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Evasion of P-gp mediated cellular efflux and permeability enhancement of HIV-protease inhibitor saquinavir by prodrug modification

Ritesh Jain, Sheetal Agarwal, Soumyajit Majumdar, Xiaodong Zhu, Dhananjay Pal, Ashim K. Mitra *

Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 5005 Rockhill Road, Kansas City, MO 64110-2499, USA

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Abstract

P-glycoprotein (P-gp) is an efflux pump responsible for limiting oral bioavailability, tissue penetration and increasing metabolism of the HIV protease inhibitor saquinavir (SQV). The objective of this study is to investigate whether prodrug derivatization of SQV to novel dipeptide prodrugs Val–Val–saquinavir (Val–Val–SQV) and Gly–Val–saquinavir (Gly–Val–SQV) targeting peptide transporters can enhance cellular permeability of saquinavir and modulate P-gp mediated efflux. Uptake and transport studies were conducted employing MDCKII-MDRI cell line at 37 °C for 10 min and 3 h, respectively. Uptake of [^{3}H]ritonavir and [^{3}H]erythromycin, utilized as model P-gp substrates, was carried out in the presence of inhibitory concentration of SQV and its peptide prodrugs. Bidirectional transport studies were conducted on MDCKII-MDR1 cells grown over membrane inserts.

Uptake of [^{3}H]erythromycin by MDCKII-MDR1 cells exhibited a four-fold increase in the presence of 75 μM SQV. However, equimolar concentrations of Val–Val–SQV and Gly–Val–SQV showed only 2.5-fold increase in [^{3}H]erythromycin uptake. Concentration dependent inhibition of [^{3}H]glycylsarcosine (Gly-Sar), a model peptide transporter substrate, was observed in the presence of SQV prodrugs. Transepithelial transport studies of Val–Val–SQV and Gly–Val–SQV exhibited an enhanced absorptive flux and reduced secretory flux relative to studies employing SQV. These results are very likely due to decreased efflux of SQV dipeptide prodrugs by P-gp. Peptide prodrug derivatization constitutes an exciting strategy to improve intestinal absorption and oral bioavailability of SQV.

Keywords: Saquinavir (SQV); P-glycoprotein; Cellular efflux; Dipeptide prodrug; MDCKII-MDR1; Transport

* Corresponding author. Tel.: +1 816 235 1615; fax: +1 816 235 5190.
E-mail address: mitraa@umkc.edu (A.K. Mitra).

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1. Introduction

Inclusion of HIV protease inhibitors (PIs) in 'highly active anti-retroviral therapy' (HAART) has substantially improved clinical outcomes of AIDS patients. However, complete eradication of HIV infection still remains an elusive goal. Sub-therapeutic concentrations of PIs in the sanctuary sites like brain, lung and bone-marrow cause persistent viral infection and may lead to viral resistance. One of the factors that may limit therapeutic efficacy of PIs is cellular efflux mediated by proteins like P-glycoprotein (P-gp) (Kim et al., 1998a,b). Saquinavir (SQV) was the first HIV protease inhibitor approved by FDA. However, SQV possesses very low oral bioavailability—ranging from 4 to 16% (Schapiro et al., 1996; Williams and Sinko, 1999). Recent investigations have demonstrated that all protease inhibitor anti-HIV drugs are substrates for P-gp, a membrane bound efflux protein that contributes significantly to their poor absorption and consequently high metabolism (Alsenz et al., 1998; Kim et al., 1998b; Polli et al., 1999; Lee, 2000).

P-gp is a 170 kDa membrane bound protein composed of two homologous halves, each containing six transmembrane domains, separated by a flexible linker polypeptide. It is an ATP driven pump responsible for the efflux of a wide variety of hydrophobic natural products, drugs and linear and cyclic peptides from the cytoplasm and cytoplasmic membrane of eukaryotic cells (Juliano and Ling, 1976; Schinkel et al., 1993; Ambudkar et al., 1999). P-gp is ubiquitously expressed on human tissues such as intestinal mucosa, brain capillary endothelial cells, biliary canaliculus and kidney tubules (Thiebaut et al., 1987). Broad substrate specificity of P-gp is a major factor responsible for sub-therapeutic levels of various drugs in blood and tissues (Varma et al., 2003). A recent report suggests that presence of this transporter on the brush border membrane of intestinal epithelium not only diminishes permeability of various therapeutic agents but also enhances the metabolism of these molecules by effluxing the drugs into the intestinal lumen or blood capillaries thereby increasing drug exposure to cellular as well as lumenal enzymes (Lown et al., 1997; Watkins, 1997; Ito et al., 1999).

It has been previously reported that inhibition of P-gp by various modulators can lead to improved absorption of drugs across intestine, kidney and blood–brain barrier (BBB) (Glisson et al., 1986; Keller et al., 1992; Hyafil et al., 1993; Bardelmeijer et al., 2000). Various agents that are P-gp inhibitors are often co-administered with P-gp substrates (therapeutic agents) results in increased bioavailability (Tolcher et al., 1996). However, systemic administration of P-gp inhibitors is limited by their toxicity resulting from high serum concentrations associated with the doses required to inhibit P-gp. Although various approaches have been explored to overcome P-gp mediated drug efflux, P-gp still remains a major barrier to oral and CNS drug absorption.

Besides efflux transporters, such as P-gp, a number of nutrient transporters are also expressed on the cellular membranes. These nutrient transporters are responsible for the influx of various nutrients and drugs into various epithelial (enterocytes) and endothelial cells (e.g. blood–brain barrier) (Tsuji and Tamai, 1996; Bolger et al., 1998; Lee, 2000; Tamai and Tsuji, 2000). Recently, transporter targeted prodrug derivatization has attracted a lot of attention amongst drug delivery scientists. Prodrugs have been designed such that the modified compounds become substrates of nutrient transporters leading to enhanced absorption of these compounds across various physiological barriers (Rousselle et al., 2000; Rouquayrol et al., 2002; Rice et al., 2003).

