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RESEARCH PAPER

The Mechanism of Lymphatic Access of Two Cholesteryl Ester Transfer Protein Inhibitors (CP524,515 and CP532,623) and Evaluation of Their Impact on Lymph Lipoprotein Profiles

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ABSTRACT

Purpose To explore the mechanism of lymphatic access of the CETP inhibitors (CETPi) CP524,515 and CP532,623 and probe their potential effect on lymph lipoprotein development.

Methods Lymphatic access mechanisms were examined via correlation of lymphatic drug transport profiles with drug affinity for lymph lipoproteins and drug solubility in representative combinations of lipoprotein lipids. The effects of the CETPi on lymph lipoprotein profiles were evaluated by ultracentrifugation and flow cytometry.

Results Both CETPi were highly lymphatically transported (22–28% of dose), and lymphatic transport was closely correlated with drug affinity for *ex-vivo* lymph lipoproteins or triglyceride emulsions and poorly related to solubility in mixtures of lipoprotein core and/or surface lipids. Both CETPi altered the kinetics of lymph lipid transport and decreased lymph lipid transport in chylomicrons.

Conclusion Lymphatic transport of the CETPi appears to reflect high affinity for the interface of lymph lipoproteins rather than solubilisation in the lipoprotein core and confirms that triglyceride solubilities >50 mg/g are not necessarily a pre-requisite for lymphatic transport. The CETPi also led to changes to lipoprotein processing in the enterocyte including a reduction in lipid transport in chylomicrons. Changes to intestinal lipoprotein profiles may contribute to the changes in systemic lipoprotein levels seen during CETPi therapy.

KEY WORDS CETP inhibitor · lipid · lymphatic transport · lipoprotein · lipid-based delivery system · intestine · absorption

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ABBREVIATIONS

CETP	cholesterol ester transfer protein
CETPi	CETP inhibitors
CM	chylomicron
HDL	high density lipoprotein
VLDL	very low density lipoprotein
TRL	TG rich lipoprotein
TG	triglyceride
PL	phospholipid
Ch	cholesterol
CE	cholesteryl ester
FA	fatty acid
DPPC	di-palmitoyl phosphatidylcholine
LCT	long chain TG
MTP	microsomal TG transfer protein
PLTP	phospholipid transfer protein
FSC	forward scatter
SSC	side scatter

INTRODUCTION

Cholesteryl ester transfer protein (CETP) is currently being explored as a target for the treatment of atherosclerosis (1,2). CETP encourages an atherogenic lipoprotein profile by promoting the transfer of cholesteryl ester (CE) from high density lipoproteins (HDL) to triglyceride-rich lipoproteins (TRL) in exchange for triglyceride (TG) (3,4). The CETP inhibitors evaluated in the current study, CP524,515 and CP532,623, belong to a series of compounds which includes torcetrapib, a CETP inhibitor withdrawn from development in 2006 when phase III studies found an increased risk of mortality in patients receiving a combination of atorvastatin and torcetrapib rather than atorvastatin alone (5). In common with many contemporary drug discovery candidates, CP532,623 and

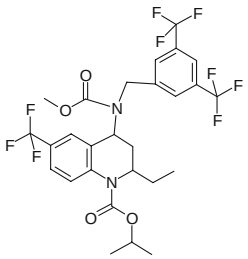
CP524,515 are poorly water soluble (both <0.03 μg/ml) and highly lipophilic properties which present a major challenge to effective development of an orally bioavailable drug product (Table I).

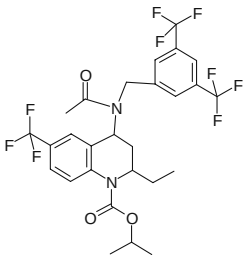
In a recent study, we found that food has a substantial positive effect on the bioavailability of CP524,515 and CP532,623 and that the major route of drug transport to the systemic circulation was via the intestinal lymphatic system after both fasted and post-prandial administration with a lipid-based formulation (6). Previous studies have suggested that a log P>4.7 and long-chain TG solubility >50 mg/g are physicochemical pre-requisites for significant intestinal lymphatic drug transport in order to facilitate drug association with the TG-rich core of lipoproteins in the enterocyte (7). Whilst the physicochemical properties of CP524,515 (Elog $D_{7.4}$ 6.13 and long-chain TG solubility >50 mg/g; Table I) are consistent with these descriptors, CP532,623 is somewhat less soluble in long-chain TG (Elog $D_{7.4}$ 5.68; TG solubility 15–20 mg/g), suggesting a potentially different mechanism of lymphatic transport. (Although not studied here, torcetrapib has a similar structure and long-chain TG solubility (15–20 mg/g) to CP532,623 (6) and therefore might be expected to exhibit similar transport properties.)

The mechanism of drug access to lymph lipoproteins and the potential impact of the CETPi on lipid absorption and lipoprotein assembly in the enterocyte (in addition to the more commonly accepted effects on systemic plasma lipoprotein profiles) was also of interest for CP532,623 and CP524,515 since drug access to lipoproteins is required for activity (CETP promotes lipid exchange between lipoproteins) (5) and because in previous studies CP532,623 appeared to reduce postprandial TG recovery in the intestinal lymph (6). In the latter studies, the greatest diminution in TG transport also occurred over the timescale of maximum CP532,623 concentrations in the intestinal lymph (6).

The current study has therefore probed the mechanisms involved in the lymphatic access of CP524,515 and CP532,623 and has examined the effect of the CETP inhibitors on intestinal lipid and lipoprotein processing. First, the effect of administration of different types and quantities of lipid formulations on the lymphatic transport of CP532,623 and CP524,515 has been studied using a lymph-cannulated rat model. Second, the affinity of CP532,623 and CP524,515 for varying combinations of lymph lipids and isolated lipoproteins has been quantified in an attempt to better understand the mechanism of lymphatic access of, in particular, the less TG-soluble CP532,623. Finally, the influence of the CETP inhibitors on lipid transport into the lymphatic system, including effects on the size, density and lipid composition of lymphatic TRL, has been evaluated and the kinetics of lipid transport from the enterocyte lymph lipid precursor pool into the lymph assessed.

Table I Physical and chemical properties of CP524,515 and CP532,623





	CP524,515	**CP532,623**
Molecular weight (Da)	614.51	598.51
Elog $D_{3.0}$ (6)	6.07	5.63
Elog $D_{7.4}$ (6)	6.13	5.68
Crystallinity in current studies[a]	Crystalline. Lath shaped, 189 μm diameter	Crystalline. Needle-like columns, 48.8 μm diameter
Soybean oil solubility (mg/ml) (6)	50.9	16.4
Melt Onset[a]	76.0°C	101.8°C

[a] Data on file, Pfizer Global Research and Development, Groton, CT

MATERIALS AND METHODS

Materials

CP524,515 and CP532,623 were kind gifts from Pfizer Inc., Groton, CT. Chloroform, acetonitrile and methanol were obtained from Merck (Germany) and were of analytical grade. Olive oil, soybean oil, Tween 80, di-palmitoyl phosphatidylcholine (DPPC), cholesterol and cholesterol oleate were obtained from Sigma-Aldrich (St Louis, MO). All other reagents were utilised as received and were of the highest grade available.

Animal Studies

All 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. The studies were conducted using male Sprague-Dawley rats (280–320 g) maintained on a standard rat diet and then fasted overnight with free access to water before experiments. Prior to commencement of surgery, rats were anaesthetised by subcutaneous injection of a combination of ketamine, xylazine and acepromazine, and anaesthesia was maintained throughout the experiment with top-up doses of ketamine and acepromazine as described previously (8). The mesenteric lymph duct and duodenum were cannulated as described previously (9) and the animals infused via the duodenum with 2.8 ml/h normal saline during a 0.5 h recovery period. After the initial recovery period, animals were infused via the duodenal cannula at a rate of 2.8 ml/h with the formulations described below. Lymph was collected continuously into tared tubes which were changed every 0.5–1 h depending on the experiment (as described below), and the total volume of lymph collected over each time period was determined gravimetrically. At the conclusion of all experiments, rats were killed via a lethal intraperitoneal dose of 1 ml sodium pentobarbitone (100 mg/ml).

Lipid Dependency of CETP Inhibitor Transport into Lymph

Lymph-cannulated rats were administered formulations containing either a long (oleic acid, OA) or medium (capric acid, CA) chain length fatty acid (FA) and total lipid doses of 0, 4 or 40 mg to assess the impact of lipid chain length and lipid dose on lymphatic drug transport. The formulations were administered at a rate of 2.8 ml/h over 0–2 h following surgical recovery and consisted of 2 mg drug (CP532,623 or CP524,515) dispersed in either 5.6 ml 0.5% Tween 80 w/v in normal saline, 4 mg OA in 5.6 ml 0.5% w/v Tween 80 in normal saline, 40 mg OA in 5.6 ml 0.2% w/v Tween 80 in normal saline or 40 mg CA in 5.6 ml of 5 mM bile salt (sodium taurocholate) in 0.1 M phosphate buffer pH 6.8. The methods used to prepare and to assess the stability of the formulations have been described previously (9,10), and the drug content of each formulation was verified by HPLC on the day of dosing. Bile salt was used as a surfactant in the CA formulation since solubilisation of CA with Tween 80 resulted in formation of unstable systems.

