Oral administration of tipranavir with long-chain triglyceride results in moderate intestinal lymph targeting but no efficient delivery to HIV-1 reservoir in mesenteric lymph nodes

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Abstract

The introduction of combination antiretroviral therapy (cART) led to substantial improvement in mortality and morbidity of HIV-1 infection. However, the poor penetration of antiretroviral agents to HIV-1 reservoirs limit the ability of the antiretroviral agents to eliminate the virus. Mesenteric lymph nodes (MLNs) are one of the main HIV-1 reservoirs in patients under suppressive cART. Intestinal lymphatic absorption pathway substantially increases the concentration of lipophilic drugs in mesenteric lymph and MLNs when they are co-administered with long-chain triglyceride (LCT). Chylomicrons (CM) play a crucial role in the intestinal lymphatic absorption as they transport drugs to the lymph lacteals rather than blood capillary by forming CM-drug complexes in the enterocytes. Thus, lipophilic antiretroviral drugs could potentially be delivered to HIV-1 reservoirs in MLNs by LCT-based formulation approach. In this study, protease inhibitors (PIs) were initially screened for their potential for intestinal lymphatic targeting using a computational model. The candidates were further assessed for their experimental affinity to CM. Tipranavir (TPV) was the only-candidate with substantial affinity to both artificial and natural CM *in vitro* and *ex vivo*. Pharmacokinetics and biodistribution studies were then performed to evaluate the oral bioavailability and intestinal lymphatic targeting of TPV in rats. The results showed similar oral bioavailability of TPV with and without co-administration of LCT vehicle. Although LCT-based formulation led to 3-fold higher concentrations of TPV in mesenteric lymph compared to plasma, the levels of the drug in MLNs were similar to plasma in both LCT-based and lipid-free formulation groups. Thus, LCT-based formulation approach alone was not sufficient for effective delivery of TPV to MLNs. Future efforts should be directed to a combined highly lipophilic prodrugs/lipid-based formulation approach to target TPV, other PIs and potentially other classes of antiretroviral agents to viral reservoirs within the mesenteric lymphatic system.

Keywords

Protease inhibitor, tipranavir, long-chain triglyceride-based formulation, mesenteric lymph nodes, HIV, intestinal lymphatic targeting

Abbreviations

cART, combination antiretroviral therapy; MLNs, mesenteric lymph nodes; LCT, long-chain triglycerides; CM, chylomicrons; PI, protease inhibitor; TPV, tipranavir; RAL, raltegravir; RTV, ritonavir; DTG, dolutegravir; SQV, saquinavir; IDV, indinavir; NFV, nelfinavir; APV, amprenavir; LPV, lopinavir; ATV, atazanavir; FPV, fosamprenavir; DRV, darunavir; CBD, cannabidiol; MTBE, methyl tertiary butyl ether; ACN, acetonitrile; DPBS, Dulbecco's phosphate buffered saline; PBS, phosphate buffered saline; ppm, parts per million; TG, triglyceride; BMI, body mass index; FDA, Food and Drug Administration; IS, internal standard; LLOQ, lower limit of quantification; SEM, standard error of the mean; BSU, Bio-Support Unit.

1. Introduction

Since the first detection of HIV-1 infection cases in the 1980s, HIV/AIDS pathophysiology and treatments have been studied and developed for decades. The introduction of combination antiretroviral therapy (cART) led to successful treatment in many cases [1, 2] and dramatically improved the morbidity and mortality of HIV-1 infection [3]. However, numerous studies demonstrated that HIV-1 remains replication-competent in patients undergoing cART treatment with undetectable plasma viral loads [4-6]. This could be due to the establishment of latent HIV-1 reservoirs in cells and various tissues at the initial stage of the infection [7, 8]. It is believed that the poor penetration of antiretroviral agents to such HIV reservoirs limit the ability of the drugs to eliminate the virus. The mesenteric lymph and mesenteric lymph nodes (MLNs) are one of the main HIV-1 reservoirs with the highest viral load reported [9] and SIV reservoirs in the nonhuman primate models [10-12]. Therefore, an effective delivery of antiretroviral drugs to mesenteric lymphatic system can contribute to eradication of HIV-1 from this important reservoir.

Most orally administered drugs following absorption from the gastrointestinal tract gain access to the systemic circulation through portal vein with a potential for hepatic first-pass metabolic loss. However, for some highly lipophilic compounds administered with lipids, intestinal lymphatic system rather than hepatic portal blood is the main route to enter the systemic blood circulation [13-15]. During the intestinal lymphatic absorption of drugs, chylomicrons (CM) play a crucial role as they transport drugs to the lymph lacteals rather than blood capillary by forming CM-drug complexes in the enterocytes [16]. A strong correlation between the intestinal lymphatic absorption and the affinity of drugs to CM has been established [17]. It is known that dietary lipids stimulate the assembly of CM [18]. We have previously shown that long-chain triglyceride (LCT)-based formulation can not only facilitates the intestinal lymphatic absorption but also leads to extremely high concentration of drugs within the mesenteric lymph and MLNs [19, 20]. This suggests that intestinal lymphatic absorption pathway substantially increases the concentration of drugs in mesenteric lymph and MLNs [21]. However, only highly lipophilic compounds with high affinity to CM could be delivered to the intestinal lymphatic system by LCT-based formulation approach. For other compounds, prodrug approach combined with LCT-based formulation was required in order to achieve substantial intestinal lymphatics targeting [22, 23].