The objective of this study is thus to investigate whether transporter targeted prodrug derivatization of SQV, a P-gp substrate, can result in avoidance of P-gp mediated efflux. To accomplish these goals peptide prodrugs of SQV, Val–Val–SQV and Gly–Val–SQV, were synthesized in our laboratory. When a substrate binds to a nutrient transporter it triggers a configurational change in the transport protein as a result of which it is translocated across the membrane and released into the cell cytoplasm. During this process, the substrate is not freely available in the inner leaflet of the cell membrane and may avoid recognition by P-gp as a substrate. To test this hypothesis, transport and uptake studies were carried out with the MDCKII-MDR1 cell line that has been genetically engineered to express high amounts of P-gp and are widely used to study P-gp mediated efflux. Thus, transport and uptake studies using this cell line demonstrate interaction of both influx (mediated by peptide transporter) as well as efflux processes (mediated by P-gp). Moreover, permeability data from MDCK was found to correlate well with human

oral bioavailability (Irvine et al., 1999; Tang et al., 2002).

2. Experimental

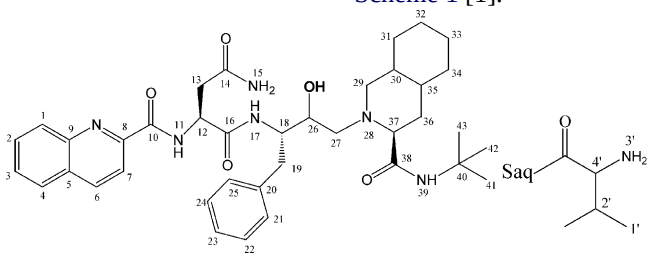
2.1. Materials

[^{3}H]Glycylsarcosine (Gly-Sar) (4.0 Ci/mmol) and [^{3}H]ritonavir (1.0 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). [^{3}H]erythromycin (80 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). [^{14}C]mannitol was purchased from Biomedical Inc. (Aurora, OH). Saquinavir mesylate was generously supplied by Hoffmann-La Roche. All the prodrugs used in the study were synthesized in our laboratory. MDCK cells, retrovirally transfected with the human *MDR1* cDNA (MDCKII-MDR1) was a gift from Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam). The growth medium Dulbecco's Modified Eagle Medium (DMEM), calf serum, minimum essential medium and non-essential amino acids were obtained from Gibco (Invitrogen, Grand Island, NY). Penicillin, streptomycin, sodium bicarbonate and HEPES were purchased from Sigma Chemical (St. Louis, MO). Culture flasks (75 cm^2 growth area), polyester Transwells (pore size 0.4 μM and 12 mm diameter) and 12-well plates were obtained from Costar (Cambridge, MA). Buffer components and other solvents were obtained from Fisher Scientific Co. (Fair Lawn, NJ).

2.2. Synthesis of Val–Val–saquinavir and Gly–Val–saquinavir

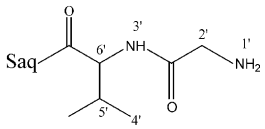
2.2.1. Synthesis of saquinavir prodrugs

The synthetic strategy is to conjugate the modification group (pro-moiety) with the hydroxyl group on saquinavir. The carboxylic group of the amino acid or peptide can form the ester linkage with saquinavir which may improve the delivery of conjugated drug through transporters expressed on various tissues. The synthetic schemes for these prodrugs include: (i) formation of amino acid anhydrides, (ii) coupling the anhydride with saquinavir, (iii) removing the N-protection group, (iv) neutralizing the conjugate, (v) coupling the conjugate with amino acid anhydride to form the peptide bond and (vi) removing the N-protection group. The overall procedure is shown in Scheme 1 [1].

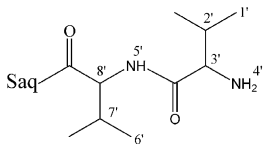


Saquinavir 1

Val-Saq 2



Gly-Val-Saq 3

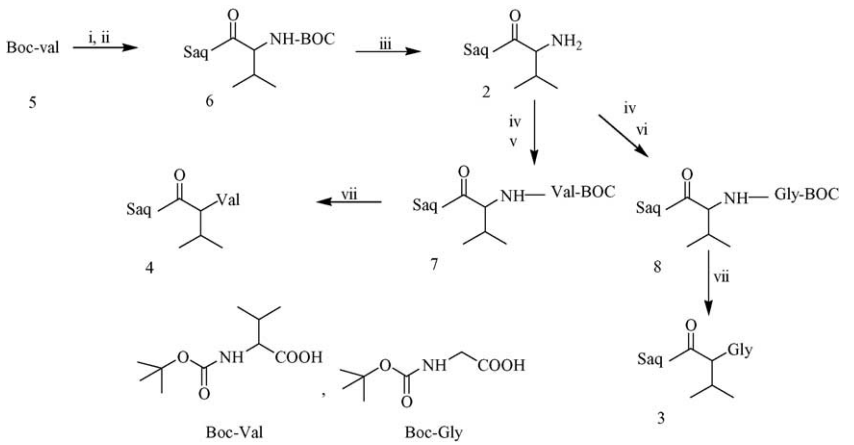


Val-Val-Saq 4

Peptide prodrugs Val–Val–SQV and Gly–Val–SQV were synthesized from Val–SQV as described below in the following sections.

2.2.2. Synthesis of Val–SQV

Synthesis of Val–SQV involves the first three steps with dicyclohexylcarbodiimide (DCC) as the coupling reagent. Boc–valine 0.33 g (1.5 mmol) and 0.16 g (0.77 mmol) of DCC were stirred for 1 h in methylene chloride under nitrogen atmosphere (Scheme 1).



