Targeted delivery of lopinavir to HIV reservoirs in the mesenteric lymphatic system by lipophilic ester prodrug approach

Chaolong Qin[1], YenJu Chu[1,2], Wanshan Feng[1], Christophe Fromont[1], Sijia He[3], Joseph Ali[1], Jong Bong Lee[1], Atheer Zgair[1,4], Mattia Berton[1,5], Sara Bettonte[1,5], Ruiling Liu[1], Lei Yang[1], Teerapong Monmaturapoj[1,6], Concepción Medrano-Padial[1,7], Allen Alonso Rodríguez Ugalde[1], Daria Vetrugno[1,8], Shi Ying Ee[1], Charles Sheriston[1], Yuntao Wu[3], Michael J. Stocks[1], Peter M. Fischer[1], Pavel Gershkovich[1,*]

[1] *School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, UK*

[2] *Tri-Service General Hospital, Medical supplies and maintenance office, National Defense Medical Center, Taipei, Taiwan*

[3] *National Center for Biodefense and Infectious Diseases, School of systems biology, George Mason University, Manassas, VA 20110, USA*

[4] *College of Pharmacy, University of Anbar, Anbar 31001, Iraq*

[5] *Department of Pharmaceutical and Pharmacological Science, University of Padova, Padova, 35100, Italy*

[6] *Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, 34190, Thailand*

[7] *Area of Toxicology, Faculty of Pharmacy, C/Profesor García González n°2, Universidad de Sevilla, 41012 Seville, Spain*

[8] *School of Pharmacy, Universita di Roma Tor Vergata, Rome 00173, Italy*

***Corresponding author**: Pavel Gershkovich, PhD
School of Pharmacy, Biodiscovery Institute (BDI)
University of Nottingham, University Park
Nottingham, UK
NG7 2RD
Tel: +44 (0) 115 846 8014
Fax: +44 (0) 115 951 3412
Email: pavel.gershkovich@nottingham.ac.uk

Highlights:

- Antiretrovirals fail to penetrate into HIV reservoirs in mesenteric lymphatic system
- Delivery of prodrugs and lopinavir to HIV reservoirs in lymphatic system
- Activated ester prodrug approach achieved the most efficient delivery of lopinavir
- This approach also improved the systemic exposure to lopinavir

Abstract

The combined antiretroviral therapy (cART) can efficiently suppress HIV replication, but the cessation of cART usually results in viral rebound, mostly due to the presence of viral reservoirs. The mesenteric lymphatic system, including mesenteric lymph nodes (MLNs), is an important viral reservoir into which antiretroviral drugs poorly penetrate. In this work, we proposed a novel lipophilic ester prodrug approach, combined with oral lipid-based formulation, to efficiently deliver lopinavir (LPV) to the mesenteric lymph and MLNs. A series of prodrugs was designed using an *in-silico* model for prediction of affinity to chylomicrons (CMs), and then synthesized. The potential for mesenteric lymphatic targeting and bioconversion to LPV in physiologically relevant media was assessed *in vitro* and *ex vivo*. Subsequently, LPV and selected prodrug candidates were evaluated for their *in vivo* pharmacokinetics and biodistribution in rats. Oral co-administration of lipids alone could not facilitate the delivery of unmodified LPV to the mesenteric lymphatic system and resulted in undetectable levels of LPV in these tissues. However, a combination of the lipophilic prodrug approach with lipid-based formulation resulted in efficient targeting of LPV to HIV reservoirs in mesenteric lymph and MLNs. The maximum levels of LPV in mesenteric lymph were 1.6- and 16.9-fold higher than protein binding-adjusted IC_{90} (PA-IC_{90}) of LPV for HIV-1 (140 ng/mL) following oral administration of simple alkyl ester prodrug and activated ester prodrug, respectively. Moreover, the concentrations of LPV in MLNs were 1.1- and 7.2-fold higher than PA-IC_{90} following administration of simple alkyl ester prodrug and activated ester prodrug, respectively. Furthermore, the bioavailability of LPV was also substantially increased following oral administration of activated ester prodrug compared to unmodified LPV. This approach, especially if can be translated to other antiretroviral drugs, has potential for reducing the size of HIV reservoirs within the mesenteric lymphatic system.

Keywords:

Lopinavir, lipophilic ester prodrug approach, chylomicrons, intestinal lymphatic transport, mesenteric lymphatic system, HIV reservoirs

1. Introduction

The combined antiretroviral therapy (cART) can efficiently reduce viral load and keep it undetectable in the blood of HIV-infected patients for the duration of treatment [1,2]. However, as soon as the therapy is interrupted, rebound viremia can occur within a few weeks [3–5]. This is partially due to the persistence of HIV-1 in anatomical and cellular viral reservoirs [6], which are established during early primary infection stage [7–9] and are extremely stable even during fully suppressive cART [10–14]. The gut-associated lymphoid tissue (GALT), including mesenteric lymph and mesenteric lymph nodes (MLNs), harbors a large proportion of body's lymphocytes [15]. During primary HIV infection, severe CD4+ T cells depletion in GALT can impair effective immune response against HIV-1, thereby contributing to poor clinical outcomes [16,17]. Initiation of early cART can partially preserve and reconstitute immune function [18], but does not prevent GALT eventually becoming one of the most important and persistent anatomical reservoirs of HIV [13,19–21].

Unfortunately, presently known antiretroviral drugs poorly permeate into lymphoid tissues and particularly low levels can be detected in MLNs, resulting in suboptimal concentrations in these important viral reservoirs [12,13,20,22]. In the past, almost all attempts to enhance the delivery of antiretroviral agents to lymph nodes (LNs) involved a general idea of subcutaneous or intramuscular injection of different types of nano-sized drug delivery systems [22–25]. The rationale for this common approach is that due to the nano-scale size of these drug delivery systems, nanoparticles are taken up preferentially by the lymphatics rather than permeate into blood capillaries. However, the main drawback of this common approach is that due to the pattern of anatomical drainage of lymph from subcutaneous space or muscle, the drug could be delivered only to peripheral LNs. Moreover, only those limited individual peripheral LNs collecting the lymph fluid directly from the local region of injection site could be reached using this approach.

In this work, a physiological pathway of absorption of dietary lipids *via* intestinal lymphatic transport was utilized for delivery of antiretroviral drug lopinavir (LPV) to the mesenteric lymphatic system. It has been previously shown in multiple studies that highly lipophilic compounds with appropriate physicochemical characteristics (Log $D_{7.4}$ > 5, long-chain triglyceride (LCT) solubility > 50 mg/mL) have substantial intestinal lymphatic transport when administered orally with lipids [26,27]. Highly lipophilic compounds can associate with chylomicrons (CMs), large lipoproteins which are assembled in the enterocytes in the presence of long-chain triglyceride or long-chain fatty acids. CMs are too large to penetrate into blood capillaries, and therefore are taken up preferentially by lymph lacteals together with the associated highly lipophilic drugs. This mechanism promotes absorption of drugs into the mesenteric lymphatic system before they reach the systemic circulation (**Fig. 1**) [15]. A linear correlation between the *in vivo* lymphatic absorption of lipophilic compounds and the degree of drug-CMs association *in vitro* has been previously reported [28]. Therefore, the degree of drug-CMs association could be used as a useful tool to select potential candidates which are likely to have intestinal lymphatic transport and therefore could be delivered to the mesenteric lymph and MLNs in an efficient and selective manner.

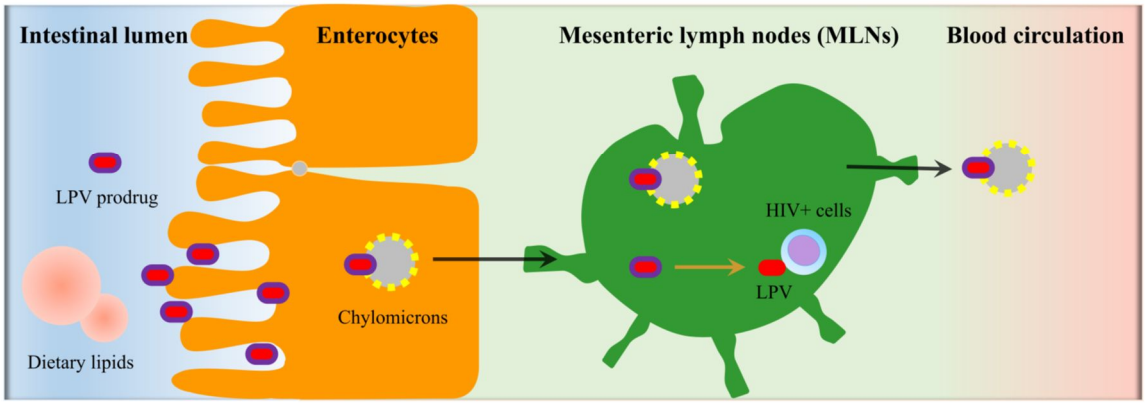


Fig. 1. Schematic diagram of delivery of LPV to the mesenteric lymphatic system. Lipophilic prodrugs of LPV have high affinity to chylomicrons (CMs) in the enterocytes, leading to intestinal lymphatic transport and high local concentrations in mesenteric lymph and MLNs. LPV released from the prodrugs by enzymatic cleavage of the ester bond exerts its pharmacological effect within the mesenteric lymph and MLNs.

Previous studies from our and other groups showed efficient delivery of highly lipophilic compounds to the mesenteric lymph and MLNs following oral administration with lipid-based formulations [26,27,29,30]. For example, it was found that the levels of cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC) in the mesenteric lymph were as high as 250- and 100-fold higher in lymph than in plasma, respectively, when co-administered with long-chain triglyceride formulation [30]. Moreover, it was also found that when the active drug does not have sufficient lipophilicity for the intestinal lymphatic transport, a lipophilic prodrug approach can still achieve efficient delivery of the drug to the mesenteric lymphatic system [29]. Recently, a number of clinically used protease inhibitors (PIs) were screened *in vitro* for their potential for intestinal lymphatic transport in unmodified form. Only tipranavir (TPV) showed some experimental association with CMs. However, *in vivo* results showed that the distribution of chemically unmodified TPV to the mesenteric lymph was moderate and to MLNs was minimal [31].

LPV has been consistently recommended as the preferred PI in second-line cART regimens [32]. However, similar to most other PIs, LPV does not possess sufficient lipophilicity for delivery to mesenteric lymph and MLNs without chemical modification [31]. Therefore, the aim of this study was to design and synthesize lipophilic ester prodrugs that possess the necessary physicochemical properties for improved association with CMs and lead to efficient delivery of LPV to HIV reservoirs within the mesenteric lymph and MLNs. The efficiency of bioconversion of inactive prodrugs to active agent (LPV) have been evaluated in physiologically relevant media. Subsequently, candidates which possess moderate to high affinity to CMs, good stability in conditions mimicking intestinal tract and rapid release to LPV in conditions mimicking the lymphatic system have been selected for *in vivo* assessment. Finally, the efficiency of the novel prodrug approach in targeting LPV to viral reservoirs in mesenteric lymphatic system has been assessed *in vivo* in rats.

2. Materials and method

2.1. Materials

Lopinavir (CAS: 192725-17-0) was purchased from Fisher Scientific (Leicestershire, UK), and cannabidiol (CAS: 13956-29-1) from THC Pharm (Frankfurt, Germany). Intralipid®, porcine liver esterases, propylene glycol, sesame oil, serum triglyceride determination kit, sodium taurocholate (NaTc), lecithin, NaF, G418 (antibiotics), propidium iodide (PI) and puromycin were all purchased from Sigma-Aldrich (Gillingham, UK or St. Louis, MO, USA). Lipofectamine 2000, Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Rat plasma was purchased from Sera Laboratories (West Sussex, UK). The HIV Rev-dependent GFP (Green fluorescent protein) indicator Rev-A3R5-GFP cells were provided by Virongy (Virginia, USA). The infectious HIV-1 molecular clone pNL4-3 was obtained from the NIH AIDS Reagent Program. HPLC analytical grade water was obtained from PURELAB® Ultra system (ELGA LabWater, UK). All other research reagents used were of HPLC analytical grade or higher.

2.2. Chemistry

2.2.1. Design of LPV prodrugs

Prodrugs **1**-**5** were designed as simple alkyl esters. For prodrug **6**, the enzymatic recognition site (acetamide) was designed to be further away from the main scaffold for better access and diketopiperazine cyclisation to liberate the drug LPV [33]. For prodrug **7**, an electronegative heteroatom (oxygen) was introduced in the β position relative to the acyl group [34]. Due to those specific features, compounds **6** and **7** will be referred in this paper as activated ester prodrugs. Chemical structures of all synthesized prodrugs are shown in **Fig. 2**.

2.2.2. Prodrugs synthesis

The synthesis of ester prodrugs was based on previously reported chemical approach with slight modifications [35]. Corresponding carboxylic acid to generate prodrugs **1**-**7** (1.59 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (1.59 mmol) and 4-dimethylaminopyridine (DMAP) (0.032 mmol) were dissolved in anhydrous dichloromethane (DCM) (10 mL) and stirred at room temperature under N_2 for 15 min before adding LPV (0.159 mmol). The reaction mixture was then allowed to stir overnight and monitored by TLC and LC-MS. The mixture was added to saturated $NH_4CH_3CO_2$ (30 mL), then extracted with ethyl acetate (20 mL × 3). The combined organic layers were washed with saturated $NaHCO_3$ (30 mL) and brine (30 mL), then dried over anhydrous $MgSO_4$, filtered, and the solvent evaporated *in vacuo*. The crude product was purified by column chromatography on silica using a gradient DCM/tetrahydrofuran (THF) 5:1. The purified fractions were pooled and fully evaporated to dryness. Final compound (20 mg) was dissolved in DMSO-d_6 for NMR analysis. The detailed methodology of characterization of the purified prodrugs is described in Supplementary Material **1**.

2.3. *In vitro* and *ex vivo* CMs association assay

2.3.1. Preparation of artificial emulsion (Intralipid®)

Intralipid® 20% was diluted with phosphate buffered saline (PBS, PH 7.4) to yield a triglyceride (TG) concentration of 1 mg/mL [36]. TG concentration was measured by serum TG determination kit using manufacturer's instructions (Sigma-Aldrich).

2.3.2. Isolation of human plasma-derived CMs

The protocol for preparation of human plasma-derived CMs was approved by the Faculty of Medicine and Health Sciences Research Ethics Committee, Queens Medical Centre, Nottingham University Hospitals (BT12102015 CBS SoP). Three healthy male participants between 30-40 years old were enrolled after providing informed consent. Study participants

received a high-fat breakfast (equivalent to a full English breakfast [29]). Three to four hours following the meal, 30 mL of blood was collected into heparinised Vacutainer® blood collection tubes, and plasma was separated by centrifugation (800 *g*, 10 min, 15°C).

