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Dipeptide Prodrug Approach to Evade Efflux Pumps and CYP3A4 Metabolism of Lopinavir

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Abstract

Oral absorption of lopinavir (LPV) is limited due to P-glycoprotein (P-gp) and multidrug resistance-associated protein2 (MRP2) mediated efflux by intestinal epithelial cells. Moreover, LPV is extensively metabolized by CYP3A4 enzymes. In the present study, dipeptide prodrug approach was employed to circumvent efflux pumps (P-gp and MRP2) and CYP3A4 mediated metabolism of LPV. Valine-isoleucine-LPV (Val-Ile-LPV) was synthesized and identified by LCMS and NMR techniques. The extent of LPV and Val-Ile-LPV interactions with P-gp and MRP2 was studied by uptake and transport studies across MDCK-MDR1 and MDCK-MRP2 cells. To determine the metabolic stability, time and concentration dependent degradation study was performed in liver microsomes. Val-Ile-LPV exhibited significantly higher aqueous solubility relative to LPV. This prodrug generated higher stability under acidic pH. Val-Ile-LPV demonstrated significantly lower affinity towards P-gp and MRP2 relative to LPV. Transepithelial transport of Val-Ile-LPV was significantly higher in the absorptive direction (apical to basolateral) relative to LPV. Importantly, Val-Ile-LPV was recognized as an excellent substrate by peptide transporter. Moreover, Val-Ile-LPV displayed significantly higher metabolic stability relative to LPV. Results obtained from this study suggested that dipeptide prodrug approach is a viable option to elevate systemic levels of LPV following oral administration

Keywords

lopinavir; LPV; dipeptide prodrug; uptake; transport; permeability; P-gp; MRP2 and metabolism

1. Introduction

LPV is a powerful anti-HIV agent that is generally indicated in highly active antiretroviral therapy (HAART) to treat HIV infection. It is currently indicated to treat HIV infection in combination with ritonavir under the trade name Kaletra®. Inclusion of LPV in HAART has

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substantially ameliorated the clinical outcomes in HIV patients. Despite potent anti-HIV efficacy, a major challenge associated with LPV delivery is its inability to generate high systemic concentrations following oral administration. LPV has produced poor oral bioavailability following single oral dosing in humans and rats. Such low oral bioavailability is mainly due to high P-gp and MRP2 mediated efflux at intestinal epithelium (Agarwal et al., 2007b) and extensive hepatic metabolism by CYP3A4 enzymes (Kumar et al., 2004).

P-gp and MRP2 are the major drug efflux pumps highly expressed on the luminal surface of intestinal epithelial cells. Due to unique localization, LPV is actively secreted back into the intestinal lumen by these efflux pumps. This process significantly reduces intestinal transport of LPV, resulting in lower oral bioavailability. In addition, LPV is extensively metabolized by CYP3A4 enzymes in the liver (Kumar et al., 1999; Kumar et al., 2004). The cumulative effects of poor aqueous solubility (40 µg/mL), efflux pumps and CYP3A4-mediated metabolism result in low to variable systemic LPV concentrations following oral administration. Hence to achieve therapeutic concentrations, large doses of LPV can be administered which may generate unacceptable systemic toxicities (Patel et al., 2013).

To improve oral absorption of LPV, various strategies have been employed. One of the common approaches includes co-administration of efflux pumps/metabolism inhibitors with LPV. However, high doses of inhibitors needed to inhibit efflux pumps/metabolic activity *in vivo* may result in unacceptable side effects. Furthermore, efflux pumps are commonly involved in extruding xenobiotics and cytotoxic molecules thereby providing cellular protection. Hence, inhibiting the normal physiological function of efflux pumps may produce serious side effects. Non-substrate strategy such as transporter targeted drug delivery has certain advantage over co-administration strategy. The former strategy not only avoids inhibition of the normal physiological function (protective) of efflux pumps but also eliminates requirement of a pharmacokinetic boosting agent. Prodrug based delivery strategy is a viable option where a transporter targeting ligand coupled LPV (Val-Ile-LPV) can bypass efflux pumps by becoming a substrate of a specific influx (nutrient) transporter highly expressed on the luminal surface of the intestinal epithelial cells (Majumdar et al., 2004).

Several influx transporters expressed on intestinal epithelium can be targeted to improve oral absorption of poorly permeable drugs such as LPV. Among these influx transporters, peptide transporters have become an attractive target for prodrug delivery approaches due to unique physiological and functional properties. These transporters are highly expressed on intestinal epithelial cells and have high capacity and broad substrate specificity. Moreover, these influx transporters can transport molecules with diverse modification in their substrate structure. Peptide transporters have been previously targeted in our laboratory to improve ocular bioavailability of acyclovir (Dias et al., 2002), ganciclovir (Gunda et al., 2006; Janoria et al., 2010; Majumdar et al., 2006; Majumdar et al., 2005; Patel et al., 2005) and intestinal absorption of LPV (Agarwal et al., 2008) and saquinavir (Jain et al., 2007). Moreover, valine and isoleucine based prodrugs are known to circumvent P-gp and MRP2 mediated efflux and CYP3A4-mediated metabolism of LPV (Agarwal et al., 2008; Patel et al., 2014a). Based on these results, we selected to target intestinal peptide transporters with Val-Ile-LPV prodrug in an attempt to improve LPV absorption.

The main objective of the present study is to determine if dipeptide prodrug approach can overcome efflux pumps (P-gp and MRP2) and CYP3A4-mediated metabolism. Extensive P-gp and MRP2-mediated efflux significantly diminishes intestinal absorption of LPV. Moreover, absorbed LPV is extensively metabolized by CYP3A4 enzymes in liver, Cumulative effect of these two processes results in lower systemic levels. Val-Ile-LPV prodrug was synthesized and identified with LCMS/MS and NMR analysis. Efficacy of Val-Ile-LPV to circumvent P-gp and MRP2 mediated cellular efflux of LPV was investigated by uptake and transport studies in MDCK-MDR1 and MDCK-MRP2 cell lines. These cell lines have been previously employed in our laboratory as *in vitro* cell culture models to study interaction of various compounds with efflux pumps (Agarwal et al., 2007b; Kwatra et al., 2010; Luo et al., 2011; Patel et al., 2014a; Patel et al., 2014b; Vadlapatla et al., 2011). Uptake studies were also conducted in the presence of cyclosporine A (P-gp substrate/inhibitor), GF 120918 (P-gp inhibitor) and MK 571 (MRP2 inhibitor) to confirm affinity of LPV/prodrug towards efflux pumps. Moreover, transepithelial transport study was conducted across colon carcinoma cell line (Caco-2), since peptide transporters are highly expressed in this cell line (Guo et al., 1999; Thwaites et al., 1993). To determine the extent of LPV and prodrug metabolism, time and concentration dependent metabolic stability studies were carried out in rat liver microsomes.