After the 2 h dosing period, rehydration with normal saline was continued throughout the experiment at a rate of 2.8 ml/h via the duodenal cannula. Lymph was collected continuously from 0 to 8 h following initiation of formulation administration, and lymph collection tubes were changed every hour. Drug and triglyceride concentrations in lymph were assessed as described below, and the mass of drug and lipid transported into the lymph over each time period was calculated from the product of the measured concentrations of drug or lipid in lymph and the volume of lymph collected.

Lipid Solubility of CETP Inhibitors and Affinity for Isolated TRL

Solubility in Lipid Combinations and Representative Lipoprotein Lipids

The solubility of CP532,623 and CP524,515 was determined in a) homogenous physical mixtures of lipids, the identity and proportions of which were chosen based on the known constituent lipids in TRL (including neutral lipid core and amphiphilic surface lipids); b) liposomes formed from amphiphilic TRL surface lipids (ie phospholipid (PL) alone or PL with cholesterol (Ch)); and c) emulsions formed from either TG and a synthetic surfactant or total (core and surface) TRL lipids. The neutral lipid core of TRL contains predominantly TG and CE, whereas the surface is composed of Ch, PL and apoproteins. The total lipid content of intestinal lymph has been suggested to comprise approximately 90% w/w TG, 7% w/w PL, 2.25% w/w Ch and 0.75% w/w CE (11), although these proportions are likely to change slightly with species, diet and fed state (11). The TG, PL and CE used in these studies were soybean oil, di-palmitoyl phosphatidylcholine (DPPC) and Ch oleate since each contains similar chain length FA to the corresponding lymph lipid (11).

Solubility in Physical Mixtures of TRL Core or Total Lipids

The mixture of TRL core lipids consisted of 99.3% w/w soybean oil, 0.12% w/w DPPC and 0.43% w/w Ch oleate (11). Total TRL lipids (including surface and core lipids) comprised 90% w/w soybean oil, 7% w/w DPPC, 2.25%

w/w Ch and 0.75% w/w Ch oleate. To prepare 1 g of each lipid mixture, an appropriate amount of each lipid component was weighed into a glass vial and vortexed for 2 min to form a homogenous mixture. After preparation of the lipid mixture, 200 mg of drug (CP524,515 or CP532,623) was added, vortexed for 1 min and incubated at 37°C. The mixture was vortexed for 1 min three times per day throughout the sampling period. At 24, 48 and 72 h the mixtures were vortexed for 10 sec and centrifuged at 5,200 g for 20 min to precipitate undissolved drug, and 1 drop of the resulting supernatant was weighed directly into a 5 ml volumetric flask, taking care not to disturb the drug pellet. Drug concentrations were measured by HPLC as described below, and the mass of drug solubilised per mass of lipid was subsequently calculated.

Solubility in Model Colloidal Species

Drug solubility in liposomes and emulsions was assessed via addition of 10 mg of drug (CP524,515 or CP532,623) to a small round-bottom glass flask containing 2 ml of a lipid solution in chloroform (see Table II for lipid concentrations). The flask was vortexed until the drug was completely dissolved and the chloroform subsequently evaporated to dryness under a steam of high purity nitrogen at 35°C using a N-EVAP 112 evaporator (Organomation, MA, USA). Once dry, 4 ml of appropriate re-suspension medium at the temperature specified in Table II was added. To prepare liposomes, the flask was sonicated on a bench-top sonicator at 40–50°C until a homogenous dispersion was formed. To prepare the emulsion, the vial was ultrasonicated with a Misonix XL 2020 ultrasonic processor (Misonix, NY) equipped with a 3.2 mm microprobe tip running at an amplitude of 240 μm and a frequency of 20 kHz for 2 min on and 1 min off until the oil was completely incorporated into the emulsion. Oil incorporation was assessed by visual inspection under polarised light. After preparation, the liposomes and emulsions were incubated for 1 h at 37°C, centrifuged at 5,200 g for 10 min at 37°C to precipitate undissolved drug and samples taken from the supernatant, taking care not to disturb the drug pellet. The concentration of drug in the supernatant was measured by HPLC and lipid concentrations using commercial enzymatic kits running on a Cobas Mira lipid analyser as described below. The mass of drug solubilised per mass of lipid was calculated from the measured drug and lipid concentrations.

Affinity for Isolated TRL (CM and VLDL)

The affinity of the CETP inhibitors for isolated TRL (mass of drug incorporated per mass of TG) was determined using an *ex-vivo* incubation method similar to previously published protocols (12). In this case, however, TRL were collected from mesenteric lymph rather than plasma, and the TRL were incubated with quantities of drug above the saturated solubility of drug in TG. To collect CM and VLDL, rats were dosed by oral gavage with 1 g of soybean oil and 1 h later were anaesthetised, and the mesenteric lymph duct and duodenum cannulated as described above (8). Lymph was then collected over 6 h into a tube containing 100 μL of heparin (1,000 U/ml), and the animals re-hydrated via continuous intraduodenal infusion of 2.8 ml/h normal saline. To separate CM, 6 ml of lymph was layered under 6 ml of normal saline in a polyallomer centrifuge tube (Beckman, CA), and the tube ultracentrifuged at 202,048 g for 1 h 35 min in a SW40Ti rotor (Beckman, CA) (13). The CM fraction formed a semi-solid plug at the top of the centrifuge tube which was removed, placed in a 12 ml polypropylene tube; CM fraction was resuspended via addition of 1 ml of normal saline. The void left in the polyallomer tube following removal of the CM was topped up with normal saline and the VLDL fraction subsequently separated by ultra-centrifugation for 16 h at 202,048 g in a SW40Ti rotor (13). After the centrifugation period, a viscous dispersion of VLDL was collected from the top of the polyallomer tube and transferred to a 12 ml polypropylene tube with a pasteur pipette. The TG concentration of the resuspended CM and VLDL fractions were measured as described below and the TG concentration adjusted to 100 mg/dl for the CM and 20 mg/dl for the VLDL preparations using normal saline. To assess the affinity of CP532,623 and CP524,515 for the CM fraction, 1 ml of a 2 mg/ml drug solution in propylene glycol was added to 4 ml of the 100 mg/dl CM dispersion, which was

Table II Conditions used for preparation of liposomes and emulsions in solubility studies

	Initial lipid solution in chloroform	Re-suspension medium
PL liposomes	20 mg/ml DPPC in 2 ml chloroform	4 ml normal saline (40–50°C)
PL and Ch liposomes	20 mg/ml lipid (96.7% DPPC, 3.3% Ch) in 2 ml chloroform	4 ml normal saline (40–50°C)
TRL lipid emulsion	20 mg/ml lipid (90% soybean oil, 7% DPPC, 3% Ch) in 2 ml chloroform	4 ml normal saline (40–50°C)
TG/synthetic surfactant emulsion	20 mg/ml soybean oil in 2 ml chloroform	4 ml of 0.5% Tween 80 in normal saline (room temperature)

vortexed for 1 min and incubated at 37°C for 1 h. For the VLDL dispersion, 0.5 ml of a 2 mg/ml drug solution in propylene glycol was added to 4 ml of the 20 mg/dl VLDL dispersion, vortexed for 1 min and incubated at 37°C for 1 h. After incubation, 3 ml of the drug and CM or VLDL mixtures were placed in polyallomer centrifuge tubes, layered under normal saline and centrifuged to obtain the CM or VLDL fraction as described above. The drug and TG concentrations of the CM and VLDL fractions collected were measured as described below and the mass of drug dissolved per mass of TG calculated.

The Effect of CP532,623 and CP524,515 on Intestinal Lipid and Lipoprotein Processing

Impact of CP532,623 and CP524,515 on Lipid Distributions Across Lipoprotein Fractions

Formulations containing 2 mg drug (CP532,623, CP524,515 or halofantrine as a model lymphatically transported comparator drug) dissolved in 40 mg oleic acid dispersed in 5.6 ml 0.2% w/v Tween 80 were infused into lymph-cannulated anaesthetised rats via intraduodenal infusion over 0–2 h. Animal procedures for cannulations and anaesthesia were as described above. Formulations were prepared as described above, and lymph was collected into tared tubes from 0–2, 2–4 and 4–6 h after initiation of formulation dosing. The volume of lymph collected was determined gravimetrically. A sample (50 μL) of the collected lymph was taken for analysis of total drug and lipid concentrations. The remainder of the lymph was then separated into different lipoprotein fractions, and the concentration of lipid and drug (CP532,623 and CP524,515) in each of the different lymph lipoprotein fractions determined. To separate lipoprotein fractions, lymph was transferred into 5 ml polyallomer tubes, the void volume in the tube filled with normal saline and tubes centrifuged at 199,740 g for 1 h 20 min at 15°C in a SW60Ti rotor. The *d* (density) <0.93 g/ml fraction (which is typically regarded as CM) (14) was recovered from the top of the tube using a pasteur pipette and transferred to a separate, tared polypropylene tube such that the volume could be determined gravimetrically. The remaining fractions were collected by piercing the base of the tube with a 5 ml syringe fitted with a 21 gauge needle and were transferred to a new polyallomer centrifuge tube which was centrifuged at 199,740 g for 16 h at 15°C in a SW60Ti rotor. After this step, the $d=0.93$–1.006 g/ml fraction (generally assumed to represent VLDL) (14) floated to the top of the tube, whereas the $d>1.006$ g/ml fraction (generally assumed to represent LDL and HDL) (14) was collected from the base of the tube with a 5 ml syringe fitted with a 21 gauge needle. The $d=0.93$–1.006 g/ml fraction (VLDL) and $d>1.006$ g/ml fraction (LDL and HDL) were also transferred to polypropylene tubes, the volume collected determined gravitrimetrically and the fractions stored at −20°C for later analysis. On the day of analysis, the lipoprotein fractions were thawed and vortexed for 1 min, and drug and TG concentrations determined as described below.