Scheme 1. Synthesis of Val–Val–saquinavir and Gly–Val–saquinavir prodrugs: (i) DCC, CH_2Cl_2, 0 °C, 1 h; (ii) saquinavir, DMAP, rt, 24 h; (iii) TFA, 0 °C, 1 h; (iv) TEA, 5 min; (v) Boc–Val, DCC, DMAP, CH_2Cl_2, rt, 24 h; (vi) Boc–Gly, DCC, DMAP, CH_2Cl_2, rt, 24 h; (vii) TFA, 0 °C, 1 h.

Then, 0.5 g (0.75 mmol) of SQV and DMAP were added dropwise into the solution and was stirred continually for 24 h at room temperature. After filtration, the solvent was removed in vacuo and the residue was purified by column chromatography with methylene chloride:methanol (6:1) as eluant. The product, Boc–Val–SQV was treated with trifluoroacetic acid (TFA) for 1 h at 0 °C. After removing TFA, the residue was co-evaporated with toluene, which generated the final product. Approximately, 0.61 g pure Val–SQV (TFA salt) is obtained as the white solid with yield of 95%. The purity of the prodrug is checked by TLC and LC–MS.

ESI-MS ($M+1$): 770.4; calculated ($C_{43}H_{59}O_6N_7$): 769.4(5). ^{1}H NMR of Val–SQV was ^{1}H NMR (DMSO): δ 8.89 (1H, d, H^{11}), 8.52 (1H, m,H^7), 8.42 (4H, bd, H^{39}, $H^{3'}$), 8.21 (2H, d, $H^{1,6}$), 8.08 (1H, m, H^4), 7.86 (1H, m, H^2), 7.74 (1H, m, H^2), 7.63 (2H, bd, $H^{15,17}$), 7.01–7.20 (5H, m, $H^{21–25}$), 5.52 (1H, m, H^{26}), 4.83 (1H, m, H^{12}), 4.58 (1H, m, H^{18}), 3.85 (1H, m, $H^{4'}$), 3.26 (1H, m, H^{29}), 2.3–2.74 (7H, m, $H^{13,19,27,37}$), 1.59–1.99 (13H, m, $H^{2'}$, $H^{30–36}$), 1.36 (9H, s, $H^{41–43}$), 1.06 (6H, dd, $H^{1'}$).

2.2.3. Synthesis of Val–Val–SQV and Gly–Val–SQV prodrugs from Val–SQV

Val–SQV (TFA salt) 0.2 g (0.23 mmol) was treated with 0.8 mL TEA for 10 min. The mixture was added to the Boc–valine anhydride solution prepared according to the procedure described above. The reaction mixture was stirred overnight. After removing the precipitate (DCU) and most of the solvent, the residue was loaded onto the silica gel column and purified with CH_2Cl_2/MeOH. About 0.21 g of Val–Val–SQV (TFA salt) was obtained as a white solid with high purity (>95%) after removing the Boc group. By adding the Val–SQV into the Boc–glycine anhydride solution as described above, the Gly–Val–SQV (TFA salt) was synthesized (yield: 93%).

2.2.3.1. Gly–Val–saquinavir. ^{1}H NMR of Gly–Val–SQV was ESI-MS ($M+1$): 827.4; calculated ($C_{45}H_{62}O_7N_8$): 826.4(7). ^{1}H NMR (DMSO): δ 8.78 (1H, d, H^{11}), 8.60 (1H, m, H^7), 8.1 (8H, bd, $H^{1,4,6,39}$, $H^{1',3'}$), 7.86 (1H, m, H^2), 7.73 (1H, m, H^3), 7.52 (2H, d, $H^{15,17}$), 7.0–7.21 (5H, m, $H^{21–25}$), 5.38 (1H, m, H^{26}), 4.81 (1H, m, H^{12}), 4.42 (1H, m, H^{18}), 3.75 (3H, m, $H^{2',6'}$), 2.18–3.21 (9H, m, $H^{13,19,27,29,37}$), 1.54–1.95 (13H, m, $H^{30–36}$, $H^{5'}$), 1.32 (9H, s, $H^{41–43}$), 0.92 (6H, s, $H^{4'}$).

2.2.3.2. Val–Val–saquinavir. ^{1}H NMR of Val–Val–SQV was ESI-MS ($M+1$): 869.4; calculated ($C_{48}H_{68}O_7N_8$): 868.5(2). ^{1}H NMR (DMSO): δ 8.78 (1H, d, H^1), 8.62 (1H, m, H^7), 8.19 (2H, d, $H^{1,6}$), 8.10 (6H, bd, $H^{1,4,39}$, $H^{4',5'}$), 7.87 (1H, m, H^2), 7.74 (1H, m, H^3), 7.39 (2H, bd, $H^{15,17}$), 6.95–7.05 (5H, m, $H^{21–25}$), 5.24 (1H, m, H^{26}), 4.83 (1H, m, H^{12}), 3.77 (5H, m,

H^{29}, $H^{3',8'}$), 1.81–3.01 (21H, m, $H^{13,19,27,30-37}$, $H^{2',7'}$), 1.28 (9H, s, H^{41}), 0.94 (12H, m, $H^{1',6}$).

2.3. Cell culture

MDCKII-MDR1 cells (passages 4–10) were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Confluency was assessed by light microscopy. Cells were passaged at 70–80% confluence using 0.25% trypsin EDTA and were seeded at a density of 50,000 cells/cm^2 on 12-well tissue culture plates or on collagen coated Transwell® inserts and were maintained in DMEM, supplemented with 10% calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% (v/v) non-essential amino acid, 3.7 g of sodium bicarbonate and 10 mM HEPES, pH 7.4. Cells were allowed to grow for 5–8 days. Integrity of monolayers formed on transwell was evaluated by monitoring [^{14}C]mannitol permeability and transepithelial electric resistance (TEER), with an epithelial volt ohmmeter (EVOM; World Precision Instruments, Sarasota, FL). TEER values of the cell monolayer were approximately 250 Ω cm^2 after correcting for the resistance imparted by filters. [^{14}C]mannitol transport was <0.5%/h ($P_{app} < 2 \times 10^{-7}$ cm s^{-1}) across the cell monolayer.

