Stereoisomeric Peptide Prodrug Modification to Improve Intestinal Absorption of Saquinavir: Synthesis and in Vitro Evaluation

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Abstract:

The objective of this study is to design and synthesize a series of stereoisomerized dipeptide prodrugs of saquinavir (SQV), and to investigate whether this modification can enhance cellular permeability of SQV. Four stereoisomeric SQV prodrugs including *L*-valine-*L*-valine-SQV (LLS), *L*-valine-*D*-valine-SQV (LDS), *D*-valine-*L*-valine-SQV (DLS) and *D*-valine-*D*-valine-SQV (DDS) were synthesized, and their solubility and cytotoxicity, as well as enzymatic and chemical hydrolysis were determined. Their affinities towards P-gp, MRP2 and peptide transporters were compared to SQV. Bidirectional transepithelial permeability was also examined in this study. Our results indicate that stereoisomeric valine-valine prodrugs exhibit improved aqueous solubility and lower cytotoxicity. SQV attached with *L*-isomers shows higher affinity for peptide transporters but lower stability and higher toxicity, whereas conjugation with *D*-isomers can enhance stability and reduce toxicity, but not be recognized by peptide transporters. Transport studies suggest that an enhanced transepithelial permeation over SQV can be achieved by these stereoisomeric dipeptide prodrugs. According to these findings, targeted stereoisomeric prodrug modification proposed in this study might be a promising strategy to improve intestinal absorption and oral bioavailability of saquinavir.

Keywords: Saquinavir (SQV), Peptide prodrug, Stereoselectivity, Synthesis, Efflux pumps.

1. **Introduction**

Saquinavir (SQV) is the first HIV protease inhibitor (PI) marketed for the treatment of AIDS in the United States. Although SQV has been reported for its potent activity against HIV, its therapeutic efficacy is limited in clinical application due to some undesirable properties like poor aqueous solubility as well as high affinity for efflux transporters and metabolic enzymes [1, 2]. In order to overcome these pharmaceutical and physicochemical barriers, "targeted prodrugs" have been developed as an attractive strategy. In comparison with classical prodrugs, which represent a nonspecific chemical promoiety attached to the active parent drug by a covalent linker, targeted prodrug approach is designed to target specific endogenous transporters and thereby to facilitate epithelial transport of these compounds [3-5]. Following the translocation across cellular membrane, the prodrug is cleaved by hydrolytic enzymes specifically to regenerate the active parent drug.

Recent advances in targeted drug delivery indicated that peptide transporters, primarily expressed in the small intestine and kidney, are attractive targets for prodrug design because they have broad substrate specificity and high capacity, especially for smaller peptides *i.e.* di and tripeptides [6-8]. On the basis of these findings, "peptidomimetic" prodrugs, designed by conjugating parent drugs to small peptides, can be recognized easily by the peptide transporter-mediated influx system and ferried across the epithelial membrane [9]. Additionally, structure modification reduces the interaction of the parent drug with efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP). Therefore enhanced cellular permeation may be achieved by cumulative effects of reducing secretion by efflux transporters and increasing absorption by influx transporters. Several peptide prodrugs of PIs have been synthesized successfully. *In vitro* permeability studies on *L*-valyl, *L*-leucyl, and *L*-phenylalanyl ester conjugates of indinavir and saquinavir demonstrated an increased translocation across Caco-2 cell monolayers [10]. Valine-valine- and glycine-valine- SQV prodrugs also exhibited 4.6- and 1.8- fold enhanced absorption relative to parent drug in rat jejunum, respectively [11]. These encouraging findings prompted us to extend prodrug study with its stereoisomeric configuration.

Different stereoisomers of chiral drug molecules possess different biological properties [12, 13]. Conjugates attached with different stereoisomeric promoieties may be featured with various physicochemical and biological properties. Most amino acids currently applied in prodrug strategy are *L*-isomers. Major advantages to *L*-configurations include their natural occurrence in the body, as well as their rapid and specific recognition by peptide transporters [14, 15]. However, most *L*-isomeric derivatives have limited chemical or enzymatic stability [15, 16]. *L*-valine-*L*-valine has been reported to be degraded completely in 15 min in Caco-2 cell homogenate (1 mg/ml) at 37°C which is much faster than the other diastereomers [17]. In contrast, *D*-amino acid prodrugs of dapsone exhibited much better resistance to enzymatic hydrolysis and showed a longer residence time *in vivo* [15]. These findings give us strong support for studying stereoisomeric peptide prodrugs of SQV.

In this study, we have described the synthesis of various stereoisomeric valine-valine prodrugs derived from SQV, and also provided a comparison of physicochemical and biological properties of these prodrug derivatives, including aqueous solubility, *in vitro* cytotoxicity, as well as chemical and enzymatic hydrolysis under physiological conditions. Additionally, affinities for efflux pumps (P-gp and MRP2) and influx peptide transporters, and permeability studies across *in vitro* cell monolayers have also been investigated. The purpose of this screening is to select the most promising stereoisomeric dipeptide prodrug that could improve cellular permeation of SQV across intestinal barriers.

2. Materials and Methods

2.1 Materials

Saquinavir mesylate was kindly donated by Hoffmann-La Roche. [14C]Erythromycin was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). [3H]glycylsarcosine ([3H]Gly-Sar) was purchased from Moravek Biochemicals (Brea, CA). GF120918 was generously provided by GlaxoSmithKline Ltd. MK571 was purchased from Biomol (Plymouth Meeting, PA). BioRad protein estimation kit was obtained from BioRad (Hercules, CA). Triton X-100, HEPES, d-glucose and all other chemicals were purchased from Sigma Chemical Co (St. Louis, MO). All chemicals were products of special reagent grade and used as such.