Impact of CP532,623 and CP524,515 on Lipoprotein Size and Density Assessed Using Flow Cytometry

Flow cytometry is a highly sensitive method by which differences in relative size (from forward scatter (FSC) measurements) and optical density (from side scatter (SSC) measurements) of particulates may be evaluated. Most commonly, flow cytometry is utilised to examine cells; however, it is applicable to any particulate analysis, and here we have utilised it as a novel method to examine the influence of CP524,515 and CP532,623 on the size and optical density of lymph lipoproteins.

Mesenteric lymph-duct cannulated rats were continuously infused via the duodenum for 5 h with formulations containing 20 mg/h oleic acid in 2.8 ml/h 0.2% Tween 80 in the presence or absence of either 1 mg/h CP532,623 or CP524,515 (experiments were performed in duplicate, and Fig. 4 shows representative plots from individual rats). Previous studies have shown that steady-state rates of lipid and drug transport into the lymph are typically obtained after 4–5 h of continuous infusion of lipid formulations (10), and therefore lymph collected over the 4–5 h period following initiation of formulation administration was used for analysis. Lymphocytes and cellular debris were subsequently removed from the lymph samples by centrifugation at 5,200 g for 3 min. Following centrifugation, no lymphocytes were observed in the remaining lymph via light microscopy or were evident in typical size and optical density ranges when lymph was analysed by flow cytometry. Chylomicron (CM) and VLDL were separated from the lymph samples using the method described above. The 4–5 h lymph, CM or VLDL samples were stored on ice and analysed by flow cytometry (BD FACSAria, San Jose, CA) within 4 h of collection. The flow cytometry gain settings were FSC 290 V and SSC 250 V.

Impact of CP532,623 and CP524,515 on the Steady-State Kinetics of Lipid Transport into the Lymph

The steady-state lymph-cannulated rat model used in these experiments has been described previously (10), and data obtained for the model lipophilic drug, halofantrine, in that study is reproduced here for comparison. The formulations used in this part of the study were identical to those described above (10). Briefly, mesenteric lymph-duct cannu-

lated rats were infused continuously via a duodenal cannula for 5 h with formulations containing 1 mg/h CP532,623, 0.1 mg/h or 1 mg/h CP524,515 or 0.1 mg/h halofantrine, 0.5 μCi/h ^{14}C oleic acid (OA) and 20 mg/h OA dispersed in 2.8 ml/h of 0.2% Tween 80 in normal saline. After 5 h, the infusion was replaced with a new infusion with the same composition but excluding the ^{14}C OA radiolabel and drug. The initial formulation was infused for 5 h, as this period was previously shown to establish steady-state rates of lipid and drug transport into the lymph (10). Removal of the ^{14}C OA radiolabel and drug from the infusion allowed examination of the washout kinetics of FA and drug transport into the lymph (which in turn reflect the rate of turnover of FA and drug from the lymph lipid precursor pool into the lymph). Lymph samples were collected continuously into tared tubes which were changed every 1 h for the first 5 h (when radiolabelled lipid and drug were included in the infusion) and then every 0.5 h for a further 4 h after infusion of ^{14}C OA radiolabel and drug ceased, i.e., 9 h in total. The volume of lymph collected during each sample period was determined gravimetrically. Drug and lipid (TG and PL) concentrations in lymph were measured using HPLC and commercial enzymatic kits running on a Cobas Mira lipid analyser, respectively, as described below. ^{14}C OA concentrations were measured by scintillation counting as described previously (9,10). Total FA concentrations in lymph were calculated using the assumption that each mole of lymph TG and PL comprised 3 and 2 moles of FA, respectively (9). Exogenous FA transport into lymph was determined as described previously via quantitation of ^{14}C OA. Endogenous FA transport into intestinal lymph was determined as the difference between the total FA transport and the exogenous FA transport in lymph (9).

Data were processed as described previously (10). Briefly, the rate of transport of total FA (endogenous plus exogenous FA) $(dX_L/dt)_{ss}$ and drug $(dD_L/dt)_{ss}$ into the lymph at steady state was calculated from the measured mass of FA and drug transported over the 4–5 h sample period. The first-order rate constants describing FA (K_X) or drug (K_D) transport from the lymph lipid precursor pool into the lymph were calculated from the gradient of a linear semi-log plot of ln (concentration of ^{14}C OA or drug in total FA in the lymph) *vs* time (h) after ceasing intraduodenal infusion of ^{14}C OA or drug. The mass of FA (X_{LP}) or drug (D_{LP}) in the lymph lipid precursor pool at steady state was then calculated using the equation

$$\frac{dX_L}{dt} = K_X.X_{LP} \quad \text{and} \quad \frac{dD_L}{dt} = K_D.D_{LP}$$

X_{LP} and D_{LP} were therefore calculated using the values of K_X and K_D obtained from the washout profiles, and the measured rate of FA (dX_L/dt) and drug (dD_L/dt) transport into lymph obtained during the 4–5 h steady-state transport period.

Analytical Methods

HPLC Analysis of Drug Concentrations

Sample Preparation for Drug Solubility in Physical Mixtures of TRL Core or Total Lipids. Fifty μl of 1 M HCl was added to one drop of weighed lipid sample and made up to 5 ml with 2:1 v/v chloroform/methanol in a volumetric flask. Ten μL of this chloroform/methanol solution was subsequently diluted 1:10 v/v with acetonitrile and the supernatant analysed by HPLC.

Sample Preparation for Solubility in Model Colloidal Species. Twenty μL samples of colloidal material were diluted 200-fold v/v with acetonitrile, vortexed for 1 min, and centrifuged at 5,200 g for 3 min, and the supernatant analysed.

Sample Preparation for Lymph and Lipoprotein Fractions. The mesenteric lymph and lipoprotein fractions were diluted 40-fold v/v (for lymph and $d = 0.93$–1.006 g/ml lipoprotein fractions), 200-fold (for $d < 0.93$ g/ml lipoprotein fraction) or 10-fold (for $d > 1.006$ g/ml lipoprotein fraction) with acetonitrile. The diluted samples were vortexed for 1 min and centrifuged at 5,200 g for 3 min, and the resulting supernatant used for HPLC analysis. The methods of sample preparation were validated by spiking blank lymph or lipoprotein fractions with CP524,515 or CP532,623 such that the final expected concentrations on dilution in acetonitrile were 0.5, 2 or 5 μg/ml. Recovery of spiked CP532,623 and CP524,515 was >90% in all cases.

UV-HPLC Assay. The validated HPLC method used to analyse CP532,623 and CP524,515 concentrations in all formulations was described previously (6). The HPLC methods for CP524,515 and CP532,623 were validated by assay of replicate ($n = 4$) quality control samples at low, medium and high concentrations (0.2, 1, 5 μg/ml) on three separate days. The assays were found to be accurate (within 10% of target concentration) and precise (co-efficient of variation <10%) for concentrations between 0.2 and 10 μg/mL. Inter-day variability in precision and accuracy was <10%, and the limit of quantitation was 200 ng/ml.

Analysis of Triglyceride, Phospholipid and Cholesterol Concentrations

Lipid (TG, PL and Ch) concentrations were analysed on a Cobas Mira lipid analyser using commercial colorimetric

enzymatic kits (triglyceride kit®, cholesterol kit®, Control for automated systems® and Precinorm U® from Roche Diagnostics, Indianapolis, IN and Wako phospholipids kit from Wako Chemicals, Richmond, VA) as described previously (15–18). Samples outside of the linear range for detection of TG, PL or Ch were diluted 2–10-fold with milli-Q and re-analysed.

Statistical Methods

Statistically significant differences were determined by ANOVA followed by Tukey's test for multiple comparisons at a significance level of $\alpha = 0.05$ using SPSS for Windows V11.5.0 (SPSS Inc, Chicago, Il).