2. Experimental Section

2.1. Materials

Unlabeled LPV was a generous gift from Abbott Laboratories Inc. (North Chicago, IL, USA) [3H]-LPV (1 Ci/mmol) was purchased from Moravsek Biochemicals (Brea, CA, USA) and utilized at 0.25 μ Ci/mL. MDCK cells, retrovirally transfected with human *MDR1* cDNA (MDCK-MDR1) and human MRP2 (MDCKII-MRP2) and wild type MDCKII (MDCK WT) cells were generously provided by Drs. A. Schinkel and P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Caco-2 (passages 20–30) was purchased from ATCC (Manassas, VA, USA). The growth medium Dulbecco's modified Eagle's Medium (DMEM), trypsin/EDTA and non-essential amino acids were obtained from Gibco (Invitrogen, Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Atlanta biological. Rat liver microsomes were procured from XenoTech LLC (Lenexa, KS, USA). Penicillin, triton X-100, HEPES, D-glucose, streptomycin, sodium bicarbonate, cyclosporin A, GF 120918, MK 571, Boc-valine, Boc-isoleucine, dichloromethane, ethyl acetate, 4-(*N,N*-dimethylamino) pyridine (DMAP), glucose, dicyclohexylcarbodiimide (DCC) sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate (Na_2HPO_4), potassium phosphate (KH_2PO_4), calcium chloride (CaCl_2), magnesium sulfate (MgSO_4) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMSO and methanol (HPLC grade) were obtained from Fisher Scientific Co. (Pittsburgh, PA, USA). Premium siliconized microcentrifuge tubes were purchased from MIDSCI (St. Louis, MO, USA) Culture flasks (75 cm^2), 12-well (3.8 cm^2 growth area/well) and transwell plates were obtained from Costar (Bedford, MA, USA). All chemical agents procured were of special reagent grade and utilized without any further purification.

2.1.1. Synthesis of Val-Ile-LPV

2.1.1.1. Synthesis of Val-Ile-LPV: Val-Ile-LPV was synthesized according to the protocol previously published from our laboratory with minor modifications (Agarwal et al., 2008). Val-Ile-LPV synthesis involved two steps a) synthesis of intermediate Ile-LPV amino acid prodrug by conjugation of isoleucine to LPV via ester bond and b) synthesis of Val-Ile-LPV by conjugating valine to Ile-LPV intermediate via amide bond. Ile-LPV was synthesized according to the procedure previously described from our laboratory (Patel et al., 2014b). To synthesize Val-Ile-LPV, commercially available Boc-Val-OH (292 mg, 1.35 mmol) and DCC (420 mg, 2.025 mmol) was dissolved in dichloromethane (6 mL) in a round bottom flask. The mixture was stirred for 1 h at 0 °C in an ice bath (mixture 1). In a separate round bottom flask, intermediate Ile-LPV (500 mg, 0.0.67 mmol) was dissolved in dichloromethane and triethylamine (2 mL) was added to resulting solution (mixture 2). This mixture was stirred for 30 min at RT under nitrogen atmosphere to activate the primary amine group of Ile-LPV. Mixture 2 was added to mixture 1 drop-wise with the help of a syringe and needle and stirred for 24 h at RT. Reaction mixture was checked for completion every 6 h with TLC and LCMS/MS technique. Following completion, the mixture was filtered and dichloromethane was evaporated under reduced pressure to obtain oily crude product. The product Boc-Val-Ile-LPV was purified by silica column chromatography using 5% methanol in dichloromethane as eluent. The yield obtained was 80%.

2.1.1.2. Deprotection of the N-Boc Group: Boc-Val-Ile-LPV was dissolved in 60% trifluoroacetic acid (TFA) in dichloromethane and stirred for 1 h at 0 °C to remove the protecting group. Following deprotection, solvent was quickly evaporated under reduced pressure to obtain oily crude product which was purified by recrystallization in cold diethyl ether. The yield obtained was 90%. The final product was stored in –20 °C until further use. The reaction scheme for the synthesis of Val-Ile-LPV is demonstrated in Fig. 1.

2.1.2. Identification and Purity of Val-Ile-LPV—Val-Ile-LPV was characterized by LCMS/MS and NMR analysis. A QTrap® LCMS/MS spectrometer (Applied Biosystems) under positive mode was applied to identify Val-Ile-LPV. LC/MS (m/z); $[M+H]^+$ obtained for Val-Ile-LPV was 841.5. The purity of prodrugs was determined using TLC and HPLC analysis. For TLC analysis, 5% methanol in dichloromethane was selected as the solvent system. For HPLC analysis, acetonitrile (solvent A) and water (solvent B) gradient was pumped through C(18) Kinetex column (100 × 4.6 mm, 2.6 μ; Phenomenex, Torrance, CA, USA). Both solvents were spiked with 0.1% formic acid. The gradient employed was 0% solvent A to 100% solvent A for 30 min, followed by 100% solvent A for another 10 min. The flow rate was 0.4 mL/min and total runtime was 40 min. The wavelength used for Val-Ile-LPV detection was 210 nm. Purity of Val-Ile-LPV was observed to be greater than 95%. $^1\text{H-NMR}$ [$(\text{CD}_3)_2\text{S=O}$] analysis was carried out to detect the main characteristic peaks of Val-Ile-LPV. The anticipated ppm values (δ) observed are: 0.78 (m, 6 H), 0.81 (m, 2 H), 0.83–0.95 (m, 6 H), 1.2–1.3 (s, 6 H), 1.5–1.59 (m, 2 H), 1.62–1.68 (m, 2 H), 1.7–1.8 (s, 6 H), 1.95 (m, 1 H), 2.54–2.75 (s, 2 H), 2.60–2.68 (m, 2 H), 2.75–2.93 (m, 4 H), 2.98–3.14 (m, 3 H), 3.76 (m, 1 H), 4.15–4.19 (m, 3 H), 4.39–4.52 (m, 2 H), 5.14 (m, 1 H), 6.92–7.02 (m, 3 H), 7.15–7.35 (m, 10 H).

2.2. Methods

2.2.1. Cell Culture—MDCK-WT, MDCK-MRP2 and MDCK-MDR1 (passage 8–10) and Caco-2 (passages 20–30) cells were grown in 75 cm² tissue culture flasks in DMEM medium supplied with high glutamine and glucose concentrations. Medium was supplemented with 10% FBS (heat inactivated), penicillin (100 units/mL) and streptomycin (100 µg/mL) and maintained at pH 7.4. Cells were incubated at 37 °C in an atmosphere of 5% CO₂ and 90% relative humidity. The medium was replaced every alternate day until cells reached 80%–90% differentiation (5–7 days for MDCK and 19–21 days for Caco2 cells).