2.2 Synthesis of SQV prodrugs

Stereoisomeric peptide prodrugs (valine-valine-SQV, VVS) were prepared from amino acid prodrugs (valine-SQV, VS) following a published procedure with modifications [18]. The synthetic procedure of *D*-valine-*L*-valine-SQV is shown in Scheme 1.
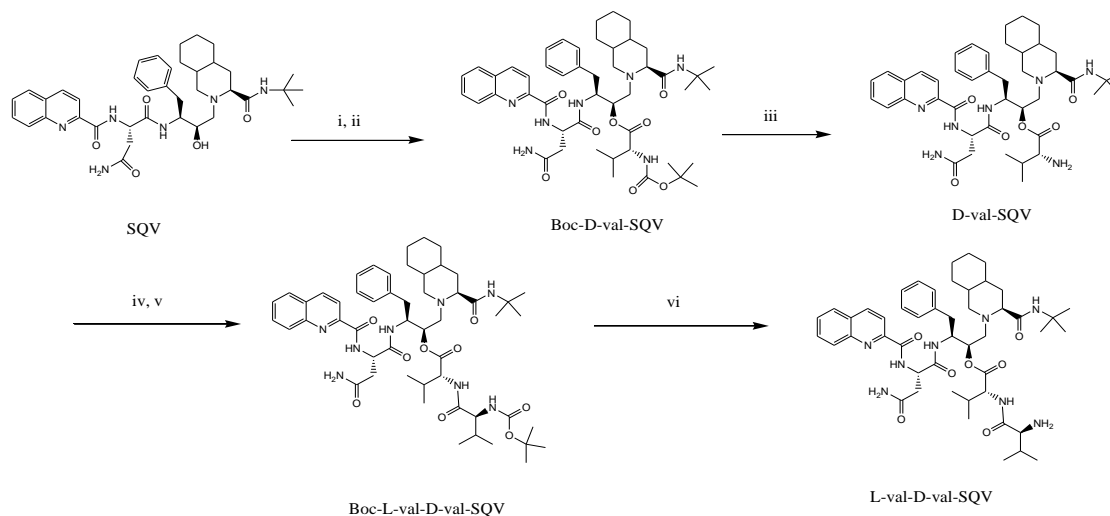
Scheme 1. Synthesis process of *L*-valine-*D*-valine-saquinavir (LDS).

Reagents and conditions: (i) Boc-*D*-valine-OH, DCC, DCM, N$_2$, 0°C, 1; (ii) TEA, DMAP, DCM, N$_2$, RT, 24 hours; (iii) TFA/ DCM (1:1), 0°C 2.5 hours; (iv) Boc-*L*- hour valine-OH, DCC, DCM, N$_2$, 0°C, 1 hour; (v) , TEA, DCM, N$_2$, RT, 24 hours; (vi) TFA/ DCM (1:1), 2.5 hours.

All the reactions were performed under anhydrous condition and protected by nitrogen. All chemicals were dessicated before reaction and anhydrous solvents were used.

2.2.1 Synthesis of amino acid prodrugs of SQV

 Stereoisomeric valine-SQV was synthesized in two steps: Conjugating SQV with N-Boc-(*L*)-valine or N-Boc-(*D*)-valine, and then deprotection of amino protecting group N-Boc. 280 mg N-Boc-(*L*)-valine or N-Boc-(*D*)-valine (1.30 mmol) and 390 mg dicyclohexylcarbodiimide (DCC, 1.95 mmol) were dissolved into 5 ml of dichloromethane (DCM) and stirred for 1 h on ice-bath (mixture A). In another round bottom flask, several drops of dry triethylamine (TEA) was added to 500 mg SQV (0.65 mmol), then a proper amount of DCM with 240 mg dimethylamino pyridine (DMAP, 1.95 mmol) were added until all SQV dissolved after 10-minute stirring (mixture B). Mixture A was removed from ice bath, and mixture B was added dropwise into mixture A while continually stirring the mixture for 24 h. Reactions were monitored with Mass Spectra to ensure complete conversion of the starting compounds to intermediates SQV-(*L*)-val-Boc or SQV-(*D*)-val-Boc. The intermediates were purified using silica column chromatography (Silica gel 60 Geduran®, 40-63 µm) with hexane and ethyl acetate (3:7) as eluent. The deprotection of the N-Boc protecting group was achieved using 10 ml of DCM and trifluoroacetic acid (TFA) (1:1) at 0 ºC for 2.5 h to get final *L*-val-SQV or *D*-val-SQV (mono-TFA salt). Crude products were purified by recrystallization using cold diethyl ether and dried in Speed Vac (SPD101B, Savant Instruments INC, Holbrook, NY) until the weight was constant. The final amino acid prodrugs were obtained as white powder with a yield of 85%.

2.2.2 Synthesis of peptide prodrugs

 Dipeptide prodrugs were synthesized using a similar procedure described previously. The starting material was 570 mg stereoisomeric valine-SQV conjugate (0.65 mmol), which was treated with 2 ml of TEA instead of DMAP, and the reaction was conducted at room temperature for 24 h. Intermediates N-Boc-valine-valine-SQV were purified using silica gel 60 column with MeOH/DCM (3:97) as eluent. The final stereoisomeric products were obtained as white dry powder with a yield of 80% (calculated from SQV).

2.2.3 Identification of the prodrugs

 Structure and purity of reaction intermediates and final compounds were confirmed by ^{1}H NMR and Mass Spectrography. ^{1}H NMR was carried out using a Varian-400MHz NMR spectrometer.

2.3 Aqueous solubility studies

Generally 5 mg of compound was added to 2 ml of distilled deionized water in a 10 ml screw-capped glass tube. These tubes were shaken mechanically at 25ºC for 24 h. At the end of experiment, the mixture was centrifuged at 15,000 ×g, 25 ºC for 10 min. The supernatant was collected and filtered through 0.45 µm membrane. The filtrate was appropriately diluted with water and drug content was measured with LC-MS/MS.

2.4 Stability studies

2.4.1 Chemical hydrolysis in buffer

Chemical hydrolysis study of SQV prodrugs was determined in buffer over a pH range of 1.4 to 10.4. Buffers containing 50 mM of hydrochloric acid/ potassium chloride (pH 1.4), phthalate (pH 3.4 and 5.4) and phosphate (pH 7.4 and 10.4) were prepared for this study and ionic strength was adjusted to 0.1 M. Prodrug solution (23 µM) was incubated at 37ºC under various pH conditions for 48 h. Aliquot (100 µl) of samples was collected at predetermined time intervals and analyzed by HPLC.

2.4.2 Enzymatic hydrolysis in cell homogenates

Caco-2 cells were isolated using a mechanical scraper, then suspended in proper volumes of chilled water and homogenized using Multipro Variable Speed Homogenizer (DREMEL, Racine, WI) for 5 min on ice bath. After centrifugation at 15,000 ×g, 4 ºC for 10 min to remove debris, protein content of the obtained supernatant was determined using BioRad protein estimation kit. Then the cell homogenates (protein content 0.25 mg protein/ml) with different prodrugs (23 µM) were incubated at 37˚C for 3 h. Samples (100 µl) were collected at predetermined time intervals and an equal volume of ice-cold acetonitrile:methanol (5:4) mixture was added to stop enzymatic hydrolysis.