The separation of CMs from human plasma was performed as previously described [28,29]. A density of 1.1 g/mL was achieved by gently mixing 4 mL of plasma with KBr (0.57 g). Standard solutions with densities of 1.006, 1.019 and 1.063 g/mL were prepared by dissolving appropriate amounts of KBr in PBS. Density gradient was built by layering standard solutions of different densities on the top of plasma in a 12-mL polyallomer ultracentrifuge tube. Samples were ultracentrifuged (SORVALL® Ultracentrifuge; TH-641 Rotor, 268,350 *g*, 35 min, 15°C) and the upper layer (approximately 1 mL) containing CMs fraction, was collected by glass Pasteur pipettes. TG concentrations were measured using the serum TG determination kit and were adjusted to 1 mg/mL TG level by PBS of 1.006 g/mL density. Standard human CMs from each volunteer were separately stored at 4°C for less than 24 h before association assay.

2.3.3. Association assay

Stock solutions of tested compounds (0.1 mM) were prepared in propylene glycol with 1% ethanol (*v*/*v*). An appropriate volume of stock solution was spiked into 1 mL of previously prepared artificial emulsion or human CMs (1 mg/mL TG) to obtain a final concentration of tested compounds of 1.75 µM [28]. The mixture was incubated at 37°C with magnetic stirring at 170 rpm. Following 1 h incubation, 0.9 mL of the mixture was added into 0.157 g of KBr to achieve a density of 1.1 g/mL. A density gradient was then built using PBS buffers with the density of 1.036, 1.019 and 1.006 g/mL. The CMs fraction was isolated by gradient density ultracentrifugation as described above and stored at -80°C until analysis.

2.4. Prodrug conversion assay

The release of LPV from the prodrugs was assessed in rat plasma and fasted state simulated intestinal fluids (FaSSIF, pH = 6.5) supplemented by esterases (mixture of esterases from porcine liver, mainly carboxylesterase, at 20 IU/mL) [29,37,38]. The preparation of FaSSIF was performed as previously reported [39]. Following pre-incubation at 37°C for 5 minutes, stock solution of tested compound (1 mM) in acetonitrile was spiked into the media to reach a final concentration of 10 µM. The mixture was incubated at 37°C, 200 rpm on a temperature-controlled orbital shaker (Thermo Scientific MaxQ4000, Waltham, MA, USA). One hundred microliters of the incubation mixture was withdrawn at pre-determined time points, and reaction was terminated by 300 µL of cooled (-20°C) acetonitrile containing 10 µL of internal standard (IS). The samples were analysed for the prodrug and LPV concentrations by means of HPLC-UV as described below. All experiments were performed in triplicates.

2.5. *In vitro* antiretroviral activity assay

2.5.1. Cell culture and virus preparation

HIV Rev-dependent GFP indicator cells Rev-A3R5-GFP [40] were cultured in RPMI 1640 plus 10% FBS supplemented with 1 µg/ml G418 and 1 µg/ml puromycin. To produce HIV-1 particles, HEK293T cells were transfected with 20 µg of the infectious HIV-1 molecular clone pNL4-3 using Lipofectamine 2000 in 100 mm cell culture dish. Virus-containing supernatant was collected at 48 h post transfection and filtered through a 0.45 µm nitrocellulose membrane.

2.5.2. Viral infection assay

For each infection, 0.3 million Rev-A3R5-GFP cells were infected with HIV-1 (NL4-3) for 2 h (150 ng p24 per infection). Cells were then washed twice and cultured in fresh media which was spiked with the working standard solutions of LPV and its prodrugs (in DMSO) to yield concentrations of 10^{-3}, 10^{-2}, 10^{-1}, 10^{0}, 10^{1}, 10^{2}, 10^{3}, 10^{4} nM. The percentage of GFP+ cells was

quantified by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) at 72 h post infection [41].

2.5.3. Prodrug stability in cell culture media

The stability of prodrugs was assessed in cell culture media (RPMI 1640 + 10% FBS). Stock solution of tested compound (1 mM) in DMSO was spiked into the media to yield a final concentration of 10 μM. The mixture was incubated at 37°C for 72 h to mimic the time frame of the antiretroviral activity assay. One hundred microliters of the incubation mixture were withdrawn at 48 and 72 h, and proteins were precipitated by 300 μL of cooled (-20°C) acetonitrile. The samples were analysed for the prodrugs and LPV concentrations by means of HPLC-UV as described below. All experiments were performed in triplicates.

2.6. TG solubility test

Tested compounds were weighed (> 5 mg) and added into sesame oil (100 μL) with constant mixing using a magnetic stirrer at 37°C for 72 h. Samples were then centrifuged in Costar Spin-X Centrifuge Tubes (Fisher Scientific, Loughborough, UK) at 2400 *g* for 20 min. The collected supernatants were diluted with ethanol (1:10^4, *v*/*v*) and analysed by means of HPLC-UV. All experiments were performed in triplicates.

2.7. Animal experiments

2.7.1. Animals

Animal welfare and all experimental procedures were reviewed and approved by the University of Nottingham Ethical Review Committee under the Animals [Scientific Procedures] Act 1986. Male Sprague Dawley rats (330-380 g) were obtained from Charles River Laboratories UK and housed at Bio Support Unit, University of Nottingham. Animals were kept in an environmentally controlled room (12:12 h light-dark cycle) with free access to food and water for at least six days before starting any procedures.

2.7.2. Pharmacokinetic study

Jugular vein catheterization surgery procedures were performed under surgical inhalation anaesthesia (2.5% isoflurane in oxygen) by implantation of silastic-polyethylene (PE-50) tubing into the right external jugular vein [29,42]. The animals were allowed to recover for two nights before pharmacokinetic experiment. Prior to the experiment day, rats were fasted overnight with free access to water. For intravenous bolus administration, LPV, prodrugs **1** and **7** were solubilised in a mixture of propylene glycol–sterile water–ethanol (70:20:10, *v*/*v*/*v*) at concentrations of 4 mg/mL, 10 mg/mL and 2.37 mg/mL, respectively. Formulations (1 mL/kg) were injected intravenously within 30 seconds *via* the jugular vein catheter followed by 0.3 mL heparinised saline (50 IU/mL). For oral administration of LPV in lipid-free group, the formulation was prepared by dissolving LPV in 100% propylene glycol (20 mg/mL). For oral administration of LPV in lipid-based group, LPV solubilised in 100% propylene glycol (20 mg/mL) was co-administered with fresh sesame oil (1 mL/kg) due to the low solubility of LPV in TG. For oral administration of prodrugs in lipid-based groups, prodrugs were solubilized in fresh sesame oil at concentrations equivalent to 20 mg/mL of LPV. All oral formulations were administered by an oral gavage (1 mL/kg), followed by 1 mL of water. Blood samples (0.25 mL) were withdrawn *via* the jugular vein catheter before dosing and at pre-determined time points following administration. Eppendorf tubes contained EDTA (1.5 M) to prevent blood coagulation and NaF (10 mg/mL) as an esterase inhibitor. Blood samples were placed on ice immediately following the collection for no longer than 5 min and plasma was separated by centrifugation (3000 *g*, at 0°C for 3 min) and stored at -80°C until analysis (except prodrug **7** which was analysed immediately). At the end of pharmacokinetic study, rats were euthanised by CO_2 inhalation, and MLNs, liver, spleen, brain and testes were harvested and stored at -80°C until analysis. The concentrations of LPV and prodrugs in plasma and tissues were determined by means of HPLC-UV. Non-compartmental analysis (NCA) was applied to

calculate pharmacokinetic parameters derived from plasma concentration-time profiles using Phoenix WinNonlin 6.3 software (Pharsight, Mountain View, CA, USA). The oral bioavailability can subsequently be calculated by comparing respective AUCs, as shown by the following equation:

$$F = \frac{AUC_{oral}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{oral}}$$ (Eq. 1).

2.7.3. Biodistribution study

Rats were fasted overnight with free access to water. Lipid-based formulations of tested compounds were prepared as described above on the day of the administration. Following oral administration of LPV or prodrugs, rats were euthanised by CO_2 inhalation at plasma t_{max} and $t_{max\text{-}1}$. Lymph sample was immediately collected from the mesenteric lymph duct. The MLNs were collected as previously reported [29,30]. Briefly, mesenteric tissues were removed from the abdominal cavity and placed in a clean weigh boat and MLNs were gently isolated from mesenteric adipose tissue. Other tissues, including spleen, brain, liver, and testes were also harvested, and stored at -80°C until analysis. All the collected tissues were homogenized (POLYTRON® PT 10-35 GT, Kinematica AG, Luzern, Switzerland) with NaF in water (10 mg/mL) (1:3, *w*/*v*) on ice bath prior to sample preparation for drug analysis. The drug and TG levels in the harvested lymph samples were analysed on the day of collection.

2.8. Analytical procedures

2.8.1. Sample preparation

All samples (plasma, lymph and tissue homogenates) underwent the same procedure of protein precipitation and liquid-liquid extraction for HPLC-UV analysis [43]. Ten microliters of IS was added to 100 µL sample, followed by 300 µL of cooled (-20°C) acetonitrile for protein precipitation and 300 µL of HPLC-grade water. A volume of 3 mL n-hexane–ethyl estate (7:3, *v*/*v*) was added as extraction solvent, then the mixture was vortex-mixed for 3 min followed by

centrifugation (1160 *g*, 10°C, 10 min). Following centrifugation, organic layer was transferred to a fresh tube and evaporated to dryness under nitrogen at 40°C (Techne DRI- Block type DB-3D, Cambridge, UK). Dry residue was reconstituted in 100 µL of 50% acetonitrile in water, vortex-mixed for 10 min, transferred to HPLC vials and 40 µL were injected into the HPLC system. The validation of prodrugs stability during sample preparation is described in Supplementary Material **1**.

2.8.2. Chromatography conditions

The HPLC-UV system consisted of a Waters Alliance 2695 separations module equipped with a Waters 996 photodiode array detector. The temperature of autosampler was maintained at 4°C and the column was kept at 40°C. Complete HPLC running conditions for all tested compounds including column, guard column and mobile phase are described in Supplementary Material **Table S1**. Empower™ 2 software was used for data acquisition and analysis.

2.9. Statistical analysis

The data are shown as mean ± standard deviation (SD). Statistical analysis between two data sets was performed by two-tailed unpaired *t*-test. One-way ANOVA followed by Dunnett's or Tukey's test was used to compare between three or more data sets. A *p*-value lower than 0.05 was considered to represent a significant difference. Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Prodrug synthesis and structural characterisation

All designed ester prodrugs were synthesized and purified. Calculated physicochemical parameters used for *in-silico* prediction of association with CMs, as well as predicted values of affinity to CMs and water solubility are shown in **Table 1**. The schematic presentation of the chemical synthetic route and the chemical structures of prodrugs are shown in **Fig. 2**. The characterisation of individual molecules is available in Supplementary Material **2**. Prodrugs **1-5** were alkyl esters with a short to medium chain fatty acid. The activated ester prodrugs **6** and **7** were then designed and synthesized following the assessment of prodrugs **1-5**.

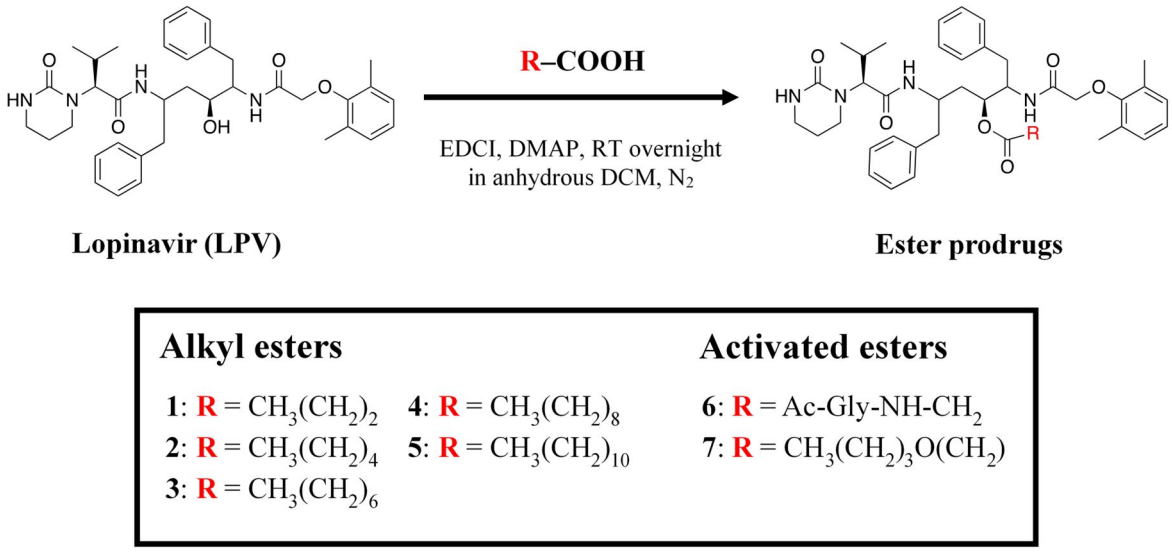


Fig. 2. Chemical structures of LPV and its prodrugs. Prodrugs **1-5** are short to medium chain fatty acid alkyl esters; Prodrugs **6** and **7** are activated esters which should promote more rapid release of LPV in the lymphatic system. EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DMAP, 4-dimethylaminopyridine; DCM, dichloromethane; RT; room temperature.

Table 1. Physicochemical parameters of LPV and its prodrugs used for *in-silico* prediction of association with CMs, as well as predicted values of affinity to CMs and water solubility.

Compounds	**Log $D_{7.4}$**	**Log P - Log $D_{7.4}$**	**PSA**	**H-acceptors**	**FRB**	**Density (g/cm^3)**	**Molar volume (cm^3)**	**H-donors**	**Predicted association (%) with chylomicrons**	**water solubility (µg/mL)**
LPV	5.88	0.38	120	9	15	1.163	540.4	4	54.6	2.1
1	6.82	1.26	126.07	10	19	1.141	612.4	3	69.6	2.9
2	8.96	0.19	126.07	10	21	1.126	645.4	3	97.4	0.007
3	9.83	0.38	126.07	10	23	1.112	678.4	3	99.2	0.00049
4	10.77	0.5	126.07	10	25	1.1	711.4	3	99.8	0.00017
5	11.54	0.79	126.07	10	27	1.089	744.5	3	99.9	0.000027
6	4.84	0.74	184.27	14	21	1.194	657.3	5	16.7	18
7	7.61	0.84	135.3	11	22	1.139	651.8	3	88.5	0.069

Physicochemical parameter values were obtained using ACD I-Lab. CMs, chylomicrons; PSA, polar surface area; FRB, freely rotatable bonds; LPV, lopinavir

3.2. Uptake of LPV and its prodrugs by CMs

The association of LPV and prodrugs **1**-**7** with artificial and natural CMs is shown in **Fig. 3A**. There was no uptake of unmodified LPV and prodrug **6** by CMs. Prodrugs **1**-**5** and **7** showed moderate to high affinity to CMs, indicating substantial potential for delivery of these prodrugs to the mesenteric lymphatic system following oral administration with lipid-based formulation. As shown in **Fig. 2**, the leaving groups of prodrugs **1**-**4** are short (4 carbons) to medium (10 carbons) chain fatty acids. The longer the fatty acid chain, the higher was the affinity to CMs for prodrugs **1**-**4**. However, further increase in the lipophilicity and molecular weight by addition of the C12 chain fatty acid (prodrug **5**) resulted in reduction in affinity to CMs, suggesting that there is a defined lipophilicity and molecular weight range which leads to optimal association with CMs.