2.2.2. Solubility Study in Distilled Deionized Water (DDW)—Saturated aqueous solubility study of Val-Ile-LPV was performed according to the published protocol from our laboratory (Agarwal et al., 2008). Briefly, saturated solution of Val-Ile-LPV was prepared in DDW in siliconized tubes and placed in shaking water bath at room temperature (RT) for 24 h. Tubes were subjected to centrifugation for 10 min at 10,000 rpm to separate undissolved prodrug. The supernatant was carefully separated, filtered through 0.45 µm membrane (Nalgene syringe filter) and analyzed with HPLC after appropriate dilutions.

2.2.3. Buffer Stability Studies—Chemical hydrolysis study of Val-Ile-LPV was conducted according to a previously published protocol from our laboratory (Luo et al., 2011). Degradation rate constant and half-life ($t_{1/2}$) values were determined at pH 5, 6 and 7.4. Val-Ile-LPV (40 µM) was dissolved in 1.5 mL of DPBS in microcentrifuge tubes and placed in shaking water bath maintained at 60 rpm and 37 °C. Aliquots (100 µL) were withdrawn at predetermined time points and stored at –80° C until further HPLC analysis. Prodrug concentrations remaining were plotted *versus* time in order to determine degradation rate constants at different pH values.

2.2.4. Cytotoxicity Studies—Cytotoxicity of LPV and Val-Ile-LPV was determined in MDCK-WT cells with MTS based cytotoxicity assay kit. Briefly, cells were seeded in 96 well plates at a density of 20,000 cells per well and maintained at 37 °C in an atmosphere of 5% CO₂ and 90% relative humidity overnight. Following day, the medium was aspirated and replaced with 100 µL of serum free medium containing various concentrations of LPV and Val-Ile-LPV (5–250 µM). Cells were then incubated for 4 h at 37 °C. After incubation, 20 µL of MTS stock solution was added to each well and incubated for 2 h at 37 °C. Cell viability was determined by measuring absorbance at 485 nm with a microplate reader (BioRad, Hercules, CA, USA). The quantity of formazan product as measured by absorbance is directly proportional to the number of viable cells in test samples.

2.2.5. Uptake Studies—Confluent cells were trypsinized and seeded at a density of 3×10^6 cells in 12 well culture plates. Medium was replaced every alternate day until cells reached confluency (6–7 days). Cellular radioactive uptake studies were carried out according to a previously published protocol from our laboratory (Patel et al., 2012a; Patel et al., 2012b). Briefly, medium was removed and cell monolayer was washed with 2 mL of DPBS (130 mM NaCl, 0.03 mM KCl, 7.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgSO₄, and 5 mM glucose, pH 7.4) three times at 37 °C (each wash of 10 min).

Uptake studies were initiated by incubating cells with radioactive solution in DPBS at 37 °C for 30 min. Following incubation, radioactive solution was quickly aspirated and plates were washed with ice-cold stop solution (210 mM KCl, 2 mM HEPES, pH of 7.4) to terminate further uptake. One mL of lysis buffer (0.1% Triton-X solution in 0.3% NaOH) was added to each well and plates were stored overnight at RT. Following day, 500 µL solutions were transferred to vials containing 3 mL of scintillation cocktail. The radioactivity associated with cells was analyzed with a scintillation counter (Beckman Instruments Inc., Model LS-6500; Fullerton, CA, USA). Uptake rate was normalized to protein amount which was quantified with a BioRad protein estimation kit. To study the effect of efflux inhibitors, cells were treated with GF 120918 (MDCK-MDR1) and MK 571 (MDCK-MRP2) for 30 min prior to the initiation of an uptake study.

Non-radioactive uptake study was also carried out to determine the extent of prodrug interaction with efflux pumps such as P-gp and MRP2. Briefly, cell monolayers (MDCK-MDR1 or MDCK-MRP2) were pre-treated with efflux inhibitors for 30 min and incubated with Val-Ile-LPV. Following incubation, prodrug solution was removed and cell monolayer was thoroughly washed with ice cold stop solution three times (each wash of 5 min) to stop further uptake process. About 500 µL of DMEM was added to each well and plates were kept at –80 °C overnight for cell lysis. On the following day, prodrug was extracted by liquid-liquid extraction technique (section 2.3.2) and analyzed with LCMS/MS technique.

2.2.6. Transport Studies—Transepithelial transport studies were carried out according to a protocol published from our laboratory (Luo et al., 2011). Briefly, Transwell® inserts (0.4 µm pore size, 12 mm insert) with transparent polyester membrane were coated with type 1 rat tail collagen (100 µg/cm²). Cells were seeded at a density of 250,000 cells per insert. Transport studies were conducted for a period of 3 h at 37 °C across a fully confluent cell monolayer. Cell monolayer was washed thoroughly with DPBS for 10 min at 37 °C, three times. For determining A-B permeability, 0.5 mL of test compound (25 µM) was added to the apical chamber of 12-well Transwell® plates. At predetermined time points (30, 60, 120 and 180 min), 100 µL sample was withdrawn from the basolateral chamber and replaced with fresh DPBS to maintain sink conditions. Samples were stored at –80 °C until further analysis with LCMS/MS. Transport studies across Caco2 cells were conducted at pH 5 and MDCK cells at pH 7.4 (Agarwal et al., 2008; Agarwal et al., 2007a).

Prior to the initiation of transport study, cell monolayer integrity was determined by measuring transepithelial electric resistance (TEER). TEER values were measured with an EVOM (epithelial volt ohmmeter from World Precision Instruments, Sarasota, FL, USA). TEER values of cell monolayers were 250 Ω*cm².

2.2.7. Microsomal Stability Studies—Rat liver microsomes were selected to determine the substrate affinity of LPV and Val-Ile-LPV towards CYP3A4. One mL of microsomal solution was generated by adding microsomal protein (0.3 mg/mL), magnesium chloride (5 mM), glucose 6-phosphate (5 mM), b-NADP1 (1 mM), and glucose 6-phosphate dehydrogenase (1 U/mL) in phosphate buffer (100 mM, pH 7.4). LPV or Val-Ile-LPV was added to the microsomal solution and incubated at 37 °C for 5 min. The metabolic reaction was initiated by adding NADPH generating system which was freshly prepared by

dissolving G-6-P, G-6-P DH and NADP in DDW. At predetermined time points, 100 μ L of sample was withdrawn and equal volume of ice-cold acetonitrile was added to arrest metabolic reaction. Samples were stored at -80°C until further analysis by LCMS/MS technique.