2.4. Stability studies in cell homogenates and DPBS

Confluent MDCKII-MDR1 cells were washed three times with Dulbecco's phosphate buffered saline (DPBS) (130 mM NaCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.03 mM KCl and 5 mM glucose). Cells were than isolated with the aid of a mechanical scrapper and suspended in two volumes of water and homogenized (Multipro variable speed homogenizer, DREMEL; Racine, WI). Suitable dilutions were made to achieve a final protein concentration of 0.25 mg/mL. Protein content was determined according to the method of Bradford (Bradford, 1976) with BioRad protein estimation kit.

An aliquot (800 μL) of the cell homogenate was incubated with 200 μL (30 μg/mL) prodrug solution at 37 °C in a shaking water bath (60 rpm). One hundred microlitres samples were withdrawn at predetermined time points and an equal volume of ice-cold acetonitrile:methanol (5:4) mixture was added to stop the enzymatic reaction. Samples were stored at −80 °C until further analysis.

Stability in DPBS was determined by incubating 200 μL prodrug solution with 800 μL DPBS with pH adjusted to 5 and 7.4. Slope of the line of "log percentage prodrug remaining versus time" plot was employed to calculate the degradation rate constants.

2.5. Uptake studies

Uptake studies were conducted with confluent cell monolayers, 6–8 days post seeding. Medium was aspirated and cells were washed three times with DPBS, pH 7.4. SQV, Val–Val–SQV and Gly–Val–SQV solutions were prepared immediately before the experiment. Concentrated stock solution of SQV and the prodrugs were prepares in DMSO. Test solutions were prepared by diluting with DPBS, pH 7.4. Final DMSO concentration in all experiments was maintained constant and did not exceed 0.5% (v/v). Uptake was initiated by adding 1 mL of drug solution (in the presence or absence of competing substrates) to the wells. Incubation was carried out over a period of 10 min at 37 °C. At the end of the incubation period, the drug solution was removed and the cell monolayer was washed three times with ice-cold stop solution. Cells were lysed overnight (1 mL 0.1%, w/v, Triton X-100 in 0.3N sodium hydroxide) at room temperature. Aliquots (500 μL) were withdrawn from each well and transferred to scintillation vials containing 5 mL scintillation cocktail. Samples were then analyzed by liquid scintillation spectrophotometry with a Beckman scintillation counter (Model LS-6500, Beckman Instruments, Inc.). Uptake was normalized to the protein content of each well. Amount of protein in the cell lysate was quantified by the method of Bradford utilizing BioRad protein estimation kit (BioRad, Hercules, CA).

2.6. Transport studies

Transport studies were conducted with MDCKII-MDR1 cell monolayers grown on twelve well transwell inserts (diameter 12 mm) for 6–7 days. Medium was aspirated and cell monolayers washed three times (10 min each wash) with DPBS, pH 7.4. Volumes of the apical and basolateral chambers were 0.5 and 1.5 mL, respectively. Transport experiments were conducted

for a period of 3 h. Concentrations used in the bi-directional studies were SQV, 10 μM; Gly–Val–SQV, 20 μM; Val–Val–SQV, 22 μM. Detection issues of the prodrug and/or parent drug in the acceptor chamber was considered for the selection of prodrug concentration. Aliquots (200 μL) were withdrawn at predetermined time intervals, i.e. 15, 30, 45, 60, 90, 120, 150, 180 min, respectively, and replaced with fresh DPBS, pH 7.4, to maintain sink conditions. Dilutions were taken into account for the calculations. Samples were stored at −80 °C until further analysis. All transport experiments were performed at 37 °C.

2.7. Analytical procedure

SQV, Val–Val–SQV and Gly–Val–SQV samples were analyzed by a reversed phase HPLC technique (Ucpinar and Stavchansky, 2003). The HPLC system was comprised of HP 1050 pump, Waters dual wavelength absorbance UV detector, and an Alcott auto sampler (model 718AL HPLC). A C(8) Luna column (250 mm × 4.6 mm; Phenomenex, Torrance, CA) was employed for the separation of analytes. Mobile phase composed of acetonitrile:water:triethylamine (55:44:1%, v/v/v) and the pH was adjusted to 6.5 with *o*-phosphoric acid. Flow rate was maintained at 0.8 mL/min and detection wavelength was set at 240 nm. Elution times for SQV, Gly–Val–SQV and for Val–Val–SQV were 8, 6 and 12 min, respectively.

2.8. Data analysis

Cumulative amounts of prodrugs (Val–Val–SQV or Gly–Val–SQV) and the parent drug, generated during transport across the cell monolayers were plotted as a function of time to determine permeability coefficient. Linear regression of the amounts transported as a function of time yielded the rate of transport across the cell monolayer (dM/dt). Rate divided by the cross-sectional area available for transport (A) generated the steady-state flux as shown in Eq. (1).

$$\text{Flux} = \frac{dM/dt}{A} \quad (1)$$

In all the transport studies, slopes obtained from the linear portion of the curve were used to calculate permeability values. Permeability was calculated by normalizing the steady-state flux to the donor concentration (C_d) of the drug or prodrug according to Eq. (2).

$$\text{Permeability} = \frac{\text{flux}}{C_d} \quad (2)$$

2.9. Statistical analysis

All experiments were conducted at least in quadruplicate ($n = 4$) and results are expressed as mean ± S.D. Statistical comparison of mean values were performed with one-way analysis of variance (ANOVA) or Student's *t*-test (Graph Pad INSTAT, Version 3.1). $^{*}p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Interaction of SQV, Val–Val–SQV and Gly–Val–SQV with P-glycoprotein

