

SYNTHESIS AND EVALUATION OF AMINO ACID ESTER CONJUGATES OF HIV PROTEASE INHIBITOR

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ABSTRACT

Objective: Ester conjugates of HIV protease inhibitor, lopinavir (LP) with various amino acids were synthesized to improve its physicochemical and pharmacokinetic profile and consequently therapeutic potential.

Materials and Methods: Conjugates of LP with amino acids; glycine, alanine, valine, and serine were prepared by dicyclohexylcarbodiimide coupling method. The synthesized compounds were characterized by nuclear magnetic resonance, mass, and Fourier-transform infrared spectroscopy and evaluated for their solubility, partition coefficient, hydrolytic stability, cytotoxicity, and permeability through Caco-2 cells.

Results: Aqueous solubility studies indicated significantly better solubility profiles of all conjugates as compared to LP. With respect to hydrolysis, all the conjugates displayed higher stability under acidic conditions while undergo hydrolysis with rise in pH. Conjugates did not exhibit cytotoxicity for concentration as high as 100 µg/ml, which indicates promising therapeutic potential. Absorptive diffusion of drug across Caco-2 cell monolayers was improved by amino acid conjugation.

Conclusion: Amino acid ester conjugates of LP not only showed better solubility but also significantly higher permeability than LP. Thus, direct conjugation of L-amino acids is a viable approach to improve oral absorption and thereby oral bioavailability of protease inhibitors.

Keywords: Amino acids, Conjugates, HIV, Hydrolysis, Lopinavir, Solubility.

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INTRODUCTION

Several strategies have been adopted for the effective treatment of HIV/AIDS since 1984. At present, the greatest success in the clinical management of HIV/AIDS has been observed with the use of antiretroviral drugs such as reverse transcriptase inhibitors and protease inhibitors (PIs). HIV PIs represent an important class of anti-HIV drugs in highly active antiretroviral therapy regimen. Although highly active, several unfavorable physicochemical and pharmacokinetic (PK) parameters limit their efficacy. Lopinavir (LP) is potent and one of the frequently administered PIs in the treatment of the HIV [1]. LP is classified as a BCS II drug (high permeability/low solubility). It has very poor bioavailability when administered orally which is due to low water solubility, limited intestinal uptake due to P-glycoprotein efflux and high first-pass metabolism by cytochrome P450 [2-5]. Hence to improve bioavailability, in most of the marketed formulations, it is given in combination with low-dose ritonavir (RTV) because RT is a well-known potent inhibitor of both P-gp and CYP3A [6-8]. However, coadministration of RT with other PIs leads to major adverse effects such as rise in lipid level, glucose, as well as gastrointestinal intolerance [9]. Hence, ritonavir free formulation strategy is needed.

Various strategies have been employed to inhibit both efflux and metabolism to improve oral absorption of PIs. Most common approaches used are coadministration of P-gp and MRP2 substrates with efflux inhibitors and a non-substrate strategy involving chemical modification of compounds such that efflux/metabolism is avoided. Transporter-targeted conjugate or prodrug derivatization is one of the non-substrate strategies which involve utilization of influx transporters facilitating transport of polar nutrients such as amino acids and peptides [10, 11]. This strategy has been successfully used in an attempt

to increase absorption of poorly absorbed drugs such as acyclovir [12], ganciclovir [13-15], saquinavir [16], and zanamivir [17]. Conjugates can be designed by coupling amino acids/peptides to compounds in such a way that they resemble the intestinal nutrients structurally and get easily absorbed by specific carrier proteins. Additional advantage of preparing such conjugates is the formation of non-toxic nutrient molecules when prodrugs are converted to parent drug and pro-moieties. As amino acids are biocompatible and easily ionizable, they have been widely used as pro-moieties in synthesis of prodrugs [18].