Several studies have demonstrated that in many cases drug combination regimens that include protease inhibitor (PI) show greater benefits of HIV-1 treatment compared to monotherapy or combination regimens without PIs [2, 3, 24, 25]. Moreover, boosted PIs have been successfully used in monotherapy and showed non-inferiority compared to cART [26]. Although PIs are gradually fading from the mainstream of HIV-1 treatment in recent years, they are still an important component of many recommended cART regimens. For instance, a raltegravir (RAL) backbone regimen is recommended as the preferred first-line regimen for neonates. In addition, ritonavir (RTV)-boosted PIs are suggested to be incorporated in a NRTI-based therapy as a preferred second-line regimen for patients with failed dolutegravir (DTG)-based treatment [27]. Targeted delivery of PIs to mesenteric lymphatic system, including mesenteric lymph and MLNs, may potentially lead to more effective treatment by increasing the exposure of this HIV-1 reservoir to PIs.

Since the association of drugs with CM determines the extent of intestinal lymphatic absorption, an *in silico* model was previously established based on multiple

physiochemical properties to predict the degree of CM association of drugs [28]. In this study, PIs were assessed for their potential of intestinal lymphatic targeting using this computational model. Although four PIs showed predicted CM association *in silico*, further experimental results indicated that tipranavir (TPV) is the only candidate which has affinity to artificial and natural CM *in vitro* and *ex vivo*. TPV is a non-peptidic PI which has high genetic barrier to drug resistance and is active for both wild and multidrug-resistant HIV-1 strains [29, 30]. Furthermore, it is a second-line agent reserved for HIV-1 infected patients previous treatment failure [31]. However, box warnings for intracranial hemorrhage and hepatotoxicity substantially constrain the actual clinical use of TPV [32]. It has been proposed that Intestinal lymphatic targeting, if successful, can potentially result in lower total required dosage, which could eventually limit these life-threating adverse effects of this compound. Accordingly, it has been hypothesized in this work that based on its physiochemical properties [33], TPV may have substantial intestinal lymphatic absorption if it is co-administered with LCT vehicle. Therefore, the aim of this study was to assess the feasibility of LCT-based formulation approach for targeting TPV to HIV-1 reservoirs within the MLNs and mesenteric lymph.

2. Materials and Methods

2.1. Materials

TPV was extracted from Aptivus® soft capsules (250 mg of TPV, Boehringer Ingelheim GmbH, Germany). Cannabidiol (CBD) was purchased from THC Pharm GmbH (Frankfurt, Germany). HPLC grade n-hexane, methyl tert-butyl ether (MTBE), ethyl acetate, acetonitrile (ACN) and ammonium acetate were purchased from Fisher Scientific (Leicestershire, UK). Intralipid®, Dulbecco's phosphate buffered saline (DPBS), potassium bromide and phosphate-buffered saline tablets (PBS, P4417-100TAB) were purchased from Sigma-Aldrich (Gillingham, UK). Pooled male Sprague Dawley rat plasma was purchased from Sera Laboratories International Ltd (West Sussex, UK). All other reagents and solvents were of HPLC grade or higher.

2.2. Extraction of TPV

An Aptivus® soft capsule was dispersed in 5 mL of warm water at 37°C. Liquid-liquid extraction was then performed 3 times using 20mL of dichloromethane. The pooled organic fractions were dehydrated with brine (30 mL) and anhydrous Na_2SO_4, and then filtered. The crude product was purified by column chromatography. The purified eluents were pooled and fully evaporated to dryness. The detailed chemical characterization of isolated and purified TPV is described in **Supplementary material 1**.

2.3. Long-chain triglyceride (LCT) solubility

The LCT solubility of TPV was assessed as previously described for other compounds [22]. Briefly, excessive amount of TPV was added to fresh sesame oil (in triplicate) and stirred using magnetic stirrer at 37°C for 72 hours. Following the incubation, the mixture was filtered using Costar Spin-X Centrifuge Tube (Fisher Scientific, Loughborough, UK) at 2,400 *g* for 5 minutes. The filtrates were then diluted 10,000-fold with 2-propanol and analyzed for TPV concentration by means of HPLC.

2.4. Association with CM-like emulsion and plasma-derived CM

2.4.1. *In silico* screening of association of PIs with CM

A previously established physicochemical properties-based computational model was applied to screen the potential affinity of different PIs to CM [28]. The screened PIs were saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), lopinavir (LPV), atazanavir (ATV), fosamprenavir (FPV), tipranaivr (TPV) and darunavir (DRV). All physicochemical properties of analyzed PIs used in *in silico* modelling were calculated using ACD/I-Lab (Advanced Chemistry Development Inc., Toronto, ON, Canada) [33].

