Available online on 15.05.2019 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

Open Access Research Article

Amino Acid Conjugation: An Approach to Enhance Aqueous Solubility and Permeability of Poorly Water Soluble Drug Ritonavir

Dr. Chabukswar Anuruddha R. [1], Dr Jagdale S.C. [1], Gandhi Preeti M.[2]*

[1] Department of Pharmaceutical Chemistry, MAEER'S Maharashtra Institute of Pharmacy, Pune-411038, India

[2] Department of Pharmaceutical Chemistry, JSPM's Jayawantrao Sawant College of Pharmacy and Research, Hadapsar, Pune-411028.

ABSTRACT

Objective: The objective of present work is to improve physicochemical and pharmacokinetic profile of poorly water soluble HIV protease inhibitor, ritonavir (RT) by preparing its amino acid conjugates.

Methods: Ester conjugates of ritonavir with various amino acids were synthesized by a simple esterification process using dicyclohexyl carbodiimide (DCC) as a coupling agent. The synthesized compounds were characterized by thin layer chromatography (TLC), fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and mass spectroscopy. All conjugates were evaluated by saturation solubility and hydrolytic stability studies. Cytotoxicity and permeability studies were conducted using Caco-2 cell line.

Results: All amino acid conjugates showed a significantly higher aqueous solubility compared to the pure RT. With respect to hydrolysis, alkaline hydrolysis of conjugates was rapid relative to acidic hydrolysis. No cytotoxicity was shown by conjugates for concentration as high as 100µg/ml, which indicates promising therapeutic potential. Permeability of RT across Caco-2 cell monolayers was improved by amino acid conjugation.

Conclusion: In vitro studies demonstrated that amino acid conjugation of RT may be an effective strategy to improve its aqueous solubility as well as permeability and can be used to improve oral absorption and thereby oral bioavailability of protease inhibitors.

Keywords: Ritonavir, amino acids, conjugates, solubility, HIV, hydrolysis

Article Info: Received 29 March 2019; Review Completed 04 May 2019; Accepted 07 May 2019; Available online 15 May 2019

Cite this article as:

Chabukswar AR, Jagdale SC, Gandhi PM, Amino Acid Conjugation: An Approach to Enhance Aqueous Solubility and Permeability of Poorly Water Soluble Drug Ritonavir, Journal of Drug Delivery and Therapeutics. 2019; 9(3):252-256
http://dx.doi.org/10.22270/jddt.v9i3.2848

***Address for Correspondence:**

Preeti M Gandhi, Department of Pharmaceutical Chemistry, JSPM's Jayawantrao Sawant College of Pharmacy and Research, Hadapsar, Pune-411028.

INTRODUCTION

Solubility behaviour of a drug is one of the key determinants of its oral bioavailability. In the pharmaceutical analysis and formulation development fields, it is most often required to increase the aqueous solubility of poorly water soluble drugs[1]. Currently, 60% of of all newly developed drugs are poorly water soluble or lipophilic in nature[2,3]. When dosed orally, insoluble drugs are not efficiently absorbed from the solid state and require specialized formulations to enhance their solubility in the gastrointestinal tract. Several techniques used to overcome challenges of poor aqueous solubility, dissolution rates and insufficient bioavailability are reduction of particle-size which includes microsizing and nanosizing, salt formation, conjugate or prodrug formation, complexation ,solid-dispersions, use of surfactants, polymorphism and micellization[4-14]. The conjuation of a drug to different carriers which is also called as a prodrug approach is a promising molecular modification by which drug pharmacokinetics, pharmacodynamics and toxicology can be modulated. Undesirable properties, including poor aqueous solubility, chemical instability, insufficient oral or local absorption, fast pre-systemic metabolism, low half-life, toxicity and local irritation are commonly resolved using the conjugation or prodrug approach. In addition, problems related to drug formulation and delivery can also be overcome by using this strategy[15]. There are around 10% marketed medicines that can be categorized as prodrugs[16-18].

Transporter targeted conjugate preparation or prodrug derivatization is one of the strategies which involve utilization of influx transporters which facilitate transport of polar nutrients such as amino acids and peptides[19,20]. This strategy has been successfully used in an attempt to increase solubility and absorption of poorly absorbed drugs such as lopinavir[20], saquinavir[21], acyclovir[22], ganciclovir[23-25] and zanamivir[26]. Conjugates can be designed by coupling amino

acids/peptides to compounds in a way to resemble the intestinal nutrients structurally and get absorbed by specific carrier proteins. Amino acids are normal dietary constituent and they are nontoxic in moderate doses as compared to other promoieties or carriers. The amino acid prodrugs improve oral delivery of drugs which have poor permeability and solubility.