2.5 Cytotoxicity assay

Cellular toxicity study was performed using Lactate Dehydrogenase (LDH) Cytotoxicity Detection Kit (Takara Bio Co. St Louis, MO) on MDCK-WT cells. Cell suspension (200 µl/well) was added in the 96-well tissue culture plate at the density of 10,000 cells/well, and then incubated at 37 ºC, 5% CO_2, 90% humidity. The medium was aspirated after 12-h post-seeding, and 200 µl of assay medium (serum-free DMEM) with different concentrations (0−150 µM) of SQV or prodrugs was added to each well. Positive control (1% Triton X-100), negative control (only assay medium) and background control (assay medium in the wells without cells) were also evaluated at the same time. After 24 hours incubation, LDH release in each of the drug treated wells was determined according to the manufacturer's protocol. Absorbance was measured at 450 nm using DTX 880 Multimode Detector (Beckman Coulter, Brea, CA).

2.6 Cell culture

MDCK-WT cells, MDCK-MDR1 cells (passages 5-12) and MDCK-MRP2 cells (passages 5-25) were seeded at a density of 40,000 cells/cm^2 in 75 cm^2 cell culture flasks and incubated at 37ºC, in a humidified atmosphere of 5% CO_2, 90% relative humidity. Cells were cultured in

DMEM containing 10% heat-inactivated FBS and passaged using TrypLE^{TM} Express Stable Trypsin Replacement at 5-7 days post-seeding. Cells were plated at 7×10^4 cells /cm^2 on 12-well tissue culture plates for uptake studies, and 2×10^5 cells /cm^2 on 12-well Transwell$^®$ inserts (diameter 12 mm, pore size 0.4µm) for transport studies.

2.7 Uptake studies

One milliliter of Dulbecco's phosphate-buffered saline (DPBS, pH 5.4) with 0.25 µCi/ml of [^{14}C]erythromycin or 0.5 µCi/ml of [^{3}H]Gly-Sar in the presence or absence of various drugs was added into each well. After the incubation period, the tracer solution was aspirated and cells were rinsed three times with ice-cold stop solution (200 mM KCl and 2 mM HEPES) to determine drug uptake. Then the cells were lysed by adding 1 ml of 0.3 N NaOH containing 0.1% Triton-X 100 solution. Cellular radioactivity was quantified using a scintillation counter (Model LS-6500; Beckman Counter, Fullerton, CA, USA) and then was normalized by amount of protein measured using BioRad protein estimation kit.

2.8 Transport studies

Cell monolayers were washed twice with transport medium (DPBS, pH 5.4) before experiment. The monolayer integrity was checked by measuring transepithelial electrical resistance (TEER) values using volt–ohm meter (EVOM-G, World Precision Instruments, Sarasota, FL). Only the monolayers with TEER values of around 600 Ω·cm^2 were used. Drug transport was initiated when drug solutions were added in donor chambers and only transport medium in receiving chambers. The final concentrations of SQV and dipeptide prodrugs were 10 µM and 25 µM, respectively. Selection of donor concentrations is dependent on the detection of SQV and prodrugs in receiver chambers. Aliquots (200 µl) were withdrawn from receiving chambers at predetermined time intervals over a period of 3 h and replaced with same volume of fresh transport medium to maintain sink conditions. Samples were analyzed using LC-MS/MS. At the end of experiments, the integrity of monolayers was checked by measuring TEER values, and samples were also taken from the donor chamber to determine whether any nonspecific adsorption had occurred.

2.9 Sample analysis

2.9.1 HPLC

Samples were analyzed by HPLC chromatography with a reversed Luna C-8 column (250 mm×4.6 mm, 5 µm; Phenomenex, Torrance, CA). The HPLC system included HP 1050 pump, Waters dual wavelength absorbance UV detector, and an Alcott HPLC autosampler 718AL. Mobile phase was composed of acetonitrile/water/triethylamine (60:39:1, v/v/v), and pH in aqueous phase was adjusted to 6.50 with *o*- phosphoric acid. Detection wavelength was set to 240 nm, and flow rate of mobile phase was 1.0 ml/min.

2.9.2 LC-MS/MS

QTrap® LC-MS/MS mass spectrometer (Applied Biosystems, Foster City, CA) equipped with Agilent 1100 Series quaternary pump (Agilent G1311A), vacuum degasser (Agilent G1379A) and autosampler (Agilent G1367A, Agilent Technology Inc., Palo Alto, CA) was utilized to analyze SQV and prodrug samples. The detection was operated in multiple-reaction monitoring (MRM) mode. The precursor and the product ion generated for SQV, VS and VVS are 671.4/570.3, 770.5/367.2, and 869.5/367.2, respectively. Samples were prepared using liquid-liquid extraction technique with ice-cold *tert*-butyl methyl ether. Verapamil (200 nM) was employed as an internal standard. Samples were loaded onto a Luna C-18 column (100×2.0 mm, 3 µm; Phenomenex Torrance, CA) and eluted with acetonitrile/water (40:60) in presence of 0.1% formic acid at a flow rate of 0.2 ml/min.

2.10 Data and statistical analysis

The apparent permeability coefficients P_{app} (cm/s) were calculated by linear regression analysis on the time course plot of amount of drugs transported across cell monolayers.

$$P_{app} = \frac{TR_{cum}/dt}{C_0 \times A} \qquad \text{Eq. 1}$$

Where TR_{cum}/dt is the flux rate of SQV or SQV prodrugs obtained from the slope of transport profile. *A* is the surface area of cell monolayers. C_0 is initial concentration of (pro)drugs in the donor chambers.

All experiments were conducted at least in triplicate and the results were expressed as mean ± SD. Statistical significance was detected using Student's *t*-test. Difference between mean values was considered significant at $p < 0.05$ and very significant at $p < 0.01$.

3. Results

3.1 Apparent aqueous solubility

Results of the solubility study presented in Table 1 demonstrate that all stereoisomeric dipeptide prodrug modification improved aqueous solubility of parent drug SQV (mesylate salt) to a certain extent. Solubilities of LLS, LDS, DLS and DDS were dramatically greater than SQV mesylate by around 2.8, 2.7, 2.6 and 2.5-fold, respectively. Amino acid prodrugs also displayed significant increase in aqueous solubility relative to SQV.

3.2 Stability studies

3.2.1 Hydrolysis in buffers

Effect of pH on hydrolysis of the SQV prodrugs was evaluated within the pH range of 1.4 to 10.4. The half lives ($t_{1/2}$) of each prodrug were estimated by plotting the natural logarithm of prodrug concentrations *versus* time (Table 2). All prodrugs showed a consistent rise in susceptibility to hydrolysis as pH was raised from acidic (pH 1-5) towards basic conditions (pH

Table 1. Apparent aqueous solubility of SQV and various stereoisomeric prodrugs.