Experiments were designed to investigate the metabolic stability of LPV and Val-Ile-LPV in liver microsomes in the presence and absence of ketoconazole (100 μ M), a potent CYP3A4 inhibitor. Prior to the initiation of the study, liver microsomal solution was incubated with ketoconazole for 15 min to inhibit CYP3A4 activity. Following incubation, the amount of LPV and Val-Ile-LPV remaining in samples was determined. In addition, time and concentration dependent metabolic stability studies were conducted to determine the degradation half-life and affinity of LPV and Val-Ile-LPV towards metabolizing enzymes.

2.2.7.1. Time Dependent Stability Studies: For time dependent metabolism studies, LPV or Val-Ile-LPV (25 μ M) were added to an activated liver microsomal solution and incubated at 37°C . This study was performed according to a previously published protocol from our laboratory (Luo et al., 2011). At predetermined time points, about 100 μ L samples were withdrawn and equal volume of ice-cold acetonitrile was added to stop further degradation. Degradation study was conducted for a period of 2 h. Samples were stored at -80°C until further analysis.

2.2.7.2. Concentration Dependent Degradation Studies: For concentration dependent degradation studies, various concentrations of LPV or Val-Ile-LPV (1–20 μ M) were incubated with activated microsomal solution for 5 min. Enzymatic degradation was arrested by adding equal amount of ice-cold acetonitrile. Samples were stored in -80°C until further analysis.

3. Sample and Data Analysis

3.1. HPLC Analysis

Aqueous solubility and buffer stability samples were analyzed with reversed phase HPLC technique. Waters 515 pump (Waters, Milford, MA, USA) connected to C(18) Kinetex column (100×4.6 mm, 2.6 μ ; Phenomenex, Torrance, CA, USA) and a UV detector (Absorbance Detector Model UV-C, RAININ, Dynamax, Palo Alto, CA, USA) was employed. Mobile phase (1:1) acetonitrile and water (0.1% trifluoroacetic acid) was used at a flow rate of 0.3 mL/min. Analytes were measured at a wavelength of 210 nm. Val-Ile-LPV eluted at approximately 9.0-9.2 min.

3.2. Sample Preparation for LCMS/MS Analysis

Transport and metabolism samples of LPV and Val-Ile-LPV were analyzed with a sensitive LCMS/MS method according to the method previously published from our laboratory (Patel et al., 2014b). Briefly, samples were extracted with liquid–liquid extraction technique with water saturated ethyl acetate as extracting solvent. Briefly, 50 μ L of verapamil (200 ng) was employed as an internal standard. Samples were vortexed with 800 μ L water saturated ethyl acetate for 2.5 min and centrifuged at 10,000 rpm for 7 min. Aliquots (600 μ L) of ethyl acetate layer was collected and evaporated under reduce pressure for 45 min. Samples were

reconstituted in 100 μ L of acetonitrile (80%) and water (20%) containing 0.1% formic acid. Ten microliters of reconstituted samples were injected into LCMS/MS.

3.3. LCMS/MS Analysis

To analyze transport samples, QTrap® LCMS/MS mass spectrometer (Applied Biosystems, Foster City, CA, USA) connected to Agilent 1100 Series quaternary pump (Agilent G1311A), vacuum degasser (Agilent G1379A) and autosampler (Agilent G1367A, Agilent Technology Inc., Palo Alto, CA, USA) was employed. LPV and Val-Ile-LPV were separated with a mobile phase of 80% acetonitrile and 20% water containing 0.1% formic acid at 0.3 mL/min flow rate. XTerra®MS C18 column (50 mm $\times$ 4.6 mm, 5.0 μ m, Waters, Milford, MA, USA) was employed for analyte separation. Chromatograms were obtained over 4 min. LPV and verapamil eluted at 3 and 1.7 min, respectively. Val-Ile-LPV eluted within 1.7–1.8 min.

Electrospray ionization (positive mode) was employed and analytes of interest were detected using multiple-reaction monitoring (MRM) mode. Precursor ion of LPV/Val-Ile-LPV was generated from the spectra which are obtained with the infusion of standard solutions to an electrospray ion source with the aid of an infusion pump. Each of these precursor ions was further subjected to collision-induced dissociation to produce product ions. Precursor/product ions obtained for LPV and verapamil were 629.398/155.183 and 455.020/150.050, respectively. Precursor/product ion obtained for Val-Ile-LPV was 841.603/155.185. Moreover, turbo ion spray setting, collision gas pressure and other operational parameters were also optimized and published from our laboratory (Patel et al., 2014b).

3.4. Permeability Analysis

Transepithelial transport rates were obtained by plotting cumulative amount of LPV/Val-Ile-LPV transported against time. Linear regression of LPV/Val-Ile-LPV transported as a function of time determines the rate of transport (dM/dt). Transport rate was further divided by the cross-sectional area (A) to determine steady-state flux [flux = (dM/dt)/A]. Steady-state flux normalized with the donor concentration (Cd) generates transepithelial permeability rates (permeability = flux/Cd)

3.5. Statistical Analysis

Uptake, transport, buffer and metabolic stability results are expressed as mean $\pm$ S.D. To determine statistical significance among groups, Student's *t*-test was employed. A difference between the mean values was considered to be statistically significant if the *p* value was 0.05.

4. Results

4.1. Solubility in DDW

Saturated aqueous solubility values of Val-Ile-LPV in DDW was found to be 339 ± 18 μ g/mL. Solubility of Val-Ile-LPV was approximately 8.5 times higher relative to LPV (40 ± 8 μ g/mL).

4.2. Buffer Stability Studies

Chemical hydrolysis of Val-Ile-LPV was determined in DPBS adjusted to various pH values *i.e.*, 4, 5.5 and 7.4. The prodrug was observed to be significantly stable under acidic conditions and degraded rapidly with rise in pH. Degradation rate constant and half life of the prodrug at various pH values are presented in Table 1. Degradation rate constant at pH 4, 5.5 and 7.4 was found to be $1.1 \pm 0.3 \times 10^{-4}$, $1.9 \pm 0.6 \times 10^{-4}$ and $15.3 \pm 1.3 \times 10^{-4} \text{ min}^{-1}$, respectively. Degradation half life at pH 4, 5.5 and 7.4 was 106 ± 19 , 69 ± 31 and 7.6 ± 0.6 h, respectively. Degradation half life displayed by Val-Ile-LPV at pH 4 was approximately 14-fold higher relative to pH 7.4. This result indicated that the prodrug is highly susceptible to alkaline hydrolysis relative to acidic hydrolysis.