RESULTS

Lipid Dependency of CP532,623 and CP524,515 Lymphatic Transport

Fig. 1 and Table III show the lymphatic transport of CP532,623 and CP524,515 after administration with a lipid-free formulation and formulations containing medium-chain (capric acid) and long-chain (oleic acid) lipids. The long-chain lipid formulation containing 40 mg oleic acid supported significantly greater lymphatic transport of both CP524,515 and CP532,623 when compared to an equal quantity (40 mg) of medium-chain lipid (capric acid). Administration of a lower (4 mg) quantity of oleic acid resulted in reduced lymphatic drug transport when compared with the 40 mg oleic acid formulation and promoted a similar extent of transport as the 40 mg capric acid formulation. As expected, lymphatic drug transport was negligible after administration of the lipid-free formulation (0.5% w/v Tween 80). The lymphatic transport of both compounds was therefore enhanced by administration with higher lipid doses and with long rather than medium chain length FA. Surprisingly, the cumulative lymphatic transport of CP524,515 (16% of the dose over 8 h) was greater than that of CP532,623 (12% of the dose over 8 h) when administered with the 4 mg oleic acid formulation, whereas the reverse was true with the higher quantity of oleic acid (40 mg), where 28% and 22% of the dose of CP532,623 and CP524,515, respectively, were recovered in the lymph over 8 h. The absorption and/or lymphatic transport of CP532,623, therefore, appeared to be more sensitive to changes in the quantity of co-administered lipid than CP524,515, at least in the current model.

Fig. 2 shows the extent and rate profiles of the lymphatic transport of CP532,623 and CP524,515 following administration with the 40 mg oleic acid formulation. Comparative data is also reproduced for halofantrine, a model drug

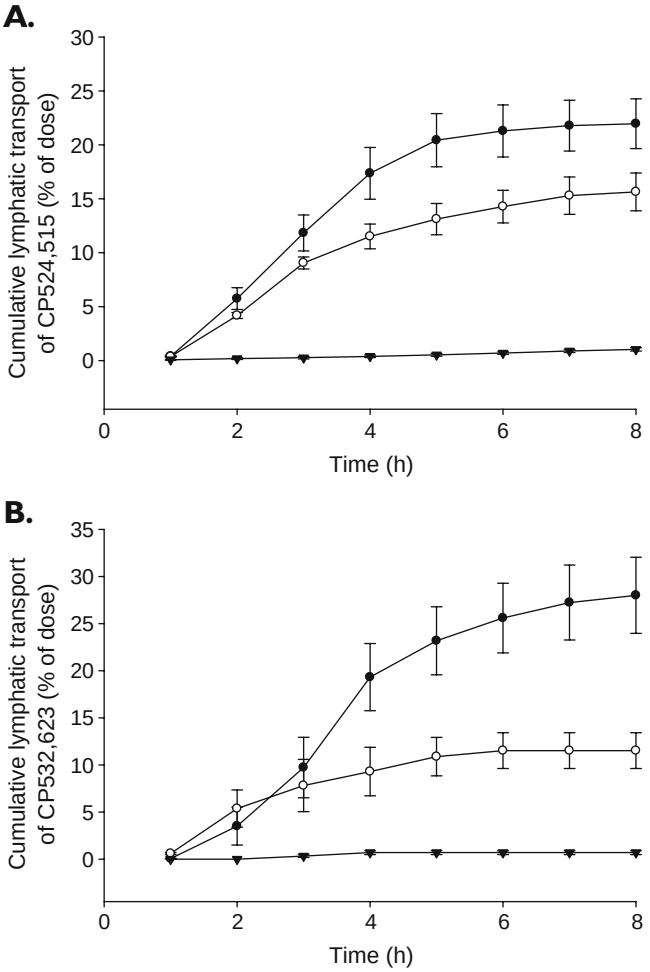


Fig. 1 Cumulative lymphatic transport of drug (% of dose) in mesenteric lymph duct cannulated, anaesthetised rats following intraduodenal infusion of formulations from 0 to 2 h. Formulations contained **A.** 2 mg CP524,515 or **B.** 2 mg CP532,623 dispersed in 5.6 ml 0.5% w/v Tween 80 (▼), 4 mg oleic acid (OA) in 5.6 ml 0.5% w/v Tween 80 (○) or 40 mg OA in 5.6 ml 0.2% w/v Tween 80 (●). Data represent mean ± SEM for $n = 4$ rats.

which shows "typical" lymphatic transport characteristics (from ref (10)). The cumulative % of the dose of both CP532,623 and CP524,515 that was recovered in the lymph over 8 h (28% and 22% of the dose, respectively) was significantly higher than that of halofantrine. Interestingly, the maximum rate of lymphatic drug transport (Fig. 2C) appeared to occur later for CP532,623 when compared to CP524,515 and halofantrine, suggestive of a delay in lymphatic transport.

The cumulative transport of TG into the lymph over 8 h following administration of CP532,623 or CP524,515 with each lipid formulation is given in Table III. Fig. 2 further shows the extent and rate profiles for TG transport in lymph when CP532,623, CP524,515 and halofantrine were administered with the 40 mg oleic acid formulation. In the animals administered CP532,623, the maximum rate of

Table III Cumulative lymphatic transport of drug (% of dose) and triglyceride (TG, mg) over 8 h in mesenteric lymph duct cannulated, anaesthetised rats following intraduodenal infusion of formulations from 0 to 2 h. Formulations contained 2 mg of CP524,515 or CP532,623 dispersed in 5.6 ml 0.5% w/v Tween 80, 4 mg oleic acid (OA) in 5.6 ml 0.5% w/v Tween 80, 40 mg OA in 5.6 ml 0.2% w/v Tween 80 or 40 mg capric acid (CA) in 5.6 ml 5 mM bile salt. Data represent mean±SEM for $n=4$ rats

	Drug transport in lymph (% of dose)		TG transport in lymph (mg)	
	CP524,515	CP532,623	CP524,515	CP532,623
0.5% Tween 80	1.0±0.2	0.7±0.2	24.1±4.2	22.1±0.2
4 mg OA in Tween 80	15.6±1.8	11.5±1.9	31.5±2.8	27.5±4.6
40 mg OA in Tween 80	21.9±2.3	28.0±4.0	37.8±4.7	36.2±2.2
40 mg CA in 5 mM bile salt	13.9±0.5	15.8±0.8	27.8±1.3	23.6±3.7

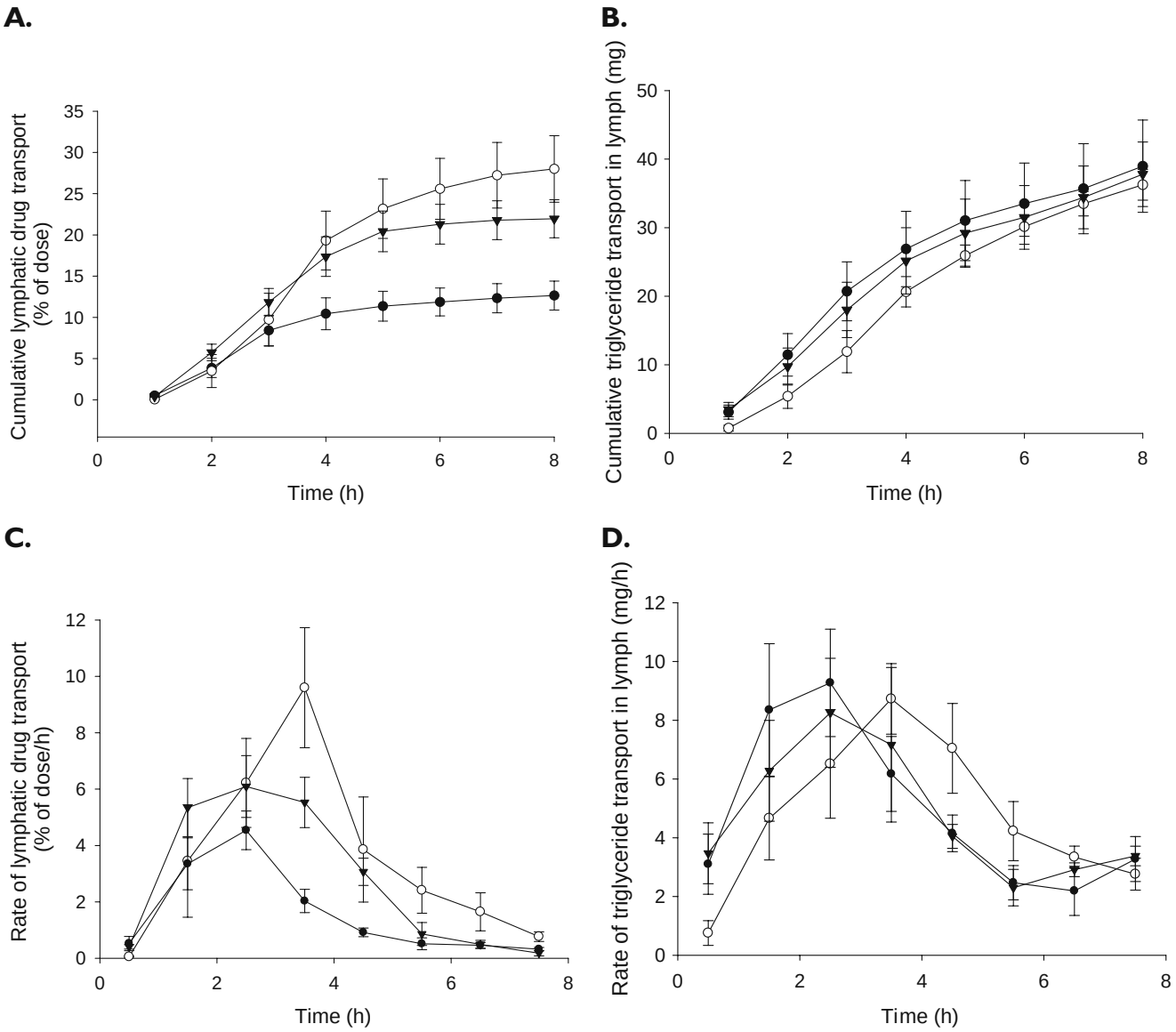


Fig. 2 Cumulative lymphatic transport of **A.** drug (% of dose) and **B.** triglyceride (mg) and the rate of lymphatic transport of **C.** drug (% of dose/h) and **D.** triglyceride (mg/h) in mesenteric lymph duct cannulated, anaesthetised rats following intraduodenal infusion of formulations from 0 to 2 h. Formulations contained 40 mg oleic acid, 5.6 ml 0.2% w/v Tween 80 in normal saline and 200 μg Halofantrine (Hf) (●), 2 mg CP532,623 (○) or 2 mg CP524,515 (▼). Data represent mean±SEM for $n=4$ rats.