	Apparent aqueous solubility (mg/ml)
SQV (mesylate salt)	1.34 ± 0.22
Dipeptide prodrugs	
LLS	$3.73 \pm 0.20^{**}$
LDS	$3.65 \pm 0.47^{**}$
DLS	$3.52 \pm 0.27^{**}$
DDS	$3.34 \pm 0.45^{**}$
Amino acid prodrugs	
LS	$2.81 \pm 0.12^{**}$
DS	$2.70 \pm 0.10^{**}$

Data represented are mean $\pm$ SD (n=4). ** $P < 0.01$, very significant difference compared with SQV (mesylate salt).

Table 2. Degradation half lives (h) for various SQV prodrugs at different pH conditions.

|	pH1.4	pH3.4	pH5.4	pH7.4	pH10.4
LLS	336.4 ± 23.7	203.2 ± 11.3	49.3 ± 0.9	10.4 ± 0.6	7.2 ± 0.2
LDS	357.2 ± 18.9	228.7 ± 23.5	55.0 ± 2.1	12.9 ± 1.4	8.0 ± 0.4
DLS	364.7 ± 21.2	242.3 ± 14.6	55.1 ± 4.3	16.0 ± 1.8	9.3 ± 0.2
DDS	412.5 ± 14.4	249.3 ± 16.3	63.1 ± 1.8	19.9 ± 1.1	11.8 ± 0.4
LS	30.2 ± 1.7	15.1 ± 1.1	5.0 ± 0.6	2.5 ± 0.3	1.9 ± 0.2
DS	43.3 ± 3.3	38.3 ± 3.6	6.9 ± 0.8	3.2 ± 0.2	2.3 ± 0.1

Data represented are mean $\pm$ SD (n=4).

7-10). The hydrolysis data indicate that SQV dipeptide conjugates are chemically more stable in comparison to amino acid prodrugs at all the pH levels. Besides, DDS appears to be most stable compared to other dipeptide conjugates.

3.2.2 Hydrolysis in cell homogenates

Concentration profile for dipeptide prodrugs in Caco-2 cell homogenates at pH 7.4 for a period of 3 h is depicted in Figure 1. Regeneration of valine-SQV and SQV demonstrates the role of esterase and peptidase enzymes in the metabolism process. LLS appears the least enzymatically stable compared to the other three peptide prodrugs (Figure 1(a)). Percentage of LLS remaining
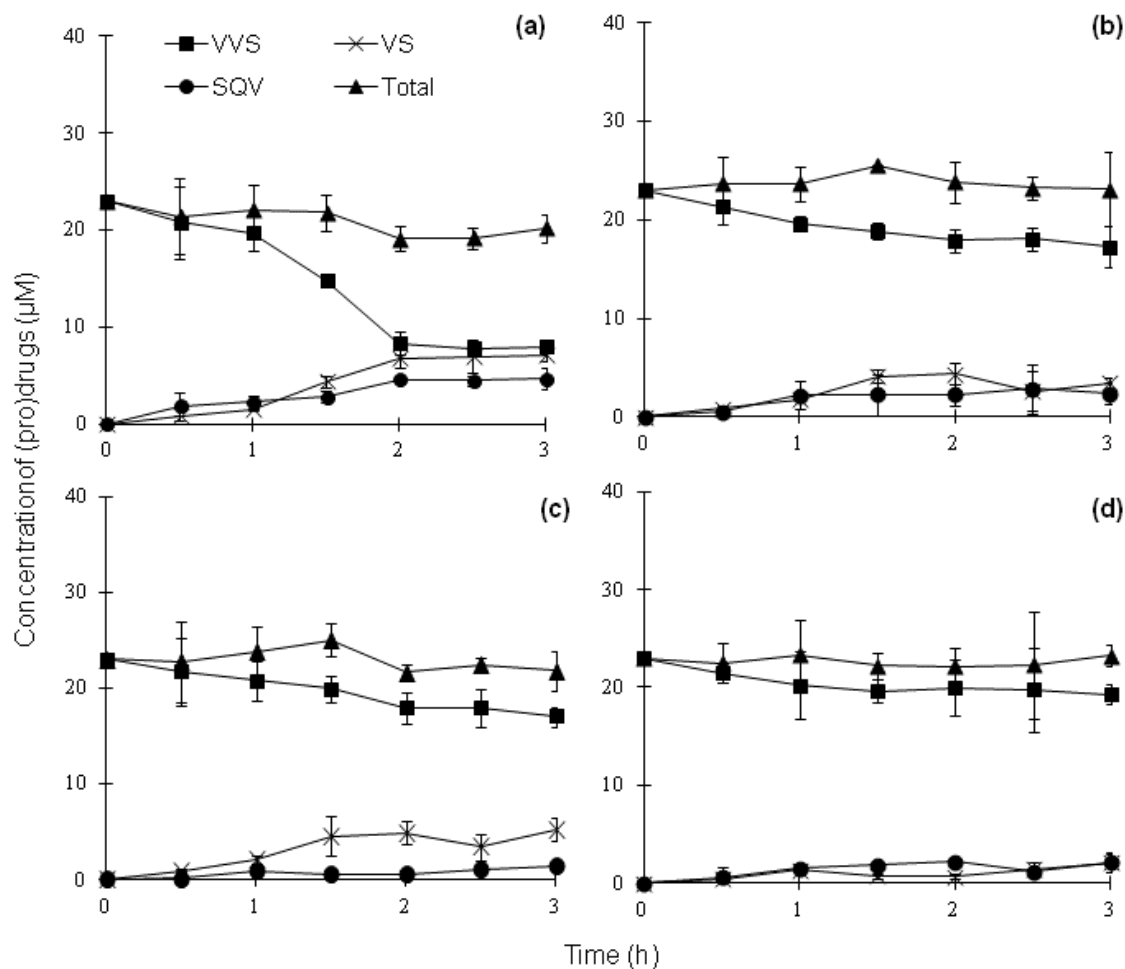
Figure 1. Degradation rate of SQV stereoisomeric dipeptide prodrugs (VVS, 23 µM) in 0.25 mg protein/ml Caco-2 cell homogenates and regeneration of amino acid prodrug (VS) and parent drug (SQV). (**a**): LLS; (**b**): LDS; (**c**): DLS; (**d**): DDS.