Ritonavir (RT) is a widely used, HIV-1 specific protease inhibitor which belongs to Class IV in the Biopharmaceutics Classification System. It is practically insoluble in water and exhibits slow intrinsic dissolution rate. Though ritonavir has favorable lipophilicity, *in vitro* permeability studies have shown that it is a substrate of P-glycoprotein. Thus, the oral absorption of ritonavir could be limited by both dissolution and permeability[27,28]. In the present study, amino acid ester conjugates of ritonavir were synthesized with a simple esterification process. These conjugates were designed to examine the effect of amino acids on solubility and permeability of RT. These conjugates were characterized by their melting points, NMR, mass, FTIR spectroscopy and elemental analysis. Solubility in distilled water was determined followed by cytotoxicity determinations. Transport studies were conducted in Caco-2 cells to compare permeability of conjugates with RT.

MATERIALS AND METHODS

Materials:

Ritonavir was obtained from Lupin Pharma Ltd (Pune, India). Boc amino acids, dimethylaminopyridine and N, N'-dicyclohexyl carbodimide, as well as all other reagents and solvents were commercially procured from Loba Chemicals Pvt. Ltd. (Mumbai, India). The purity of the synthesized compounds were confirmed by thin layer chromatography using precoated TLC plates (Merck, 20×20, 60F 254). Melting points were recorded in open capillary tubes and are uncorrected. FTIR spectra were recorded in Bruker FT-IR spectrometer (Model – Alpha).^{1}H NMR spectra were recorded using Bruker AVANCE III 500 MHz (AV 500) multi nuclei solution NMR Spectrometer using $CDCl_3$ as the solvent and TOPSPIN 3.2 software. Electron-spray ionization mass spectra (ESI–MS) were recorded on Bruker Impact II UHR-TOF Mass spectrometer system by electron ionization (EI) technique. This method used in positive mode gives either $(M+H)^+$ and/or $(M+Na)^+$ signal.

Methods:

Synthesis of ester conjugates of ritonavir

Step I: Reaction of Boc protected amino acids with lopinavir:

To the well stirred and cooled solution of ritonavir (0.57 g, 0.8 mmol) in dichloromethane dimethylaminopyridine (0.19 g, 1.6 mmol) was added to activate the secondary hydroxyl group of RT and continuously stirred for 10min at 0°C under anhydrous conditions. Then Boc-amino acid (valine, glycine, alanine and serine 1.6 mmol) and N, N-Dicyclohexyl carbodimide (0.49 g, 2.4 mmol) were added to the reaction mixture. The mixture was stirred at 0°C for one hour and at room temperature for the next 90 hours. To ensure complete conversion of reactants to product the reaction mixture was analysed by TLC. The mixture was filtered and dried over Na_2SO_4. The solvent was evaporated under reduced pressure at room temperature to obtain dried product.

Step II: Deprotection of the N-Boc Group

Boc-amino acid-RT was treated with 1:1 trifluroacetic acid/ dicholoromethane (TFA/CH_2Cl_2) mixture at 0°C for about 2 h. The solvent was evaporated under vacuum. The crude oily product, amino acid-RT, was purified by recrystallization from cold diethyl ether.

Characterization of conjugates

Conjugates were characterized by FT-IR, ^{1}H 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 and kept at room temperature (RT) for 24 h in a mechanical shaker. Solutions were centrifuged for 10 min to separate undissolved conjugates. The supernatant was separated, filtered and analyzed by UV spectrophotometer at 239nm after appropriate dilutions[29]. The samples were studied in triplicate and the results were presented as the mean values.

In Vitro Hydrolysis Study:

Chemical hydrolysis study of prodrugs was carried out in an aqueous buffer solution at pH 1.2 and pH 7.4 at 37 °C using hydrochloric acid and phosphate buffer respectively and degradation rate constant and half-life (t1/2) of conjugates were determined. 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 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 conjugates. Pseudo first order rate constants for the degradation (Kobs) and half-life (t1/2) were calculated[30,31].

Cytotoxicity Studies

MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay was carried out to determine cytotoxicity of conjugates in Caco-2 cells. The amount of MTT cleaved is directly proportional to the number of viable cells present, which is quantified by colorimetric methods. Briefly the compounds were dissolved in DMSO and serially diluted with complete medium to get the concentrations a range of test concentration. DMSO concentration was kept < 0.1% in all the samples. Caco2 cells maintained in appropriate conditions were seeded in 96 well plates and treated with different concentrations of the test samples (5–200 µg/ml) and incubated at 37 °C, 5% CO2 for 4 hours. MTT reagent was added to the wells and incubated for 2 hours at 37 °C; the dark blue formazan product formed by the cells was dissolved in DMSO. Cell viability was determined by measuring absorbance at 550nm.