2.4.2. Preparation of protein-free artificial CM-like emulsion (Intralipid®)

Intralipid® 20% was diluted with phosphate buffered saline (PBS) to generate a 1 mg/mL concentration of triglyceride (TG) emulsion as previously described [28].

2.4.3. Isolation of human plasma-derived CM

The isolation of human plasma-derived CM was performed as previously described [20, 22]. The protocol for isolation of human plasma-derived CM emulsion was approved by the Faculty of Medicine and Health Sciences Research Ethics Committee, Queens Medical Centre, Nottingham University Hospitals (Ethics reference number: BT12102015). Healthy male volunteers between the age of 28-33 years old and body mass index (BMI) of 18.5-25.0 were enrolled in the study. Participants receiving prescribed or over-the-counter medicines within 1 week before the study were excluded from the enrolment. On the day of the study, high-fat meal (equivalent to full English breakfast) was provided to volunteers. Within the interval of 3 to 4 hours following the meal, 50 mL of blood was withdrawn using K2-EDTA tubes (Vacutainer® Blood Collection Tubes, Fischer Scientific, Loughborough, UK), and plasma was obtained by centrifugation (1,160 *g*, 15°C, 10 minutes).

CM isolation was performed based on the previous reports with slight modifications [17, 20, 22]. Briefly, 4 mL of plasma was mixed with 0.57g potassium bromide (KBr) to achieve a density of 1.1 mg/mL. A density gradient was built on the top of the plasma layer with densities of 1.006, 1.019 and 1.063 g/mL using a 3 mL syringe with bent needle (23G x 1 inch). The samples were then ultra-centrifuged (SORVALL Discovery 100SE; TH-641 Rotor, 268,350 *g*, 15°C, 35 minutes). The upper layers containing CM fraction were collected into 1.5 mL Eppendorf tubes using glass Pasteur pipettes. The collected CM emulsion was diluted with PBS to generate TG concentration of 1 mg/mL. A TG enzyme kit (Sigma Aldrich, Dorset, UK) was used to measure the TG concentration in collected CM emulsion based on manufacturer's instructions. The CM emulsion was kept in 4°C for up to 24 hours until the association assay.

2.4.4. CM association assay

The experiments for the uptake by artificial CM-like emulsion (all tested PIs) and human CM (TPV only) were performed as previously described with minor modifications [17, 20, 22]. Briefly, stock solutions at a concentration of 1 mg/mL were prepared in propylene glycol-ethanol (99:1, v/v). Two milliliters of artificial CM-like emulsion or human CM at TG concentration of 1 mg/mL were used in association assay. Stock solutions were spiked into the emulsion to obtain a final concentration of 1.75 µM of the tested compound in the experimental medium. The samples were then incubated at 37°C for 1 hour with continuous stirring at 170 rpm. Following the incubation, artificial CM-like emulsion or human CM were isolated by means of density gradient ultracentrifugation as described above.

2.5. Animal experiments

2.5.1. Animals

The protocols for pharmacokinetic and biodistribution experiments in this study were reviewed and approved by the University of Nottingham Ethical Committee in accordance with the Animals [Scientific Procedures] Act 1986. Male Sprague Dawley rats (Charles River Laboratories, UK) weighing 275-300 g were housed in Bio Support Unit, University of Nottingham in a controlled-temperature environment with 12 h light/dark cycles and were allowed free access to food and water.

2.5.2. Preparation of TPV formulations

Lipid-free solution formulations for intravenous and oral administration were prepared by dissolving TPV in propylene glycol-sterile water-ethanol (70:20:10, v/v/v) vehicle to

achieve concentrations at 1 mg/mL and 5 mg/mL, respectively. For preparation of LCT-based formulation for oral administration, TPV was dissolved in sesame oil at a concentration of 5 mg/mL. The vial containing the LCT-based formulation was filled with nitrogen and protected from light and air to avoid oxidation of the sesame oil.

2.5.3. Pharmacokinetic study

Right jugular vein cannulation surgery was performed under general gaseous anesthesia (2.5% isoflurane in oxygen) [20, 22, 23]. Following the surgery, the animals were allowed to recover for 2 nights. Animals were fasted for up to 16 hours prior to the drug administration with free access to water. Rats were divided into 3 treatment groups: IV bolus of TPV at a dose of 1 mg/kg, oral administration of TPV at a dose of 5 mg/kg in lipid-free or LCT-based formulations. Blood samples were collected from the cannula at pre-determined time points (pre-administration, 5 and 15 minutes, 0.5, 1, 2, 4, 8, 12, 18 and 24 hours following IV bolus; 1, 2, 3, 4, 5, 6, 8, 10, 12, 18 and 24 hours following oral administration) into EDTA-contained tubes. Blood samples were centrifuged at 1,160 *g* at 10°C for 10 minutes to obtain plasma. The levels of TPV were determined in the plasma by means of a validated HPLC-UV method as described below. Pharmacokinetic parameters were calculated by non-compartmental analysis using Phoenix WinNonlin 6.3 software (Pharsight, Mountain View, CA, USA).