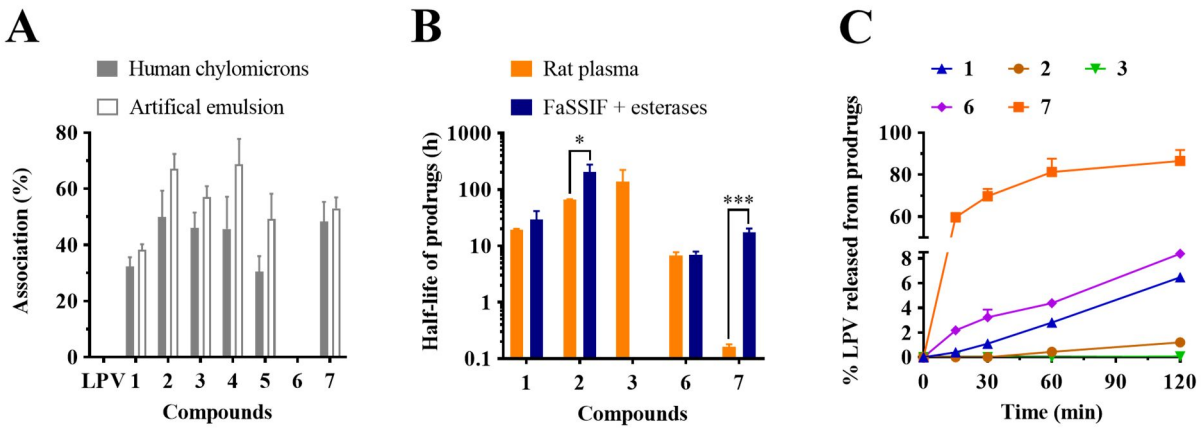


Fig. 3. ***In vitro*** **and** ***ex vivo*** **assessment for LPV and its prodrugs.** (**A**) The association (%) of LPV and its prodrugs with human chylomicrons and artificial emulsion (Intralipid®), representing their potential for the delivery to the mesenteric lymphatic system (n = 5). The final concentration of each tested compound in incubation media was 1.75 µM [28]; (**B**) The degradation half-lives of the respective prodrugs in fasted state simulated intestinal fluid (FaSSIF) with added esterases (20 IU/mL) and in rat plasma, representing the stability of prodrugs in the intestinal tract and lymphatic system, respectively (n = 3). Two-tailed unpaired *t*-test was used for statistical analysis, *, $p < 0.05$; ***, $p < 0.001$. (**C**) The relative percentage of released active drug LPV from its corresponding prodrugs in rat plasma (n = 3). Data are expressed as mean ± SD.

3.3. *In vitro* and *ex vivo* biotransformation of prodrugs

In this study, the stability of prodrugs and release of the active drug LPV were conducted in rat plasma and fasted state simulated intestinal fluid with added esterase activity (FaSSIF, representing intestinal tract environment). Plasma contains similar composition and levels of enzymes to lymph fluid in many species (including humans and rats) [44,45]. Therefore, rat plasma was chosen as an acceptable surrogate to represent lymph fluid in these studies. Similar approach was implemented in previous work by us [29] and other groups [46,47].

The degradation half-lives of the respective prodrugs in both matrixes are shown in **Fig. 3B**. The degradation half-lives of prodrug **3** in FaSSIF, as well as prodrugs **4** and **5** in both matrixes could not be reliable calculated due to their extremely high stabilities. Alkyl ester prodrug **1** showed efficient release of LPV in plasma compared to prodrugs **2** and **3** (**Fig. 3C**). Activated ester prodrugs **6** and **7** were substantially less stable in plasma than alkyl ester prodrugs. In particular, prodrug **7** was relatively stable in FaSSIF (half-life: 17.3 ± 3.0 h) but rapidly converted (half-life: 0.16 ± 0.02 h) to LPV in plasma (**Fig. 3B** and **3C**). This is in line with previous literature suggesting that insertion of a heteroatom in the 3rd position (β position) of acyl moiety in the carboxyl esters can enhance plasma and lymph carboxylesterases (CEs) sensitivity [34]. Based on combined CMs association data, plasma and intestinal stability assessments, prodrugs **1** and **7** were selected for subsequent antiretroviral activity and *in vivo* pharmacokinetics and biodistribution studies.

3.4. Antiretroviral activities of LPV and selected prodrugs

The 50% inhibition concentration (IC_{50}) of LPV and selected prodrugs against HIV-1 (NL4-3) were derived from released GFP signals where the cell lines responded to infectious HIV-1 stimuli (Supplementary Material **Fig. S1**). The IC_{50} of both prodrugs increased after chemical modification, compared to that of unmodified LPV (**Fig. 4A**). This, combined with the data of LPV release from prodrugs in cell culture media (**Fig. 4B and 4C**), suggests that efficient

release of active drug from prodrugs is necessary for pharmacological effect of LPV to take place in the lymphatic system.

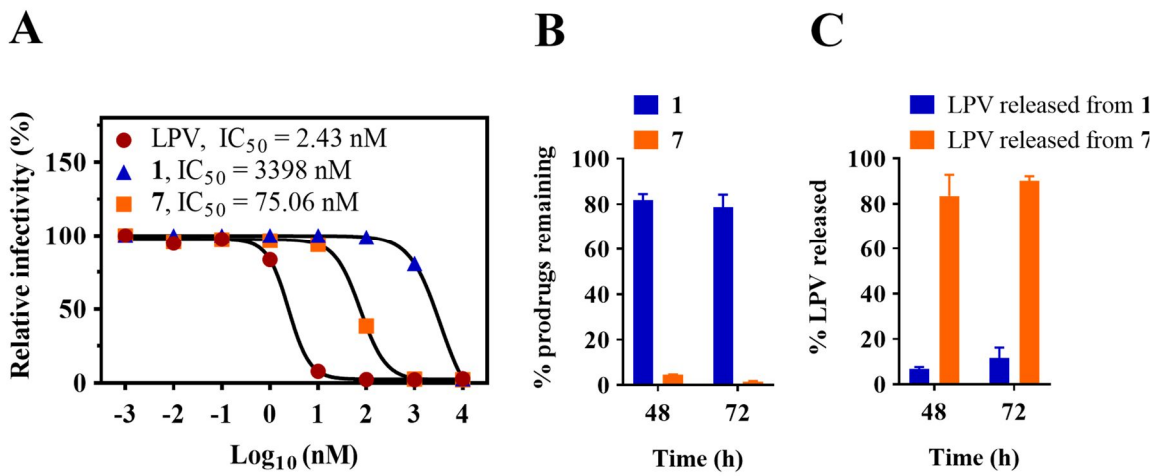


Fig. 4 Assessment of the antiretroviral efficacy of LPV and selected prodrugs. (**A**) *In vitro* anti-HIV-1 activity of LPV and its prodrugs following 72 h incubation. Inhibition curves and IC_{50} were generated from plotting relative infectivity (%) against drug concentrations using GraphPad Prism version 7.04; (**B**) The relative percentage of prodrugs remaining; (**C**) the relative percentage of released LPV from its corresponding prodrugs in cell culture media at 48 and 72 h. Data are shown as mean ± SD, n = 3.

3.5. Triglyceride solubility of LPV and selected prodrugs

Triglyceride (TG) solubility was tested for LPV and prodrugs selected for *in vivo* studies. The TG solubility for LPV and prodrug **7** were 1.8 ± 0.1 and 24.2 ± 1.4 mg/mL, respectively (mean ± SD, n = 3). Prodrug **1** solubility was above 50 mg/mL (n = 3).

3.6. Plasma pharmacokinetics of LPV and selected prodrugs

The *in vivo* plasma pharmacokinetic profiles of LPV and its prodrugs were obtained following intravenous and oral (in lipid-free and lipid-based formulation) administrations in rats (**Fig. 5**). The pharmacokinetic parameters derived from these plasma concentration-time profiles are shown in **Table 2** and **Table 3**. A significant difference between half-lives of LPV was observed in oral lipid-based group versus intravenous or oral lipid-free group (**Fig. 5A** and **5B**; **Table 2**), suggesting flip-flop kinetics following oral administration in lipidic formulation,

most probably due to delayed gastric emptying [48]. The oral bioavailability values of LPV were not significantly different between oral administrations of LPV in lipid-free and lipid-based groups (**Table 2**).

A long elimination half-life of prodrug **1**, as well as relatively low levels of released LPV following single intravenous bolus administration of prodrug **1** have been observed in plasma (**Fig. 5C** and **Table 3**). The very long circulation time of prodrug **1** was also observed in plasma up to 3 days when dosed orally, indicating there could be a re-distribution of this lipophilic prodrug between plasma and deep tissue compartments. Importantly, in parallel with prolonged systemic exposure to prodrug **1**, a continuous release of LPV was observed in plasma (**Fig. 5D**). Oral administration of short alkyl ester prodrug **1** resulted in relatively high bioavailability of unchanged prodrug (**Table 3**). However, the absolute bioavailability of released LPV was lower than following administration of unmodified LPV (**Table 2**), most probably due to slow release of LPV from the prodrug.

On the other hand, in case of activated ester prodrug **7**, the elimination half-life of prodrug in systemic circulation was extremely short following single intravenous bolus administration (**Fig. 5E** and **Table 3**). When prodrug **7** was orally administered with lipids, only the released LPV could be detected in systemic blood circulation (**Fig. 5F**), suggesting that the ester bond of prodrug **7** was rapidly hydrolyzed, and most probably while still in the lymphatic system. This is in agreement with the rapid release of active drug in plasma *ex vivo* (**Fig. 3C**). The oral bioavailability of released LPV following oral administration of prodrug **7** was nearly 2-fold higher than following administration of unmodified LPV in lipid-free formulation or with lipids (**Table 2**).

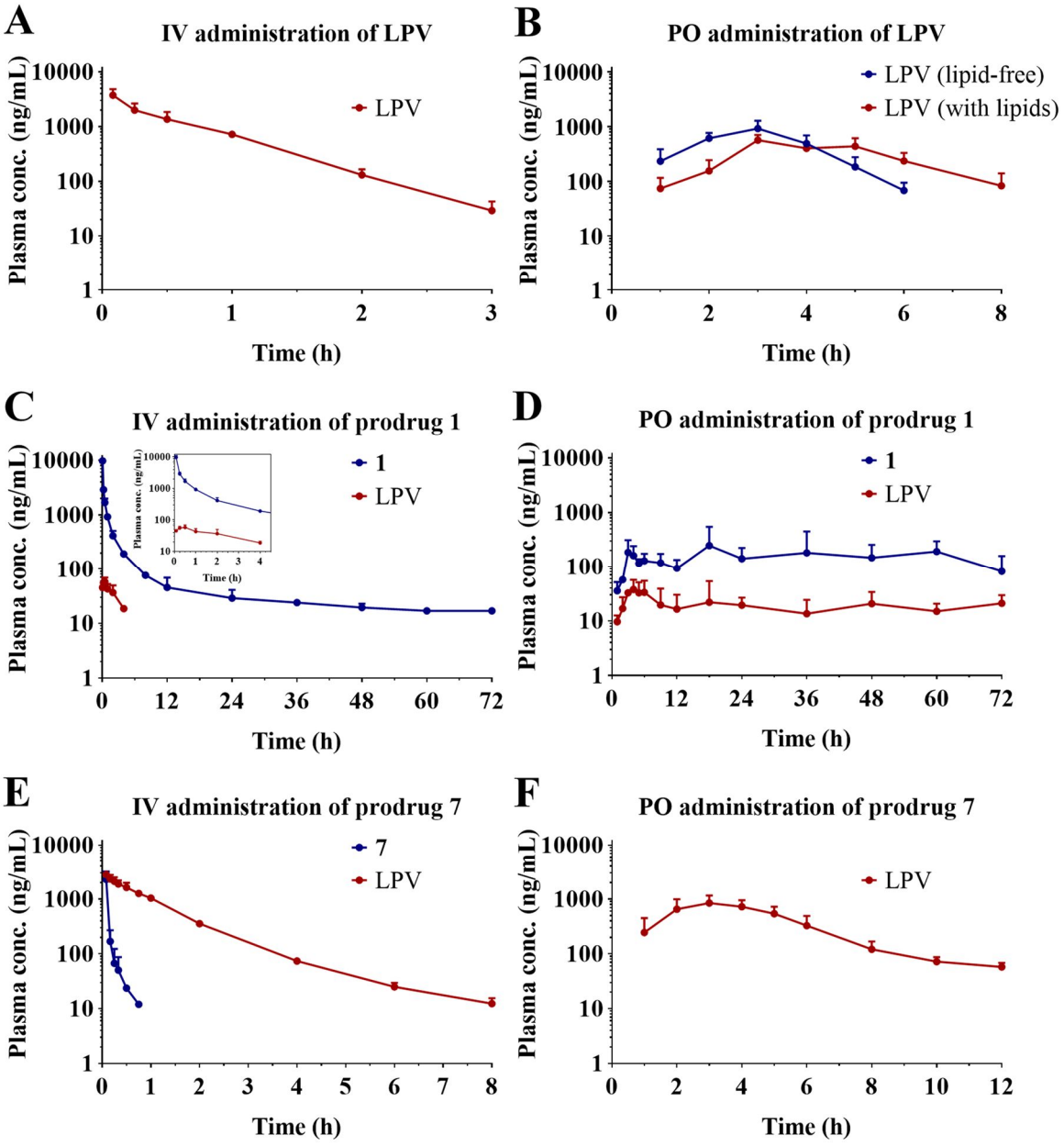


Fig. 5. Plasma pharmacokinetic profiles of LPV and selected prodrugs in rats. Plasma concentration-time curves following: (**A**) intravenous bolus administration of LPV (4 mg/kg, n = 4); (**B**) oral administration of LPV in lipid-free formulation and with lipids (20 mg/kg, n = 4); (**C**) intravenous administration of prodrug **1** (10 mg/kg, n = 3); (**D**) oral administration of prodrug **1** in lipid-based formulation (equivalent to 20 mg/kg of LPV, n = 7); (**E**) intravenous administration of prodrug **7** (equivalent to 2 mg/kg of LPV, n = 3); (**F**) oral administration of prodrug **7** in lipid-based formulation (equivalent to 20 mg/kg of LPV, n = 6). Data are expressed as mean ± SD.