The quantity of formazan product as measured by absorbance is directly proportional to the number of viable cells in test samples.

Permeability/Transport studies:

Permeability studies were conducted across Caco-2 cell monolayers grown on 12 well Transwell® inserts. Cell monolayers were washed thoroughly with DPBS (pH7.4) for 15 min at 37 °C, three times. 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 RT or conjugate solution was added to the apical chamber and 1.5ml of transport buffer was added to the basolateral side of the Transwell® plates. 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 spectrophotometry at a wavelength of 239nm [32].

The apical-to-basolateral permeability coefficient (Papp in cm/second) was calculated according to following equation:

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

where Q is the cumulative amount of drug/conjugate appearing in the basolateral compartment, A is the surface area of insert(cm^2), C_o – the initial concentration of drug/conjugate in apical compartment (mg/mL)[33].

Statistical Analysis

Solubility and cytotoxicity study results were expressed as mean ± S.D. Student's t-test was applied to determine the statistical significance among groups. P values ≤0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Chemistry

The ritonavir ester conjugates were synthesized by dicyclohexyl carbodiimide (DCC) coupling method using dimethylaminopyridine as a catalyst. Amino acids, glycine, alanine, valine and serine were identified as carriers for the synthesis of conjugates. All conjugates were evaluated by solubility, physicochemical characterization and hydrolytic studies. Purity of synthesized conjugates was determined by thin layer chromatography (TLC). The products were obtained in good yields (82-90%). The structures of compounds were confirmed by FTIR, 1H NMR and Mass spectroscopic data. The IR spectra of these compounds show characteristic C=O stretching bands around 1730–1740 cm^{-1} and C-O stretching bands around 1223–1251 cm^{-1} for the ester functional group. 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:

(2*S*,3*S*,5*S*)-5-(2-(3-((2-isopropylthiazol-4-yl)methyl)-3-methylureido)-3 methylbutanamido)-1,6-diphenyl-2-(((thiazol-5ylmethoxy)carbonyl)amino)hexan-3-yl glycinate (RTG)

%Yield: 90.2; **UV (λmax)**: (MeOH) 239 nm, **IR (cm^{-1}):** 3323.51 (N-H str. amine), 2969.66(C-H str. aromatic), 1735.25 (C = O str. ester), 1223.88 (C-O str. ester), 1642.26, 1520.59 (C = C str. aromatic) **^{1}H NMR (500 MHz, $CDCl_3$)** δ 0.84-0.88 (d,6H), 1.02-1.07(d,6H), 1.25-1.29 (d,6H), 1.62-1.64 (d,2H), 1.93-1.94 (d,1H), 2.10 (d,1H), 2.93-2.94 (d,2H), 3.21 (s,2H), 3.47 (d,1H),4.04-4.41 (m,3H), 5.19 (s,2H), 6.71-6.73 (d,1H), 7.13-7.27 (m,10H), 7.85-7.86 (d,1H), 8.80 (s,1H) **ESI-MS:** 778.7 $(M+H)^+$

(2*S*,5*S*)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-(3-methyl-2-(2-oxotetrahydropyrimidin-1(2*H*)-yl)butanamido)-1,6-diphenylhexan-3-yl alaninate (RTA)

%Yield: 84.4; **UV (λmax):** (MeOH) 238 nm, **IR (cm^{-1}):** 3342.75 (N-H str. amine), 2966.47 (C-H str. aromatic), 1738.16 (C = O str. ester), 1251.60 (C-O str. ester), 1640.65, 1516.39 (C = C str. aromatic) **^{1}H NMR (500 MHz, $CDCl_3$)** δ 0.85-0.91 (d, 6H), 1.11-1.18(d, 6H), 1.68-1.72 (d, 2H), 1.91-1.93(d,2H), 2.69 (s,3H), 2.92-2.93 (d,2H), 3.21 (s,2H), 3.44-3.45(s,1H,OH), 3.62-3.66 (d,2H), 5.27-5.29(d,1H), 6.72-6.74 (d,1H),7.20-7.26 (m,10H),7.78-7.79 (d,1H), 8.70-8.71 (d,1H) **ESI-MS**: 792.9 $(M+H)^+$

(2*S*,5*S*)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-(3-methyl-2-(2-oxotetrahydropyrimidin-1(2*H*)-yl)butanamido)-1,6-diphenylhexan-3-yl valinate (RTV)