Uptake of [^{3}H]erythromycin (0.25 μCi/mL), by MDCKII-MDR1 cells, was studied in the presence of equimolar concentration of SQV, Val–Val–SQV and Gly–Val–SQV (75 μM). A four-fold increment in the cellular uptake of [^{3}H]erythromycin was observed in the presence of 75 μM SQV. However, equimolar concentrations of SQV prodrugs produced only 2.5-fold increase in cellular uptake of [^{3}H]erythromycin (Fig. 1). These results indicate that prodrug modification results in decreased affinity of the prodrug molecule for the efflux transporter. Similar studies were carried out with [^{3}H]ritonavir (0.5 μCi/mL) as a P-gp substrate and SQV and its prodrugs as inhibitors. The results demonstrate that greatest inhibition was seen with SQV confirming our earlier observation that peptide prodrugs of SQV have reduced affinity for the efflux pump, P-gp (Fig. 2).

3.2. Uptake of [^{3}H]Gly-Sar in the presence of SQV, Val–Val–SQV, Gly–Val–SQV by MDCKII-MDR1 cells

These studies were carried out to investigate the interaction of SQV, Val–Val–SQV and Gly–Val–SQV with the peptide transporter. Uptake of [^{3}H]Gly-Sar (0.5 μCi/mL), in the presence of unlabeled Gly-Sar,

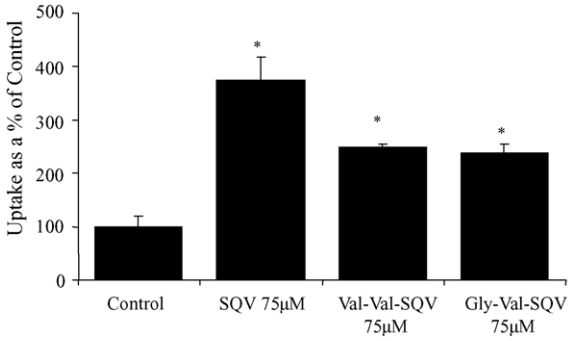


Fig. 1. Cellular uptake of [^{3}H]erythromycin (0.25 μCi/mL) by MDCKII-MDR1 cell monolayers in the absence (control) and presence of SQV, Val–Val–SQV and Gly–Val–SQV. Increased uptake [^{3}H]erythromycin was observed in presence of SQV as compared to equimolar concentration of prodrugs. Statistically significant difference ($^*p < 0.05$) was observed in uptake between prodrugs and equimolar concentrations of SQV. Values are mean ± S.D. ($n = 4$).

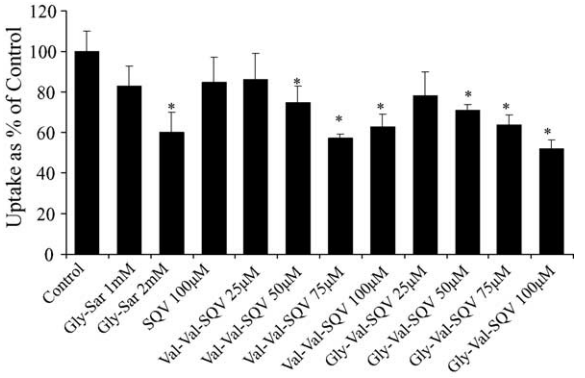


Fig. 3. Uptake of [^{3}H]Gly-Sar (0.5 μCi/mL) by MDCKII-MDR1 cells in the absence (control) or presence of 1 and 2 mM unlabeled Gly-Sar, SQV (100 μM), different concentrations of Gly–Val–SQV and Val–Val–SQV. $^*p < 0.05$ represents statistically significant difference as compared to control. Data expressed as mean ± S.D. ($n = 4$).

at concentrations of 1 and 2 mM, demonstrated a concentration dependent reduction in cellular accumulation of [^{3}H]Gly-Sar. Unlabeled Gly-Sar at concentrations of 1 and 2 mM reduced the uptake of [^{3}H]Gly-Sar from 0.033 to 0.026 pmol/(min mg) (20%) and 0.019 pmol/(min mg) (41%), respectively (Fig. 3). Uptake of [^{3}H]Gly-Sar was significantly decreased in the presence of Val–Val–SQV (100 μM) and Gly–Val–SQV (100 μM), whereas equimolar concentration of SQV did not demonstrate any significant inhibition on [^{3}H]Gly-Sar uptake (Fig. 3). All the prodrugs exhibited concentration dependent inhibition of [^{3}H]Gly-Sar uptake (Fig. 3). Higher percentage inhibition of [^{3}H]Gly-Sar uptake by SQV prodrugs, compared to equimolar concentrations of unlabeled Gly-Sar, indicates that SQV prodrugs may possess even higher affinity for the peptide transporter compared to Gly-Sar, a well known peptide transporter substrate.

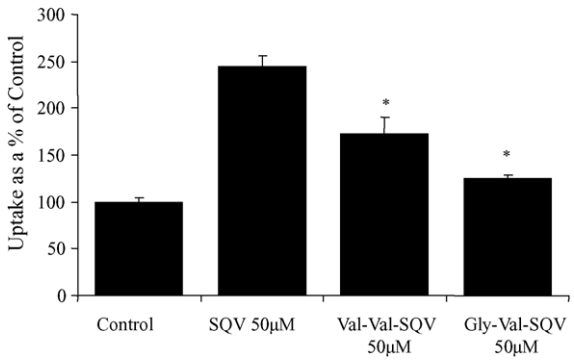


Fig. 2. Cellular uptake of [^{3}H]ritonavir (0.5 μCi/mL) by MDCKII-MDR1 cell monolayers in the absence (control) and presence of SQV, Val–Val–SQV and Gly–Val–SQV. Increased uptake [^{3}H]ritonavir was observed in presence of SQV as compared to equimolar concentration of prodrugs. Statistically significant difference ($^*p < 0.05$) was observed in uptake between prodrugs and equimolar concentrations of SQV. Values are mean ± S.D. ($n = 4$).