Each point represents mean ± SD (n=3).

in 0.25 mg/ml cell homogenates after 3-hour incubation was around 35%, while more than 70% of DLS, DDS, and LDS were detected in the samples. Table 3 displays the estimated degradation rate constants and half-lives obtained from linear regression of pseudo-first-order plots of prodrug concentration *versus* time in cell homogenate. DDS exhibited the highest enzymatic stability with a half life of 12.6 h. LLS showed 7-fold less stability compared to DDS. LDS and DLS showed similar moderate stability in response to enzymatic hydrolysis in Caco-2 cell homogenates.

Table 3. Degradation rate constants (K_d) and half lives ($t_{1/2}$) for various SQV prodrugs in 0.25 mg protein/ml Caco-2 cell homogenates.

	K_d (h^{-1})	$t_{1/2}$ (h)
LLS	0.4145 ± 0.0488	1.7 ± 0.2
LDS	0.0948 ± 0.0052	7.3 ± 0.4
DLS	0.0930 ± 0.0062	7.5 ± 0.5
DDS	0.0548 ± 0.0039	12.6 ± 0.9

Data represented are mean ± SD (n=3).

3.3 Cytotoxicity studies

   LDH is a stable cytoplasmic enzyme present in most cells. It is released into cell culture medium upon damage of the cell membrane and can be detected to represent damage in cells. Results obtained after 24 hours-exposure of different amounts of SQV (pro)drugs (5 µM to 150 µM) to MDCK-WT cells are depicted in Figure 2. SQV and its prodrugs display cellular toxicity in a concentration-dependant manner. In comparison to SQV, significantly lower cellular toxicity was induced from prodrugs especially at higher concentrations. Two *D*-valine conjugate DDS displayed the least toxic on MDCK-WT cells at all concentration levels in this study.

3.4 Affinity for efflux pumps

   Accumulation of [^{14}C] erythromycin (0.25 µCi/ml), a model substrate for both P-gp and MRP2, was determined in the presence of SQV or its peptide derivatives in MDCK cells to evaluate the affinity of prodrugs for P-gp or MRP2 efflux proteins. Results depicted in Figure 3 show that uptake of [^{14}C]erythromycin in MDCK-MDR1 cells in the presence of 75 µM of drugs were 286.7 ± 15.7% (SQV), 209.7 ± 10.0% (LLS), 214.9 ± 5.8% (LDS), 182.0 ± 17.5% (DLS), and 219.4 ± 8.7% (DDS), respectively. Similar results were also found in MDCK-MRP2 cells (Figure 3). Accumulation of [^{14}C]erythromycin was reduced significantly from 3-fold with SQV to less than 2-fold with various SQV stereoisomeric dipeptide prodrugs in both MDR1 and MRP2 cells.

3.5 Recognition by peptide transporters

   Gly-Sar is a good substrate for peptide transporters. Figure 4 shows that accumulation of [^{3}H]Gly-Sar was inhibited in the presence of 1 mM unlabeled Gly-Sar or 75 µM dipeptide (*L*-val-*L*-val) in both transfected MDCK cells. Dipeptide conjugates LLS, LDS, and DLS (75 µM) displayed significant inhibition to [^{3}H]Gly-Sar uptakes to the range of 65%-75% relative to control in MDCK-MDR1 cells, whereas SQV and DDS did not show any significant inhibition on [^{3}H]Gly-Sar uptake. Results in MDCK-MRP2 cells also exhibit similar inhibition effects on

accumulation of [3H]Gly-Sar. Percentage of [3H]Gly-Sar uptake observed for LLS, LDS, DLS, and DDS compared to the control was 73.4%, 72.3%, 73.9% and 94.5%, respectively.

3.6 Transepithelial permeability determination

To determine whether stereoisomeric prodrug modifications may actually improve absorption across intestinal epithelium, both apical-to-basolateral (A-B) and basolateral-to-apical (B-A) permeabilities for SQV and its prodrugs were measured across MDCK-MDR1 and MDCK-MRP2 cell monolayers. The transport studies were performed at pH 5.4 to reduce chemical hydrolysis since all dipeptide prodrugs of SQV showed higher stability in comparison to pH 7.4 (Table 2). Bidirectional transport profiles of SQV were compared in the presence or absence of 5 µM P-gp inhibitor GF120918 and 50µM MRP inhibitor MK571 (Figure 5). The A-B flux of SQV was less than B-A flux across both MDR1 and MRP2 cells. The presence of GF120918 and MK571 reduced the efflux ratio between B-A and A-B directions significantly to around 1.0 (Table 4).

Table 4. Apparent permeabilities (P_{app}) of SQV and stereoisomeric prodrugs on apical-to-basolateral direction (A-B) and basolateral-to-apical direction (B-A) across MDCK-MDR1 and MDCK-MRP2 cell monolayers.

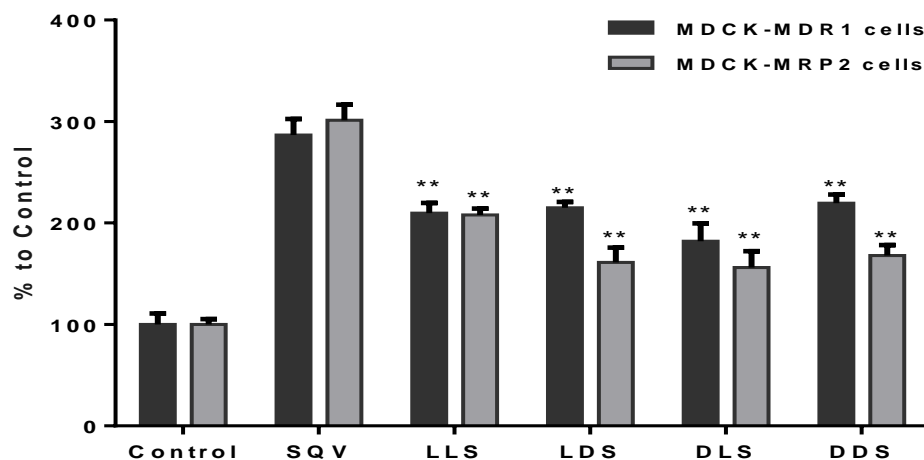|	MDCK-MDR1 cells			MDCK-MRP2 cells		
	P_{app}(A-B) (cm/s, $\times10^{-6}$)	P_{app}(B-A) (cm/s, $\times10^{-6}$)	Efflux ratio	P_{app}(A-B) (cm/s, $\times10^{-6}$)	P_{app}(B-A) (cm/s, $\times10^{-6}$)	Efflux ratio
SQV	0.90±0.16	4.06±0.48	4.50	2.47±0.30	7.51±0.24	3.04
SQV+MK571(50µM)	ND	ND	ND	5.95±1.60	5.97±0.81	1.00
SQV+GF120918(5µM)	2.66±0.21	3.03±0.33	1.14	ND	ND	ND
LLS	1.66±0.09	2.27±0.24	1.37	4.98±0.45	5.72±1.18	1.15
LDS	1.52±0.31	2.13±0.57	1.40	4.60±1.35	6.08±1.10	1.32
DLS	2.08±0.43	2.69±0.43	1.29	5.10±0.98	5.90±1.15	1.16
DDS	1.35±0.14	2.52±0.12	1.87	4.66±0.57	6.61±0.23	1.42