%Yield: 82.6; **UV (λmax):** (MeOH) 238 nm, **IR (cm^{-1}):** 3338.44 (N-H str. amine), 2965.64 (C-H str. aromatic), 1740.44 (C = O str. ester), 1230.71 (C-O str. ester), 1520.99(C = C str. aromatic) **^{1}H NMR** (500 MHz, $CDCl_3$) 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**: 821.38 $(M+H)^+$

(2*S*,5*S*)-2-(2-(2,6-dimethylphenoxy)acetamido)-5-(3-methyl-2-(2-oxotetrahydropyrimidin-1(2*H*)-yl)butanamido)-1,6-diphenylhexan-3-yl serinate (RTS)

%Yield: 85.3; **UV (λmax):** (MeOH) 239 nm, **IR (cm^{-1}**): 3413.33 (N-H str. amine), 3288.55 (O-H str), 2966.47(C-H str. aromatic), 1730.58 (C = O str. ester), 1251.60 (C-O str. ester), 1640.65, 1516.39 (C = C str. aromatic) **^{1}H NMR** (500 MHz, $CDCl_3$) 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:** 808.9$(M+H)^+$

Solubility study:

Solubility studies were carried out in distilled water. All amino acid conjugates of ritonavir exhibited higher solubility compared to parent drug (Table 1). Serine conjugate of ritonavir showed maximum water solubility. Currently, RT is administered to HIV-1 infected patients in the form of approximately 43% v/v alcoholic solution (NORVIR) as it is practically insoluble in water. Such high alcohol concentrations may not be suitable for pediatric patients. In contrast, amino acid conjugates might offer potential advantages due to their higher solubility relative to RT.

Table 1: Physicochemical and kinetic data for the hydrolysis of ritonavir conjugates at different pH at 37^{0}C

Compound	Rf Value*	Solubility (mg/ml)	pH 1.2		pH 7.4	
			Kobs (h^{-1})	t1/2 (h)	Kobs (h^{-1})	t1/2 (h)
RT	4.12	0.025± 0.001				
RTG	2.69	0.37 ± 0.05	0.046	14.86	0.096	7.19
RTA	3.15	0.35 ± 0.03	0.039	17.35	0.075	9.14
RTV	3.32	0.58 ± 0.04	0.032	21.40	0.072	9.51
RTS	2.19	0.75 ± 0.06	0.050	13.84	0.107	6.45

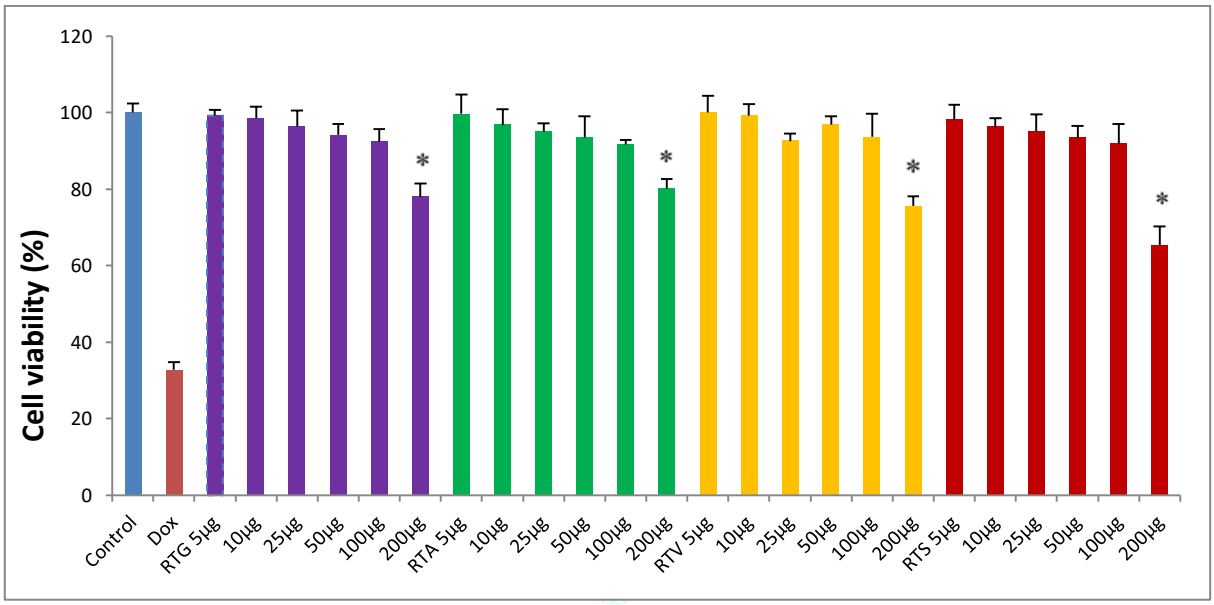
*TLC (Chloroform:Ethyl acetate:Acetone (50:20:30).

