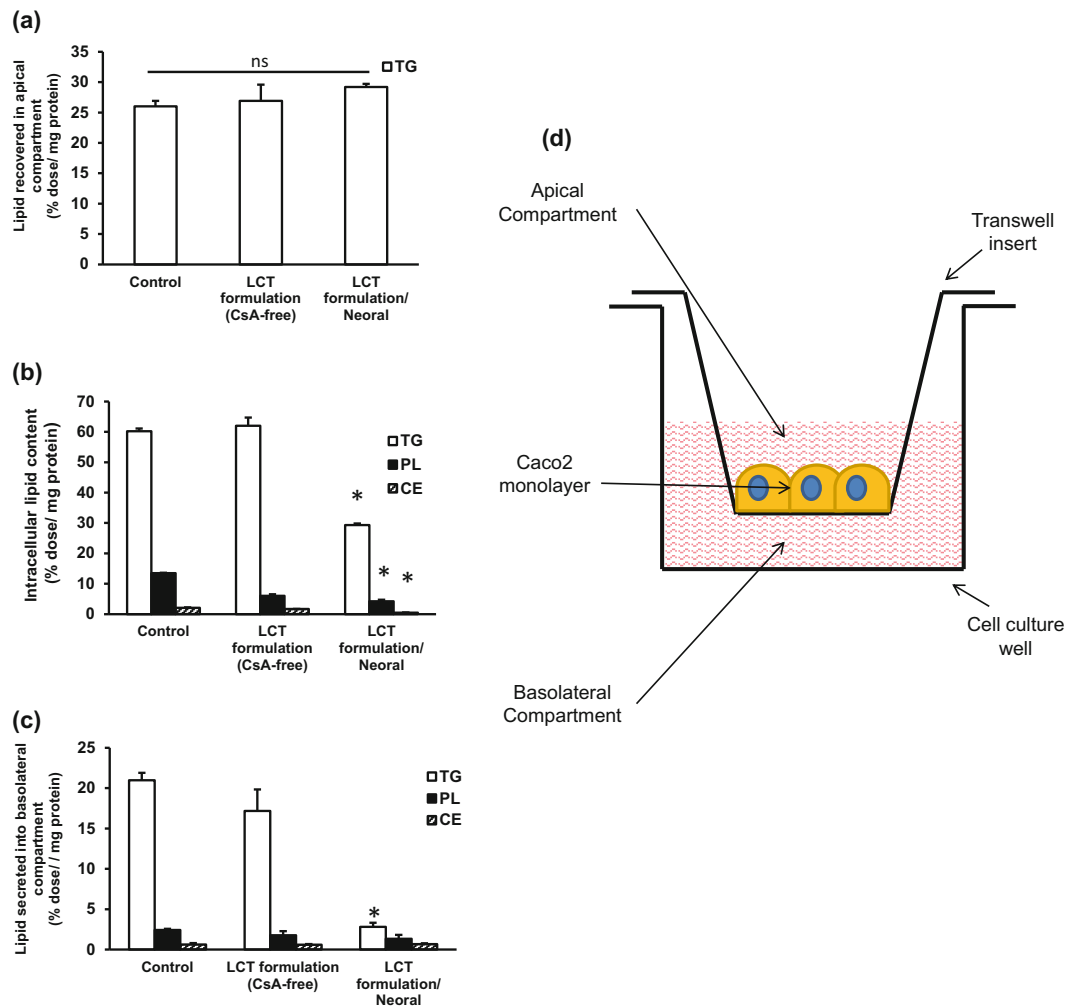


Fig. 5 Effect of cyclosporine A on uptake, intracellular processing and output of lipids in Caco2 cells *in vitro*. Caco2 cells were treated with digested FaSSIF, cyclosporine A-free LCT formulation and cyclosporine A-containing LCT formulation (Neoral®) for 20 h, then analysed for lipid and lipoprotein content in (a) the apical compartment, (b) intracellularly and (c) in the basolateral compartment. (d) schematic representation of the experimental set up, whereby Caco2 cells were grown on semi-permeable filters for 19–23 days before treatment. TG: triglyceride, PL: phospholipid, CE: cholesterol ester.