Data represented are mean ± SD (n=3). ND: not determined

Furthermore, comparison of transepithelial transport between SQV and stereoisomeric prodrugs was evaluated by determining apparent permeability coefficients (P_{app}) in both transport directions. Results in Table 4 indicate that A-B permeability of SQV was significantly enhanced by stereoisomeric prodrug modification, and B-A permeability was decreased on both MDR1 and MRP2 cell monolayers. The efflux ratios for prodrugs are in the range of 1.3 to 1.9 on MDCK-MDR1 cells and 1.1 to 1.4 on MDCK-MRP2 cells, respectively, which are much lower than that of SQV (4.50 on MDCK-MDR1 and 3.04 on MDCK-MRP2), but comparable to the values in the presence of P-gp inhibitor (1.14) and MRP inhibitor (1.00).
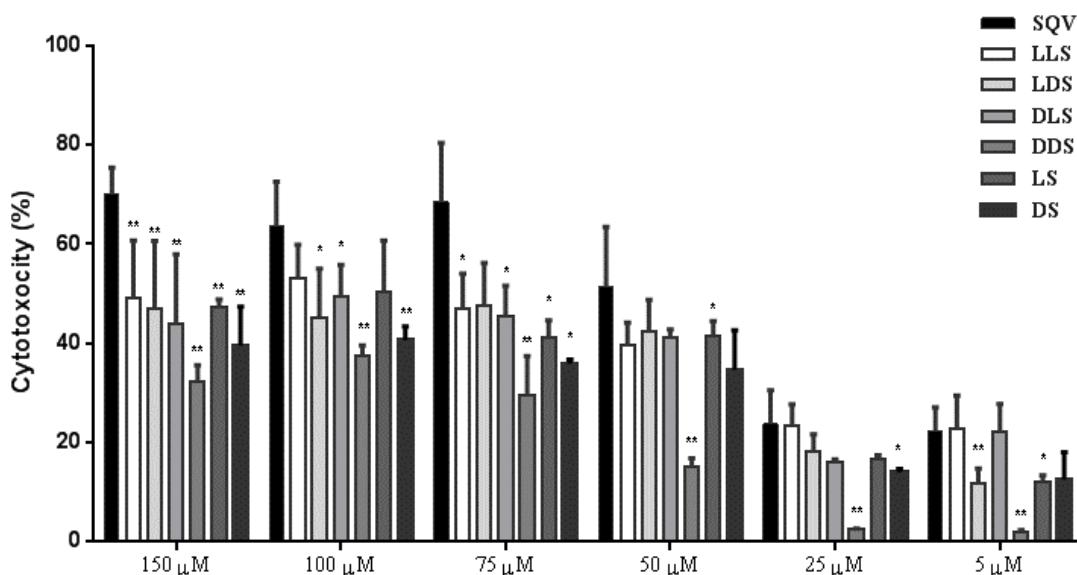

Figure 2. LDH cytotoxicity assay of SQV and stereoisomeric prodrugs after 24-hour incubation on MDCK-WT cells.

Each point represents mean ± SD (n=4). * $P < 0.05$, significant difference compared with SQV at same concentration. ** $P < 0.01$, very significant difference compared with SQV at same concentration.

Figure 3. Uptake of [^{14}C]erythromycin (0.25 µCi/ml) in the absence (Control) or presence of 75 µM of SQV and its prodrugs in MDCK-MDR1 and MDCK-MRP2 cells.

Each point represents mean ± SD (n=4). ** *P*<0.01, very significant difference compared with uptake of [^{14}C]erythromycin in the presence of SQV.
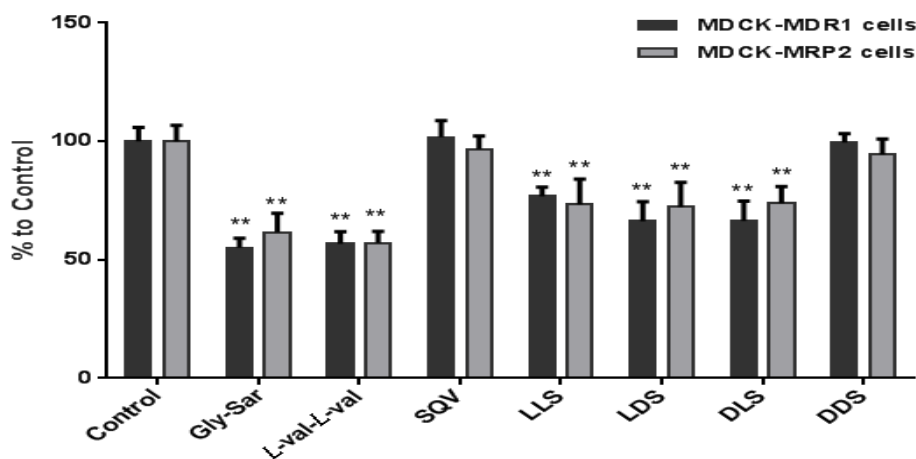
Figure 4. Uptake of [^{3}H] Gly-Sar (0.5 µCi/ml) in the absence (control) or presence of unlabeled Gly-Sar (1 mM), *L*-val-*L*-val (75 µM), SQV (75 µM) or SQV prodrugs (75 µM) in MDCK-MDR1 and MDCK-MRP2 cells.