In the present study, conjugates of LP with amino acids, namely glycine, alanine, valine, and serine were synthesized with a simple esterification process to examine the effect of amino acids on solubility and in circumventing P-gp-mediated cellular efflux of LP. The synthesized conjugates were characterized by spectral data (ultraviolet [UV], IR, proton magnetic resonance, and mass) and evaluated for solubility, hydrolytic stability, and cytotoxicity. Transport studies were conducted in Caco-2 cells to compare permeability of conjugates with LP since efflux proteins (Pgp and MRP2) and peptide transporters are well expressed and characterized in Caco-2 cells.

MATERIALS AND METHODS

Materials

LP was obtained from Lupin Pharma Ltd. (Pune, India). Boc amino acids, dimethylaminopyridine and N, N'-dicyclohexylcarbodiimide, as well as all other reagents and solvents were commercially procured from Loba Chemicals Pvt. Ltd. (Mumbai, India). The purity of the synthesized compounds was confirmed by thin-layer chromatography (TLC) using precoated TLC plates (Merck, 20 × 20, 60F 254). Melting points were recorded in open capillary tubes and

are uncorrected. Fourier transform infrared (FTIR) spectra were recorded in Bruker FTIR spectrometer (Model - Alpha). ^1H nuclear magnetic resonance (NMR) spectra were recorded using Bruker AVANCE III 500 MHz (AV 500) multinuclei solution NMR spectrometer using CDCl_3 as the solvent and TOPSPIN 3.2 software. Electrospray ionization mass spectra were recorded on Bruker Impact II UHR-TOF mass spectrometer system by electron ionization technique. This method used in positive mode gives either $(\text{M} + \text{H})^+$ and/or $(\text{M} + \text{Na})^+$ signal. The UV spectrophotometer used for determining the partition coefficient and hydrolysis studies of the compounds was Shimadzu-1800 double beam spectrometer.

Methods

Synthesis of ester conjugates of lopinavir

Step I: Reaction of Boc protected amino acids with lopinavir

To a well stirred and cooled solution of LP (500 mg, 0.8 mmol) in dichloromethane, dimethylaminopyridine (190 mg, 1.6 mmol) was added to activate the secondary hydroxyl group of LP and continuously stirred for 10 min at 0°C under anhydrous conditions. Then, Boc amino acid (glycine, alanine, valine, and serine, 1.6 mmol) and N, N-dicyclohexylcarbodiimide (490 mg, 2.4 mmol) were added to the reaction mixture over 30 min. The reaction mixture was thereafter, allowed to stir at 0°C for 1 h and at room temperature for next 48 h. The reaction mixture was analyzed by TLC to ensure complete conversion of reactants to product. The mixture was filtered and after drying over Na_2SO_4 , the solvent was evaporated under reduced pressure at room temperature.

Step II: Deprotection of the N-Boc Group

Boc amino acid LP was treated with 1:1 TFA- CH_2Cl_2 mixture at 0°C for about 2 h. The solvent was evaporated under vacuum. The crude oily product, amino acid-LP, was purified by recrystallization in cold diethyl ether. The reaction scheme for the synthesis of LP-amino acid ester conjugates is shown in Scheme 1.

Characterization of conjugates

Conjugates were characterized with FTIR, ^1H NMR, and mass spectroscopy. The purity was determined using TLC and physicochemical parameters were determined.