2.5.4. Biodistribution study

Animals were fasted up to 16 hours prior to the drug administration. LCT-based and lipid-free formulations of TPV were prepared as described above and administered by oral gavage at a dose of 5 mg/kg. Following the administration of TPV, the rats were euthanized at predetermined time points according to the time of maximum plasma concentrations observed in pharmacokinetic study (2 and 3 hours following administration, $t_{max\text{-}1h}$ and t_{max}). The lymph samples were collected from the superior mesenteric lymph duct immediately after confirming the death of the animals. The assessment of the association of TPV with lipoproteins in rat lymph fluid is described in **Supplementary material 2**. The MLNs were also collected as previously described [19, 34]. All biological samples were kept at -80°C until analysis for TPV levels by means of a validated HPLC-UV method as described below.

2.6. Bioanalytical procedures

2.6.1. Sample preparation for HPLC analysis

The stock solutions of TPV and CBD (internal standard, IS) were prepared at the concentration of 1 mg/mL in ACN and kept at -20°C. Working standard solutions of TPV were prepared by diluting the stock solution with ACN in a series of concentrations of 50, 100, 250, 500, 1,000, 5,000, 10,000, 50,000, 100,000 and 150,000 ng/mL. CBD stock solution was diluted with ACN to generate working solution at a concentration of 100 µg/mL. For the preparation of calibration curve samples, aliquots of 117 µL blank rat plasma were mixed with 13 µL TPV working solutions in a borosilicate glass culture tubes (Fischer Scientific, UK). Thirteen microliters IS working solution was spiked into 130 µL sample, followed by protein precipitation with 390 µL of ice-cold ACN (-20°C). Liquid-liquid extraction was performed using 5 mL MTBE and vortex-mixing for 5 minutes. Samples were centrifuged at 1,160 *g* at 10°C for 10 minutes. The upper organic layer was then transferred to a fresh tube and evaporated to dryness under a stream of nitrogen gas at 40°C (Techne DRI-Block type DB-3D,

Cambridge, UK). The dry residue was reconstituted with 130 µL of ACN-water (1:1, v/v) followed by vortex-mixing for 5 minutes. Following a brief centrifugation (1,160 *g*, 10°C for 1 minute), 90 µL of the clear solution was injected into HPLC system. All biological samples (plasma, lymph and tissues homogenates) generated from pharmacokinetic and biodistribution studies have undergone the same procedure as described above.

2.6.2. Analytical conditions

The analytical conditions used for the initial screening of affinity of PIs to artificial CM are described in **Supplementary material 3**. The HPLC-UV system consisting of a Waters Alliance 2695 separations module coupled with Waters 996 photodiode array detector was used for analysis of *in vitro*, *ex vivo* and *in vivo* samples containing TPV in this study. The autosampler was maintained at 5°C and the column temperature was 45°C. Chromatographic separation was performed using Waters Atlantis C18 4.6 x 150 mm, 5 µm particle size column (Elstree, Herts, UK) equipped with a 2 x 4 mm, 3 µm particle size guard column (Phenomenex, Macclesfield, UK). The mobile phase was composed of ammonium acetate buffer (10 mM, pH adjusted to 4.2 with glacial acetic acid) and ACN in a ratio of 20:80 (v/v) with a 0.4 mL/min flow rate. The analytes were monitored at 263 nm for TPV and 220 nm for CBD. Data were recorded and analyzed using Empower™ 2 software. The bioanalytical assay was validated for selectivity, sensitivity and linearity according to the US Food and Drug Administration (FDA) guidelines for bioanalysis [35]. The lower limit of quantification (LLOQ) of TPV was found to be 5 ng/mL. The linearity of the calibration curves was validated over the range of 5-15000 ng/mL. The details of the validation of bioanalytical assay are described in **Supplementary material 4**.

2.7. Statistical analysis

One-way ANOVA followed by Tukey's or Dunnett's multiple-comparisons tests, or two-tailed unpaired t-test were used where appropriate. All values were expressed as mean ± standard error of the mean (SEM). A significant difference was stated when a *p* value was below 0.05. The statistical analyzes were performed using GraphPad Prism version 7.04 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Assessment of intestinal lymphatic targeting potential of tipranavir (TPV)

Association of drugs with chylomicrons (CM) in enterocytes plays a crucial role in the intestinal lymphatic targeting. To investigate the potential of intestinal lymphatic targeting of different protease inhibitors (PIs), the affinity of PIs to CM was predicted using a previously established *in silico* model [28]. Of the 10 PIs assessed *in silico*, ritonavir (RTV), nelfinavir (NFV), lopinavir (LPV) and TPV showed mild to moderate (>10 %) potential for association with CM (**Figure 1A**). However, when these selected candidates were screened *in vitro* for association with artificial CM-like emulsion (Intralipid®), the only PI that showed measurable experimental association with Intralipid® at the initial screening was TPV. The association values of TPV with artificial and natural human plasma-derived CM are summarized in **Figure 1B**. Although TPV has low triglyceride (TG) solubility (5.9 ± 0.3 mg/mL), the association of TPV with artificial lipid particles and natural CM was substantial (31.6% and 66.7%, respectively), suggesting a potential for intestinal lymphatic targeting when administered orally with lipids. Interestingly, the affinity of TPV for the human CM was significantly higher than for artificial lipid particles ($p < 0.0001$).