lymphatic TG transport (Fig. 2D) appeared to occur later (4 h) than after administration of formulations containing CP524,515 or halofantrine (3 h). These trends were particularly evident in the data collected at early time points (1–3 h), when the rate of lymphatic lipid transport was up to 50% lower than that observed after administration of CP524,515 or the control compound, halofantrine. The delay in lymphatic TG transport in the group administered CP532,623 was consistent with the delay in lymphatic drug transport of CP532,623 (Fig. 2C).

Fig. 3 shows the ratio of the mass of drug transported into lymph and the mass of TG in the lymph (mg/g) over time following intraduodenal administration of CP532,623 or CP524,515 in formulations consisting of 0.5% w/v Tween 80, 4 mg oleic acid in 0.5% w/v Tween 80 or 40 mg oleic acid in 0.2% w/v Tween 80. A dashed line depicting the maximum solubility of CP524,515 (51 mg/g) and CP532,623 (16 mg/g) in long-chain TG (as determined previously (6)) is also shown to enable comparison of the mass ratio of drug/TG in the lymph (mg/g) and the maximum solubility of the drugs in long-chain TG. Following administration with the lipid-free (0.5% w/v Tween 80) formulation, the mass ratios of drug/TG in lymph were low for both drugs. As the dose of lipid was increased from 4 to 40 mg oleic acid, the mass ratios of drug/TG in the lymph increased. For CP524,515, the maximum ratio of drug/TG in the lymph (mg/g) after administration with 40 mg oleic acid was approximately 2.5, fold lower than the solubility (mg/g) of CP524,515 in long-chain TG *in vitro*. In contrast, the maximum mass ratio of CP532,623/TG (mg/g) in the lymph was significantly greater ($\alpha < 0.05$) than the maximum triglyceride solubility of CP532,623, suggesting that a driver other than solubilisation within TG was responsible for lymphatic drug access.

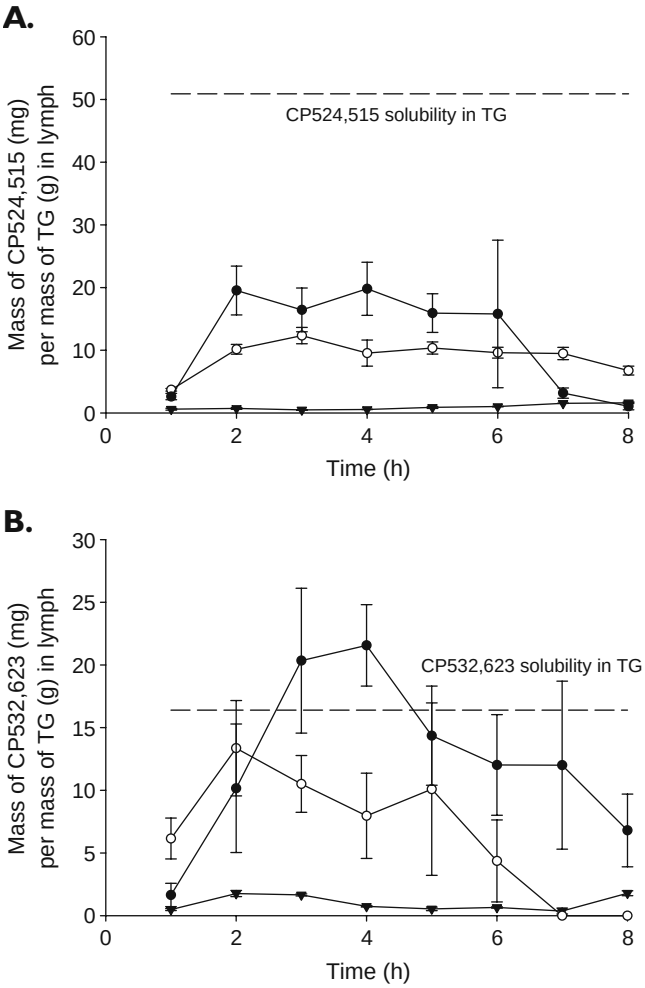


Fig. 3 Mass of drug per mass of triglyceride (TG) (mg/g) in the mesenteric lymph over time in mesenteric lymph duct cannulated, anaesthetised rats following intraduodenal infusion of **A.** 2 mg CP524,515 or **B.** 2 mg CP532,623 dispersed in 5.6 ml 0.5% w/v Tween 80 (▼), 4 mg oleic acid (OA) in 5.6 ml 0.5% w/v Tween 80 (○) or 40 mg OA in 5.6 ml 0.2% w/v Tween 80 (●). The maximum solubility of CP524,515 and CP532,623 in long-chain triglyceride (soybean oil) are also represented by a dashed line on the plots. Data represent mean ±SEM for $n = 4$ rats.

Lipid Solubility of CP524,515 and CP532,623 and Affinity for Isolated TRL

Table IV documents the affinity of the CETP inhibitors for isolated CM or VLDL and their solubility in combinations of lipids which represent the different components of TRL. The solubility of CP524,515 and CP532,623 in medium (miglyol)- and long (soybean oil)-chain TG are reproduced from a previous publication (6). CP524,515 was highly soluble in both medium- and long-chain TG, whereas the solubility of CP532,623 in both vehicles was approximately 2–3-fold lower, presumably reflecting the lower melting point of CP524,515 (Table I). The solubility of CP532,623 and CP524,515 in simple homogenous mixtures of lipids representative of those contained in the core or core plus surface constituents of TRL was similar to their respective solubilities in long-chain TG. In contrast, the masses of CP524,515 and CP532,623 solubilised per mass of TG in TRL were considerably higher (4-fold and 10-fold respectively). Drug affinity for TRLs therefore appeared to be driven by additional forces beyond simple lipid solubility.

In an attempt to identify the rationale behind the affinity of CP524,515 and, in particular, CP532,623 for TRL, the solubility of both compounds in a series of lipid preparations designed to replicate different components of lipoproteins was determined (Table IV). The mass of CP532,623 and CP524,515 that could be incorporated per mass of lipid in phospholipid (PL) and PL/cholesterol (Ch) liposomes (i.e. liposomes comprising lipids reflecting the surface lipids of TRL) was relatively low and not consistent with the high affinity for isolated TRL. However, when an emulsion was prepared containing similar types

Table IV Mass of drug (CP524,515 or CP532,623) solubilised per mass of lipid (mg/g) following incubation at 37°C with miglyol, soybean oil, physical mixtures of lipids similar to those contained within a triglyceride-rich lipoprotein (TRL) core or surface, isolated chylomicrons (CM) or very low density lipoproteins (VLDL), liposomes formed from phospholipid (PL) and cholesterol (Ch), an emulsion containing concentrations of lipids similar to those found in lymph TRL and a soybean oil emulsion formed with a synthetic surfactant. The ratio of CP524,515:CP532,623 solubility in each lipid mixture is also given. The constituent parts of the lipid mixtures are described in the method section. Data represent mean±SEM ($n=4$ to 8).

	CP524,515 (mg/g lipid)	CP532,623 (mg/g lipid)	Ratio CP524,515 to CP532,623
Miglyol[a]	164.9±0.9 ($n=4$)	61.2±0.9 ($n=4$)	2.7
Soybean oil[a]	50.9±0.0 ($n=4$)	16.4±0.4 ($n=4$)	3.1
TRL core lipids	34±5 ($n=4$)	15±2 ($n=4$)	2.3
TRL core and surface lipids	70±1 ($n=4$)	26±1 ($n=4$)	2.7
Isolated CM	195±37 ($n=5$)	163±36 ($n=5$)	1.2
Isolated VLDL	148±31 ($n=4$)	124±13 ($n=4$)	1.2
PL liposomes	44±6 ($n=8$)	23±3 ($n=8$)	1.9
PL and Ch liposomes	39±6 ($n=4$)	30±3 ($n=4$)	1.3
Emulsion with similar lipids to TRL	80±1 ($n=4$)	131±13 ($n=4$)	0.6
Emulsion with soybean oil and synthetic surfactant (Tween 80)	166±19 ($n=4$)	111±5 ($n=4$)	1.5

[a] Data taken from reference (6)

and concentrations of lipids to lymph (90% w/w soybean oil, 7% w/w di-palmitoyl phosphatidylcholine and 3% w/w cholesterol), the mass of CP532,623 dissolved in the emulsion per mass of lipid (131 mg/g) was high (higher in fact than that of CP524,515 (80 mg/g)). The affinity of both CP532,623 and CP524,515 for a simple emulsion prepared with soybean oil and synthetic surfactant was also high and similarly consistent with drug affinity for isolated TRL (Table IV) and drug transport in lymph *in vivo* (Fig. 3). CP524,515 and CP532,623, therefore, had a higher affinity for isolated lymph lipoproteins or colloidal structures with a TG-rich core and amphiphilic surface when compared to homogenous mixtures of lipids.