Solubility study

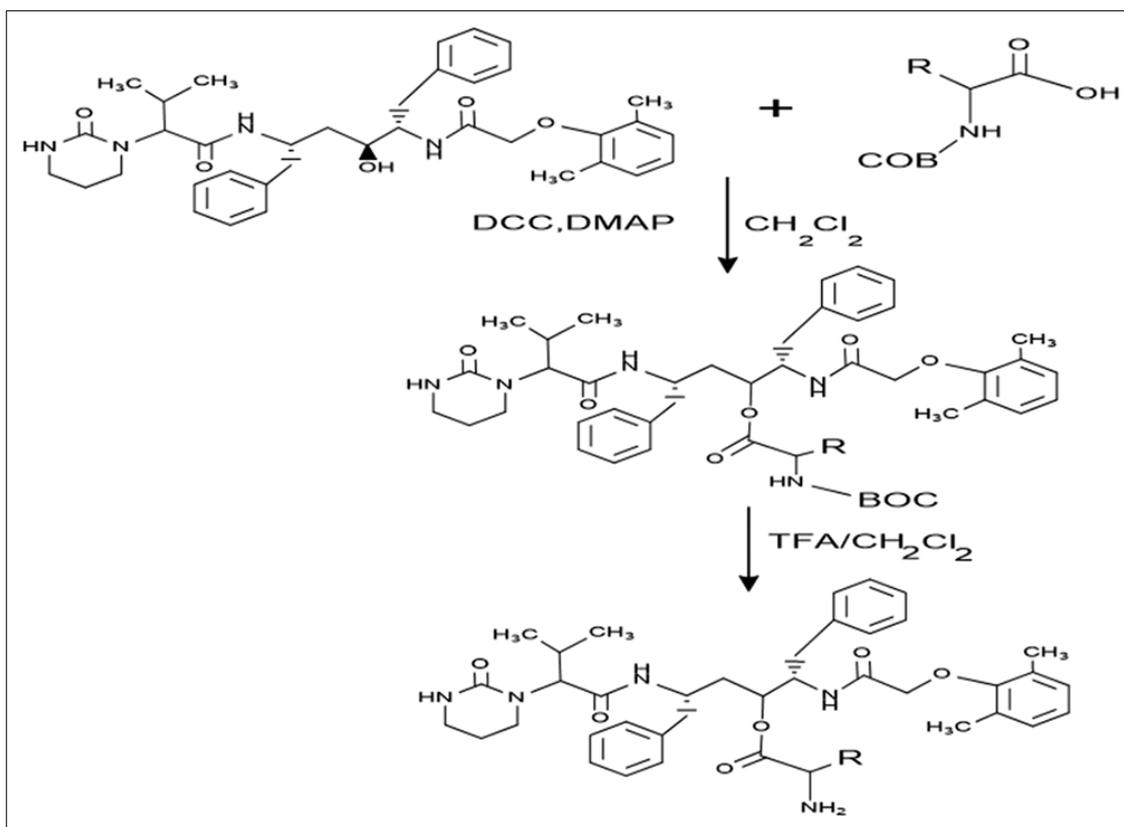
Saturated solutions of drug and conjugates were prepared in distilled water in 25 mL conical flasks. The sealed flasks were placed in a mechanical shaker at room temperature (RT) for 24 h. and were centrifuged for 10 min to separate undissolved conjugates. The supernatant was separated, filtered, and analyzed by UV spectrophotometer at 259 nm after appropriate dilutions. The samples were studied in triplicate and the results were presented as the mean values [19, 20].

Determination of partition coefficient

The partition coefficient of the LP and synthesized conjugates was determined by shake flask method in n-octanol and water. The drug/conjugate 10 mg was added to 10 ml of aqueous phase followed by addition of 10 ml of n-octanol. The contents were thoroughly shaken for 2 h at room temperature and left for 1 h. The conjugate concentration in aqueous phase and organic phase was determined by UV spectrophotometer at 259 nm and partition coefficient was calculated [21].

In vitro hydrolysis

The hydrolysis kinetics of conjugates was studied in aqueous buffer solution at pH 1.2 and pH 7.4 at 37°C using hydrochloric acid and phosphate buffer, respectively. Solutions of 10 mg of conjugate prepared in 90 mL of hydrochloric acid buffer (pH 1.2) or phosphate buffer (pH 7.4) were kept in screw-capped tubes and then placed in shaking water bath (60 rpm) at 37°C . At predetermined time points, aliquots were withdrawn from tubes and analyzed by UV spectrophotometer for the amount of drug released after the hydrolysis of prodrugs. Pseudo-first-order rate constants (Kobs) were calculated with equation, Kobs =