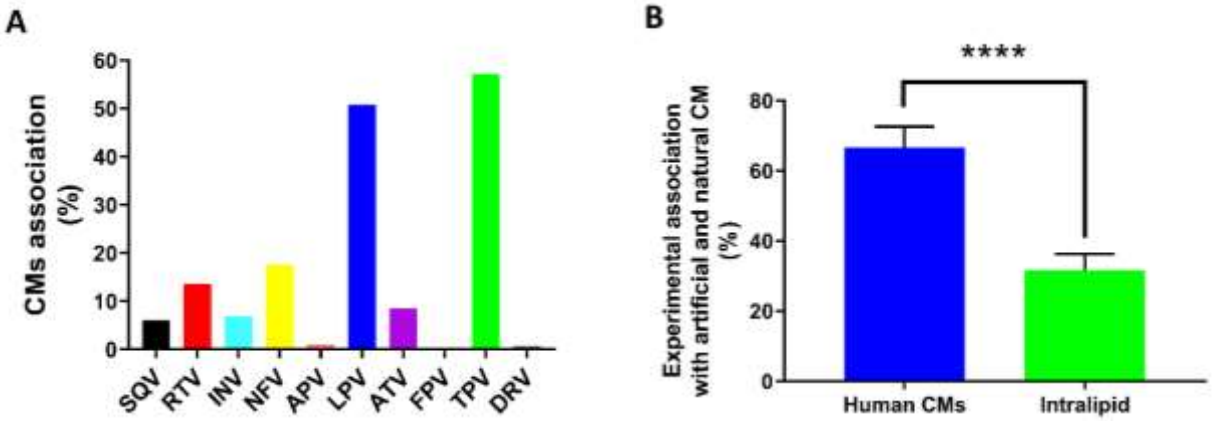

Figure 1. The predicted CM association of tested PIs and the experimental CM association of TPV. (A) The screening for CM association of PIs using *in silico* model. (B) Association of TPV with artificial CM-like emulsion (Intralipid®, n = 22) and human CM (n=9), mean ± SEM. ****, $p < 0.0001$.

3.2. Plasma pharmacokinetics of TPV following intravenous bolus (IV), and oral administration in lipid-free and long-chain triglyceride (LCT)-based formulations

Pharmacokinetic profiles of TPV were assessed following single IV and oral gavage administrations in LCT-based and lipid-free formulations in rats. The plasma concentration-time profiles of TPV are presented in **Figure 2**. **Table 1** summarizes the pharmacokinetic parameters derived from these pharmacokinetic profiles. Both oral groups share similar area under the curve (AUC_{inf}). The absolute oral bioavailability of lipid-free group is similar to the LCT-based group (36% and 44%, respectively). Furthermore, the TPV reaches similar maximum plasma concentration (C_{max}) when administered with or without lipids.

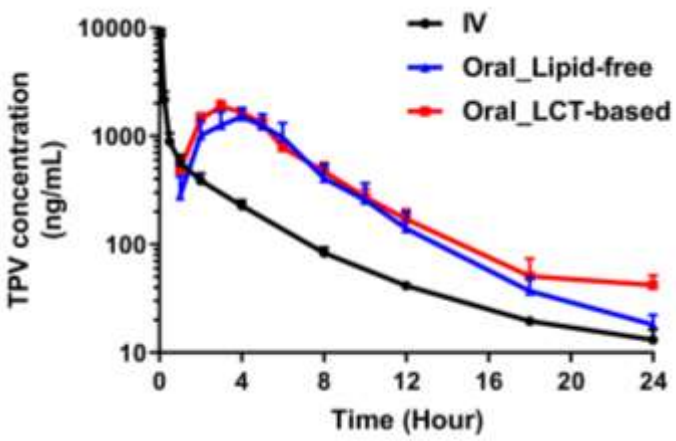

Figure 2. Plasma concentration-time pharmacokinetic profiles of TPV following IV (1 mg/kg, n = 5) and oral administration in lipid-free and LCT-based formulations (5 mg/kg, n = 3 for lipid-free group and n=6 for LCT-based group), mean ± SEM.