Table 2. Plasma pharmacokinetic (PK) parameters of LPV following administration of LPV and its prodrugs to rats (mean ± SD).

	PK parameters of LPV following administration of LPV			**PK parameters of LPV following administration of prodrug 1**		**PK parameters of LPV following administration of prodrug 7**	
Route of administration	***i.v.* (n = 4)**	***p.o.* (lipid-free, n = 4)**	***p.o.* (with lipids, n = 4)[a]**	***i.v.* (n = 3)**	***p.o.* (in lipids, n = 7)**	***i.v.* (n = 3)**	***p.o.* (in lipids, n = 6)[b]**
AUC_{inf} (h·ng/mL)	2176 ± 403	2483 ± 649	2231 ± 396	232 ± 44	-	2913 ± 261	4100 ± 731
$AUC_{0\rightarrow t}$ (h·ng/mL)	2157 ± 407	2419 ± 676	2031 ± 375	144 ± 23	1163 ± 677	2884 ± 253	3868 ± 714
C_0 or C_{max} (ng/mL)	5171 ± 1796	920 ± 360	591 ± 112	63 ± 7	50 ± 26	3421 ± 385	927 ± 181
t_{max} (h)	-	3.0 ± 0.0	3.5 ± 1.0	0.3 ± 0.1	4.3 ± 1.0	-	3.3 ± 1.0
$t_{1/2}$ (h)	0.4 ± 0.1	0.8 ± 0.2	1.5 ± 0.6**	3.1 ± 2.0	96.6 ± 72.4	1.6 ± 0.3	2.8 ± 0.6
V_{ss} (L/kg)	1.18 ± 0.41	-	-	-	-	-	-
CL (L/h/kg)	1.89 ± 0.36	-	-	-	-	-	-
F_{oral} (%)	-	22.8 ± 6.0[c]	20.5 ± 3.6[c]	-	10.8 ± 6.3[d]	-	37.3 ± 6.7[c] **

AUC_{inf}, area under the curve from time zero to infinity; $AUC_{0\rightarrow t}$, area under the curve from time zero to the last sampling time point; C_0, concentration extrapolated to time zero; C_{max}, maximum observed concentration; t_{max}, time to reach peak concentration; $t_{1/2,}$ half-life; V_{ss}, volume of distribution at steady state; CL, clearance; F_{oral}, oral bioavailability.

[a] One-way ANOVA followed by Dunnett's test was used for statistical analysis, **, $p < 0.01$ compared to $t_{1/2}$ obtained following *i.v.* administration of LPV.

[b] One-way ANOVA followed by Tukey's test was used for statistical analysis, **, $p < 0.01$ compared to F_{oral} (%) obtained following *p.o.* administration of LPV in lipid-free formulation and with lipids.

[c] Calculated based on the AUC_{inf}.

[d] Calculated based on the $AUC_{0\rightarrow t}$.

Table 3. Plasma pharmacokinetic (PK) parameters of prodrugs **1** and **7** following administration of LPV prodrugs to rats (mean ± SD).

	PK parameters of prodrug 1 following administration of prodrug 1		**PK parameters of prodrug 7 following administration of prodrug 7**	
Route of administration	***i.v.* (n = 3)**	***p.o.* (in lipids, n = 7)**	***i.v.* (n = 3)**	***p.o.* (in lipids, n = 6)**
AUC_{inf} (h·ng/mL)	8210 ± 407	-	1061 ± 290	-
$AUC_{0\rightarrow t}$ (h·ng/mL)	6631 ± 443	10208 ± 4077	1056 ± 292	-
C_0 or C_{max} (ng/mL)	17857 ± 2680	220 ± 110	33701 ± 9384	-
t_{max} (h)	-	3.6 ± 0.8	-	-
$t_{1/2}$ (h)	64.5 ± 1.3	-	0.3 ± 0.1	-
V_{ss} (L/kg)	48.09 ± 5.69	-	0.10 ± 0.04	-
CL (L/h/kg)	1.22 ± 0.06	-	2.34 ± 0.61	-
F_{oral} (%)[a]	-	69.3 ± 27.7	-	-

AUC_{inf}, area under the curve from time zero to infinity; $AUC_{0\rightarrow t}$, area under the curve from time zero to the last sampling time point; C_0, concentration extrapolated to time zero; C_{max}, maximum observed concentration; t_{max}, time to reach peak concentration; $t_{1/2}$, half-life; V_{ss}, volume of distribution at steady state; CL, clearance; F_{oral}, oral bioavailability.

[a] Calculated based on the $AUC_{0\rightarrow t}$

3.7. Biodistribution of LPV and selected prodrugs

For the assessment of drug distribution to the mesenteric lymph and MLNs following oral administration, t_{max} observed in plasma during pharmacokinetic studies and 1 h before t_{max} (t_{max-1}) were considered as the two important sampling time points. As previously reported [29,30], the levels of drugs in mesenteric lymphatic system are usually higher at t_{max-1}, compared to t_{max}. In addition to the mesenteric lymph and MLNs, other tissues representing important HIV reservoirs were harvested and analysed for prodrugs and LPV concentrations at the same time points, as well as following the completion of the pharmacokinetic studies (**Fig. 6**). The concentrations of LPV were below the limit of detection in both mesenteric lymph and MLNs following administration of unmodified LPV, even in presence of lipids (**Fig. 6A**). However, high TG levels were observed in mesenteric lymph fluid (Supplementary Material **Fig. S2**). This indicates that despite the fact that lipids were efficiently absorbed, unmodified LPV could not be delivered to the mesenteric lymphatic system even when co-administered with lymphotropic lipids.

On the other hand, oral administration of prodrug **1** in lipid-based formulation resulted in high levels of the prodrug in the mesenteric lymphatic system (**Fig. 6B** and **6C**). The concentrations of prodrug **1** were 52- and 55-fold higher in mesenteric lymph and MLNs, respectively, compared to plasma at t_{max-1} (**Fig. 6B**), whereas relatively low levels of released LPV were observed (**Fig. 6C**). Although the concentrations of prodrug **1** were substantially higher in mesenteric lymph and MLNs compared to plasma, slow release of active drug in lymphatic system seems to be the limitation of simple alkyl ester prodrug approach.

Following oral administration of activated ester prodrug **7** in lipid-based formulation, high concentrations of the prodrug were observed in mesenteric lymph and MLNs (**Fig. 6D**), which is in line with its high affinity to CMs (approximately 50%) (**Fig. 3A**). Importantly, administration of prodrug **7**, featuring an oxygen in the β position relative to the acyl group,

led to an impressive improvement of delivery of active LPV to the mesenteric lymphatic system. The levels of LPV in mesenteric lymph and MLNs following oral administration of prodrug **7** were 10.3- and 6.8-fold higher, respectively, compared to administration of prodrug **1** (**Fig. 7A** and **7B**). Importantly, the levels of LPV were above the protein binding-adjusted IC_{90} (PA-IC_{90}) (140 ng/mL) in both lymph fluid and MLNs for both prodrugs. The maximum levels of LPV in mesenteric lymph were 1.6- and 16.9-fold higher than PA-IC_{90} of LPV for HIV-1 (140 ng/mL) following oral administration of simple alkyl ester prodrug and activated ester prodrug, respectively. The concentrations of LPV in MLNs were 1.1- and 7.2-fold higher than PA-IC_{90} following administration of simple alkyl ester prodrug and activated ester prodrug, respectively. Moreover, substantial levels of released LPV were observed in MLNs even at 12 h following oral administration of prodrug **7** (**Fig. 6E**). Therefore, this approach not only leads to the delivery of the prodrug to the mesenteric lymphatic system, but also to efficient release of the active drug LPV at the site of action within this viral reservoir.

It should be noted that high levels of LPV and prodrugs could be also detected in liver following oral administrations of LPV and prodrugs (**Fig. 6**). The concentrations of prodrug **1** (but not prodrug **7**) were detectable in spleen (**Fig. 6B**), but no LPV could be detected in this tissue (**Fig. 6C**) following oral administration of prodrug **1**. Interestingly, the levels of LPV could be observed in spleen at t_{max} after administration of prodrug **7** (**Fig. 6E**), probably due to improved systemic exposure to LPV (**Fig. 5F** and **Table 2**). The drug levels in other harvested tissues representing viral reservoirs (such as brain and testes) were below the limit of detection.

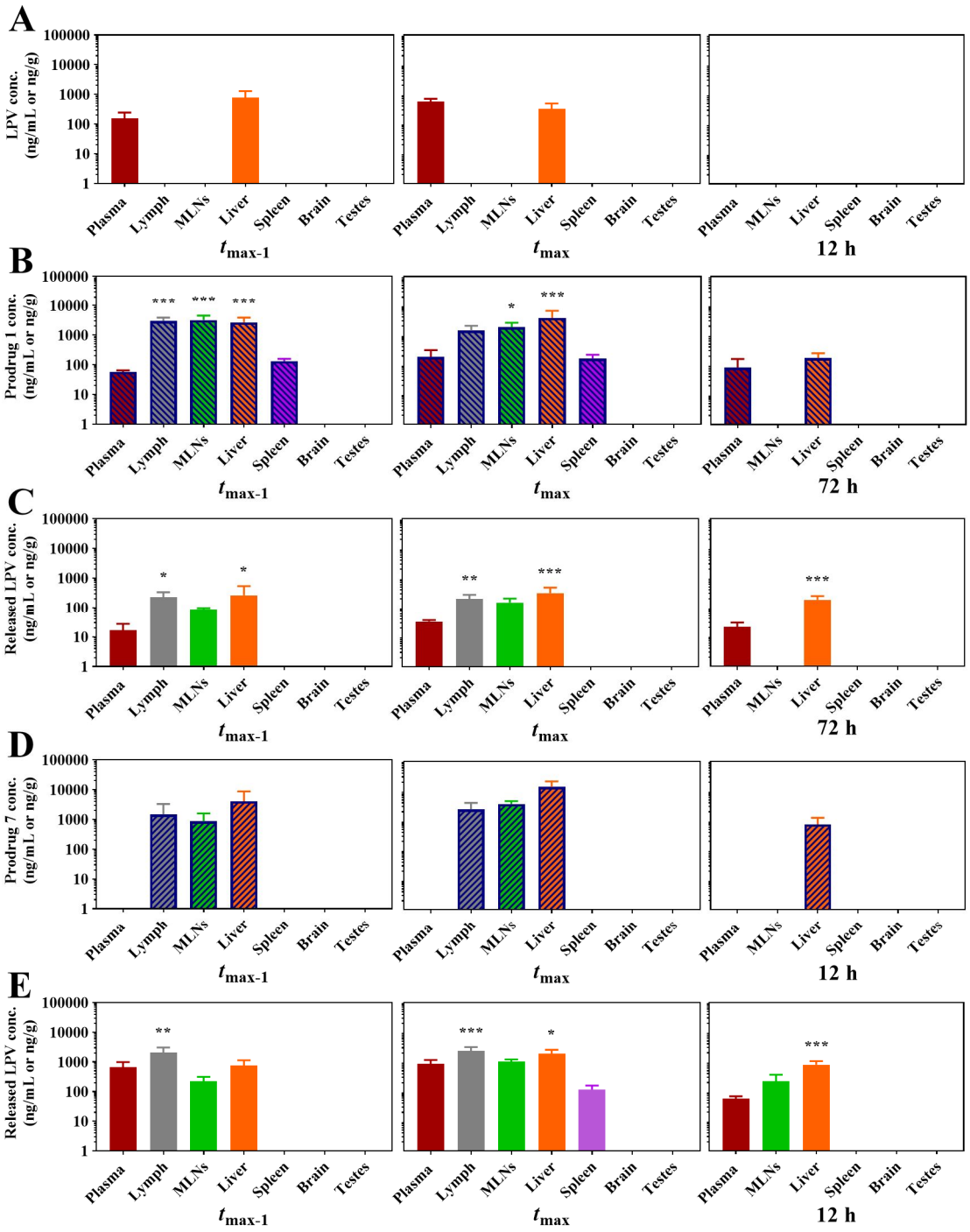


Fig. 6. Biodistribution profiles of LPV and selected prodrugs. Rats were administered a 20 mg/kg LPV-equivalent dose of LPV, prodrugs **1** and **7** with lipids by an oral gavage. Biodistribution profiles of (**A**) LPV following administration of unmodified LPV (n = 4); (**B**) prodrug **1** and (**C**) released LPV from prodrug **1** following administration of prodrug **1** (n = 8); (**D**) prodrug **7** and (**E**) released LPV from prodrug **7** following administration of prodrug **7** (n = 4). Data are expressed as mean ± SD. Plasma concentrations were generated in pharmacokinetic studies. MLNs, mesenteric lymph nodes. One-way ANOVA followed by Dunnett's test and two-tailed unpaired *t*-test were used for statistical analysis, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

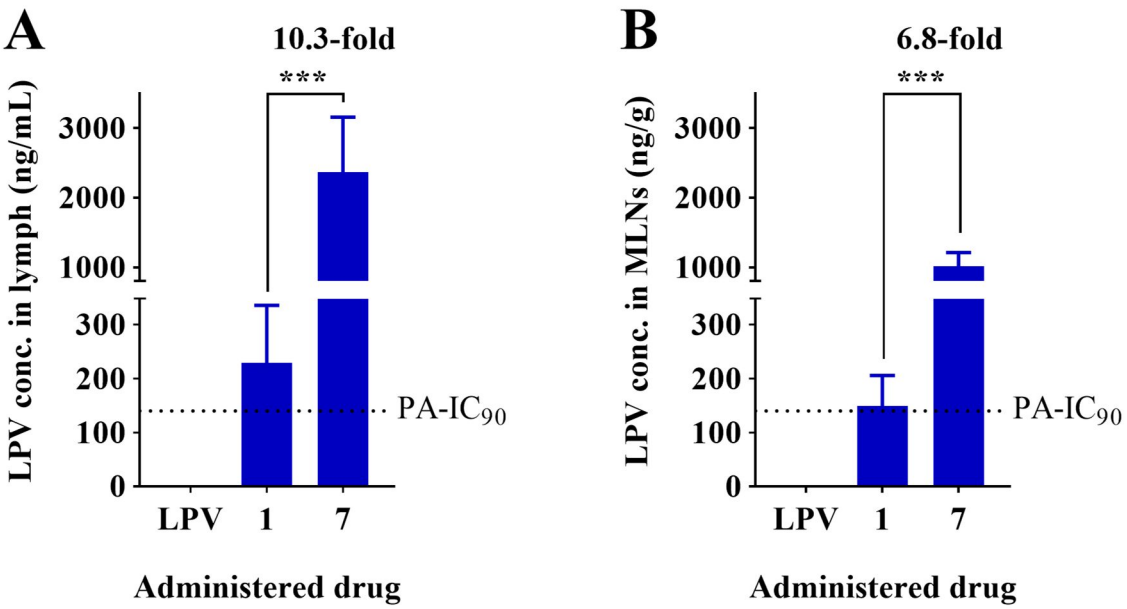


Fig. 7. Maximum concentrations of LPV in mesenteric lymphatic system. Concentrations of LPV in (**A**) mesenteric lymph fluid (ng/mL) and (**B**) MLNs (ng/g) were obtained following oral administration of LPV (n = 4), prodrugs **1** (n = 8) and **7** (n = 4) with lipids during biodistribution studies. Limit of detection for lymph fluid and MLNs were 100 ng/mL and 80 ng/g, respectively. The dotted line represents the plasma PA-IC_{90} of LPV (140 ng/mL). Data are expressed as mean ± SD (***, $p < 0.001$ by two-tailed unpaired *t*-test). MLNs, mesenteric lymph nodes. PA-IC_{90}, protein binding-adjusted concentration required for 90% viral inhibition.