4.3. Cytotoxicity Studies

Cytotoxicity of LPV and Val-Ile-LPV in MDCK-WT cells was performed with MTT assay prior to the initiation of uptake and transport studies. Serum free medium was employed for this study. Medium containing no test compounds and organic solvents was selected as control. Medium containing 0.1% triton-X was employed as positive control. As demonstrated in Fig. 2, assay medium containing 2% DMSO did not show any cytotoxicity following 4 h of incubation. Positive controls displayed significant cytotoxic effects relative to control. Triton-X (0.1%) produced 90% reduction in absorbance relative to control. LPV appears to be non-cytotoxic in the concentration range of 5-50 μM . However, at 100 and 250 μM , LPV generated significant cytotoxicity to MDCK-WT cells. A 22 and 30% reduction in the absorbance was observed at 100 and 250 μM concentration of LPV. Similarly, the prodrug did not produce any cytotoxic effect in the concentration range of 5-50 μM . At 100 and 250 μM concentrations, the prodrug generated 22 and 34% reduction in absorbance indicating significant cytotoxic effects.

4.4. Cellular Uptake Studies

To determine the extent of LPV and Val-Ile-LPV interaction with P-gp and MRP2, we carried out [^3H]-LPV cellular uptake in MDCK-MDR1 and MDCK-MRP2 cells. These cell lines represent an excellent *in vitro* cell culture model alternative to Caco-2 for high throughput drug screening (Tang et al., 2002a, b). Results obtained from this study are depicted in Fig. 3. [^3H]-LPV uptake elevated significantly in the presence of cold LPV (50 μM) in both MDCK-MDR1 and MDCK-MRP2 cells. A 3.2 and 2.5-fold enhancement in the uptake process was observed in the presence of unlabelled LPV in MDCK-MDR1 and MDCK-MRP2 cells. Interestingly, [^3H]-LPV uptake did not alter significantly in the presence of Val-Ile-LPV (50 μM) in both MDCK-MDR1 and MDCK-MRP2 cells.

Non-radiolabeled uptake was conducted with Val-Ile-LPV (50 μM) in MDCK-MDR1 and MDCK-MRP2 cells in the presence of P-gp and MRP2 inhibitors (Table 2). Val-Ile-LPV uptake did not alter significantly in the presence of P-gp inhibitors in MDCK-MDR1 cells. Similar results were obtained for Val-Ile-LPV uptake in MDCK-MRP2 cells in the presence of MK 571 (25 and 75 μM).

Furthermore, [^3H]-GlySar uptake in the presence of Val-Ile-LPV (50 μM) in MDCK-MDR1 and MDCK-MRP2 cells was performed to determine prodrug interaction with peptide

transporters. Results obtained from this study are depicted in Fig. 4. [3H]-GlySar uptake diminished dramatically in the presence of cold GlySar (10 mM). Approximately, 80% reduction in the uptake was observed in the presence of cold GlySar. Val-Ile-LPV (50 μ M) also produced significant inhibition in the uptake of [3H]-GlySar. A 40% inhibition in the uptake was observed in the presence of Val-Ile-LPV in both MDCK-MDR1 and MDCK-MRP2 cells.

4.5. Transepithelial Transport Studies

Transepithelial transport of Val-Ile-LPV and LPV was carried out in the absorptive direction (apical to basolateral, A-B) in MDCK-MDR1 and MDCK-MRP2 cells. Previously, we have reported that peptide transporters and efflux pumps (MDR1 and MRP2) are expressed on the apical surface of these cell lines (Agarwal et al., 2007a; Agarwal et al., 2007b). Moreover, we have also demonstrated that MDR1 and MRP2 efflux pumps play a significant role in diminishing A-B transport of LPV (Agarwal et al., 2008; Agarwal et al., 2007b; Patel et al., 2014b). Hence, to determine the efficacy of Val-Ile-LPV to circumvent efflux pumps by translocating through peptide transporters, A-B transepithelial transport study was undertaken. Results obtained from this study are presented in Fig. 5. A-B permeability rate of Val-Ile-LPV across MDCK-MDR1 and MDCK-MRP2 cells was significantly higher relative to LPV. A-B permeability rates generated by LPV and Val-Ile-LPV across MDCK-MDR1 cells were $2.37 \pm 0.13 \times 10^{-6}$ and $1.09 \pm 0.07 \times 10^{-5}$ cm/s, respectively. A 4.6-fold enhancement in the A-B permeability rate was observed for Val-Ile-LPV relative to LPV. Similarly, a 4-fold higher A-B permeability rate was generated by Val-Ile-LPV compared to LPV across MDCK-MRP2 cells. A-B permeability values exhibited by LPV and Val-Ile-LPV across MDCK-MRP2 cells were $2.7 \pm 0.08 \times 10^{-6}$ and $1.06 \pm 0.01 \times 10^{-5}$ cm/s, respectively. Significant enhancement in the A-B permeability rates suggests that Val-Ile-LPV may have lower substrate affinity towards P-gp and MRP2 relative to LPV.

In order to confirm the role of peptide transporters in the transport of Val-Ile-LPV across MDCK-MDR1 and MDCK-MRP2 cells, A-B transport study was conducted in the presence of GlySar, a model substrate of peptide transporters. Results obtained from this study are depicted in Fig. 5. Transport of Val-Ile-LPV diminished significantly in the presence of GlySar in both MDCK-MDR1 and MDCK-MRP2 cells. A-B permeability values of Val-Ile-LPV in the presence of 10 and 20 mM of GlySar in MDCK-MDR1 cells were $7.1 \pm 0.2 \times 10^{-6}$ and $3.7 \pm 0.2 \times 10^{-6}$ cm/s, respectively. A 1.6 and 2.9-fold reduction in the A-B transport of Val-Ile-LPV was observed in the presence of 10 and 20 mM of GlySar in MDCK-MDR1 cells. Similarly, A-B permeability of Val-Ile-LPV diminished by 1.5 and 3.0-fold in the presence of 10 and 20 mM concentrations of GlySar in MDCK-MRP2 cells. A-B permeability values of Val-Ile-LPV in the presence of 10 and 20 mM of GlySar in MDCK-MDR1 cells were $7.2 \pm 0.2 \times 10^{-6}$ and $3.5 \pm 0.2 \times 10^{-6}$ cm/s, respectively. Significant reduction in the apparent A-B permeability rates in the presence of GlySar clearly suggests that Val-Ile-LPV is an excellent substrate of peptide transporters.

We also carried out A-B transport of LPV and Val-Ile-LPV across Caco2 cells. This cell line was selected as it represents an excellent *in vitro* cell culture model to determine intestinal

permeability. Results obtained from this study are depicted in Fig. 6. A-B permeability rate of Val-Ile-LPV was significantly higher relative to LPV. Permeability values of LPV and Val-Ile-LPV were found to be $2.6 \pm 0.1 \times 10^{-6}$ and $11.3 \pm 0.7 \times 10^{-6}$ cm/s, respectively. A 4.4-fold enhancement in the prodrug transport was observed relative to LPV. Moreover, we performed transport studies in the presence of GlySar to confirm prodrug translocation through peptide transporters. Permeability rate of VAL-Ile-LPV in the presence of 10 and 20 mM GlySar was $7.2 \pm 0.3 \times 10^{-6}$ and $3.6 \pm 0.2 \times 10^{-6}$ cm/s, respectively. A 1.6 and 3.1-fold reduction in the A-B transport of the prodrug was observed in the presence of 10 and 20 mM GlySar. These results clearly suggest that Val-Ile-LPV is an excellent substrate of peptide transporter and may therefore generate higher systemic levels following oral administration.