Scheme 1: Scheme for synthesis of lopinavir ester conjugates. R=H, $-\text{CH}_3$, $-\text{CH}(\text{CH}_3)_2$, $-\text{CH}_2\text{OH}$ (glycine, alanine, valine, and serine)

$2.303/t \times \log(a/a-x)$, where "a" is initial concentration of conjugate, "x" is the amount of conjugate hydrolyzed, and "t" is time in minutes. The corresponding half-life ($t_{1/2}$) was then obtained from the equation: $t_{1/2} = 0.693/K_{obs}$ [22, 23].

Cytotoxicity studies

Cytotoxicity of conjugates was determined in Caco-2 cells by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay based cytotoxicity kit. MTT is a pale yellow substrate which is cleaved by living cells to yield a dark blue formazan product. The amount of MTT cleaved is directly proportional to the number of viable cells present. Briefly, the compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with complete medium to a range of test concentration. DMSO concentration was kept <0.1% in all the samples. Caco2 cells were seeded in 96-well plates and treated with different concentrations of the test samples (5–200 µg/ml) and incubated at 37°C for 4 h. MTT reagent was added to each well and incubated for 2 h at 37°C. The dark blue formazan product formed by the cells was dissolved in DMSO and absorbance was read at 550 nm.

Permeability/transport studies

Transport studies were conducted with Caco-2 cell monolayers grown on 12 well Transwell® inserts. Cell monolayers were washed with Dulbecco's phosphate buffered saline (pH 7.4) twice for 15 min at 37°C. Transport studies were conducted across Caco-2 cells for a period of 3 h at 37°C. For determining apical-to-basolateral permeability (A-B), 0.5 mL LP or conjugate solution was added to the apical chamber of 12-well Transwell® plates while the basolateral side of the inserts contained 1.5 ml volume of transport buffer. Aliquots were withdrawn at predetermined time intervals (30, 60, 120, and 180 min) from the receiver chamber and replaced with fresh DPBS pH 7.4 to maintain sink conditions. The concentrations of the test compounds in the transport medium were immediately analyzed by UV spectrophotometer at a wavelength of 259 nm [24].

The apical-to-basolateral permeability coefficient (P_{app} in cm/second) was calculated according to the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}$$

Where, $\Delta Q/\Delta t$ is the rate of drug/conjugate flux (mol/s) from donor into the receiver compartment at steady state, A – the monolayer area (cm²), and C_0 – the initial concentration of drug/conjugate in apical compartment (mg/mL) [25].

Statistical analysis

Solubility, buffer stability, and cytotoxicity study results were expressed as mean ± standard deviation; Student's *t*-test was applied to determine the statistical significance among groups. $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Chemistry

The LP ester conjugates were synthesized by the dicyclohexylcarbodiimide coupling method. Amino acids, glycine, alanine, valine, and serine were identified as carriers for the synthesis of LP ester conjugates. All conjugates were subjected to solubility, physicochemical characterization, and hydrolytic studies. Purity of the synthesized prodrugs was ascertained by melting point and TLC. The products were obtained in good yields (78%–90%). The structures of compounds were confirmed by FTIR, ¹H NMR, and mass spectroscopic data. The IR spectra of these compounds show characteristics C = O stretching bands around 1731–1750 cm⁻¹ and C-O stretching bands around 1237–1243 cm⁻¹ for the ester functionality. The ¹H NMR spectra of synthesized conjugates show the chemical shift values and signals characteristic to the functionalities of the amino acids, leading to the confirmation of the structures assigned to the target conjugates.

The mass spectroscopic analysis gives the (M + H)⁺ peak confirming molecular weight of the targeted compounds.