4. Discussion

The GALT (including mesenteric lymphatic system) is the largest viral reservoir in HIV-infected individuals [13,19–21]. Limited penetration of antiretroviral drugs to the mesenteric lymphatic system is a significant barrier for HIV eradication from these important viral reservoirs [12,13,20,22]. In this study, we aimed to deliver antiretroviral drug (LPV) to the mesenteric lymph and MLNs by novel lipophilic ester prodrug approach. The rationale behind this approach is based on physiological process of intestinal lipid absorption and transport.

4.1. LPV prodrugs with high potential for delivery to mesenteric lymphatic system

Initially, we assessed the potential of unmodified LPV for intestinal lymphatic transport. The affinity of drugs to CMs is usually highly predictive for intestinal lymphatic transport *in vivo* [28,36]. Although *in-silico* modelling predicted moderate affinity of LPV to CMs (**Table 1**), the experimental association of the drug with CMs was negligible, in accordance with the relatively low experimental lipophilicity (log P = 1.7 [49]). Therefore, increasing the lipophilicity of the molecule by adding fatty acid to form ester prodrugs was considered a reasonable approach to promote high affinity to CMs. We chemically masked the polar and ionisable hydroxyl group within the LPV scaffold to obtain highly lipophilic alkyl ester prodrugs. Results indicate that increasing the lipophilicity dramatically improved the degree of association with CMs by 30% to 50%, compared to the unmodified LPV (**Fig. 3A**). Previous studies demonstrated that the increased association of highly lipophilic prodrugs with CMs resulted in efficient delivery of drugs to the mesenteric lymph and MLNs, compared to the unmodified moderately lipophilic active drug [29].

4.2. *In vitro* screening and selection of prodrug candidates for *in vivo* studies

In order to efficiently deliver drugs to the mesenteric lymphatic system by a prodrug approach, two critical factors have to be considered in the process of prodrug design and assessment. First, the ideal prodrug should have good chemical and enzymatic stability in the gastrointestinal

tract prior to absorption. Second, the prodrug should rapidly and efficiently release the active drug as soon as it reaches the lymphatic system. The good stability of prodrugs in FaSSIF (**Fig. 3B**) suggests that all these prodrugs are likely to remain at least partially intact in the intestinal tract before they reach the enterocytes where association with CMs takes place. Therefore, given the similar stability of all synthesized prodrugs in this study in the intestinal environment, the stability in rat plasma (as an appropriate surrogate to lymph) becomes a critical aspect for screening of potential candidates and their selection for *in vivo* studies. Among the alkyl ester prodrugs, prodrug **1** (shortest alkyl ester) demonstrated the most efficient release of LPV in plasma (**Fig. 3C**), and therefore was selected as a candidate for *in vivo* studies. Following the results of the *in vivo* assessment of prodrug **1** (discussed below), activated ester prodrugs were designed, synthesized and evaluated *in vitro* before potential selection for *in vivo* studies. The release of LPV from activated ester prodrug **7** was more efficient than from prodrug **1** (86.6% vs 6.5%) at 2 h time point (**Fig. 3C**), therefore this prodrug was chosen for the subsequent studies. Taken together, high affinity to CMs, good stability in intestinal tract environment and rapid conversion to active drug in lymphatics were considered the major requirements in selection of prodrugs for *in vivo* studies.

4.3. *In vitro* antiretroviral activity of the selected prodrugs and released LPV

The *in vitro* antiretroviral efficacy tests confirm the need for the release of LPV from the prodrugs for the efficacy against HIV-1 (**Fig. 4A**). This is not surprising, as LPV's hydroxyl group is a critical functional group for the inhibition of HIV-1 protease [50]. Prodrug **7** showed significantly higher anti-HIV activity compared to prodrug **1**, indicating the antiretroviral activity of prodrugs in cell culture media was in fact closely linked to the amount of released LPV (**Fig. 4C**). Previously published report suggests similar tendency for zidovudine prodrugs [51]. To note, the *in vitro* IC_{50} cannot be directly translated to *in vivo* IC_{50}, as LPV is highly plasma protein bound drug (>99% [52]). It has been reported previously that a highly effective

viral inhibition is achieved at a plasma concentration higher than protein binding-adjusted IC_{90} (PA-IC_{90}) of LPV for wild-type HIV-1 (140 ng/mL) [53].

4.4. Pharmacokinetics of selected prodrugs

The pharmacokinetic profiles following intravenous and oral administrations of prodrug **1** exhibited a prolonged exposure to the prodrug and released LPV in blood (**Fig. 5C** and **5D**). The long circulation time of the prodrug is likely due to the tissue distribution of highly lipophilic prodrug **1**, which is similar to other lipophilic, lymphatically transported compounds [54,55]. Compared to relatively low released levels of LPV from prodrug **1** in systemic circulation (**Fig. 5C** and **5D**), activated ester prodrug **7** achieved higher systemic exposure to LPV *in vivo* (**Fig. 5E** and **5F**).

The increase in oral bioavailability of LPV achieved in this work is comparable to other studies showing the increase in oral bioavailability of LPV using other approaches [56–58]. In a study carried out by Patel *et al.* [56], a proliposome formulation of LPV led to increase in bioavailability by 2.24-fold compared to pure LPV after oral administration in rats. Furthermore, Patel *et al.* [57] and Negi *et al.* [58] reported 2.97- and 3.56-fold enhancement in oral bioavailability using solid self-nanoemulsifying drug delivery system (S-SNEDDS) and solid lipid nanoparticles (SLNs), respectively, compared to pure LPV following oral administration in rats. In clinical practice, LPV is co-administered with a low dose of ritonavir, a strong inhibitor of cytochrome P450 3A4 (CYP3A4), which results in increased plasma exposure to LPV. However, co-administration of ritonavir with other drugs may lead to serious drug-drug interactions (DDIs) mediated by inhibition of CYP3A4 [59,60]. Therefore, the prodrug approach described in this work can lead, in addition to targeting viral reservoirs in mesenteric lymph and MLNs (discussed below), to higher oral bioavailability of LPV without DDIs issues in HIV-infected patients undergoing ritonavir-boosted lopinavir (LPV/r) regimen.

4.5. Delivery of prodrugs and LPV to the viral reservoirs in the mesenteric lymphatic system

Our results showed that the levels of LPV were below the limit of detection in both mesenteric lymph and MLNs when unmodified LPV was administered orally, even in conditions facilitating lymphatic transport (with lipids) (**Fig. 6A**). This indicates that oral administration of unmodified LPV does not result in substantial intestinal lymphatic transport and therefore cannot achieve protective concentration in lymphatic system to prevent HIV-1 replication. It is clear that the concentrations of prodrugs **1** and **7** in mesenteric lymph and MLNs were substantially higher than in plasma (**Fig. 6B** and **6D**). However, the levels of released LPV were higher in mesenteric lymphatic system following administration of prodrug **7** compared to prodrug **1** (**Fig. 7**), probably due to the different LPV release rates (**Fig. 3C**). A similar pattern of results was obtained in our previous work with anticancer compounds bexarotene and retinoic acid, showing more efficient delivery of active drugs to the mesenteric lymphatic system by activated ester prodrug approach compared to simple alkyl ester prodrug [29]. Although the extent of cumulative lymph transport was not directly measured in this study, we would expect, based on lymph and plasma concentrations, the total amount of prodrug to access the lymph to be similar to that described previously for simple esters [61] and lower than we have described previously for cannabidiol and Δ9-tetrahydrocannabinol [30]. The advantage of the approach taken here, however, is that more effective release of active drug from prodrug in case of activated ester leads to high levels of LPV in mesenteric lymph and MLNs.

Despite the importance of antiretroviral drugs delivery to viral reservoirs such as mesenteric lymph and MLNs, there is a very limited number of studies in which the delivery of LPV to the intestinal lymphatic system has been attempted. A number of publications have assessed the potential of solid-lipid nanoparticles (SLNs) to improve the delivery of LPV to the mesenteric lymphatic system [58,62–64]. It has been reported that the concentrations of LPV

observed in MLNs were 3.4- [62] and 2.6-fold [63] higher, respectively, compared to those in plasma 1 h following oral administration of LPV-loaded SLNs in rats. Other studies showed that optimized SLNs approaches achieved 6.6- [64] and 9.1-fold [58] higher levels of LPV in mesenteric lymph fluid, respectively, compared to plasma 2 h following oral administration of LPV-loaded SLNs. Although it seems that SLNs indeed can increase the concentrations of LPV in both mesenteric lymph and MLNs, it is unclear if LPV in these tissues is free or is still encapsulated within the SLNs flowing through the intestinal lymphatics to the systemic circulation. In the current study, we achieved 6.2- and 2.8-fold higher levels of released LPV in mesenteric lymph, as well as 4.6- and 1.2-fold in MLNs, compared to those in plasma 3 h following oral administration of lipophilic ester prodrugs **1** and **7**, respectively (**Fig. 6C** and **6E**). We have shown previously that drug associated with CMs (approach implemented in this work) has full pharmacological access to lymphocytes [30], probably due to the activity of lipoprotein lipase (LPL) interacting with Apo-C-II proteins on chylomicrons surface [65]. As noted above, an effective viral inhibition is achieved at LPV concentration higher than PA-IC_{90} [53]. The levels of free released LPV from equivalent dose of both prodrugs **1** and **7** in mesenteric lymphatic system substantially exceeds the critical PA-IC_{90} of 140 ng/mL (**Fig. 7**). Moreover, these efficient levels were extended up to 12 h in MLNs in the case of prodrug **7** (**Fig. 6E**).

5. Conclusion

In this study, we proposed a highly lipophilic prodrug approach, combined with oral lipid-based drug delivery to selectively and efficiently deliver LPV to the difficult-to-penetrate HIV reservoirs in mesenteric lymphatic system. LPV has negligible CMs association, which indeed results in the lack of substantial delivery of unmodified LPV to the mesenteric lymphatic system even when administered with lipids promoting intestinal lymphatic absorption. Simple alkyl ester prodrug approach resulted in substantially higher levels of the prodrug in mesenteric lymphatic system compared to plasma, but poor release of active drug within the lymphatic system. On the other hand, an activated ester prodrug approach showed good stability of the prodrug in the intestinal tract and rapid release of LPV in lymphatic system, thereby leading to high levels of LPV in the MLNs and mesenteric lymph. Moreover, oral administration of activated ester prodrug also increased the oral bioavailability of LPV by nearly 2-fold, and substantially prolonged the circulation time of LPV. This approach, especially if can be translated to other antiretroviral drugs, has potential for reducing the size of HIV reservoirs within the mesenteric lymphatic system.

Declaration of interests

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors would like to thank the Bio-Support Unit (BSU) team in University of Nottingham for excellent technical assistance.

Reference

[1] S.M. Hammer, K.E. Squires, M.D. Hughes, J.M. Grimes, L.M. Demeter, J.S. Currier, J.J. Eron, J.E. Feinberg, H.H. Balfour, L.R. Deyton, J.A. Chodakewitz, M.A. Fischl, J.P. Phair, L. Pedneault, B.-Y. Nguyen, J.C. Cook, A Controlled Trial of Two Nucleoside Analogues plus Indinavir in Persons with Human Immunodeficiency Virus Infection and CD4 Cell Counts of 200 per Cubic Millimeter or Less, New England Journal of Medicine. 337 (1997) 725–733. https://doi.org/10.1056/nejm199709113371101.

[2] R.M. Gulick, J.W. Mellors, D. Havlir, J.J. Eron, C. Gonzalez, D. McMahon, D.D. Richman, F.T. Valentine, L. Jonas, A. Meibohm, E.A. Emini, J.A. Chodakewitz, P. Deutsch, D. Holder, W.A. Schleif, J.H. Condra, Treatment with Indinavir, Zidovudine, and Lamivudine in Adults with Human Immunodeficiency Virus Infection and Prior Antiretroviral Therapy, New England Journal of Medicine. 337 (1997) 734–739. https://doi.org/10.1056/nejm199709113371102.

[3] R.T. Davey, N. Bhat, C. Yoder, T. Chun, J.A. Metcalf, R. Dewar, V. Natarajan, R.A. Lempicki, J.W. Adelsberger, K.D. Miller, J.A. Kovacs, M.A. Polis, R.E. Walker, J. Falloon, H. Masur, D. Gee, M. Baseler, D.S. Dimitrov, A.S. Fauci, H.C. Lane, HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression, Proceedings of the National Academy of Sciences. 96 (1999) 1–6. https://doi.org/10.1073/pnas.96.26.15109.

[4] SMART, CD4+ Count–Guided Interruption of Antiretroviral Treatment, New England Journal of Medicine. 355 (2006) 2283–2296. https://doi.org/10.1056/nejmoa062360.

[5] P.G. Cardiello, E. Hassink, J. Ananworanich, P. Srasuebkul, T. Samor, A. Mahanontharit, K. Ruxrungtham, B. Hirschel, J. Lange, P. Phanuphak, D.A. Cooper, A Prospective, Randomized Trial of Structured Treatment Interruption for Patients with Chronic HIV Type 1 Infection, Clinical Infectious Diseases. 40 (2005) 594–600. https://doi.org/10.1086/427695.

[6] E. Eisele, R.F. Siliciano, Redefining the Viral Reservoirs that Prevent HIV-1 Eradication, Immunity. 37 (2012) 377–388. https://doi.org/10.1016/j.immuni.2012.08.010.Redefining.

[7] J. Ananworanich, A. Schuetz, C. Vandergeeten, I. Sereti, M. De, R. Rerknimitr, R. Dewar, M. Marovich, F. Van Griensven, R. Sekaly, S. Pinyakorn, N. Phanuphak, R. Trichavaroj, W. Rutvisuttinunt, N. Chomchey, R. Paris, S. Peel, V. Valcour, F. Maldarelli, N. Chomont, N. Michael, P. Phanuphak, J.H. Kim, Impact of Multi-Targeted Antiretroviral Treatment on Gut T Cell Depletion and HIV Reservoir Seeding during Acute HIV Infection, PLoS ONE. 7 (2012) e33948. https://doi.org/10.1371/journal.pone.0033948.

