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 expressed and characterized in Caco-2 cells.

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-dicyclohexylcarbodimide, 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
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 CDCl₃ 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 (M + H)⁺ and/or (M + 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 Na₂SO₄, 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-CH₂Cl₂ mixture at 0°C for about 2 h. The solvent was evaporated under reduced pressure and dried over Na₂SO₄. The mixture was filtered and after drying over Na₂SO₄, the solvent was evaporated under reduced pressure at room temperature.

**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 =

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Scheme 1: Scheme for synthesis of lopinavir ester conjugates. R = H, -CH₂-C (CH₃)₂-C₂H₂OH (glycine, alanine, valine, and serine)
signals characteristic to the functionalities of the amino acids, leading to 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-diphenyhexan-3-yl glycinate lopinavir glycine conjugate (LPG)

- \(\%Yield: 78.5; Low melting solid; UV (\(\lambda_{max}\)) (MeOH) 261 nm, IR (cm\(^{-1}\))
- 3432.74 (N-H str. amine), 3066.04 (C-H str. aromatic), 1739.32 (C = O str. ester), 1283.52 (C-O str. ester), 1447.91(C = C str. aromatic)
- \(\delta 0.75–1.26 (m, 3H), 1.85–2.13 (m, 3H), 3.90–4.27 (m, 2H), 7.08–7.17 (m, 3H), 7.30–7.35 (m, 3H), 7.52–7.57 (m, 3H), 7.60–7.63 (m, 3H)\)

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

- \(\%Yield: 85.7; Low melting solid; UV (\(\lambda_{max}\)) (MeOH) 260 nm, IR (cm\(^{-1}\))
- 3320.06 (N-H str. amine), 3066.04 (C-H str. aromatic), 1739.32 (C = O str. ester), 1283.52 (C-O str. ester), 1447.91(C = C str. aromatic)
- \(\delta 0.75–1.26 (m, 3H), 1.85–2.13 (m, 3H), 3.90–4.27 (m, 2H), 7.08–7.17 (m, 3H), 7.30–7.35 (m, 3H), 7.52–7.57 (m, 3H), 7.60–7.63 (m, 3H)\)

### 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

**RESULTS AND DISCUSSION**

### Chemistry

The LP ester conjugates were synthesized by the dicarboxylic anhydride 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 compounds was 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 signal characteristics to the functionalities of the amino acids, leading to the formation of the structures assigned to the target conjugates.

**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 hydrophobic 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Rf value*</th>
<th>Log p</th>
<th>Solubility (mg/ml)</th>
<th>pH 7.4 Kobs (h⁻¹)</th>
<th>t1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP</td>
<td>C₃₈H₅₆N₂O₇</td>
<td>628.80</td>
<td>0.74</td>
<td>5.31</td>
<td>0.045±0.005</td>
<td>0.1394</td>
<td>4.97±0.2</td>
</tr>
<tr>
<td>LPG</td>
<td>C₄₀H₅₅N₇O₈</td>
<td>685.85</td>
<td>0.82</td>
<td>3.94</td>
<td>0.63±0.02</td>
<td>0.1239</td>
<td>5.59±0.4</td>
</tr>
<tr>
<td>LPA</td>
<td>C₄₀H₅₇N₇O₈</td>
<td>699.87</td>
<td>0.83</td>
<td>3.16</td>
<td>0.59±0.01</td>
<td>0.1136</td>
<td>6.10±0.4</td>
</tr>
<tr>
<td>LPV</td>
<td>C₄₁H₆₁N₇O₈</td>
<td>727.93</td>
<td>0.85</td>
<td>4.09</td>
<td>0.90±0.06</td>
<td>0.1283</td>
<td>5.40±0.3</td>
</tr>
<tr>
<td>LPS</td>
<td>C₄₀H₅₃N₅O₇</td>
<td>715.87</td>
<td>0.79</td>
<td>3.02</td>
<td>1.35±0.12</td>
<td>0.1239</td>
<td>5.59±0.4</td>
</tr>
</tbody>
</table>

Molecular formula for LPG: C₄₀H₅₅N₇O₈

*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

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.

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.

REFERENCES