2.8 mg, Fig. 4) and the commercially available cyclosporine A formulation Neoral® (20.9 ± 6.7 mg, Fig. 4). These results confirm that the inhibitory effect of cyclosporine A on lymph TG was not limited to the in-house formulations, but also occurred with a widely used clinical product.

Cyclosporine A is a known inhibitor of P-gp efflux protein and a correlation between excipient-mediated P-gp inhibition and inhibition of lipoprotein assembly has been reported *in vitro*, which may suggest a link between these two biochemical processes [17]. However, it must be noted that from this current work it is not possible to elucidate the precise mechanism of the cyclosporine induced lymph TG inhibition and, in addition to P-gp, metabolism by enzymes including cytochrome P450 may also play a role. Long term administration of cyclosporine A clinically has been linked with various dyslipidaemias, including increased plasma triglyceride and cholesterol plasma concentrations [33]. There is limited data

available regarding the mechanism of such cyclosporine A-induced dyslipidaemias and whether this may be linked to a decrease in lymph TG levels is unclear.

Overall, these findings indicate that a combination of vehicle and drug effects should be taken into consideration when predicting lymphatic drugs transport and that certain drugs, through their inhibitory effects on the lymph TG, may impair their own lymphatic transport.

Lymphatic cyclosporine A transport has been previously reported to be quite low. Approximately 2.2% of the administered dose was transported in the lymph from an olive oil based formulation [34] and ~2% from a micellar solution [35]. The current study is in agreement with these previous reports, with just over 2% of the dose transported in the lymph after 8 h in the case of both LCFA and LCT formulations. However, this study goes a step further by establishing the non-linearity of the relationship between cyclosporine A dose

and transport. This effect is clearly seen in Fig. 3b, when no further increase in lymph cyclosporine A was observed despite increasing the dose more than two fold (2.2 to 5.1 mg). This non-linear relationship should be taken into account when interpreting studies that predict the lymphatic transport of cyclosporine A.

The final part of the current study was an investigation into the mechanism of the effects of cyclosporine A on lymph TG, using an *in vitro* Caco2 cell model. This model has been well established as a reliable model to predict lipid processing *in vivo* [4, 17, 18]. Caco2 cells are capable of secreting chylomicrons on exposure to lipids, reflecting the behaviour of enterocytes *in vivo* [18]. In addition, the method employed in the current study involved pre-treating the formulations with physiological media before application to cells, which further improves the biological relevance of the study [2]. The lipid and lipoprotein levels were measured at the apical and basolateral sides of the cell model, as well as intracellularly.

Cyclosporine A is a known inhibitor of P-gp, however, there was no difference in fatty acid uptake at the apical side between the cyclosporine A-containing LCT formulation (Neoral) and the cyclosporine A free LCT formulation. This suggests that P-gp inhibition at the apical side does not play a critical role in the effect of cyclosporine A on lymph TG. In fact, there is increasing evidence that fatty acid uptake into enterocytes is by an active mechanism *via* membrane fatty-acid binding proteins, as well as passive diffusion, which may at least partly explain this observation [36].

However, the *in vitro* data in this study strongly suggest that the effects of cyclosporine A occur at an intracellular level (processing/assembly) and to an even greater extent at a basolateral level (secretion) (Fig. 5c). TG output in the *in vitro* Caco2 model was significantly reduced after treatment with Neoral®, compared to the cyclosporine A free LCT formulation. This is in agreement with a previous study that reported a decrease in chylomicron secretion with increasing cyclosporine A in Caco2 cells [17]. Furthermore, this *in vitro* data correlates well with our findings *in vivo*, *i.e.* that cyclosporine A treatment reduced lymph TG output. This correlation highlights the usefulness of the Caco2 model for investigations into lymph transport.

CONCLUSIONS

In summary, this study presents several key findings. Firstly, aqueous solubility and LCT solubility are superior predictors of lymphatic drug transport compared to log P. Secondly, lipophilic drugs, including cyclosporine A, can exert negative effects on lymph TG output, overriding the effects of the lipid formulations. Thirdly, cyclosporine A displays a non-linear dose-lymph transport relationship, due at least in part to its inhibitory effect on intestinal lymph TG. Finally, mechanistic

studies carried out *in vitro* help to advance our understanding of the mechanisms behind these effects of cyclosporine A and allow us to hypothesise that the disruption of intracellular lipid processing and/or lipid secretion are the key processes affected.

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