3.3. Stability of SQV, Val–Val–SQV and Gly–Val–SQV in transport buffers and MDCKII-MDR1 cell homogenates

Stability of Val–Val–SQV and Gly–Val–SQV was determined in MDCKII-MDR1 cell homogenates, DPBS, pH 5, and DPBS, pH 7.4, for 48 h. Half-lives ($t_{1/2}$) of Gly–Val–SQV in MDCK-MDR1 cell homogenates, DPBS, pH 5, and DPBS, pH 7.4, were 1.75 ± 0.11, 34.5 ± 0.85 and 13.1 ± 0.33 h, respectively. The half-life of Val–Val–SQV, in MDCK-MDR1 cell homogenates, DPBS, pH 5, and DPBS, pH 7.4, are 6.6 ± 0.56, 94.8 ± 21.4 and 18.3 ± 1.4 h, respectively. Degradation studies suggest that some percentage of SQV is generated from the prodrugs during the uptake and transport experiments. Appreciable degradation

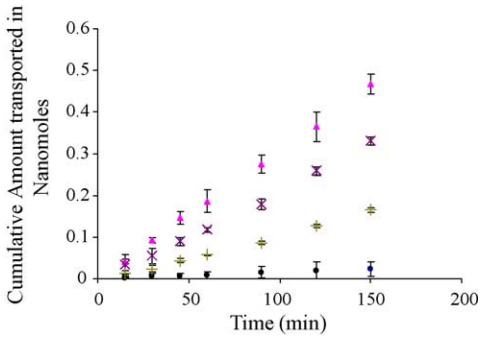


Fig. 4. Bidirectional transepithelial transport of SQV (10 μM) across MDCKII-MDR1 cell monolayers: (●) apical to basolateral (AP–BL) direction; (▲) basolateral to apical (BL–AP) direction; (+) AP–BL transport in presence of cyclosporine; (×) BL–AP transport in presence of cyclosporine. Values are expressed as mean ± S.D. ($n = 4$).

was not observed for SQV in buffer and MDCKII-MDR1 cell homogenate.

3.4. Transport of SQV across MDCKII-MDR1 cells

Transepithelial bidirectional transport of SQV across MDCKII-MDR1 cells demonstrate that transport of SQV in the absorptive direction (AP–BL) direction, was significantly lower ($p < 0.05$) than that in secretory (BL–AP) direction (Fig. 4). Apparent permeability (P_{app}) of SQV from BL to AP direction was $1.99 \pm 0.17 \times 10^{-5}$ and from AP to BL direction was $4.63 \pm 0.25 \times 10^{-7}$ cm s^{-1} a 40-fold difference. This asymmetric permeation is due to the involvement of the apically polarized P-gp efflux transporter, for which SQV is a very good substrate. In the presence of 10 μM cyclosporine, secretory permeability (P_{app}) of SQV significantly reduced from $1.99 \pm 0.17 \times 10^{-5}$ to $1.33 \pm 0.06 \times 10^{-5}$ cm s^{-1} and absorptive permeability increased from $4.63 \pm 0.25 \times 10^{-7}$ to $6.80 \pm 0.34 \times 10^{-6}$ (Fig. 4).

3.5. Transport of Val–Val–SQV and Gly–Val–SQV across MDCKII-MDR1 cells

Transepithelial transports of Val–Val–SQV and Gly–Val–SQV were studied across MDCKII-MDR1 cell monolayers. During the transport process degradation of prodrug can take place in the transport buffer (in the donor compartment), cell cytoplasm (when the drug molecule diffuses inside the cell) or in the receiver chamber after the drug has been transported. In vitro stability studies of the prodrugs involving transport buffers and cell homogenates demonstrate that some quantity of SQV can be regenerated during the transport process. During the transport of Val–Val–SQV and Gly–Val–SQV hydrolysis products, Val–SQV and SQV, were observed. Intact prodrug transported and breakdown products, formed during the transport study, were analyzed. Cumulative amount of drug transported (the sum of prodrug and regenerated parent drug) was plotted as a function of time. Apparent permeabilities (P_{app}) were determined from the linear portion of the cumulative amount versus time plot. AP–BL permeabilities of SQV from Val–Val–SQV ($2.39 \pm 0.16 \times 10^{-6}$ cm s^{-1}) and Gly–Val–SQV ($7.67 \pm 1.04 \times 10^{-7}$ cm s^{-1}) were enhanced significantly as compared to that of SQV ($4.63 \pm 0.25 \times 10^{-7}$ cm s^{-1}) (Fig. 5). Similarly, BL–AP permeabilities of SQV from Val–Val–SQV ($1.28 \pm 0.23 \times 10^{-5}$ cm s^{-1}) and Gly–Val–SQV ($4.72 \pm 0.59 \times 10^{-6}$ cm s^{-1}) were significantly lower than SQV ($1.99 \pm 0.17 \times 10^{-5}$ cm s^{-1}) (Fig. 5). Such enhanced AP–BL transport of SQV prodrugs can be attributed to an active transport mechanism and/or decreased efflux resulting from reduced affinity of these prodrugs for P-gp. These results are consistent with our earlier observations that SQV increased cellular uptake of [^{3}H]erythromycin and [^{3}H]ritonavir to a greater extent relative to prodrugs.