Each point represents mean ± SD (n=4). ** *P*<0.01, very significant difference compared with control.
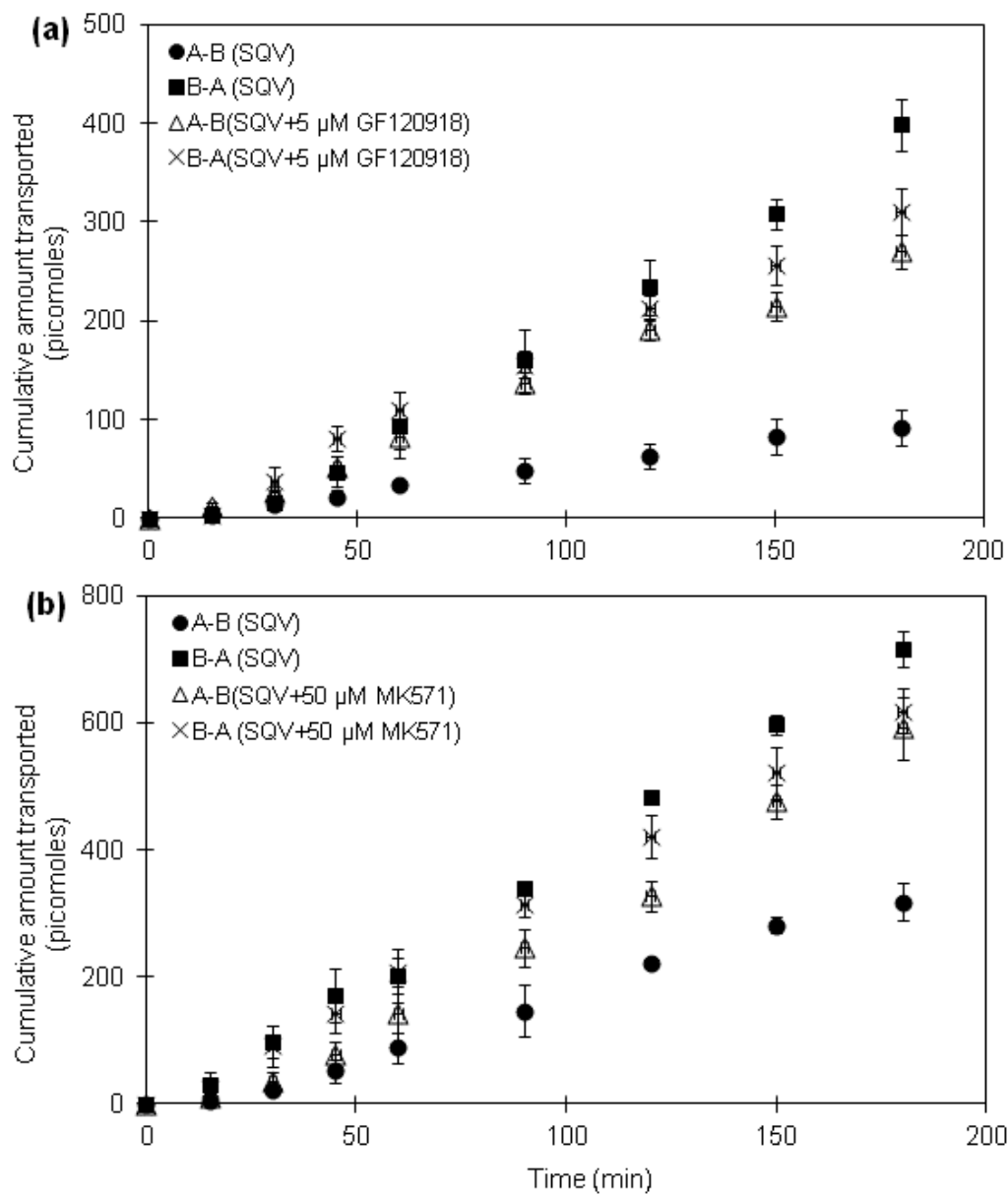
Figure 5. Bidirectional transepithelial transport of SQV (10 µM) across MDCK-MDR1 (**a**) or MDCK-MRP2 (**b**) cell monolayers.

Each point represents mean ± SD (n=3).

4. **Discussion**

   Apparent aqueous solubilities of all stereoisomeric val-val-SQV increased to more than 2-fold over SQV mesylate salt, and 1000-fold over SQV which is predicted as 2.47 µg/ml at 25°C (DrugBank database: DB01232). This increased solubility can be explained by three dimensional structure modification of SQV by amino acids. Generally the formation of hydrogen bonds between compounds and water molecules is considered to have strong influence on physical properties like aqueous solubility. The high hydrogen-bonding potential and the presence of charged amino groups in the promoiety val-val may dramatically contribute to the hydrophilicity of the conjugates. Similar results are obtained by MSX-4, a valine ester prodrug of the adenosine A2A receptor antagonist MSX-2, with an enhanced solubility to more than 73 times over its parent drug [19].

   A good prodrug candidate after oral administration should be stable enough in gastrointestinal (GI) tract, and then be absorbed across intestinal mucosa and converted to intact parent drug following its transport. Degradation of peptide prodrug in GI tract can proceed either by enzyme-catalyzed hydrolysis or *via* a base-catalyzed hydrolysis yielding amino acid prodrug or parent drug directly. Therefore chemical and enzymatic stability studies will help in screening the ideal prodrug candidate for the further research. Results in Table 2 indicate that all stereoisomeric valine-SQV conjugates underwent acid/base hydrolysis in the pH range of 1.4 to 10.4. Chemical stability enhanced with the increased number of isomers in promoieties, suggesting that dipeptide conjugates are chemically more stable than amino acid conjugates. These results are consistent with previous observations [20, 21]. In comparison to *L*-valine conjugates, the attachment of *D*-valine with SQV exhibited higher chemical stability under various pH conditions. The $t_{1/2}$ values of DS at different pH are either higher or comparable to that of LS, and DDS displayed the longest degradation half lives at all pH conditions and is considered to be the most stable compound among all prodrugs. Desiderio *et al.* also reported this stereoselective chemical stability. Approximately 42% of *D,L*-adenosylmethionine and 26% of *L,L*-adenosylmethionine were left after 14 days- incubation in sodium phosphate buffer (pH 2.5), respectively, indicating *L,L*-diastereoisomer showed a stronger chemical hydrolysis than the *D,L*-form [22]. Since the physical pH of major portion of drug absorption in GI tract is ranged from 5 to 8, dipeptide conjugates of SQV with longer residence time in intestine could be better candidates for oral drug delivery in comparison to amino acid prodrugs.