[8] J. Ananworanich, C.P. Sacdalan, S. Pinyakorn, N. Chomont, M. De Souza, T. Luekasemsuk, A. Schuetz, S.J. Krebs, R. Dewar, L. Jagodzinski, S. Ubolyam, R. Trichavaroj, S. Tovanabutra, S. Spudich, V. Valcour, I. Sereti, N. Michael, M. Robb, P. Phanuphak, J.H. Kim, N. Phanuphak, Virological and immunological characteristics of HIV-infected individuals at the earliest stage of infection, Journal of Virus Eradication. 2 (2016) 43–48.

[9] T. Schacker, S. Little, E. Connick, K. Gebhard, Z. Zhang, J. Krieger, J. Pryor, D. Havlir, J.K. Wong, R.T. Schooley, D. Richman, Productive Infection of T Cells in Lymphoid Tissues during Primary and Early Human Immunodeficiency Virus Infection, The Journal of Infectious Diseases. 183 (2001) 555–562.

https://doi.org/10.1086/318524.
[10] T.-W. Chun, L. Stuyver, S.B. Mizell, L.A. Ehler, J.A.M. Mican, M. Baseler, A.L. Lloyd, M.A. Nowak, A.S. Fauci, Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy, Proceedings of the National Academy of Sciences. 94 (1997) 13193–13197. https://doi.org/10.1073/pnas.94.24.13193.
[11] D. Finzi, M. Hermankova, T. Pierson, L.M. Carruth, C. Buck, R.E. Chaisson, T.C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D.D. Ho, D.D. Richman, R.F. Siliciano, Identification of a Reservoir for HIV-1 in Patients on Highly Active Antiretroviral Therapy, Science. 278 (1997) 1295–1300. https://doi.org/10.1126/science.278.5341.1295.
[12] R. Lorenzo-redondo, H.R. Fryer, T. Bedford, E. Kim, J. Archer, S.L.K. Pond, Y. Chung, S. Penugonda, J.G. Chipman, V. Fletcher, Courtney, T.W. Schacker, M.H. Malim, A. Rambaut, A.T. Haase, A.R. McLean, S.M. Wolinsky, Persistent HIV-1 replication maintains the tissue reservoir during therapy, Nature. 530 (2016) 51–56. https://doi.org/10.1038/nature16933.
[13] C. V Fletcher, K. Staskus, S.W. Wietgrefe, M. Rothenberger, C. Reilly, J.G. Chipman, G.J. Beilman, A. Khoruts, A. Thorkelson, T.E. Schmidt, J. Anderson, K. Perkey, M. Stevenson, A.S. Perelson, D.C. Douek, A.T. Haase, T.W. Schacker, Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues, Proceedings of the National Academy of Sciences. 111 (2014) 2307–2312. https://doi.org/10.1073/pnas.1318249111.
[14] J.D. Siliciano, J. Kajdas, D. Finzi, T.C. Quinn, K. Chadwick, J.B. Margolick, C. Kovacs, S.J. Gange, R.F. Siliciano, Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting, Nature Medicine. 9 (2003) 727–728. https://doi.org/10.1038/nm880.
[15] A. Zgair, J.C.M. Wong, P. Gershkovich, Targeting Immunomodulatory Agents to the Gut-Associated Lymphoid Tissue, in: Neuro-Immuno-Gastroenterology, Springer International Publishing Switzerland, 2016: pp. 237–261. https://doi.org/10.1007/978-3-319-28609-9.
[16] J.M. Brenchley, T.W. Schacker, L.E. Ruff, D.A. Price, J.H. Taylor, G.J. Beilman, P.L. Nguyen, A. Khoruts, M. Larson, A.T. Haase, D.C. Douek, CD4 + T Cell Depletion during all Stages of HIV Disease Occurs Predominantly in the Gastrointestinal Tract, The Journal of Experimental Medicine. 200 (2004) 749–759. https://doi.org/10.1084/jem.20040874.
[17] M. Guadalupe, E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. Mcneil, S. Dandekar, Severe CD4 + T-Cell Depletion in Gut Lymphoid Tissue during Primary Human Immunodeficiency Virus Type 1 Infection and Substantial Delay in Restoration following Highly Active Antiretroviral Therapy, Journal of Virology. 77 (2003) 11708–11717. https://doi.org/10.1128/JVI.77.21.11708.
[18] R. Rajasuriar, E. Wright, S.R. Lewin, Impact of antiretroviral therapy (ART) timing on chronic immune activation/inflammation and end-organ damage, Current Opinion in HIV and AIDS. 10 (2015) 35–42. https://doi.org/10.1097/coh.0000000000000118.
[19] S. Siddiqui, S. Perez, Y. Gao, L. Doyle-Meyers, B.T. Foley, Q. Li, B. Ling, Persistent Viral Reservoirs in Lymphoid Tissues in Suppressive Antiretroviral Therapy, Viruses. 11 (2019) 105. https://doi.org/10.3390/v11020105.
[20] J.D. Estes, C. Kityo, F. Ssali, L. Swainson, K.N. Makamdop, G.Q. Del Prete, S.G. Deeks, P.A. Luciw, J.G. Chipman, G.J. Beilman, T. Hoskuldsson, A. Khoruts, J. Anderson, C. Deleage, J. Jasurda, T.E. Schmidt, M. Hafertepe, S.P. Callisto, H. Pearson, T. Reimann, J. Schuster, J. Schoephoerster, P. Southern, K. Perkey, L. Shang, S.W. Wietgrefe, C. V. Fletcher, J.D. Lifson, D.C. Douek, J.M. McCune, A.T. Haase,

T.W. Schacker, Defining total-body AIDS-virus burden with implications for curative strategies, Nature Medicine. 23 (2017) 1271–1276. https://doi.org/10.1038/nm.4411.
[21] M. Horiike, S. Iwami, M. Kodama, A. Sato, Y. Watanabe, M. Yasui, Y. Ishida, T. Kobayashi, T. Miura, T. Igarashi, Lymph nodes harbor viral reservoirs that cause rebound of plasma viremia in SIV-infected macaques upon cessation of combined antiretroviral therapy, Virology. 423 (2012) 107–118. https://doi.org/10.1016/j.virol.2011.11.024.
[22] J.P. Freeling, R.J.Y. Ho, Anti-HIV drug particles may overcome lymphatic drug insufficiency and associated HIV persistence, Proceedings of the National Academy of Sciences. 111 (2014) 2512–2513. https://doi.org/10.1073/pnas.1406554111.
[23] J.P. Freeling, J. Koehn, C. Shu, J. Sun, R.J.Y. Ho, Long-acting three-drug combination anti-HIV nanoparticles enhance drug exposure in primate plasma and cells within lymph nodes and blood, AIDS. 29 (2015) 1727. https://doi.org/10.1097/QAD.0000000000000421.
[24] J.P. Freeling, J. Koehn, C. Shu, J. Sun, R.J.Y. Ho, Anti-HIV Drug-Combination Nanoparticles Enhance as Well as Triple-Drug Combination Levels in Cells Within Lymph Nodes and Blood in Primates, AIDS Research and Human Retroviruses. 31 (2015) 107–114. https://doi.org/10.1089/aid.2014.0210.
[25] L.A. Mcconnachie, L.M. Kinman, J. Koehn, J.C. Kraft, S. Lane, W. Lee, A.C. Collier, R.J.Y. Ho, Long-Acting Profile of 4 Drugs in 1 Anti-HIV Nanosuspension in Nonhuman Primates for 5 Weeks After a Single Subcutaneous Injection, Journal of Pharmaceutical Sciences. 107 (2018) 1787–1790. https://doi.org/10.1016/j.xphs.2018.03.005.Long-Acting.
[26] W.N.A. Charman, V.J. Stella, Estimating the maximal potential for intestinal lymphatic transport of lipophilic drug molecules, International Journal of Pharmaceutics. 34 (1986) 175–178. https://doi.org/10.1016/0378-5173(86)90027-x.
[27] N.L. Trevaskis, W.N. Charman, C.J.H. Porter, Lipid-based delivery systems and intestinal lymphatic drug transport: A mechanistic update, Advanced Drug Delivery Reviews. 60 (2008) 702–716. https://doi.org/10.1016/j.addr.2007.09.007.
[28] P. Gershkovich, A. Hoffman, Uptake of lipophilic drugs by plasma derived isolated chylomicrons: Linear correlation with intestinal lymphatic bioavailability, European Journal of Pharmaceutical Sciences. 26 (2005) 394–404. https://doi.org/10.1016/j.ejps.2005.07.011.
[29] J.B. Lee, A. Zgair, J. Malec, T.H. Kim, M.G. Kim, J. Ali, C. Qin, W. Feng, M. Chiang, X. Gao, G. Voronin, A.E. Garces, C.L. Lau, T.H. Chan, A. Hume, T.M. McIntosh, F. Soukarieh, M. Al-Hayali, E. Cipolla, H.M. Collins, D.M. Heery, B.S. Shin, S.D. Yoo, L. Kagan, M.J. Stocks, T.D. Bradshaw, P.M. Fischer, P. Gershkovich, Lipophilic activated ester prodrug approach for drug delivery to the intestinal lymphatic system, Journal of Controlled Release. 286 (2018) 10–19. https://doi.org/10.1016/j.jconrel.2018.07.022.
[30] A. Zgair, J.B. Lee, J.C.M. Wong, D.A. Taha, J. Aram, D. Di Virgilio, J.W. McArthur, Y.K. Cheng, I.M. Hennig, D.A. Barrett, P.M. Fischer, C.S. Constantinescu, P. Gershkovich, Oral administration of cannabis with lipids leads to high levels of cannabinoids in the intestinal lymphatic system and prominent immunomodulation, Scientific Reports. 7 (2017) 1–12. https://doi.org/10.1038/s41598-017-15026-z.
[31] Y. Chu, C. Qin, W. Feng, J. Ali, C. Sheriston, B. Ling, M. Stocks, P. Fischer, P. Gershkovich, Targeting tipranavir to mesenteric lymph nodes (MLNs) for improved treatment of HIV/AIDS, in: 23rd International AIDS Conference, 2020.
[32] World Health Organization, UPDATE OF FIRST- AND SECOND-LINE ANTIRETROVIRAL REGIMENS, 2019.

[33] J.M. Férriz, J. Vin, Prodrug Design of Phenolic Drugs, Current Pharmaceutical Design. 16 (2010) 2033–2052. https://doi.org/10.2174/138161210791293042.
[34] T.L. Huang, T. Shiotsuki, T. Uematsu, B. Borhan, Q.X. Li, B.D. Hammock, Structure activity relationships for substrates and inhibitors of mammalian liver microsomal carboxylesterases, Pharmaceutical Research. 13 (1996) 1495–1500. https://doi.org/10.1023/A:1016071311190.
[35] B. Neises, W. Steglich, Simple Method for the Esterification of Carboxylic Acids, Angewandte Chemie International Edition in English. 17 (1978) 522–524. https://doi.org/10.1002/anie.197805221.
[36] P. Gershkovich, J. Fanous, B. Qadri, A. Yacovan, S. Amselem, A. Hoffman, The role of molecular physicochemical properties and apolipoproteins in association of drugs with triglyceride-rich lipoproteins: in-silico prediction of uptake by chylomicrons, Journal of Pharmacy and Pharmacology. 61 (2009) 31–39. https://doi.org/10.1211/jpp/61.01.0005.
[37] J. Stappaerts, S. Geboers, J. Snoeys, J. Brouwers, J. Tack, P. Annaert, P. Augustijns, Rapid conversion of the ester prodrug abiraterone acetate results in intestinal supersaturation and enhanced absorption of abiraterone : In vitro , rat in situ and human in vivo studies, EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS. 90 (2015) 1–7. https://doi.org/10.1016/j.ejpb.2015.01.001.
[38] G. Xue-jiao, F. Xue-jiao, Q. Bin, G. Zhi-qiang, A lipophilic prodrug of Danshensu : preparation , characterization , and in vitro and in vivo evaluation, Chinese Journal of Natural Medicines. 15 (2017) 355–362. https://doi.org/10.1016/S1875-5364(17)30056-0.
[39] M. Marques, Dissolution Media Simulating Fasted and Fed States, Dissolution Technologies. 11 (2004) 16. https://doi.org/10.14227/DT110204P16.
[40] Y. Wu, M.H. Beddall, J.W. Marsh, Rev-Dependent Indicator T Cell Line, Current HIV Research. 5 (2007) 394–402. https://doi.org/10.2174/157016207781024018.
[41] Y. Fu, S. He, A.A. Waheed, D. Dabbagh, Z. Zhou, B. Trinité, Z. Wang, J. Yu, D. Wang, F. Li, D.N. Levy, H. Shang, E.O. Freed, Y. Wu, PSGL-1 restricts HIV-1 infectivity by blocking virus particle attachment to target cells, Proceedings of the National Academy of Sciences. 117 (2020) 9537–9545. https://doi.org/10.1073/pnas.1916054117.
[42] A. Zgair, J.C.M. Wong, J.B. Lee, J. Mistry, O. Sivak, K.M. Wasan, M.H. Ivo, D.A. Barrett, C.S. Constantinescu, P.M. Fischer, P. Gershkovich, Dietary fats and pharmaceutical lipid excipients increase systemic exposure to orally administered cannabis and cannabis-based medicines, American Journal of Translational Research. 8 (2016) 3448–3459.
[43] C. Qin, W. Feng, Y. Chu, J.B. Lee, M. Berton, S. Bettonte, Y.Y. Teo, M.J. Stocks, P.M. Fischer, P. Gershkovich, Development and validation of a cost-effective and sensitive bioanalytical HPLC-UV method for determination of lopinavir in rat and human plasma, Biomedical Chromatography. 34 (2020) e4934. https://doi.org/10.1002/bmc.4934.
[44] M.Y.Z. Fanous, A.J. Phillips, J. a Windsor, Mesenteric lymph: the bridge to future management of critical illness, Journal of the Pancreas. 8 (2007) 374–399.
[45] J. Lindena, W. Küpper, I. Trautschold, Catalytic Enzyme Activity Concentration in Thoracic Duct , Liver , and Intestinal Lymph of the Dog , the Rabbit , the Rat and the Mouse, Clinical Chemistry and Laboratory Medicine. 24 (1986) 19–33. https://doi.org/10.1515/cclm.1986.24.1.19.
[46] S. Han, L. Hu, T. Quach, J.S. Simpson, N.L. Trevaskis, C.J.H. Porter, Profiling the Role of Deacylation-Reacylation in the Lymphatic Transport of a Triglyceride-