The Effect of CP532,623 and CP524,515 on Intestinal Lipid and Lipoprotein Processing

Effect on Lipid Distribution in Different Lipoprotein Fractions

The distribution of CP532,623, CP524,515, halofantrine, TG and PL across the different lipoprotein fractions in lymph (<0.93 g/ml, 0.93–1.006 g/ml and >1.006 g/ml density fractions which represent CM, VLDL and LDL/HDL, respectively) following administration of the 40 mg oleic acid formulation is shown in Table V. The most significant difference in lipid distribution was a notable decrease in lipid transport (both TG and PL) in CM and corresponding increase in transport in LDL/HDL in the groups administered CP532,623 or CP524,515 when compared to halofantrine. Lipid transport in the 0.93–1.006 g/ml VLDL fraction was seemingly unaffected. The distribution of the drugs across the lipoprotein fractions largely reflected lipid transport with the exception that significantly more CP532,623 and less CP524,515 were associated with the $d>1.006$ g/ml fraction (i.e. LDL/HDL) when compared to halofantrine.

The Effect of CP532,623 and CP524,515 on Lipoprotein Size and Density Assessed Using Flow Cytometry

Fig. 4 shows the flow cytometry profiles for whole lymph and CM and VLDL lymph fractions obtained from rats administered 20 mg of oleic acid/h for 5 h alone or with either 1 mg/h of CP532,623 or CP524,515. Flow cytometry provides an indication of the relative size (from forward scatter (FSC) measurements) and optical density (from side scatter (SSC) measurements) of particulate material such as cells or (in this case) lymph lipoproteins. The histograms in Fig. 4 depict the number of lipoproteins associated with each FSC or SSC co-ordinate, whereas the contour plots show the relative frequency of lipoproteins detected with each SSC and FSC value. The FSC *vs* SSC contour plot (Fig. 4A control) for lymph collected from control animals (which were not infused with drug) show two populations of lipoproteins with distinctly different SSC/optical density (Fig. 4C control) and overlapping FSC/size (Fig. 4B control) values (although the more dense lipoproteins were on average slightly larger in size). CM separated from the control animals (Fig. 4D control) were within the size (FSC) and optical density (SSC) range of the larger and more optically dense lipoprotein fractions in lymph. In contrast, the VLDL separated from the control animals (Fig. 4E control) overlapped the two different populations.

In the lymph of animals infused with CP524,515 (Fig. 4A CP524,515) and CP532,623 (Fig. 4A CP532,623) there were again two different populations of lipoproteins with overlapping size (FSC) but distinctly different optical density (SSC). However, a smaller proportion of the lipoproteins

Table V % of the mass of drug (CP524,515, CP532,623 or halofantrine), triglyceride or phospholipid associated with each lipoprotein fraction (density <0.93 g/ml, 0.93–1.006 g/ml or >1.006 g/ml) in lymph collected over 6 h from mesenteric lymph duct cannulated, anaesthetised rats following intraduodenal infusion of formulations containing 2 mg drug and 40 mg oleic acid dispersed in 5.6 ml 0.2% w/v Tween 80 over 0–2 h. Data represent mean±SEM for $n=4$ rats.

	$d<0.93$g/ml fraction	$d=0.93$–1.006g/ml fraction	$d>1.006$g/ml fraction
A.% of the mass of drug in each fraction			
CP524,515	60.7±11.0	36.5±9.9	2.9±1.1
CP532,623	64.8±5.8	25.8±4.7	9.4±1.1[a]
Halofantrine	67.8±2.5	25.8±2.4	6.4±1.2[b]
B.% of the mass of triglyceride in each fraction			
CP524,515	56.3±1.2[c]	30.4±1.8	13.2±0.6[c]
CP532,623	55.9±6.1	28.4±5.9	15.6±0.2[c]
Halofantrine	68.9±2.6	27.0±4.8	4.1±0.2
C.% of the mass of phospholipid in each fraction			
CP524,515	49.2±2.3[c]	30.6±0.6	20.2±1.7[c]
CP532,623	49.5±3.2	29.2±2.9	21.2±0.3[c]
Halofantrine	55.0±2.0	34.2±3.0	10.8±1.1

[a] Significantly higher ($\alpha<0.05$) than the % of the dose of halofantrine and CP524,515 which were associated with the same fraction

[b] Significantly higher ($\alpha<0.05$) than the % of the dose of CP524,515 which was associated with the same fraction and lower than the % of the dose of CP532,623 associated with this fraction

[c] Significantly different ($\alpha<0.05$) from the % of triglyceride or phospholipid associated with the fraction in the group administered halofantrine

were contained within the more optically dense and larger population. The CM (Fig. 4D) and VLDL (Fig. 4D) separated from the lymph obtained from the animals infused with CP524,515 and CP532,623 was also different and contained more of the smaller, low optical density lipoproteins. The reduction in the number of large optically dense lipoproteins in the groups administered CP524,515 and CP532,623 was consistent with the lower contribution to lipid transport by CM observed in the lipoprotein fractions separated by ultracentrifugation (Table V)

Impact of CP532,623 and CP524,515 on the Steady-State Kinetics of Lipid Transport into the Lymph

The kinetics of lipid transport from the lymph lipid precursor pool into the lymph following intraduodenal administration of halofantrine, CP532,623 or CP524,515 and 20 mg oleic acid/h are summarised in Table VI. The dose dependency of the effect of CP524,515 on the kinetic parameters was also explored via administration of either 0.1 mg/h or 1 mg/h of CP524,515. Halofantrine does not have any reported effects on lipid absorption or transport, and the halofantrine group is therefore included for comparative purposes. The latter data set is reproduced from reference (10).

The steady-state rate of lymphatic transport of exogenous FA and the mass of exogenous FA in the lymph lipid precursor pool was substantially lower ($\alpha<0.05$) in the group administered CP532,623 when compared to the control (halofantrine) group. The rate of lymphatic transport of endogenous FA and the mass of endogenous FA in the lymph lipid precursor pool, however, was similar. The decrease in exogenous lipid transport led to an increase in the relative proportion of endogenous FA compared to exogenous FA in the lymph and lipid pool and a reduction in the rate of lymphatic transport of total FA and the mass of total FA in the lymph lipid precursor pool in the group administered CP532,623. The rate constant describing the turnover of FA from the lipid pool to lymph (K_X) was not significantly different following administration of CP532,623 or halofantrine.

In contrast to the group administered CP532,623, the rate of exogenous, endogenous and total FA transport into the lymph was not significantly different in the groups administered 0.1 mg/h or 1 mg/h CP524,515 when compared to the control (halofantrine) group (Table VI). The proportion of exogenous and endogenous lipids in the lymph and lipid pool were also unchanged in the groups administered CP524,515. The lipid turnover rate constant (K_X), however, was significantly greater, and the mass of FA (endogenous, exogenous and total) in the lymph lipid precursor pool significantly lower in rats administered 1 mg/h CP524,515 when compared to halofantrine or CP532,623. CP524,515, therefore, resulted in the generation of a smaller lymph lipid precursor pool that turned over more rapidly when compared to that observed after administration of CP532,623 or halofantrine. Interestingly, the effect of CP524,515 on the kinetics of lipid turnover and lymphatic transport was dose-dependent and was essentially abolished