Spectral data

(2S,5S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-(3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl glycinate lopinavir glycine conjugate (LPG)

%Yield: 90.1; Low melting solid; UV (λ_{max}): (MeOH) 261 nm, IR (cm⁻¹): 3342.74 (N-H str. amine), 3029.75 (C-H str. aromatic), 1750.03 (C = O str. ester), 1237.49 (C-O str. ester), 1558.02 (C = C str. aromatic) 1444.7 H-C-H Bend (asymmetric bend,-CH₃) ¹H NMR (500 MHz, CDCl₃) δ 0.75–0.86(m, 6H), 1.25–1.32(m, 2H), 1.68–1.71 (d,2H), 1.88–1.90 (d, 2H), 2.87–2.88 (m, 2H), 3.17–3.21 (m,3H), 3.64–3.69 (m, 2H), 3.71–3.85 (m,3H), 6.85–7.10 (m, 3H), 7.14–7.34 (m, 10H) electrospray ionization mass spectrometry (ESI-MS): 686.4 (M + H)⁺.

(2S,5S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-(3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl alaninate lopinavir alanine conjugate (LPA)

%Yield: 87.4; Low melting solid; UV (λ_{max}): (MeOH) 259 nm, IR (cm⁻¹): 3327.92 (N-H str. amine), 2927.44 (C-H str. aromatic), 1731.49 (C = O str. ester), 1237.52 (C-O str. ester), 1521.50 (C = C str. aromatic) 1447.91 H-C-H Bend (asymmetric bend,-CH₃) ¹H NMR (500 MHz, CDCl₃) δ 0.80–0.85(d, 6H), 1.25 (d, 3H), 1.72–1.75(d, 2H), 1.88–1.91(m,2H), 2.15–2.18 (m, 6H), 2.71–2.87 (m,4H), 2.90 (d,2H) 3.12–3.19 (m,2H), 6.69–6.71(d, 1H), 6.85–6.87(d, 3H), 6.96–7.28(m, 10H) ESI-MS: 700.4 (M + H)⁺.

(2S,5S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-(3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl valinate lopinavir valine conjugate (LPP)

%Yield: 85.7; Low melting solid; UV (λ_{max}): (MeOH) 260 nm, IR (cm⁻¹): 3320.06 (N-H str. amine), 3066.04 (C-H str. aromatic), 1735.32 (C = O str. ester), 1243.00 (C-O str. ester), 1516.58 (C = C str. aromatic), 1446.18 H-C-H Bend (asymmetric bend,-CH₃) ¹H NMR (500 MHz, CDCl₃) δ 1.14–1.16 (d, 6H), 1.25–1.34 (d, 6H), 1.72–1.74(d, 2H), 1.86–1.92 (m, 6H), 2.11(s, 6H), 2.27–2.31(s,2H), 3.75(s, 2H), 4.05–4.26 (m,2H), 4.39 (s,1H), 6.71–6.82 (m, 3H), 6.95–7.27(m, 10H) ESI-MS: 728.4 (M + H)⁺.

(2S,5S)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-(3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl)butanamido)-1,6-diphenylhexan-3-yl serinate lopinavir serine conjugate (LPS)

%Yield: 78.5; Low melting solid; UV (λ_{max}): (MeOH) 261 nm, IR (cm⁻¹): 3320.42 (N-H str. amine), 2928.76 (C-H str. aromatic), 1739.34 (C = O str. ester), 1239.23 (C-O str. ester), 1565.58 (C = C str. aromatic) 1446.18 H-C-H Bend (asymmetric bend,-CH₃) ¹H NMR (500 MHz, CDCl₃) δ 0.81–0.83 (d,6H), 1.72–1.75(d, 2H), 1.90–1.92 (d, 2H), 2.12–2.19(m,1H), 2.29 (s,2H), 3.03–3.10 (m,3H), 3.18–3.20 (m,1H), 3.50–3.43 (m, 2H), 3.70(s,2H), 6.71–6.72 (d,1H), 6.93–7.00 (m,2H), 7.13–7.17(m,1H), 7.20–7.30(m,10H) ESI-MS: 716.4 (M + H)⁺.