   Enzymatic hydrolysis of SQV dipeptide prodrugs in rat intestinal homogenates has been performed in our laboratory [23]. However, it is very difficult to isolate intestinal mucosa from animal intestine for preparation of homogenates. Therefore application of epithelial cell homogenates may provide a reliable way to investigate drug hydrolysis in intestine. Human colon adenocarcinoma cell line Caco-2 cells was used in metabolic hydrolysis study because it has been reported to be particularly abundant in many esterases like carboxylesterases and peptidases including aminopeptidases, dipeptidyl peptidases, endopeptidase, and membrane dipeptidase [24-26]. These hydrolytic enzymes play an important role in the degradation of dipeptide prodrug to parent drug when it is absorbed across the intestinal mucosa. Hydrolysis pattern of SQV dipeptide prodrugs (Figure 1) is observed to be compliant to the mechanism of

metabolic degradation as demonstrated by the previous study [21]. VVS was degraded to VS by peptidases and further to SQV by esterases. Regeneration of SQV directly from dipeptide conjugates is also possible but the amount is very limited. Incorporation of *D*-isomer enhances the enzymatic stability of conjugation significantly. The half-lives of DLS and LDS are 4.4-fold and 4.3-fold longer than that of LLS, respectively. DDS exhibits the most stability in cell homogenate, with a 7.4-fold longer half-life over LLS. This stereoselective enzymatic stability may due to the different affinities to hydrolases. These stereoisomers can interact with hydrolases with different kinetic parameters since they have different three-dimensional structures. Results in this study indicate that LLS is an excellent substrate for hydrolases. Incorporation of *D*-valine might reduce the recognition by hydrolases, thus it decreases the interaction between conjugates and active site of the enzymes. Correspondingly, DDS displayed significantly enhanced stability in cell homogenates.

All prodrugs displayed similar or much lower toxicity in comparison to parent drug SQV (Figure 2). Additionally, the toxicity induced by prodrugs was stereoselective on MDCK-WT cells. *D*-isomer conjugates, especially DDS, showed relatively lower cytotoxicity than other prodrugs. This stereoselecitive toxicity may be explained by the results of enzymatic stability. Less enzymatic hydrolysis would lead to less cellular accumulation of SQV from prodrug degradation, consequently contribute to less cytotoxicity because SQV is more toxic than prodrugs.

Drug efflux proteins P-gp and MRP2, which are highly expressed in the intestinal mucosa, could be responsible for the low oral bioavailability of SQV [27, 28]. Peptide prodrugs have been reported by our laboratory for their partial avoidance of efflux pump mediated transport [18, 29, 30]. However, modification of the three-dimensional structure may affect the interaction of the substrate with the enzyme significantly. In this regard, comparison of the affinity of SQV and stereoisomeric valine-valine prodrugs towards efflux transporters was examined employing human *MDR1* and *MRP2* gene-transfected MDCK cell lines. These two cell lines are widely employed as *in vitro* models of intestinal epithelium for screening drug permeability [31-33]. Cellular uptake of [^{14}C]erythromycin was enhanced to 2.9-fold over the control with the administration of 75 µM SQV on MDCK-MDR1 cells, suggesting that the binding sites on P-gp were competitively occupied by SQV, thereby the cellular accumulation of another P-gp substrate erythromycin increased. However, equimolar amount of prodrugs exhibited significant reduction of [^{14}C]erythromycin uptake compared to SQV (Figure 3). Similar extent of inhibition was also observed on MDCK-MRP2 cells. These differential inhibitory activities indicate that all four stereoisomeric peptide modifications possess lower affinity for both P-gp and MRP2 relative to SQV, and consequently result in reduced cellular accumulation of [^{14}C]erythromycin.

Conversely, recognition of prodrugs by peptide transporters was the primary objective of our study. The presence of peptide transporters has been reported on MDCK cells [34]. Uptake of [^{3}H]Gly-Sar, a typical substrate for peptide transporters, was inhibited by unlabeled Gly-Sar and val-val (Figure 4), suggesting the expression of peptide transporters on both MDCK-MDR1 and MDCK-MRP2 cell lines. Interaction between SQV prodrugs and peptide transporters is observed to be stereoselective. It is evident by the diminished cellular uptake of [^{3}H]Gly-Sar in the

presence of LLS, LDS, and DLS, except two *D*-isomer conjugate DDS. These results are consistent with the previous studies that peptides containing *L*-amino acids showed higher interactions with peptide transporters than those containing *D*-amino acids [35, 36]. Our findings in uptake studies clearly demonstrate that stereoisomeric prodrugs LLS, LDS, and DLS could be translocated into cells by peptide transporter-mediated influx as well as bypass efflux transporters, whereas DDS may only evade efflux pumps and did not show reasonable affinity for the peptide transporters.

Comparison of transepithelial flux of SQV on absorptive (apical-to-basolateral, A-B) and secretive (basolateral-to-apical, B-A) directions demonstrates the influence of efflux transporters which are located on apical membrane of cells. Results in Figure 5 and Table 4 exhibited a much lower absorptive transport of SQV compared to excretive transport. This asymmetric permeation is due to the involvement of efflux transporters present on apical cell membrane. Similar bidirectional transepithelial flux of SQV was obtained in the presence of GF120918 or MK571, inhibitors for P-gp and MRP2 respectively. It confirms that efflux activities of P-gp and MRP2 proteins are one of the major obstacles which limit intestinal absorption of SQV. Both MDCK-MDR1 and MDCK-MRP2 cell lines are originated from same wild type MDCK cells. However, the apparent permeabilities of peptide prodrugs across MDCK-MDR1 are different from MDCK-MRP2 monolayers (Table 4). It suggests a different capacity of peptide translocation in these two cell lines. Previous studies in our laboratory have reported that transfection of human efflux genes like *MDR1* influences the functional activities and biological expression of endogenous transporters such as peptide transporters [37, 38]. According to P_{app} values summarized in Table 4, the bidirectional translocation of all prodrugs displayed insignificant asymmetry across both transfected MDCK cell lines. This increment may be due to combined effects of higher solubility and stability, less affinity for the efflux transporters and better recognition by peptide transporters. Among all stereoisomeric peptide prodrugs, the efflux ratio of DDS exhibited the least difference from SQV, suggesting its translocation across both MDCK cells is not as efficient as other isomeric conjugates. Even though DDS showed excellent chemical and enzymatic stability which keeps more intact peptide prodrugs remaining in the 3 hours-transport study, the failure to be recognized by peptide transporters might attribute to its relatively low transepithelial permeation.

Our present study suggests that stereoisomeric peptide might be an attractive promoiety employed in targeted prodrug design. All valine-valine-saquinavir stereoisomeric modifications exhibit similar enhanced aqueous solubility as well as reduced affinity for efflux transporters, but significant stereoselectivity in cellular toxicity, chemical and enzyme-catalyzed hydrolysis, and recognizition by peptide transporters. Conjugation of *L*-valine to SQV illustrates higher affinity towards peptide transporters but poor stability and higher toxicity, whereas incorporation of *D*-valine displays good stability and lower toxicity but weak binding with peptide transporters. The results in this research indicate that *D*-valine-*L*-valine-SQV and *L*-valine-*D*-valine-SQV might be potential targeted prodrug candidates which improve intestinal absorption of saquinavir and enhance oral bioavailability.

5. Acknowledgements

6. References

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