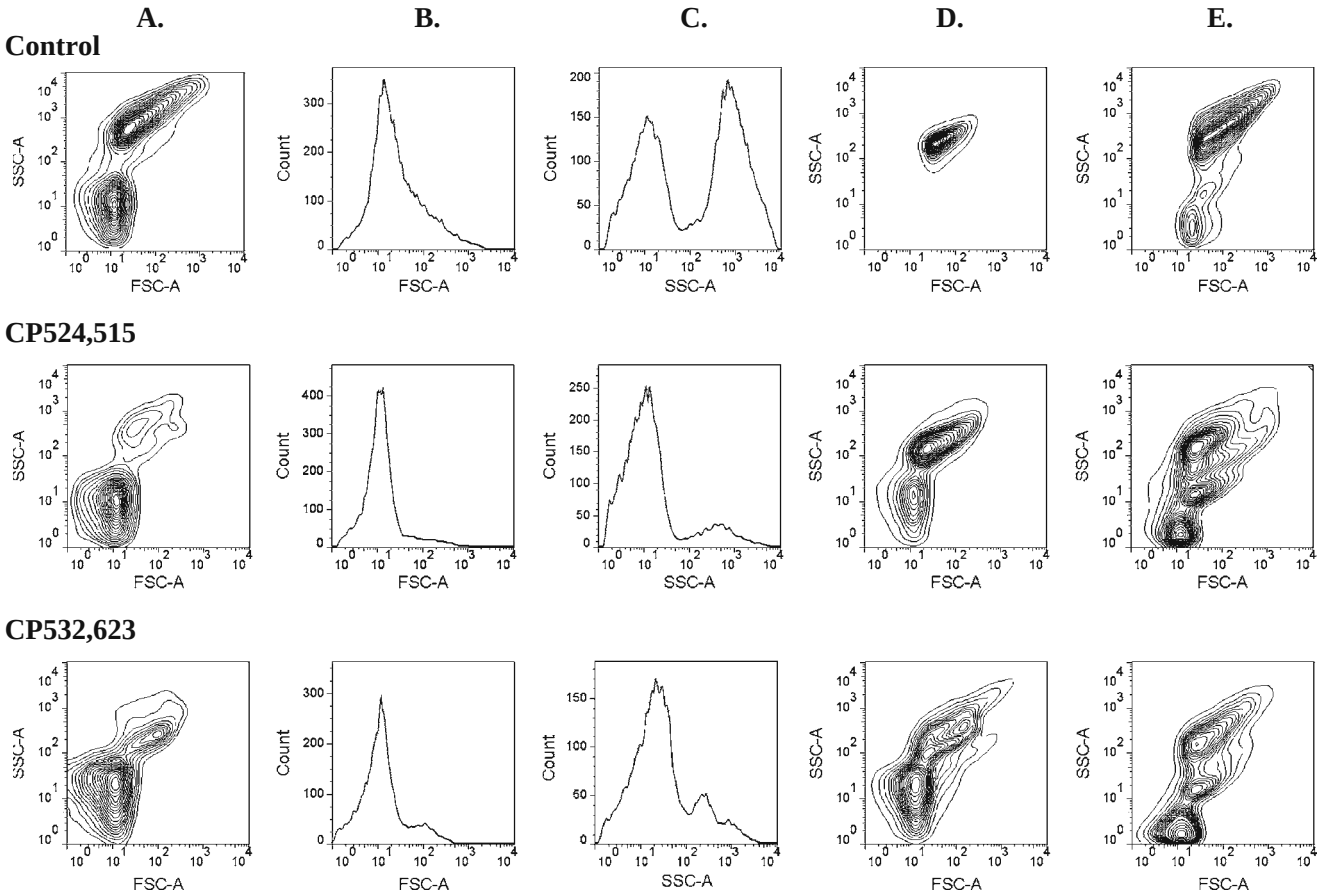


Fig. 4 Flow cytometry contour plots of forward *versus* side scatter and forward and side scatter frequency histograms of mesenteric lymph (**A**–**C**), chylomicrons (**D**) and VLDL (**E**) collected from rats administered 20 mg oleic acid (OA)/h dispersed in 0.2% w/v Tween 80 with either no drug, with 1 mg/h CP524,515 or 1 mg/h CP532623 for 5 h via continuous intraduodenal infusion.

in the group administered 0.1 mg/h (rather than 1 mg/h) CP524,515.

Table VII shows the results for the kinetics of drug transfer into the lymph (rather than the lipid data in Tables VI) during steady-state administration of CP524,515, CP532,623 and halofantrine with 20 mg/h oleic acid. The rate of lymphatic transport (% of dose/h) of all three drugs was remarkably similar. However, a significantly ($\alpha < 0.05$) lower fraction of the dose of CP524,515 and CP532,623 was resident in the lymph lipid pool at steady state, and the turnover rate constants were significantly higher when compared to halofantrine. The turnover rate constant describing CP532,623 transport from the lipid pool into the lymph (0.72 h^{-1}) was essentially the same as the corresponding rate constant for FA turnover in the same group (0.67 h^{-1}). In contrast, the turnover rate constants describing CP524,515 (0.72 h^{-1}) and halofantrine (0.43 h^{-1}) turnover from the lipid pool into the lymph were significantly lower than the corresponding rate constants for FA (1.05 and 0.59 h^{-1} in the presence of CP524,515 and halofantrine, respectively). For halofantrine, this has been suggested to result from enterocyte-based metabolism and therefore drug removal from the lipid pool (38). Similar differences between lipid and drug turnover rate constants suggest that enterocyte-based metabolism may also occur for CP524,515, although further studies are required to confirm this possibility.

DISCUSSION

Recent studies have shown that CP524,515 and CP532,623 are substantially transported to the systemic circulation via the lymphatic system following oral administration to greyhound dogs. In these previous studies, CP532,623 was remarkably highly lymphatically transported considering its modest solubility in long-chain TG, and also appeared to reduce lymphatic lipid transport in the greyhound dog model. The current studies were, therefore, conducted to

Table VI The turnover rate constants describing lipid (K_X) transport from the lymph lipid precursor pool into the lymph, the rate of transport of total, exogenous and endogenous fatty acid (FA) into the lymph, the proportion of FA in the lymph and lymph lipid precursor pool which is from endogenous sources and the mass of total, exogenous and endogenous FA in the lymph lipid precursor pool at steady state in mesenteric lymph duct cannulated, anaesthetised rats (Mean±SEM) following 5 h of continuous intraduodenal infusion of formulations administered at a rate of 1 μCi/h ^{14}C oleic acid and 0.1 mg/h of Halofantrine ($n=4$) or 1 mg/h CP532,623 ($n=4$) or 1 mg/h of CP524,515 ($n=3$) or 0.1 mg/h CP524,515 ($n=3$) dissolved in 20 mg/h oleic acid in 2.8 ml/h of 0.2% w/v Tween 80

	0.1 mg/h Halofantrine	1 mg/h CP532,623	1 mg/h CP524,515	0.1 mg/h CP524,515
K_X	0.59 ± 0.06	0.67 ± 0.02	1.05 ± 0.05[c]	0.48 ± 0.01
Rate of total FA transport in lymph (μmol/h)	49.79 ± 2.55	42.95 ± 1.75[a]	49.18 ± 0.88	46.02 ± 0.70
Rate of exogenous FA transport in lymph (μmol/h)	32.13 ± 2.44	22.43 ± 0.57[a]	33.59 ± 1.23	30.60 ± 2.19
Rate of endogenous FA transport in lymph (μmol/h)	17.67 ± 2.59	20.52 ± 1.82	15.59 ± 0.35[b]	15.42 ± 1.49[b]
Proportion endogenous of total FA in the lipid pool and lymph	0.54 ± 0.02	0.72 ± 0.02	0.52 ± 0.02	0.56 ± 0.03
Mass of total FA in the lipid pool (μmol)	89.67 ± 16.63	64.84 ± 3.00[a]	47.95 ± 2.19[a, b]	96.43 ± 2.71
Mass of exogenous FA in the lipid pool (μmol)	56.89 ± 8.81	34.06 ± 1.68[a]	32.71 ± 1.80[a]	64.49 ± 5.41
Mass of endogenous FA in the lipid pool (μmol)	32.78 ± 8.78	30.78 ± 2.62	15.24 ± 0.75[a, b]	31.94 ± 2.70

[a] Significantly lower than the same parameter in the group administered halofantrine ($\alpha < 0.05$)

[b] Significantly lower than the same parameter in the group administered CP532,623 ($\alpha < 0.05$)

[c] Significantly higher than the same parameter in the groups administered CP532,623, halofantrine or 0.1 mg/h CP524,515 ($\alpha < 0.05$)

further explore the mechanisms involved in lymphatic transport of CP524,515 and CP532,623 and to evaluate their potential effects on lymphatic lipid transport.

Consistent with previous studies in greyhound dogs (6), CP532,623 and CP524,515 were both significantly transported into the lymphatic system after oral administration to rats, and lymphatic drug transport was enhanced by administration with greater lipid masses and with long- rather than medium-chain lipids (19). Interestingly, the mass of CP523,623 transported in the lymph per mass of long-chain TG (Fig. 3) was significantly greater than the maximum solubility of CP532,623 in TG. This provided a first indication that mechanisms beyond simple solubilisation within the TG-rich core of TRL may have been responsible for the high levels of lymphatic transport of the moderately lipophilic CP532,623 (Table IV).