4.6. Liver Microsomal Stability Studies

The extent of LPV and Val-Ile-LPV metabolism was studied in rat liver microsomes (0.3 mg/mL). Results obtained from this study are depicted in Fig. 7. Amount of LPV remaining in the microsomal preparation was about 2.3 times higher relative to control (LPV in microsomal preparation without ketoconazole). Interestingly, amount of Val-Ile-LPV obtained in microsomal solution in the presence and absence of ketoconazole was almost similar. This result indicates that the peptide prodrug may have lower affinity towards CYP3A4 enzymes relative to LPV.

4.6.1. Time Dependent Degradation Studies—Degradation rate constant of LPV and Val-Ile-LPV was determined by conducting time dependent metabolic stability study in liver microsomal preparation. Degradation rate constant generated by the prodrug was significantly lower relative to LPV. Degradation rate constant of Val-Ile-LPV and LPV was 3.3 ± 0.3 and $6.0 \pm 0.5 \times 10^{-3} \text{ min}^{-1}$. The half life of Val-Ile-LPV was found to be 1.8-fold higher than LPV. This result suggests that the prodrug has lower affinity towards metabolizing enzymes relative to LPV.

4.6.2. Concentration Dependent Degradation Studies—Concentration dependent metabolism study was carried out to compare affinity of LPV and Val-Ile-LPV towards metabolizing enzymes. In this study, various concentrations (0.5–40 μM) of LPV or prodrug were incubated in liver microsomes (0.3 mg/mL) for 5 min. The data generated was fitted to a Michaelis-Menten kinetic model to determine K_m (affinity constant) and V_{max} values. Val-Ile-LPV displayed approximately 3-fold lower affinity towards metabolizing enzymes relative to LPV. K_m values observed for Val-Ile-LPV and LPV was approximately 17 ± 4 and $6.0 \pm 0.9 \mu\text{M}$, respectively. V_{max} values observed for Val-Ile-LPV and LPV were 12 ± 1 and $8.1 \pm 0.4 \text{ nmoles/min/mg protein}$, respectively. Furthermore, we calculated the intrinsic clearance (V_{max}/K_m) of LPV and Val-Ile-LPV. LPV and Val-Ile-LPV displayed intrinsic clearance values of 1.4 and 0.7 mL/min/mg protein, respectively. A 2-fold lower intrinsic clearance was observed with by Val-Ile-LPV relative to LPV.

5. Discussion

LPV is a highly effective anti-HIV agent recommended for HIV patients. Despite high efficacy, low to variable oral bioavailability of LPV is a major challenge. The major factors

responsible for poor oral absorption include poor aqueous solubility, significant efflux by P-gp and MRP2 at intestinal epithelium and extensive CYP3A4-mediated metabolism. In the present study, we employed dipeptide prodrug approach (non-substrate strategy) to circumvent efflux pumps and CYP3A4 metabolism of LPV. We selected to target peptide transporters due to their high expression on the apical surface of intestinal epithelial cells. Val-Ile-LPV dipeptide prodrug was synthesized and substrate affinity towards efflux pumps and peptide transporters was investigated.

We first examined the aqueous solubility of Val-Ile-LPV. The prodrug displayed significantly higher saturated aqueous solubility (8.5 times) relative to LPV. Such significant enhancement in the aqueous solubility may offer better formulations for oral absorption. Moreover, LPV is currently administered to HIV-1 patients as 40% v/v alcoholic solution due to practical insolubility in water. Such high alcohol concentrations may not be suitable for pediatric patients. In addition, alcohol has been demonstrated to induce CYP3A activity, an enzyme primarily responsible for the metabolic degradation of LPV (Feierman et al., 2003). Induction of CYP3A levels may result in increased LPV metabolism thereby, lowering systemic availabilities. Hence, Val-Ile-LPV may offer significant pharmaceutical and delivery related advantages due to higher aqueous solubility relative to LPV.

We determined chemical hydrolysis of Val-Ile-LPV at various pH values (Table 1). An ideal prodrug for oral administration should display significant stability in gastrointestinal tract and be available at higher concentrations for intestinal absorption. Following oral administration, Val-Ile-LPV can undergo acid/base catalyzed chemical hydrolysis to yield intermediate amino acid prodrug (Ile-LPV) or drug (LPV) directly. Hence, it is very important to determine the rate of Val-Ile-LPV hydrolysis under acidic and slightly basic conditions. Results obtained from chemical hydrolysis study suggested that the prodrug degrades rapidly with rise in pH. Degradation half life observed at acidic pH was several fold higher relative to pH 7.4. Val-Ile-LPV displayed degradation half life of approximately 7.5 h at pH 7.4. Therefore, longer residence times between pH 4 to 7.4 render Val-Ile-LPV a potential advantage for oral delivery of LPV.

Cytotoxicity of LPV and Val-Ile-LPV was measured prior to the initiation of uptake and transport studies (Fig. 2). LPV and prodrug were observed to be non-cytotoxic between the concentration range of 5 to 50 μ M. However, both compounds displayed significant cytotoxic effects at 100 and 250 μ M concentrations. Hence, we carried out all cellular uptake and transporter studies at concentrations $\leq$ 50 μ M concentrations. Drug efflux pumps such as P-gp and MRP2 have been reported to play a major role in oral disposition of a wide range of therapeutic agents (Murakami and Takano, 2008; Takano et al., 2006; Yokooji, 2013). These efflux pumps are highly expressed on the luminal side of intestinal epithelial cells. LPV has been reported to be an excellent substrate of both P-gp and MRP2 (Agarwal et al., 2007b). Hence, it is very crucial to circumvent these efflux pumps in order to generate higher intestinal absorption following oral administration. We carried out [3 H]-LPV cellular accumulation in the presence of cold LPV and Val-Ile-LPV in MDCK-MDR1 and MDCK-MRP2 cells (Fig. 3). Cellular uptake of [3 H]-LPV elevated dramatically in the presence of unlabelled LPV in both cell lines. This result suggests that LPV possess high substrate affinity towards P-gp and MRP2, consistent with previously published reports. Moreover,

significant enhancement in the uptake process also indicates that P-gp and MRP2 are functionally active in MDCK-MDR1 and MDCK-MRP2 cells, respectively. Interestingly, the uptake process remained unaltered in the presence of Val-Ile-LPV in both the cell lines. This result suggests that the prodrug might have lower substrate affinity towards P-gp and MRP2 relative to LPV. This result is consistent with other dipeptide prodrugs previously synthesized in our laboratory (Agarwal et al., 2008).