Mimetic Prodrug, Pharmaceutical Research. 32 (2015) 1830–1844. https://doi.org/10.1007/s11095-014-1579-9.
[47] V. Bala, S. Rao, P. Li, S. Wang, C.A. Prestidge, Lipophilic Prodrugs of SN38 : Synthesis and in Vitro Characterization toward Oral Chemotherapy, Molecular Pharmaceutics. 13 (2016) 287–294. https://doi.org/10.1021/acs.molpharmaceut.5b00785.
[48] P. Gershkovich, E.K. Wasan, M. Lin, O. Sivak, C.G. Leon, J.G. Clement, K.M. Wasan, Pharmacokinetics and biodistribution of amphotericin B in rats following oral administration in a novel lipid-based formulation, Journal of Antimicrobial Chemotherapy. 65 (2009) 101–108. https://doi.org/10.1093/jac/dkp140.
[49] J. Ford, S.H. Khoo, D.J. Back, The intracellular pharmacology of antiretroviral protease inhibitors, Journal of Antimicrobial Chemotherapy. 54 (2004) 982–990. https://doi.org/10.1093/jac/dkh487.
[50] G.S.K.K. Reddy, A. Ali, M.N.L. Nalam, S.G. Anjum, H. Cao, R.S. Nathans, C.A. Schiffer, T.M. Rana, Design and Synthesis of HIV-1 Protease Inhibitors Incorporating Oxazolidinones as P2/P2′ Ligands in Pseudosymmetric Dipeptide Isosteres, Journal of Medicinal Chemistry. 50 (2007) 4316–4328. https://doi.org/10.1021/jm070284z.Design.
[51] J.-F. Liotard, M. Mehiri, A. Di Giorgio, N. Boggetto, M. Reboud-Ravaux, A.-M. Aubertin, R. Condom, N. Patino, AZT and AZT-monophosphate Prodrugs Incorporating HIV-protease Substrate Fragment: Synthesis and Evaluation as Specific Drug Delivery Systems, Antiviral Chemistry and Chemotherapy. 17 (2006) 193–213. https://doi.org/10.1177/095632020601700404.
[52] A. Gulati, F.D. Boudinot, P.M. Gerk, Binding of Lopinavir to Human 1-Acid Glycoprotein and Serum Albumin, Drug Metabolism and Disposition. 37 (2009) 1572–1575. https://doi.org/10.1124/dmd.109.026708.
[53] R.L. Murphy, S. Brun, C. Hicks, J.J. Eron, R. Gulick, M. King, A.C. White, C. Benson, M. Thompson, H.A. Kessler, S. Hammer, R. Bertz, A. Hsu, A. Japour, E. Sun, ABT-378/ritonavir plus stavudine and lamivudine for the treatment of antiretroviral-naive adults with HIV-1 infection : 48-week results, AIDS. 15 (2001) F1–F9. https://doi.org/10.1097/00002030-200101050-00002.
[54] P. Gershkovich, A. Hoffman, Effect of a high-fat meal on absorption and disposition of lipophilic compounds : The importance of degree of association with triglyceride-rich lipoproteins, European Journal of Pharmaceutical Sciences. 32 (2007) 24–32. https://doi.org/10.1016/j.ejps.2007.05.109.
[55] L. Hu, S. Han, C.J.H. Porter, N.L. Trevaskis, The Impact of Lymphatic Transport on the Systemic Disposition of Lipophilic Drugs, Journal of Pharmaceutical Sciences. 102 (2013) 2395–2408. https://doi.org/10.1002/jps.23597.
[56] G.M. Patel, P.K. Shelat, A.N. Lalwani, QbD based development of proliposome of lopinavir for improved oral bioavailability, European Journal of Pharmaceutical Sciences. 108 (2016) 50–61. https://doi.org/10.1016/j.ejps.2016.08.057.
[57] G. Patel, P. Shelat, A. Lalwani, Statistical modeling , optimization and characterization of solid self-nanoemulsifying drug delivery system of lopinavir using design of experiment, Drug Delivery. 23 (2016) 3027–3042. https://doi.org/10.3109/10717544.2016.1141260.
[58] J.S. Negi, P. Chattopadhyay, A.K. Sharma, V. Ram, Development and evaluation of glyceryl behenate based solid lipid nanoparticles (SLNs) using hot self-nanoemulsification (SNE) technique, Archives of Pharmacal Research. 37 (2014) 361–370. https://doi.org/10.1007/s12272-013-0154-y.
[59] Y. Gong, S. Haque, P. Chowdhury, T.J. Cory, S. Kodidela, M.M. Yallapu, J.M.

Norwood, S. Kumar, Pharmacokinetics and pharmacodynamics of cytochrome P450 inhibitors for HIV treatment, Expert Opinion on Drug Metabolism & Toxicology. 15 (2019) 417–427. https://doi.org/10.1080/17425255.2019.1604685.
[60] A. Stolbach, K. Paziana, H. Heverling, P. Pham, A Review of the Toxicity of HIV Medications II : Interactions with Drugs and Complementary and Alternative Medicine Products, Journal of Medical Toxicology. 11 (2015) 326–341. https://doi.org/10.1007/s13181-015-0465-0.
[61] A. Coert, J. Geelen, J. De Visser, van der V. J, The pharmacology and metabolism of testosterone undecanoate (TU), a new orally active androgen, Acta Endocrinologica. 4 (1975) 789–800. https://doi.org/10.1530/acta.0.0790789.
[62] P.R. Ravi, R. Vats, V. Dalal, A.N. Murthy, A hybrid design to optimize preparation of lopinavir loaded solid lipid nanoparticles and comparative pharmacokinetic evaluation with marketed lopinavir/ritonavir coformulation, Journal of Pharmacy and Pharmacology. 66 (2014) 912–926. https://doi.org/10.1111/jphp.12217.
[63] P.R. Ravi, R. Vats, Comparative pharmacokinetic evaluation of lopinavir and lopinavir-loaded solid lipid nanoparticles in hepatic impaired rat model, Journal of Pharmacy and Pharmacology. 69 (2017) 823–833. https://doi.org/10.1111/jphp.12716.
[64] S.J. Negi, P. Chattopadhyay, K.S. Ashok, V. Ram, Development of solid lipid nanoparticles (SLNs) of lopinavir using hot self nano-emulsification (SNE) technique, European Journal of Pharmaceutical Sciences. 48 (2013) 231–239. https://doi.org/10.1016/j.ejps.2012.10.022.
[65] J. Goldberg, A. Scheraldi, K. Yacoub, U. Saxena, L. Bisgaier, ApoC-II Activation of Lipoprotein Lipase, Journal of Biological Chemistry. 265 (1990) 4266–4272.

Supplementary Materials to:

Targeted delivery of lopinavir to HIV reservoirs in the mesenteric lymphatic system by lipophilic ester prodrug approach

Chaolong Qin[1], YenJu Chu[1,2], Wanshan Feng[1], Christophe Fromont[1], Sijia He[3], Joseph Ali[1], Jong Bong Lee[1], Atheer Zgair[1,4], Mattia Berton[1,5], Sara Bettonte[1,5], Ruiling Liu[1], Lei Yang[1], Teerapong Monmaturapoj[1,6], Concepción Medrano-Padial[1,7], Allen Alonso Rodríguez Ugalde[1], Daria Vetrugno[1,8], Shi Ying Ee[1], Charles Sheriston[1], Yuntao Wu[3], Michael J. Stocks[1], Peter M. Fischer[1], Pavel Gershkovich[1,*]

[1] *School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, UK*

[2] *Tri-Service General Hospital, Medical supplies and maintenance office, National Defense Medical Center, Taipei, Taiwan*

[3] *National Center for Biodefense and Infectious Diseases, School of systems biology, George Mason University, Manassas, VA 20110, USA*

[4] *College of Pharmacy, University of Anbar, Anbar 31001, Iraq*

[5] *Department of Pharmaceutical and Pharmacological Science, University of Padova, Padova, 35100, Italy*

[6] *Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, 34190, Thailand*

[7] *Area of Toxicology, Faculty of Pharmacy, C/Profesor García González n°2, Universidad de Sevilla, 41012 Seville, Spain*

[8] *School of Pharmacy, Universita di Roma Tor Vergata, Rome 00173, Italy*

***Corresponding author**: Pavel Gershkovich, PhD
School of Pharmacy, Biodiscovery Institution (BDI)
University of Nottingham, University Park
Nottingham, UK
NG7 2RD
Tel: +44 (0) 115 846 8014
Fax: +44 (0) 115 951 3412
Email: pavel.gershkovich@nottingham.ac.uk

Supplementary Material 1.

Methods

Characterization of prodrugs

The ester prodrugs were synthesized following the procedure described in previous report [1] with main difference being chromatography on silica was conducted with a mixture of DCM/THF (5:1) as eluents. ^{1}H NMR and ^{13}C NMR spectra (Bruker 400 Ultrashield Spectrometer) were recorded at 400 and 100 MHz, respectively, at ambient temperature. Chemical shifts (δ) were reported in parts per million (ppm) relative to DMSO-d_6 (^{1}H, δ = 2.50 ppm; ^{13}C, δ = 39.52 ppm [2]). ^{1}H NMR data was reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and coupling constants (*J*) were recorded in Hz. The spectra were analysed using MestReNova 12.0.1 software. High-resolution mass spectrometry was performed using a Bruker MicroTOF II with electrospray ionisation (ESI). The liquid chromatograph-tandem mass spectrometry (LC-MS) system consisted of a Shimadzu UFLCXR module equipped with an Applied Biosystems API2000. An analytical C18 Gemini-NX column (50 mm × 2 mm I.D.) with a particle size of 3μm (Phenomenex, Macclesfield, UK) was used at 40°C to accomplish separation. All compounds were monitored at 220 nm (channel 2) and 254 nm (channel 1). Short gradient method: pre-equilibration for 1 min at 5% solvent B, then increase to 98% B in 2 min, 98% B for 2 min, decrease to 5% B in 0.5 min, then 5% B for 1 min. Long gradient method: pre-equilibration for 1 min at 5% B, then 5% B for 0.5 min and increase to 98% B in 8 min, 98% B for 2 min, decrease to 5% B in 0.5 min, then 5% for 1 min. Both methods underwent at flow rate of 0.5 mL/min. Solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile. Thin-layer chromatography (TLC) was performed using Silica gel 60 Å F254 plates (Merck, Darmstadt, Germany). Compounds were purified *via* column chromatography

using ISOLUTE SPE columns (Flash Silica II 5g/25mL) (Biotage, Hengoed, UK). Reagents and solvents for prodrugs characterization were purchased from Sigma-Aldrich (Gillingham, UK) and Fisher Scientific (Leicestershire, UK), and were used without further purification.

Validation of the stability of prodrugs in rat plasma

The stability assessment was performed by spiking prodrug solution in acetonitrile into rat plasma supplemented with 10 mg/mL of NaF. For the validation of prodrug **1**, samples were kept on ice for 5 min, followed by centrifugation (3000 *g*, 0°C, for 3 min) to mimic the procedure of blood collection and plasma separation. Following centrifugation, the 'separated plasma' samples were placed on ice for 1 h to mimic the time frame of temporary storage of samples during pharmacokinetic studies, and then kept frozen at -80°C (for 24 h) until analysis. For the validation of prodrug **7**, plasma samples were kept on ice for 5 min and immediately centrifuged (3000 *g*, 0°C, for 3 min). Following centrifugation, proteins were immediately precipitated by 300 µL cooled (-20°C) acetonitrile and samples were processed for HPLC analysis. Sample preparation procedure and chromatography conditions are described in section 2.8 of the main manuscript. All experiments were performed in triplicates (data are shown as mean ± SD). Tested prodrugs were stable at these conditions (97.6 ± 6.6% remaining of prodrug **1**; 92.3 ± 8.0% remaining of prodrug **7**).

Validation of the stability of prodrug 7 in homogenized MLNs

Prodrug **7** solution was spiked into homogenized blank MLNs with water (1:3, *w/v*) and placed on ice for 10 min (mimicking the time frame of lymph nodes isolation procedure), then stored in dry ice for 1 h and transferred to -80°C (for 24 h) until analysis. Tested homogenates were thawed at ambient temperature (containing 10 mg/mL of NaF), following by homogenization

on ice bath within 30 second to mimic the time frame of homogenization procedure. Sample preparation procedure and chromatography conditions were described in section 2.8 of the main manuscript. All experiments were performed in triplicates (data are shown as mean ± SD). Prodrug **7** was stable in homogenized MLNs (93.9 ± 9.8% remaining).

Supplementary Material 2.

Characterization of ester prodrugs of lopinavir

1 (lopinavir butyl ester), (3S)-2-(2-(2, 6-dimethylphenoxy) acetamido)-5-((R)-3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl) butanamido)-1, 6-diphenylhexan-3-yl butyrate was synthesized using LPV and butyric acid as the reactants. The purity of the final compound was more than 98% by HPLC-UV, white solid (85% yield), melting point 54.0-56.0°C. H^1 NMR (400 MHz, DMSO-d_6): δ 7.60-7.70 (m, 2H), 7.10-7.27 (m, 10H), 6.99-7.04 (m, 2H), 6.90-6.96 (m, 1H), 6.30 (s, 1H), 5.06-5.14 (m, 1H), 4.61-4.72 (m, 1H), 4.34 (d, J = 10.96 Hz, 1H), 4.13-4.24 (m, 1H), 4.10 (s, 2H), 2.96-3.07 (m, 2H), 2.54-2.93 (m, 6H), 2.29-2.44 (m, 2H), 2.16 (s, 6H), 2.00-2.09 (m, 1H), 1.46-1.76 (m, 6H), 0.92 (t, J = 7.31 Hz, 3H), 0.83 (d, J = 6.4 Hz, 3H), 0.75 (d, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.31, 169.45, 167.68, 155.45, 154.69, 138.91, 138.41, 130.27, 129.12, 129.04, 128.79, 127.99, 127.9, 126.07, 125.61, 124.21, 72.74, 70.18, 61.46, 50.83, 46.64, 37.32, 37.18, 35.55, 25.40, 21.62, 19.59, 18.66, 18.00, 15.9, 13.5. HR-MS (ESI^+): *m/z* $[M+ H]^+$ calculated for $C_{41}H_{55}N_4O_6$, 699.4116, found 699.4102.