Recent studies by Gershkovich *et al.* have shown that drug affinity for *ex vivo* TG-rich lipoproteins may be used as a more accurate predictor of the potential for intestinal lymphatic transport than simple indicators of lipophilicity such as log D or TG solubility. With the exception of benzpyrene, however, in all previously reported studies, significant lymphatic transport was only evident after administration of molecules with both high lipid solubility and high log D. (12,20). Bearing in mind the moderate lipid solubility of CP532,623, a series of studies was therefore conducted to elucidate the components of lymph lipoproteins which were responsible for the lymphatic transport of the CETP inhibitors. Consistent with the high recovery of CP532,623 and CP524,515 within TRL in lymph, the mass of CP532,623 and CP524,515 which could be incorporated into *ex-vivo* TRL was high and far greater than drug solubility in long-chain TG or in mixtures of lipids based on the composition of TRL. The mass of drug that could be solubilised per mass of lipid in liposomes formed with amphiphilic TRL surface lipids was also

Table VII The turnover rate constants describing drug (halofantrine, CP532,623 or CP524,515) (K_D) transport from the lymph lipid precursor pool into the lymph, the rate of drug transport into lymph (% of dose/h) and the mass of drug in the lymph lipid precursor pool at steady state in mesenteric lymph duct cannulated, anaesthetised rats (Mean±SEM) following 5 h of continuous intraduodenal infusion of formulations administered at a rate of 1 μCi/h ^{14}C oleic acid, 0.1 mg/h of halofantrine ($n=4$) or 1 mg/h of CP532,623 ($n=4$) or 1 mg/h of CP524,515 ($n=3$) dissolved in 20 mg/h oleic acid in 2.8 ml/h of 0.2% w/v Tween 80

	0.1 mg/h Halofantrine	1 mg/h CP532,623	1 mg/h CP524,515
K_D	0.43 ± 0.06	0.72 ± 0.04[a]	0.75 ± 0.02[a]
Rate of drug transport in lymph (% of dose/h)	22.02 ± 3.98	21.83 ± 0.69	22.82 ± 1.29
Mass of drug in the lipid pool (% of hourly dose)	51.22 ± 7.14	30.90 ± 1.32[a]	30.92 ± 2.18[a]

[a] Significantly different from the same parameter in the group administered halofantrine ($\alpha < 0.05$)

relatively low. Association of CP532,623 and CP524,515 with lipoproteins was, therefore, unlikely to have been facilitated by solubilisation in TRL core lipids or surface lipids alone (although a proportion of the drug mass may be associated with both these components). Rather, the high affinity of CP532,623 and CP524,515 for isolated TRL was more closely correlated with the mass of drug which could be incorporated into a lipid emulsion formed using TRL core and surface lipids or soybean oil and Tween 80 (i.e. a synthetic emulsion). The colloidal character of TRL and the presence of an interfacial stabiliser therefore appears to dictate the unique affinity of CP532,623 and CP524,515 for TRL. *In vivo*, CP532,623 might therefore be expected to be orientated across the oil/water interface of colloidal TRL, whereas CP524,515, which has significantly greater affinity for long-chain TG and TRL core lipids, might be expected to associate with both core lipids and interfacial components. It is interesting to speculate that the affinity of CP532,623 and CP524,515 for the lipoprotein interface may be linked with access to the target (CETP) present on the surface of HDL or TRL (1), although further experimentation is required to confirm this possibility.

From the perspective of predictive methods for lymphatic transport, the current studies are consistent with those of Gershkovich (12) and confirm the utility of drug affinity for *ex-vivo* lipoproteins as an indicator of lymphatic transport. However, the current data also indicate that in some cases drug solubility in simple emulsions formed with long-chain TG (soybean oil) and Tween 80 may also be predictive of the potential for lymphatic transport. In comparison to *ex-vivo* incubation in lipoproteins, this method is quicker, has high throughput and does not require separation of lipoproteins from plasma or lymph. In contrast to this suggestion, however, Gershkovich has shown for certain other probe compounds that drug solubility in an artificial TG emulsion (Intralipid®) may not be highly predictive of drug association with TRL (and therefore lymphatic transport) (12,20). Further studies are required to determine whether drug solubility in TG emulsions can be used to predict the extent of drug association with TRL and transport into the lymph, and whether the optimum composition of these TG emulsions is critical to applicability.

The Impact of CP532,623 and CP524,515 on Intestinal Lipid and Lipoprotein Processing

In recent greyhound dog studies, administration of CP532,623 with food appeared to inhibit TG transport into the lymph when compared with administration of CP524,515. Similar effects were replicated in the current study where administration of CP532,623 with 40 mg of oleic acid delayed TG transport into the lymph when compared with administration of the same lipid dose with a model lymphatically transported drug (halofantrine) or with CP524,515 (Fig. 2). CP532,623 and CP524,515 also influenced the kinetics of lipid transport from the lymph lipid precursor pool into the lymph under steady-state conditions (Table VI), including changes to the size of the lymph lipid precursor pool and the rate of turnover of lipid from the lymph lipid precursor pool to the lymph (Table VIII). Both CP532,623 and CP524,515 also increased lipid (TG and PL) transport in lymph via HDL (d>1.006 g/ml fraction) and reduced the proportion of lipids carried by CM (d<0.93 g/ml fraction) (Table V). Consistent with these trends, the flow cytometry profiles obtained from rats administered CP532,623 and CP524,515 showed a reduction in the prevalence of a population of large and optically dense lipoproteins that share similar scattering characteristics to CM.

Whilst the mechanisms by which these changes were mediated have not been studied here, potential molecular targets include effects mediated by CETP, microsomal TG

Table VIII Summary of the effects of CP524,515 and CP532,623 on intestinal lipid and lipoprotein processing

	CP532,623	CP524,515
Steady state conditions		
Mass of exogenous and endogenous lipids in the lipid pool	Reduced	Reduced
Mass of drug in the lipid pool	Reduced	Reduced
Rate of lipid transport in lymph	Reduced	No change
Rate of exogenous lipid transport in lymph	Reduced	No change
Rate of endogenous lipid transport in lymph	No change	No change
Ratio of exogenous to endogenous lipids in lipid pool and lymph	Reduced	No change
Rate of lipid turnover from the lipid pool into the lymph	No change	Increased
Proportion of larger, more optically dense lymph lipoproteins	Decreased	Decreased
Non-steady-state conditions		
Rate of lipid transport in lymph	Delayed	No change
Proportion of TG and PL carried by HDL rather than VLDL and CM (separation by centrifugation)	Increased	Increased

transfer protein (MTP) (21–23) or phospholipid transfer protein (PLTP) (24). PLTP shares a similar structure and activity to CETP (25–27) and was recently shown to influence lipoprotein processing in the liver and intestine (28,29). MTP facilitates transfer of TG, PL and Apo B out of the SER membrane to the SER lumen, resulting in the subsequent formation of TRL (21–23). However, torcetrapib and other analogs related to CP532,623 and CP524,515 do not appear to inhibit PLTP-mediated PL transfer (1) or MTP (Pfizer data on file), and CETP therefore appears to be the more likely target since CP532,623 and CP524,515 are known inhibitors of CETP. Treatment with the CETP inhibitor, torcetrapib (in combination with atorvastatin) has also been shown to significantly attenuate the post-prandial increase in plasma CM and VLDL and to increase post-prandial plasma HDL2 in humans with type IIB hyperlipidemia (when compared to no treatment or treatment with atorvastatin alone). CETP-deficient humans also display a diminished or delayed post-prandial increase in plasma TG (30–32). Similarly, CETP increases post-prandial triglyceridemia and delays triacylglycerol plasma clearance in transgenic mice which express human CETP (33). These changes are consistent with the effect of CP532,623 on lymph TG transport following administration with a meal or lipid dose to dogs and rats and the effect of CP524,515 and CP532,623 on lymph lipoprotein profiles in the current study.

A recent study has also examined the influence of CETP deficiency on lipid storage, degradation and re-synthesis in adipocytes (34). In this study, CETP deficiency led to decreased TG synthesis, increased CE synthesis, reduced TG and CE degradation and inefficient transport of TG and CE from the endoplasmic reticulum (ER) to cytoplasmic storage droplets. This resulted in the accumulation of TG and CE in the ER rather than in storage droplets. CETP was therefore suggested to facilitate TG and CE transport from the ER to cytoplasmic storage droplets, in a role analogous to MTP. Inhibition of a similar function in the small intestine of rats would explain, at least in part, the effects of CP532,623 and CP524,515 on intestinal lipid and lipoprotein processing in the current study. Rats and dogs do not, however, express functional CETP in plasma (which accounts for the high plasma HDL levels and resistance to atherosclerosis in these species) (35,36), and there are conflicting reports as to whether CETP is expressed in other tissues in rats (28,37). CETP expression in the intestine of rats does not appear to have been studied specifically. The current findings warrant further investigation as effects on lipid and lipoprotein processing within tissues, in addition to the well-known effects of the CETP inhibitors in plasma, may contribute to the activity and toxicity profiles of CETP inhibitors in the treatment of atherosclerosis.

CONCLUSION

The current data suggest that the surprisingly high lymphatic transport of CP532,623 (given its relatively moderate lipid solubility) is driven in part by affinity for the colloidal interface of lymph lipoproteins rather than simple solubilisation in core lipids and that lipid solubility >50 mg/g is not a pre-requisite for significant intestinal lymphatic transport. CP532,623 and CP524,515 also appear to influence the kinetics of lipid transport into the lymph and decrease the proportion of lymph TG and PL transported by TRL and increase the proportion transported by HDL. These findings suggest that an effect of the CETPi on lipoprotein assembly in the enterocyte may, in addition to their well established effects on lipid transfer between lipoproteins in the systemic circulation (3,4), contribute to the changes in plasma lipoprotein profiles that occur after oral administration of CETP inhibitors.

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