We further carried out the uptake of Val-Ile-LPV in the absence and presence of known P-gp and MRP2 inhibitors (table 2). The uptake process remained unaltered in the presence of P-gp inhibitors such as cyclosporine A and GF 120918 in MDCK-MDR1 cells. This result confirms that Val-Ile-LPV does not display substrate affinity towards P-gp. Similarly, prodrug uptake did not alter significantly in the presence MRP2 inhibitor. No significant enhancement in the uptake process confirms that Val-Ile-LPV is a poor substrate of MRP2. Based on these observations, Val-Ile-LPV is anticipated to generate higher intestinal absorption relative to LPV. We also investigated the affinity of prodrug towards peptide transporters by carrying out [³H]-GlySar uptake in MDCK-MDR1 and MDCK-MRP2 cells (Fig. 4). [³H]-GlySar uptake diminished significantly in the presence of cold GlySar in both cell lines. This result indicates that peptide transporters are highly expressed and functionally active in these cells. Similarly, the uptake process was inhibited significantly in the presence of the prodrug in both cell lines. This result indicates that Val-Ile-LPV possess substantial substrate affinity towards peptide transporters.

Previously, we have reported bidirectional transpeithelial transport of LPV in MDCK-MDR1 and MDCK-MRP2 cells (Patel et al., 2014b). P-gp and MRP2 are predominantly expressed on the apical surface of MDCK-MDR1 and MDCK-MRP2 cells, respectively. Hence, a classical substrate of these efflux pumps would generate higher permeability rates from secretive (B-A) relative to absorptive (A-B) direction. A-B and B-A permeability rates of LPV across MDCK-MDR1 and MDCK-MRP2 cells have been determined (Patel et al., 2014b). Apparent permeability of LPV from absorptive and secretive directions in MDCK-MDR1 cells were found to be $2.37 \pm 0.13 \times 10^{-6}$ and $5.8 \pm 0.5 \times 10^{-6}$ cm/s, respectively. Absorptive and secretive permeability values of LPV across MDCK-MRP2 cells were observed to be $2.7 \pm 0.08 \times 10^{-6}$ and $6.0 \pm 0.1 \times 10^{-6}$ cm/s, respectively. These results indicate significant P-gp and MRP2-mediated cellular efflux of LPV in the absorptive (A-B) direction. Hence, we carried out A-B transport study to determine the potential of Val-Ile-LPV to circumvent P-gp and MRP2 mediated cellular efflux of LPV. Circumvention of these efflux pumps would be apparent provided that A-B permeability of Val-Ile-LPV is significantly higher compared to LPV. As observed in Fig. 5, Val-Ile-LPV generated about 4-fold higher A-B permeability values relative to LPV in both MDCK-MDR1 and MDCK-MRP2 cells. This result confirms that Val-Ile-LPV is not recognized as a substrate by efflux pumps such as P-gp and MRP2. Moreover, the absorptive permeability of Val-Ile-LPV diminished significantly in the presence of cold GlySar (Fig. 5). This result confirms our previous observation that the prodrug is recognized as an excellent substrate by peptide transporters.

Several dipeptide prodrugs (valine-valine-LPV and glycine-valine-LPV) have been previously developed and investigated for their potential in evading P-gp and MRP2

mediated cellular efflux of LPV in our laboratory (Agarwal et al., 2008). These prodrugs generated significantly higher transport in the absorptive direction in both MDCK-MDR1 and MDCK-MRP2 cells relative to LPV. Val-Ile-LPV produced in this study displayed similar efficacy as valine-valine-LPV in improving LPV transport across P-gp and MRP2 overexpressing cells. Interestingly, Val-Ile-LPV was found superior to glycine-valine-LPV prodrug and generated higher A-B permeability rates across MDCK-MDR1 and MDCK-MRP2 cells. Importantly, A-B permeability values generated by Val-Ile-LPV across MDCK-MDR1 and MDCK-MRP2 cells were significantly higher than several amino acid prodrugs of LPV previously developed in our laboratory (Patel et al., 2014b). Based on these observations, Val-Ile-LPV is expected to generate higher intestinal transport due to evasion of efflux pumps and translocation by high capacity peptide transporters. Moreover, we carried out A-B transport of LPV and Val-Ile-LPV across Caco2 cells, a well established cell culture model to determine intestinal absorption (Fig. 6). Val-Ile-LPV generated significantly higher transport rates in the absorptive direction relative to LPV. Moreover, the transport rate was found to diminish significantly in the presence of cold GlySar. Hence, Val-Ile-LPV not only displays higher aqueous solubility but also efficiently evades efflux pumps. As a result, this prodrug is expected to generate superior permeability relative to LPV.

In addition to efflux pumps, extensive first-pass metabolism is another major contributor of lower systemic availability of LPV following oral administration. LPV is primarily metabolized by CYP3A4 enzyme. Hence, to elevate systemic concentrations, ritonavir is co-administered to inhibit CYP3A4-mediated metabolism of LPV. Previously, non-substrate strategy has been employed to evade CYP3A4-mediated metabolism of substrate drugs in our laboratory. For instance, several dipeptide prodrugs of LPV have displayed significant stability towards metabolic degradation relative to LPV itself (Agarwal et al., 2008). Stereoisomeric prodrugs of saquinavir have generated lower degradation rate constants in liver microsomal solution relative to saquinavir (Wang et al., 2012). Sodium dependent vitamin C transporter targeted ascorbic acid prodrugs produced lower affinity towards CYP3A4 relative to saquinavir (Luo et al., 2011). These results clearly suggest the potential of non-substrate prodrug approach in lowering CYP3A4-mediated metabolism of LPV and saquinavir.

We carried out various metabolic stability studies to determine the efficacy of Val-Ile-LPV to evade CYP3A4-mediated metabolism of LPV. First, we investigated the stability of LPV and Val-Ile-LPV in liver microsomal solution in the presence and absence of ketoconazole (CYP3A4 inhibitor) (Fig. 7). In the presence of ketoconazole, the amount of LPV remaining in the liver microsomal solution was approximately 2.3-fold higher compared to control (microsomal degradation without ketoconazole). Interestingly, the amount of Val-Ile-LPV in the microsomal preparation remained unaltered in the absence and presence of ketoconazole. This result suggests that Val-Ile-LPV may have lower substrate affinity towards metabolizing enzymes relative to LPV. Time dependent metabolic study demonstrated that the degradation rate constant of Val-Ile-LPV is significantly lower compared to LPV. This result suggests that Val-Ile-LPV might remain intact in the microsomal solution for a longer time period relative to LPV. Degradation rate constants for Val-Ile-LPV was observed to be $6.0 \pm 0.5 \times 10^{-3} \text{ min}^{-1}$, a 1.82-fold increase relative to LPV ($3.3 \pm 0.3 \times 10^{-3} \text{ min}^{-1}$). This

result suggest that Val-Ile-LPV possess superior metabolic stability compared to LPV. Various dipeptide prodrugs of LPV developed in our laboratory displayed higher metabolic stability relative to LPV. Val-Ile-LPV (1.82-fold increase) displayed higher metabolic stability compared to valine-valine-LPV (increase of 1.48 fold) (Agarwal et al., 2008). However, Val-Ile-LPV possessed slightly lower metabolic stability compared to glycine-valine-LPV (increase of 2.44-fold) (Agarwal et al., 2008).