2 (lopinavir hexyl ester), (3S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-((S)-3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl hexanoate was synthesized using LPV and hexanoic acid as the reactants. The purity of the final compound was more than 98% by HPLC-UV, white solid (80% yield), melting point 52.0-54.0°C. H^1 NMR (400 MHz, DMSO-d_6): δ 7.58-7.70 (m, 2H), 7.10-7.26 (m, 10H), 6.99-7.04 (m, 2H), 6.91-6.96 (m, 1H), 6.29 (s, 1H), 5.06-5.12 (m, 1H), 4.60-4.69 (m, 1H), 4.33 (d, J = 10.97 Hz, 1H), 4.12-4.22 (m, 1H), 4.09 (s, 2H), 2.96-3.05 (m, 2H), 2.54-2.92 (m, 6H), 2.30-2.43 (m, 2H), 2.15 (s, 6H), 1.89-1.99 (m, 1H), 1.55-1.76 (m, 4H), 1.19-1.32 (m, 6H), 0.80-0.86 (m, 6H), 0.74

(d, J = 6.65 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.44, 169.44, 167.44, 155.43, 154.65, 138.91, 138.39, 130.26, 129.11, 129.03, 128.79, 128.00, 127.9, 126.08, 125.62, 124.22, 72.73, 70.16, 61.44, 50.83, 46.64, 37.31, 37.20, 33.63, 30.72, 25.41, 24.19, 21.81, 21.62, 19.59, 18.67, 15.89, 13.77. HR-MS (ESI$^+$): *m/z* [M+ H]$^+$ calculated for $C_{43}H_{59}N_4O_6$, 727.4429, found 727.4415.

3 (lopinaivr octyl ester), (3S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-((S)-3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl decanoate was synthesized using LPV and octanoic acid as the reactants. The purity of the final compound was more than 95% by HPLC-UV, colourless oil (65% yield). H^1 NMR (400 MHz, DMSO-d_6): δ 7.59-7.66 (m, 2H), 7.10-7.27 (m, 10H), 6.99-7.04 (m, 2H), 6.90-6.96 (m, 1H), 6.28 (s, 1H), 5.06-5.12 (m, 1H), 4.60-4.69 (m, 1H), 4.34 (d, J = 11.39 Hz, 1H), 4.12-4.19 (m, 1H), 4.09 (s, 2H), 2.96-3.06 (m, 2H), 2.54-2.92 (m, 6H), 2.34-2.41 (m, 2H), 2.15 (s, 6H), 1.99-2.05 (m, 1H), 1.55-1.74 (m, 4H), 1.19-1.30 (m, 10H), 0.80-0.85 (m, 6H), 0.74 (d, J = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.44, 169.42, 167.66, 155.43, 154.64, 138.90, 138.38, 130.26, 129.10, 129.03, 128.79, 128.00, 127.9, 126.1, 125.62, 124.23, 72.72, 70.15, 61.44, 50.83, 46.63, 37.33, 37.22, 33.70, 31.11, 28.88, 25.41, 24.62, 23.55, 21.62, 19.59, 18.66, 15.89, 13.77. HR-MS (ESI$^+$): *m/z* [M+ H]$^+$ calculated for $C_{45}H_{63}N_4O_6$, 755.4742, found 755.4737.

4 (lopinavir decanyl ester), (3S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-((S)-3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl dodecanoate was synthesized using LPV and decanoic acid as the reactants. The purity of the final compound was more than 95% by HPLC-UV, colourless oil (89% yield). H^1 NMR (400 MHz, DMSO-d_6): δ 7.58-7.67 (m, 2H), 7.10-7.26 (m, 10H), 6.99-7.04 (m, 2H), 6.90-6.96 (m, 1H), 6.29 (s,

1H), 5.05-5.12 (m, 1H), 4.61-4.70 (m, 1H), 4.34 (d, *J* = 11.07 Hz, 1H), 4.13-4.23 (m, 1H), 4.09 (s, 2H), 2.96-3.07 (m, 2H), 2.53-2.93 (m, 6H), 2.32-2.41 (m, 2H), 2.15 (s, 6H), 1.98-2.09 (m, 1H), 1.53-1.74 (m, 4H), 1.18-1.28 (m,14H), 0.81-0.86 (m, 6H), 0.74 (d, *J* = 6.65 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.42, 169.43, 167.65, 155.44, 154.64, 138.90, 138.37, 130.24, 129.10, 129.02, 128.78, 127.98, 127.89, 126.09, 125.61, 124.22, 72.74, 70.15, 61.43, 50.82, 46.63, 37.34, 37.26, 33.70, 31.26, 28.92, 28.88, 28.52, 25.40, 24.52, 22.08, 21.62, 19.59, 18.65, 15.90, 13.92. HR-MS (ESI$^+$): *m/z* [M+ H]$^+$ calculated for $C_{47}H_{67}N_4O_6$, 783.5055, found 783.5035.

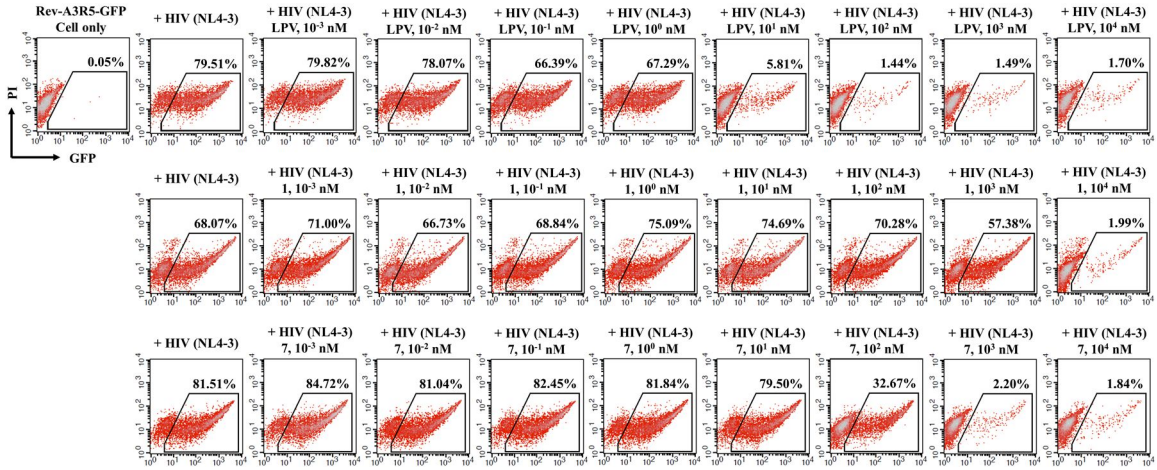
5 (lopinavir dodecanyl ester), (3S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-((S)-3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl dodecanoate was synthesized using LPV and dodecanoic acid as the reactants. The purity of the final compound was more than 95% by HPLC-UV, colourless oil (85% yield). H^1 NMR (400 MHz, DMSO-d_6): δ 7.58-7.66 (m, 2H), 7.11-7.28 (m, 10H), 6.99-7.04 (m, 2H), 6.90-6.96 (m, 1H), 6.29 (s, 1H), 5.05-5.12 (m, 1H), 4.60-4.71 (m, 1H), 4.33 (d, *J* = 11.06 Hz, 1H), 4.12-4.22 (m, 1H), 4.09 (s, 2H), 2.97-3.05 (m, 2H), 2.54-2.92 (m, 6H), 2.34-2.41 (m, 2H), 2.15 (s, 6H), 1.99-2.07 (m, 1H), 1.55-1.74 (m, 4H), 1.18-1.26 (m, 18H), 0.80-0.86 (m, 6H), 0.74 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.42, 169.42, 167.65, 155.43, 154.64, 138.90, 138.37, 130.24, 129.11, 129.02, 128.78, 127.98, 127.89, 126.08, 125.42, 124.21, 72.75, 70.15, 61.93, 50.82, 46.64, 37.35, 37.27, 33.71, 31.29, 28.99, 28.88, 28.71, 28.52, 25.41, 24.52, 22.09, 21.62, 19.59, 18.65, 15.90, 13.93. HR-MS (ESI$^+$): *m/z* [M+ H]$^+$ calculated for $C_{49}H_{71}N_4O_6$, 811.5368, found 811.5374.

6 (lopinavir N-Acetylglycylglycinyl ester), (3S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-((R)-3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl acetylglycylglycinate was synthesized using LPV and 2-(2-acetamidoacetamido) acetic acid as the reactants. The purity of the final compound was more than 95% by HPLC-UV, colourless oil (46% yield). H^1 NMR (400 MHz, DMSO-d_6): δ 8.32 (t, J = 5.9 Hz, 1H), 8.20 (t, J = 5.9 Hz, 1H), 7.70 (d, J = 9.7 Hz, 1H), 7.63 (d, J = 9.1 Hz, 1H), 7.10-7.25 (m, 10H), 7.00-7.02 (m, 2H), 6.90-6.94 (m, 1H), 6.28 (s, 1H), 5.05-5.09 (m, 1H), 4.70-4.77 (m, 1H), 4.33 (d, J = 11 Hz, 1H), 4.14-4.28 (m, 1H), 3.85-4.06 (m, 2H), 3.70-3.72 (m, 2H), 2.98-3.03 (m, 2H), 2.56-2.90 (m, 6H), 2.15 (s, 6H), 2.02-2.06 (m, 1H), 1.54-1.73 (m, 4H), 1.24-1.39 (m, 2H), 1.83 (s, 3H), 0.82 (d, J = 6.4 Hz, 3H), 0.74 (d, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.82, 169.69, 169.60, 169.30, 167.89, 155.42, 154.92, 138.87, 138.49, 130.32, 129.16, 129.01, 128.75, 127.99, 127.90, 126.05, 125.61, 124.13, 73.91, 70.28, 61.44, 50.51, 46.65, 41.98, 40.81, 37.28, 37.20, 29.03, 25.39, 22.44, 21.61, 19.63, 18.66, 15.96. HR-MS (ESI^+): *m/z* $[M+H]^+$ calculated for $C_{43}H_{57}N_6O_8$, 785.4232, found 785.4223.

7 (lopinavir butoxyacetyl ester), (2S,3S,5R)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-((S)-3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl 2-butoxyacetate was synthesized using LPV and 2-butoxyacetic acid as the reactants. The purity of the final compound was more than 98% by HPLC-UV, white solid (87% yield), melting point 72.0-75.0°C. H^1 NMR (400 MHz, DMSO-d_6): δ 7.68 (d, J = 9.1 Hz, 2H), 7.63 (d, J = 9.8 Hz, 1H), 7.11-7.28 (m, 10H), 7.00-7.02 (m, 2H), 6.91-6.95 (m, 1H), 6.30 (s, 1H), 5.13-5.17 (m, 1H), 4.66-4.73 (m, 1H), 4.35 (d, J = 11 Hz, 1H), 4.12-4.25 (m, 1H), 4.03 (quintet, J = 7.0 Hz, 2H), 3.46-3.51 (m, 2H), 2.99-3.04 (m, 2H), 2.57-2.92 (m, 6H), 2.14 (s, 6H), 1.99-2.07 (m, 1H), 1.55-1.77 (m, 4H), 1.15-1.53 (m, 6H), 0.82-0.86 (m, 6H), 0.75 (d, J = 6.7 Hz, 3H). ^{13}C

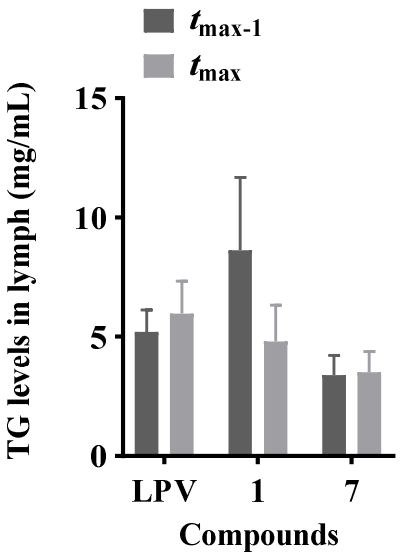
NMR (100 MHz, DMSO-d_6): δ 169.90, 169.53, 167.74, 155.44, 154.62, 138.86, 138.34, 130.29, 129.12, 129.02, 128.78, 128.01, 127.91, 126.11, 125.65, 124.24, 73.41, 70.44, 70.13, 61.46, 50.74, 46.68, 37.28, 37.22, 33.63, 31.22, 25.42, 21.63, 20.75, 19.59, 18.73, 18.67, 15.89, 13.66. HR-MS (ESI^+): *m/z* $[M+H]^+$ calculated for $C_{43}H_{58}N_4O_7$, 743.4378, found 743.4384.

Supplementary Material Fig. S1.



Supplementary Material Fig. S1. HIV-1 (NL4-3) infectivity assay was performed using Rev-A3R5-GFP cell line after drug (10^{-3}–10^{4} nM) treatment. The percentage of GFP+ cells was measured by flow cytometry at 72 h post infection.

Supplementary Material Fig. S2



Supplementary Material Fig. S2. Assessment of the TG levels in biodistribution studies in rats. Triglyceride (TG) levels obtained in mesenteric lymph following oral administration of LPV (n = 4) and prodrugs **1** (n = 7) and **7** (n = 4) with lipids (mean ± SD).

Supplementary Material Table S1. HPLC running conditions.

Compounds	Moblie phase (%)		Column [a]	Flow rate (mL/min)	UV length (nm)	Internal standard	Reconstitution solvent [b]
	Acetonitrile	water					
1, **6**, **7**	Gradient 1[c]		C18	0.3	211	CBD[g]	50%
LPV, **2**	Gradient 1[d]		C18	0.3	211	CBD	50%
3	Gradient 1[e]		C18	0.5	211	CBD	50%
4, **5**	Gradient 3[f]		C18	0.5	211	CBD	50%

[a] C18: Gemini C18 2.0 × 150 mm, 110Å, 5 µm particle size, coupled with a guard column (2 mm × 4mm) with a particle size of 3 µm (Phenomenex, Macclesfield, UK).

[b] Reconstitution solvent expressed as % of acetonitrile in HPLC-grade water.

[c] Gradient 1: Acetonitrile 50% at 0-9.5 min, increase to 65% at 9.5-11.5 min, 65% at 11.5-20 min, decrease to 50% at 20-21 min, 50% at 21-25 min.

[d] Gradient 2: Acetonitrile 50% at 0-9.5 min, increase to 70% at 9.5-11.5 min, 70% at 11.5-20 min, decrease to 50% at 20-21 min, 50% at 21-25 min.

[e] Gradient 3: Acetonitrile 50% at 0-6.5 min, increase to 80% at 6.5-10 min, 80% at 10-15 min, decrease to 50% at 15-16 min, 50% at 16-24 min.

[f] Gradient 4: Acetonitrile 50% at 0-6.5 min, increase to 85% at 6.5-10 min, 85% at 10-15 min, decrease to 50% at 15-16 min, 50% at 16-24 min.

[g] CBD: cannabidiol

Reference

[1] B. Neises, W. Steglich, Simple Method for the Esterification of Carboxylic Acids, Angew. Chemie Int. Ed. English. 17 (1978) 522–524. https://doi.org/10.1002/anie.197805221.

[2] H.E. Gottlieb, V. Kotlyar, A. Nudelman, NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities, J. Org. Chem. 62 (1997) 7512–7515. https://doi.org/10.1021/jo971176v.