Solubility and partition coefficient

Solubility studies were carried out in distilled water. All amino acid conjugates exhibited higher solubility compared to parent drug. The observed increase in aqueous solubility of the investigated conjugates was accompanied by a decrease in lipophilicity relative to the parent drug (Table 1). Hence, it is evident that esterification of the hydroxyl group with different amino acids modifies the physicochemical properties of LP. Serine conjugate of LP showed maximum solubility and minimum partition coefficient relative to other amino acid conjugates.

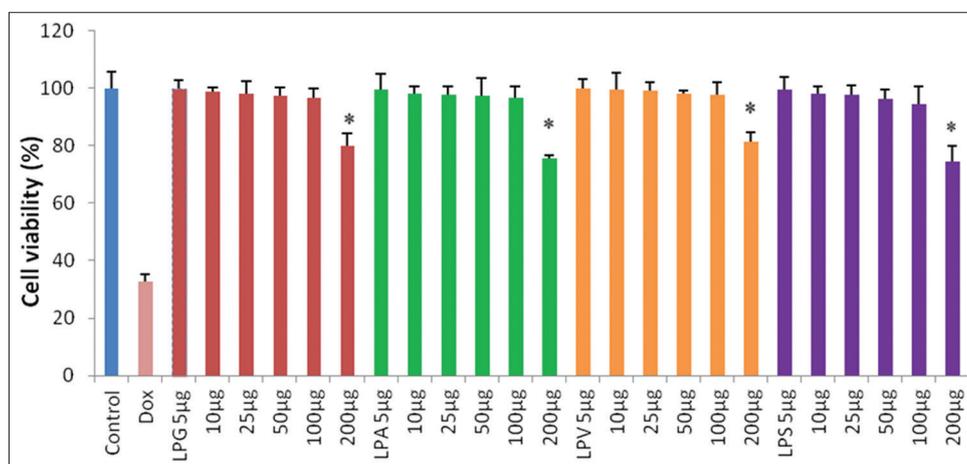
Chemical stability

Hydrolysis kinetics of LP amino acid conjugates was studied in aqueous buffer solution at pH 1.2 and pH 7.4. Conjugates showed negligible hydrolysis in acidic buffer solution (data not shown) compared to phosphate buffer pH 7.4. All the conjugates displayed higher stability under acidic conditions while undergo base-catalyzed hydrolysis. The

Table 1: Physicochemical and kinetic data of lopinavir and its conjugates in aqueous phosphate buffer solution

Compound	Molecular formula	Molecular weight	Rf value*	Log p	Solubility (mg/ml)	pH 7.4	
						Kobs (h ⁻¹)	t _{1/2} (h)
LP	C ₃₈ H ₅₂ N ₆ O ₇	628.80	0.74	5.31	0.045±0.005		
LPG	C ₄₀ H ₅₅ N ₇ O ₈	685.85	0.82	3.94	0.63±0.02	0.1394	4.97±0.2
LPA	C ₄₁ H ₅₇ N ₇ O ₈	699.87	0.83	3.16	0.59±0.01	0.1239	5.59±0.4
LPV	C ₄₃ H ₆₁ N ₇ O ₈	727.93	0.85	4.09	0.90±0.06	0.1136	6.10±0.4
LPS	C ₄₀ H ₅₃ N ₅ O ₇	715.87	0.79	3.02	1.35±0.12	0.1283	5.40±0.3

*Mobile phase for Thin layer chromatography of lopinavir and its conjugates is ethyl acetate: methanol (9.8:0.2). TLC: Thin-layer chromatography, LP: Lopinavir, LPG: Lopinavir glycine conjugate, LPA: Lopinavir alanine conjugate, LPV: Lopinavir valine conjugate, LPS: Lopinavir serine conjugate

**Fig. 1: Cytotoxicity result of lopinavir conjugates in Caco-2 cells. *Statistically significant. P < 0.05 with respect to control**