Concentration dependent metabolic studies demonstrated that Val-Ile-LPV possesses 3-fold lower affinity (km) towards metabolizing enzymes relative to LPV. Moreover, the intrinsic clearance of Val-Ile-LPV was approximately 2-fold lower compared to LPV. Results obtained from metabolic study demonstrate that prodrugs have significantly lower degradation rate and affinity towards CYP3A4 enzymes compared to LPV. Based on these results, first-pass metabolism of Val-Ile-LPV is expected to be significantly lower relative to LPV. Consequently, systemic concentrations of Val-Ile-LPV may be significantly higher following oral administration.

4. Conclusions

Dipeptide prodrug of LPV, Val-Ile-LPV was synthesized and successfully evaluated for its potential to evade efflux pumps and metabolizing enzymes. Val-Ile-LPV exhibited superior aqueous solubility and permeability profiles across P-gp and MRP2 overexpressing cells relative to LPV. Moreover, Val-Ile-LPV demonstrated excellent metabolic stability compared to LPV. Results obtained from the present study suggest that dipeptide prodrug might be a feasible option in improving pharmacokinetic properties of LPV. In summary, Val-Ile-LPV prodrug approach seems to be a viable strategy to improve oral absorption of LPV and overcome the possibility of resistance development due to chronic LPV administration. In further studies, anti-HIV activity of intact Val-Ile-LPV and CYP3A4 metabolites, plasma protein binding and oral pharmacokinetics will be reported.

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Highlights

- To provide dipeptide prodrug approach to circumvent P-gp and MRP2-mediated efflux of anti-HIV drug, lopinavir
- To provide interaction of dipeptide prodrug with peptide transporters
- To provide a strategy to overcome CYP3A4-mediated metabolism of lopinavir
- To provide a viable option for improving oral absorption of lopinavir

Fig. 1.

Synthetic scheme for Val-Ile-LPV prodrug: i) Boc-isoleucine and DCC in dichloromethane, 1 h at 0 °C; LPV and DMAP in dichloromethane, 15 min at RT and mixture stirred for 48 h at RT. ii) 60% TFA in DCM, 1 h at 0 °C. iii) Boc-valine and DCC in dichloromethane, 1 h at 0 °C; Ile-LPV and triethylamine in dichloromethane, 30 min at RT and mixture stirred for 24 h at RT; iv) 60% TFA in DCM, 1 h at 0 °C.

Fig. 2.

Cellular cytotoxicity of LPV (grey bar) and Val-Ile-LPV (striped bar) in MDCK-WT cells following 4 h incubation. Each data point is expressed as mean $\pm$ standard deviation (n=8). Absorbance is expressed as percentage of control (serum/drug free medium). Asterisk (**) represents significant difference from the control ($p < 0.05$).

Fig. 3. Cellular uptake of [3H]-LPV in MDCK-MDR1 cells (empty bars) and MDCK-MRP2 cells (filled bars) in the absence and presence of LPV (50 μM) and Val-Ile-LPV (50 μM) in DPBS (pH 7.4) at 37 °C. Each data point is expressed as mean ± standard deviation (n=4). Uptake is expressed as percentage of control ([3H]-LPV). Asterisk (**) represents significant difference from the control (p < 0.05).

Fig. 4.

Cellular uptake of [3H]-GlySar in MDCK-MDR1 cells (empty bars) and MDCK-MRP2 cells (filled bars) in the absence and presence of cold GlySar (10 mM) and Val-Ile-LPV (50 μM) in DPBS (pH 7.4) at 37 °C. Each data point is expressed as mean ± standard deviation (n=4). Uptake is expressed as percentage of control ([3H]-GlySar). Asterisk (**) represents significant difference from the control (p < 0.05).

Fig. 5.

A-B permeability of LPV and Val-Ile-LPV across MDCK-MDR1 (empty bars) and MDCK-MRP2 (filled bars) cells. Each data point is expressed as mean $\pm$ standard deviation ($n=4$). Asterisk (**) represents significant difference from the control (LPV) ($p < 0.05$).

Fig. 6.

A-B permeability of LPV and Val-Ile-LPV across Caco2 cells. Each data point is expressed as mean $\pm$ standard deviation (n=4). Asterisk (**) represents significant difference from the control (LPV) (p < 0.05).

Fig. 7.

Amount of LPV or Val-Ile-LPV remaining after 15 min incubation in rat liver microsomes (0.3 mg/mL) in the absence (empty bars) and presence (filled bars) of ketoconazole (100 μM). Data points are expressed as mean ± SD (n=4). Asterisks (**) represent significant difference from the control (in the absence of ketoconazole, $p < 0.05$).

Table 1

Degradation rate constants and half lives of Val-Ile-LPV at various pH values.

pH 4		pH 5.5		pH 7.4	
$k \times 10^{-4}$ (min ⁻¹)	t _{1/2} (h)	$k \times 10^{-4}$ (min ⁻¹)	t _{1/2} (h)	$k \times 10^{-4}$ (min ⁻¹)	t _{1/2} (h)
1.1 ± 0.3	106 ± 16	1.9 ± 0.6	69 ± 31	15.3 ± 1.3	7.6 ± 0.6

Table 2

Non-radiolabelled uptake of Val-Ile-LPV in MDCK-MDR1 cells in the absence and presence of cyclosporine A and GF 120918 and MDCK-MRP2 cells in the absence and presence of MK 571 in DPBS (pH 7.4) at 37 °C. Each data point is expressed as mean $\pm$ standard deviation (n=4). Uptake is expressed as percentage of control (Val-Ile-LPV). Asterisk (**) represents significant difference from the control ($p < 0.05$).

Cell type	Compounds	Uptake as % control (Val-Ile-LPV)
MDCK-MDR1 cells	Val-Ile-LPV	100 $\pm$ 3
	GF 120918 (2 μ M)	96 $\pm$ 11
	Cyclosporine A (10 μ M)	96 $\pm$ 2
MDCK-MRP2 cells	Val-Ile-LPV	100 $\pm$ 3
	MK 571 (25 μ M)	104 $\pm$ 4
	MK 571 (75 μ M)	102 $\pm$ 2