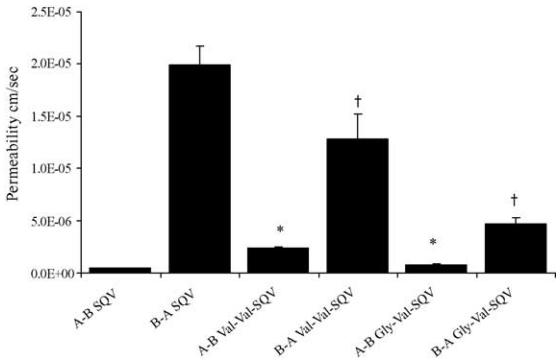


Fig. 5. Apparent permeability of SQV, Val–Val–SQV and Gly–Val–SQV in apical to basolateral direction and basolateral to apical direction across MDCK-MDR1 cells. A statistically significant difference ($^*p < 0.05$) in AP–BL direction and ($^\dagger p < 0.05$) in BL–AP direction as compared to SQV was observed.

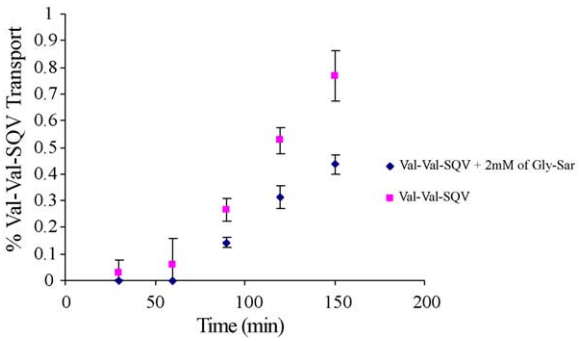


Fig. 6. Percentage transport of Val–Val–SQV in presence of 2 mM Gly-Sar across MDCK-MDR1 cells.

3.6. Transport of Val–Val–SQV in presence of Gly-Sar

Transport of Val–Val–SQV was conducted in the presence of 2 mM Gly-Sar across MDCK-MDR1 cells. Significant inhibition in Val–Val–SQV transport was observed in presence of 2 mM Gly-Sar (Fig. 6). A two-fold reduction in total percentage Val–Val–SQV transported, at the end of 150 min, was observed in the presence of Gly-Sar.

4. Discussion

The primary objective of this study is to demonstrate whether the dipeptide conjugates of protease inhibitor SQV can circumvent P-gp mediated efflux. This finding has great clinical implications and will aid in enhancing intestinal absorption and oral bioavailability of poorly absorbed PIs. In a recent work from our laboratory, we have demonstrated that modification of quinidine (a well know P-gp substrate), to Val–quindine targeting nutrient transporter, can bypass P-gp mediated efflux (Jain et al., 2004). Recently, considerable attention has been directed towards developing strategies to overcome the permeability barrier exerted by P-gp mediated efflux. Overcoming the barrier presented by P-gp will not only aid in enhancing drug absorption in the gut but will also lead to increased drug permeability across various other biological barriers such as the blood–brain barrier. In the present study, we have demonstrated that by targeting nutrient transporters the prodrugs with reduced affinity for P-gp can be designed.

Cellular uptake of [^{3}H]erythromycin showed a four-fold enhancement in the presence of SQV, whereas an equimolar concentration of Val–Val–SQV and Gly–Val–SQV demonstrated only 2.5-fold increment in [^{3}H]erythromycin uptake (Fig. 1). This differential inhibition by equimolar concentration of SQV and its derivatives indicates that the dipeptide prodrugs have reduced affinity for P-gp. Similar results were obtained when cellular uptake of [^{3}H]ritonavir was determined in the presence of equimolar concentrations of SQV and its prodrugs. Uptake of [^{3}H]erythromycin was studied in the presence of 75 μM Val–Val–SQV and Gly–Val–SQV, whereas [^{3}H]ritonavir uptake was evaluated in the presence of 50 μM of the prodrugs. Two different concentrations were employed to examine the effect of prodrug concentration on P-gp inhibition. Concentration dependent uptake studies (data not included in the manuscript) demonstrated that Val–Val–SQV saturates P-gp at 50 μM, whereas Gly–Val–SQV saturates P-gp at 75 μM. Consequently, when used at 75 μM both prodrugs exhibit similar extent of inhibition, whereas, differential inhibition is observed at lower concentrations (Figs. 1 and 2). Furthermore, uptake studies were conducted to study whether the SQV prodrugs are substrates for peptide transporters. Uptake of [^{3}H]Gly-Sar (0.5 μCi/mL), in the presence of unlabeled Gly-Sar, at concentrations of 1 and 2 mM, demonstrated a concentration dependent decrease in cellular accumulation of [^{3}H]Gly-Sar. Such diminished cellular uptake in the presence of unlabeled Gly-Sar suggests expression of peptide transporters on the apical membrane of MDCKII-MDR1 cells (Fig. 3). This is consistent with earlier reports on the expression of peptide transporters on MDCK cells (Putnam et al., 2002). Uptake of [^{3}H]Gly-Sar was also reduced by 35% in the presence of 100 μM Val–Val–SQV and by 42% in the presence of 100 μM Gly–Val–SQV. However, equimolar concentration of SQV did not demonstrate any inhibition of [^{3}H]Gly-Sar uptake (Fig. 3). Also, Val–Val–SQV and Gly–Val–SQV demonstrated concentration dependent inhibition of [^{3}H]Gly-Sar uptake exhibiting highest inhibition at 100 μM (Fig. 3). Greater percentage inhibition of [^{3}H]Gly-Sar uptake by prodrugs, compared to equimolar concentrations of unlabeled Gly-Sar, indicates that prodrugs may possess higher affinity for the peptide transporter relative to Gly-Sar. Several earlier reports indicated that valine ester prodrugs are substrates of

peptide transporters, e.g. valine esters of ganciclovir (Sugawara et al., 2000) and acyclovir (Balimane et al., 1998) which resulted in increased oral bioavailability of the parent drugs. High transport capacity, broad substrate specificity and dense expression of peptide transporter in intestine and other biological barrier render the peptide transporter an ideal candidate for drug targeting. Our results suggest that Val–Val–SQV and Gly–Val–SQV may be excellent substrates for the peptide transporter expressed on apical membrane of MDCKII-MDR1 cells, whereas SQV is not.