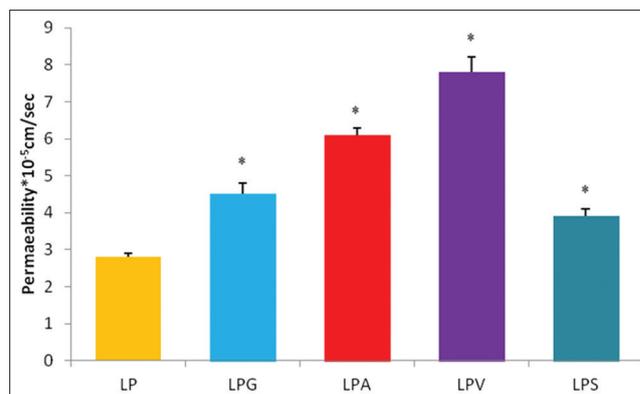
chemical degradation of ester conjugates of LP followed the first-order kinetics. The rate constants (Kobs) and the corresponding half-lives (t_{1/2}) for the respective conjugates were calculated from the linear regression equations correlating the log concentration of the residual conjugates versus time. The results are summarized in Table 1. Degradation half-lives of conjugates at pH 7.4 ranged between 4.9 and 6.1 h.

Cytotoxicity studies

Cytotoxicity of conjugates was determined in Caco-2 cells with MTT assay to check their safety. Results obtained from this study are demonstrated in Fig. 1. Blank medium (without any drug) was used as control. Doxorubicin was used as positive control. DMSO concentration was kept < 0.1% in all the samples. Conjugates did not exhibit any cell cytotoxicity up to a concentration of 100 µg/ml. However, they were observed to be significantly cytotoxic at 200 µg/ml. Based on these results, to prevent cytotoxic effects of conjugates, permeability studies were carried out at concentrations of ≤50 µg/ml.

Permeability study

Human colon adenocarcinoma cell line (Caco-2) is one of most frequently used and best-established cell lines for the determination of drug permeability across intestinal membranes. Caco-2 cells have been reported to express both P-gp and peptide transporters. Hence, the permeability of LP and its ester conjugates was estimated across the apical cell layer of this cell line. Apparent permeability generated by glycine, alanine, valine, and serine conjugates of LP across Caco-2 cells in A-B direction was 4.5×10^{-5} , 6.1×10^{-5} , 7.0×10^{-5} and 3.9×10^{-5} cm/s, respectively, whereas for LP, it was found to be 2.9×10^{-5} cm/s. Apical-to-basolateral permeability of LPG, LPA, LPV, and LPS was about 1.6, 2.2, 2.5 and 1.4-fold higher relative to LP (Fig. 2). All the amino acid conjugates tested showed higher permeability compared to LP which may be due to their reduced recognition by efflux carriers (P-gp) compared to LP. This study also indicated that synthesized amino acid ester conjugates are good substrates for the amino acid transporters expressed on the intestinal barrier and, hence, may get translocated efficiently resulting in higher oral bioavailability.

**Fig. 2: Permeability of conjugates as compared to drug in Caco-2 cells. *Statistically significant. P < 0.05 with respect to control (lopinavir)**

CONCLUSION

The present study utilizes amino acids to prepare ester conjugates of LP to improve its physicochemical and PK profile and consequently therapeutic effectiveness. Direct conjugation of amino acids not only improved solubility but also led to enhancement in absorptive flux of LP across Caco-2 cells. This study confirms that the amino acid conjugation approach has the potential to improve oral absorption and thereby oral bioavailability of PIs.

AUTHORS' CONTRIBUTIONS

Preeti Gandhi has carried out experiments and analyzed results. Dr. A. R. Chabukswar guided this research work. The authors are thankful to the principal and management of JSPM's Jayawantrao Sawant College of Pharmacy and Research and Maharashtra Institute of Pharmacy, Pune, for providing necessary facilities to carry out present research work.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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