Chemical Stability:

Amino acid ester conjugates of ritonavir degraded rapidly at higher pH relative to lower pH. All the conjugates displayed higher stability under acidic conditions while undergo base catalyzed hydrolysis. The hydrolytic reconversion rate to parent drug increased with decreasing side chain length as well as increasing pH. The chemical degradation of ester conjugates of ritonavir followed 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 vs. time. Degradation half-lives of conjugates at pH 1.2 ranged between 13.8-21.4h while at pH 7.4 ranged between 6.4-9.5h. The results are summarized in Table 1.

Cytotoxicity Studies

Cytotoxicity of RT and conjugates was determined in Caco-2 cells with MTT assay. Results obtained from this study are demonstrated in fig. 1. Blank medium (without any drug) was used as control. Doxorubicin (500µg/ml) was used as positive control. DMSO concentration was kept < 0.1% in all the samples. Conjugates did not exhibit any cell cytotoxicity upto a concentration of 100 µg/ml while RT generated cytotoxic effects at 100 µg/ml. Conjugates were found to be significantly cytotoxic at 200 µg/ml. Based on these results permeability studies were carried out at concentrations ≤50 µg/ml to prevent cytotoxic effects of conjugates.

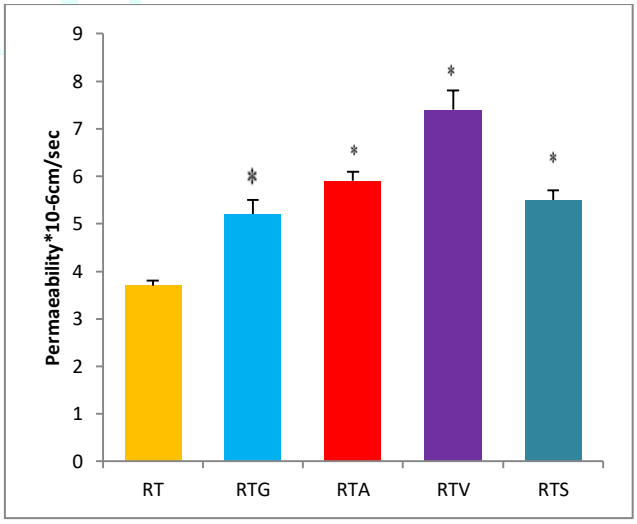


*** Statistically significant. P < 0.05 with respect to control.**

Figure 1: Cytotoxicity result of ritonavir conjugates in Caco-2 cells.

Permeability study:

The permeability of RT and its ester conjugates was estimated across the apical cell layer of human colon adenocarcinoma cell line (Caco-2). Caco-2 is one of most frequently used cell lines for the determination of drug permeability across intestinal membranes. Caco-2 cells have been reported to express P-gp and peptide transporters. Apparent permeability generated by ritonavir across Caco-2cells in A-B direction was 3.7×10^{-6} whereas for glycine, alanine, valine and serine conjugates were 5.2×10^{-6}, 5.9×10^{-6}, 7.4×10^{-6} and 5.5×10^{-6} cm/s respectively. Apical to basolateral permeability of RTG, RTA, RTV and RTS was about 1.4, 1.6, 2.0 and 1.5-fold higher relative to RT (fig.2). All the amino acid conjugates tested showed higher permeability compared to RT which may be due to their reduced recognition by efflux carriers (P-gp) relative to RT. Thus it is concluded that synthesized amino acid ester conjugates are good substrates for the amino acid transporters expressed on the intestinal barrier and may be translocated efficiently resulting in higher oral bioavailability.



***Statistically significant. P < 0.05 with respect to control (RT).**

Figure 2: Permeability of conjugates as compared to drug in Caco-2 cells.

CONCLUSION

The present study utilizes amino acids to prepare ester conjugates of ritonavir in order to improve its physicochemical and pharmacokinetic profile and consequently therapeutic effectiveness. Direct conjugation of amino acids not only improved solubility but also led to enhancement in absorptive flux of ritonavir. This study confirms that the amino acid conjugation approach has the potential to improve oral absorption and thereby oral bioavailability of protease inhibitors.

ACKNOWLEDGEMENTS

The authors are thankful to the Principal & management of JSPM's Jayawantrao Sawant College of Pharmacy & Research and Maharashtra Institute of Pharmacy, Pune for providing necessary facilities to carry out present research work.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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