Table 1. Pharmacokinetic parameters of TPV following IV (1 mg/kg, n = 5) and oral administrations in lipid-free and LCT-based formulations (5 mg/kg, n = 3 for lipid-free group and n = 6 for LCT-based group) administration, mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison post-hoc analysis and two-tailed unpaired t-test was used to assess statistical difference among groups.

Route of administration	IV (n=5)	Oral	
		LCT-based (n=6)	Lipid-free (n=3)
AUC_{inf} (h*ng/mL)	4,873 ± 577	10,618 ± 1,093	8,733 ± 771
C_0 (ng/mL)	17,586 ± 1828	-	-
C_{max} (ng/mL)	-	1,937 ± 204	1,916 ± 98
$t_{1/2}$ (h)	5.21 ± 0.51	4.11 ± 0.4	3.57 ± 0.73
CL (mL/h/kg)	273 ± 45	-	-
V_{ss} (mL/kg)	903 ± 204	-	-
F_{oral} (%)	-	44 ± 4	36 ± 3

3.3. Biodistribution of TPV to the mesenteric lymph and MLNs following oral administration

The drug distribution to the mesenteric lymphatic system at plasma t_{max} time point and one hour prior to t_{max} (t_{max-1h}) was assessed following oral administration of TPV in LCT-based and lipid-free formulations to rats. The concentrations of TPV in plasma, lymph fluid and MLNs of LCT-based group at t_{max-1h} and t_{max} are shown in **Figure 3A-B**. The levels of TPV in mesenteric lymph were three-fold higher compared to plasma at both t_{max-1h} and t_{max} following oral administration. This suggests that the intestinal lymphatic transport plays a certain role in the absorption of TPV following oral administration with LCT. The concentrations of TPV in mesenteric lymph nodes (MLNs) and plasma of lipid-free group at t_{max-1h} and t_{max} are shown in **Figure 3C-D**. To note, mesenteric lymph is translucent and invisible without oral administration of lipids and therefore could not be collected for this group. The levels of TPV in plasma were comparable to MLNs at both time points in lipid-free group, as well as in LCT-based group.