To further test the hypothesis that prodrug modification can bypass P-gp mediated efflux and increase absorption, transepithelial transport studies were conducted employing MDCK-MDR1 cells. A classical indication of P-gp involvement in transport kinetics is the difference in permeation rates of P-gp substrates in the apical to basolateral and basolateral to apical directions (Polli et al., 2001). If a compound is a substrate for P-gp, apical to basolateral transport (AP–BL) is considerably lower than that from the basolateral to apical direction (BL–AP). Transepithelial bi-directional transport studies of SQV across MDCKII-MDR1 cells revealed that transport studies of SQV in the absorptive direction (AP–BL) direction, was significantly lower than that in secretory (BL–AP) direction with an efflux ratio (BL P_{app}/AP P_{app}) of 40 (Fig. 4). This asymmetric permeation is due to the presence of apically polarized P-gp efflux pump, for which SQV is a very good substrate. In the presence of 10 μM cyclosporine, a P-gp inhibitor, secretory permeability (P_{app}) of SQV was significantly decreased from $1.99 \pm 0.17 \times 10^{-5}$ to $1.33 \pm 0.06 \times 10^{-5}$ cm s^{-1} and absorptive permeability increased from $4.63 \pm 0.25 \times 10^{-7}$ to $6.80 \pm 0.34 \times 10^{-6}$ (Fig. 4). Such decrease in secretory transport and increase in absorptive transport is due to the inhibitory effect of cyclosporine on P-gp mediated efflux of SQV.

Gly–Val–SQV and Val–Val–SQV also showed asymmetric SQV permeation across the cell monolayer however with a flux ratio of only 6. This decrease in efflux ratio of SQV prodrugs as compared to SQV (flux ratio 40) is attributed to increased absorptive flux (AP–BL) and decreased secretory flux (BL–AP). In vitro stability studies of the prodrugs involving transport buffers and cell homogenates demonstrate that some quantity of SQV can be regenerated during the transport process. Asymmetric permeability of SQV prodrugs is possibly due to SQV regeneration from the prodrugs (which is again a good substrate for P-gp) during the course of a transport study. Two- to five-fold increment in the absorptive permeability of the dipeptide prodrug is probably due to involvement of an active peptide transport system. A decrease in secretory permeability may be attributed to the lower affinity of these prodrugs for P-gp (Table 1). As compared to SQV, Val–Val–SQV showed increased AP–BL and not much decrease in BL–AP transport which can be attributed to various factors such peptide transporter mediated translocation (Fig. 6), lower efflux by P-gp, higher lipophilicity and higher stability in the buffer and cell homogenates. In contrast, Gly–Val–SQV showed marginal increase in AP–BL transport and tremendous decrease in BL–AP direction as compared to SQV, which can be attributed to its lower efflux by P-gp and lower lipophilicity (compared to both SQV and Val–Val–SQV). Additionally, the prodrugs may possess differential affinity for the basolateral peptide transporter, which may contribute towards the differences in AP–BL and BL–AP transport.

Table 1
Apparent permeabilities (P_{app}) of SQV and its peptide prodrugs across MDCKII-MDR1 cells

Compound	P_{app} (AP–BL) (cm s^{-1})	P_{app} (BL–AP) (cm s^{-1})
SQV	4.63×10^{-7} (±0.25)	1.99×10^{-5} (±0.17)
Val–Val–SQV	2.39×10^{-6} (±0.16)	1.28×10^{-5} (±0.23)
Gly–Val–SQV	7.67×10^{-7} (±1.04)	4.72×10^{-6} (±0.59)
SQV + cyclosporine	6.80×10^{-6} (±0.34)	1.33×10^{-5} (±0.06)

To further test the hypothesis that increased AP–BL transport of Val–Val–SQV was due to peptide transporter mediated influx, transport studies were conducted in presence of Gly-Sar. In presence of 2 mM Gly-Sar, there is a significant decrease in Val–Val–SQV transport (Fig. 6). These results together with uptake results clearly show that SQV prodrugs not only bind to peptide transporter but also are translocated by the peptide transporter. Thus, peptide prodrug derivatization of SQV is a viable strategy to bypass P-gp mediated efflux such that the oral absorption of these poorly absorbed drugs can be significantly enhanced. Ratio between prodrug affinity for the efflux pump and active nutrient transporter will determine whether a drug molecule can circumvent P-gp mediated efflux.

If a molecule has greater affinity for nutrient transporter than it will preferentially bind to the nutrient transporter and will get translocated across the cell membrane. Prodrug molecules are not freely available for binding to efflux pump and consequently P-gp fails to recognize the prodrug molecule and will result in bypassing P-gp mediated efflux. Circumventing P-gp mediated efflux will not only increase absorption across intestinal mucosa but will also decrease the repetitive exposure to metabolism in intestinal mucosa. In vitro data from our studies indicate that prodrug modification of SQV leads to partial avoidance of its P-gp mediated efflux, and enhanced absorption due to the involvement of nutrients transporters. Two- to five-fold enhancement in absorption of SQV through peptide prodrug design indicates that potentially lower doses can be administered orally to AIDS patients.

In this study, we have demonstrated for the first time that peptide prodrug modification of SQV to Val–Val–SQV and Gly–Val–SQV can lead to an increased permeability across P-gp overexpressing MDCKII-MDR1 cells. This increase in permeability, under the experimental conditions employed, can be attributed partially to P-gp's failure to recognize these compounds as substrates, and partially due to involvement of peptide transport system. Thus, transporter targeted prodrug modification of P-gp substrates could lead to shielding of these drug molecules from efflux pump. This strategy may also lead to enhanced drug delivery across biological membranes expressing efflux pumps in general and in chemotherapy of drug resistant tumors in particularly.

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