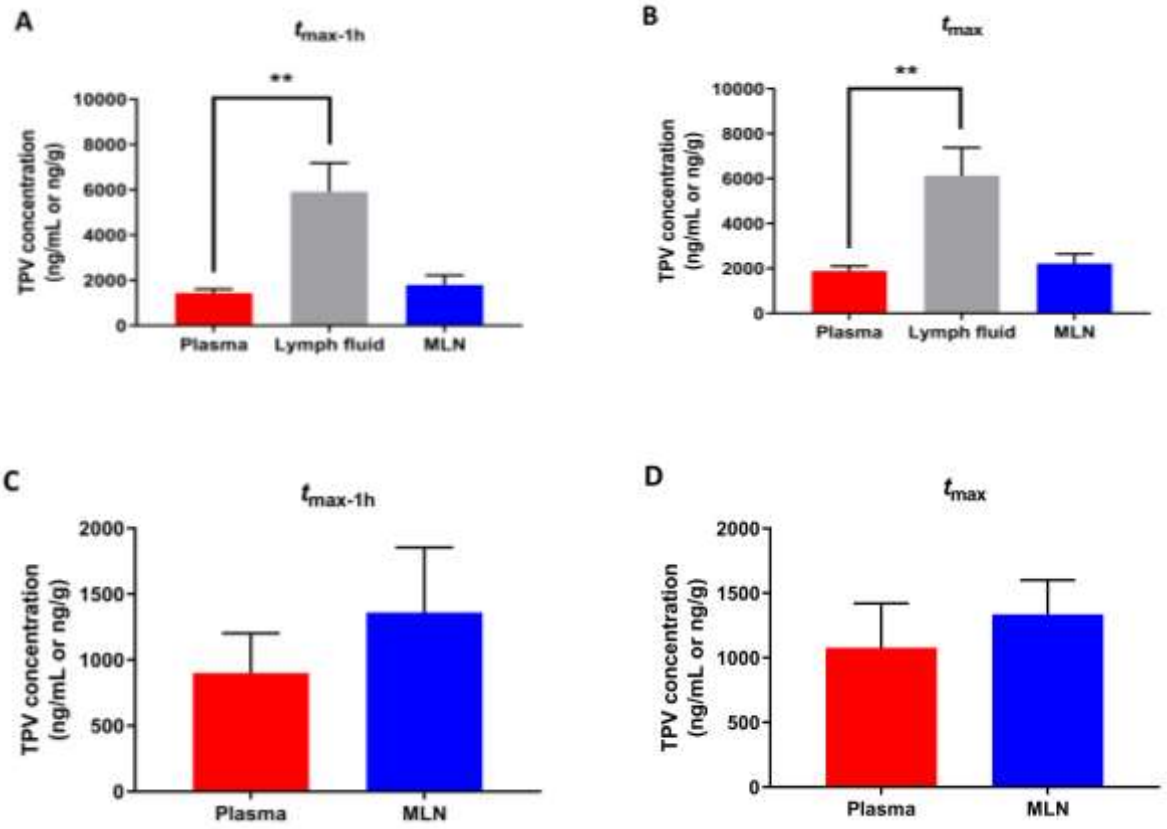

Figure 3. Distribution of TPV to plasma (obtained from pharmacokinetic study), mesenteric lymph fluid and MLNs following oral administration of TPV (5 mg/kg) in LCT-based (fresh sesame oil) and lipid-free formulations to rats. (A) Concentration of TPV in plasma (n=5), lymph fluid and MLNs (n=8 for both groups) two hours (one-hour prior to t_{max}, (t_{max-1h})) following oral administration of TPV in LCT-based formulation. (B) Concentration of TPV in plasma (n=5), lymph fluid and MLNs (n=9 for both groups) three hours (t_{max}) following oral administration of TPV in LCT-based formulation. (C) Concentration of TPV in plasma and MLNs (n=4 for each group) at t_{max-1h} following oral administration of TPV in lipid-free formulation. (D) Concentration of TPV in plasma and MLNs (n=4 for each group) at t_{max} following oral administration of TPV in lipid-free formulation. One-way ANOVA followed by Dunnett's multiple comparisons was used for statistical analysis for (A) and (B). Two-tailed unpaired t-test was used for statistical analysis for (C) and (D). All values are expressed as mean ± SEM. **, p < 0.01.

4. Discussion

The latent HIV-1 reservoirs, including anatomical and cellular viral reservoirs, represent a substantial barrier for eradication of the virus [36]. The poor penetration of antiretroviral drugs to HIV-1 reservoirs limits the therapeutic efficacy and could be one of the reasons for difficulty to achieve functional cure [37, 38]. Mesenteric lymph nodes (MLNs) are believed to be the largest HIV-1 reservoir [39]. In this work, protease inhibitors (PIs) were assessed for their potential to be delivered to the HIV-1 reservoir in mesenteric lymphatic system. Since tipranavir (TPV) is the only candidate that showed experimental association with chylomicrons (CM), in this work we have attempted to deliver TPV to viral reservoir within the mesenteric lymphatic system using long-chain triglyceride (LCT)-based formulation approach.

4.1. Assessment of intestinal lymphatic targeting potential of TPV

Delivering PIs or other antiretroviral drugs to difficult-to-penetrate viral reservoirs, especially mesenteric lymphatic system, could result in better treatment outcomes of HIV-1 infection. Intestinal lymphatic targeting is potentially a promising approach as it can not only increase the plasma exposure to antiretroviral drugs, but also efficiently deliver the drugs to viral reservoirs within the mesenteric lymph and MLNs [15]. Since association of drugs with CM in the enterocytes is a key step in the intestinal lymphatic targeting of drugs, in the current study, FDA-approved PIs [40] were screened *in silico* for their predicted affinity to CM based on their physicochemical properties [28]. We found that ritonavir (RTV), nelfinavir (NFV), lopinavir (LPV) and TPV showed mild to moderate potential for association with CM based on *in silico* prediction (**Figure 1A**). NFV was excluded from further assessment as it has been withdrawn from clinical use due to high-level genotoxic drug contamination found in 2007 [41, 42] and the marketing authorization in the European Union has been terminated since 2013 [43]. Remaining compounds were then further screened for their experimental association with Intralipid®. Intralipid® is an artificial lipid-rich emulsion which has similar compositions and particle size to CM with the exception of absence of apolipoproteins on the surface of artificial particles. It has been extensively used as a surrogate for natural CM in our previous studies [20, 22, 28]. Despite *in silico* prediction results, TPV was the only compound that had measurable experimental affinity to artificial emulsion (31.6%) and was therefore suitable for the next level of assessment with human plasma-derived CM. Interestingly, the association of TPV with natural CM was substantially higher (66.7%) compared to artificial emulsion (**Figure 1B**). This was an unusual phenomenon, as for vast majority of assessed compounds in the past the association is driven by solubility in triglyceride (TG) and lipophilicity, and is therefore similar for artificial emulsion and natural CM [20, 22, 23, 28]. This probably indicates that TPV's affinity to CM is driven, at least partially, by a different mechanism from most other assessed compounds, which are widely believed to associate with the lipophilic CM core [13, 44]. It is likely that surface apolipoproteins (which are present on natural CM but not on artificial emulsion) play an important role in association of TPV with natural plasma-derived CM. Recently, drugs' affinity to the interfacial region and the surface apolipoproteins have been reported to play a certain role [28, 45]. In addition, previously proposed LCT solubility above 50 mg/mL threshold [14] has been recently suggested as not an absolute requirement for intestinal lymphatic transport [45]. In this study, the LCT solubility of TPV was measured to be far below the 50 mg/mL, but substantial association with artificial CM-like emulsion and even more so with natural plasma-derived human CM were still observed.

4.2. Plasma pharmacokinetics of TPV following intravenous bolus, oral administration in lipid-free and LCT-based formulation

LCT vehicle is known to facilitate the transport of highly lipophilic drugs through the intestinal lymphatic system [46, 47]. In this study, sesame oil was used as LCT-based formulation. It was demonstrated in multiple works that sesame oil is a powerful vehicle for facilitation of intestinal lymphatic transport of lipophilic compounds [19, 22, 23]. Moreover, we have recently shown that sesame oil is superior to pre-digested artificial formulations in promoting intestinal lymphatic transport of cannabidiol [48]. Although previous reports suggest that administration of TPV with a high-fat meal could enhance the oral bioavailability in humans [49, 50], our results showed similar oral bioavailability of TPV with and without co-administration of LCT vehicle in rats (**Figure 2 and Table 1**). To the best of our knowledge there are no previous reports about the effect of LCT-based formulations on oral bioavailability of TPV in rats. One study reported oral bioavailability of TPV in rats of 30 % (similar to the finding in our work) following administration with lipid-free formulation, but co-administration of lipids was not assessed in that work [51].

4.3. Biodistribution of TPV to mesenteric lymph fluid and MLNs following oral administration in LCT-based formulation

Although the LCT-based formulation showed no beneficial effect on the oral bioavailability of TPV, our results suggest that TPV indeed has some intestinal lymphatic absorption following oral administration with LCT, as suggested by about 3-fold higher concentrations of the drug in mesenteric lymph fluid compared to plasma (**Figure 3A-B**). However, despite the substantial affinity to CM (**Figure 1B**) and moderate intestinal lymphatic absorption, LCT-based formulation approach alone was not sufficient for effective delivery of TPV to MLNs, as the concentration of the drug in MLNs were similar to plasma in both LCT-based and lipid-free formulation groups (**Figure 3B, D**). It should be emphasized that MLNs rather than lymph fluid are the primary reservoirs of HIV-1. Therefore, for the eradication of the virus from these reservoirs, the antiretroviral drugs should be efficiently delivered primarily to MLNs, while lymph fluid has secondary importance [9, 52, 53]. Our previous studies showed that LCT-based formulation approach alone could achieve very high concentrations of some drugs in MLNs [19], while for other less lipophilic compounds a combined approach of chemical lipophilic prodrug modification with LCT-based formulation was required for efficient MLNs targeting [22, 23]. Thus, a combination of both prodrug and LCT-based formulation approaches looks like a more promising way forward for targeting TPV and other PIs to viral reservoirs within the mesenteric lymphatic system compared to LCT-based formulation only.

5. Conclusion

In this study, tipranavir (TPV) was found to be the only compound with experimental affinity to chylomicrons (CM) among other screened protease inhibitors (PIs). Long-chain triglyceride (LCT)-based formulation approach results in 3-fold higher concentrations of TPV in mesenteric lymph compared to plasma. However, despite substantial association with CM and considerable drug concentration in mesenteric lymph, the levels in MLNs, the primary viral reservoir, were similar to the concentrations in plasma. Therefore, LCT-based formulation approach alone does not lead to effective targeting of TPV to HIV-1 reservoirs in MLNs. Future efforts should be directed to a combined lipophilic prodrugs/lipid-based formulation approach to target TPV, other PIs and potentially other classes of antiretroviral agents to viral reservoirs within the mesenteric lymphatic system.

DECLARATION OF INTERESTS

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1. The predicted CM association of tested PIs and the experimental CM association of TPV. (A) The screening for CM association of PIs using in silico model. (B) Association of TPV with artificial CM-like emulsion (Intralipid®, n = 22) and human CM (n=9), mean ± SEM. ****, $p < 0.0001$.

Figure 2. Plasma concentration-time pharmacokinetic profiles of TPV following IV (1 mg/kg, n = 5) and oral administration in lipid-free and LCT-based formulations (5 mg/kg, n = 3 for lipid-free group and n=6 for LCT-based group), mean ± SEM.

Figure 3. Distribution of TPV to plasma (obtained from pharmacokinetic study), mesenteric lymph fluid and MLNs following oral administration of TPV (5 mg/kg) in LCT-based (fresh sesame oil) and lipid-free formulations to rats. (A) Concentration of TPV in plasma (n=5), lymph fluid and MLNs (n=8 for both groups) two hours (one-hour prior to tmax, (tmax-1h)) following oral administration of TPV in LCT-based formulation. (B) Concentration of TPV in plasma (n=5), lymph fluid and MLNs (n=9 for both groups) three hours (tmax) following oral administration of TPV in LCT-based formulation. (C) Concentration of TPV in plasma and MLNs (n=4 for each group) at tmax-1h following oral administration of TPV in lipid-free formulation. (D) Concentration of TPV in plasma and MLNs (n=4 for each group) at tmax following oral administration of TPV in lipid-free formulation. One-way ANOVA followed by Dunnett's multiple comparisons was used for statistical analysis for (A) and (B). Two-tailed unpaired t-test was used for statistical analysis for (C) and (D). All values are expressed as mean ± SEM. **, $p < 0.01$.