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Circumventing P-Glycoprotein-Mediated Cellular Efflux of Quinidine by Prodrug Derivatization

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Abstract: The objective of this study is to investigate whether transporter-targeted prodrug derivatization of quinidine, a model P-glycoprotein (P-gp) substrate, can circumvent P-gp-mediated efflux. The L-valine ester of quinidine (val-quinidine) was synthesized in our laboratory. Uptake and transport studies were carried out using the MDCKII-MDRI cell line at 37 °C for 10 min and 3 h, respectively. [^{3}H]Ritonavir and cyclosporine were also used as model P-gp substrates to delineate the kinetics of translocation of val-quinidine across the MDCKII-MDRI cell monolayer. The rate of uptake of [^{3}H]ritonavir by MDCKII-MDRI cells exhibited a 2-fold increase in the presence of 75 μM quinidine, but 75 μM val-quinidine did not demonstrate any effect on [^{3}H]ritonavir uptake. The rate of transport of quinidine from the basolateral to the apical membrane [$(18.3 \pm 1.25) \times 10^{-6}$ cm s^{-1}] and from the apical to the basolateral membrane [$(6.5 \pm 0.66) \times 10^{-6}$ cm s^{-1}] exhibited a 3-fold difference. However, transport of val-quinidine from the apical to the basolateral membrane [$(5.13 \pm 0.49) \times 10^{-6}$ cm s^{-1}] and from the basolateral to the apical membrane [$(6.17 \pm 1.28) \times 10^{-6}$ cm s^{-1}] did not demonstrate any statistically significant difference. Moreover, cyclosporine, a potent P-gp substrate and/or inhibitor, did not alter the transport kinetics of val-quinidine. The rates of uptake of [^{3}H]Gly-Sar and various amino acid model substrates were reduced in the presence of 200 μM val-quinidine. Results from this study clearly indicate that prodrug derivatization of quinidine into val-quinidine can overcome P-gp-mediated efflux. Val-quinidine once bound to a peptide or amino acid transporter is probably not recognized and cannot be accessed by the P-gp efflux pump. Transporter-targeted prodrug derivatization seems to be a viable strategy for overcoming P-gp-mediated efflux.

Keywords: Efflux; prodrug; P-glycoprotein; val-quinidine

Introduction

P-Glycoprotein (P-gp) is a member of the large ATP binding cassette superfamily of transport proteins also termed traffic ATPases.[1] 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.[2−4]

P-gp is ubiquitously expressed on human tissues such as intestinal mucosa, brain capillary endothelial cells, biliary

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canaliculus, and kidney tubules.[5] The broad substrate specificity of P-gp is a major factor responsible for subtherapeutic levels of various drugs in blood and tissues.[6] A recent report suggests that the presence of this transporter on the brush border membrane of intestinal epithelium not only diminishes the 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 the level of exposure of the drug to cellular as well as luminal enzymes.[7−9] Thus, oral bioavailability of drugs which are P-gp substrates, such as anticancer, anti-HIV, and calcium channel-blocking agents, is limited by this efflux pump. Similarly, overexpression of P-gp by tumor cells imparts multidrug resistance. Several anticancer agents such as paclitaxel, vincristine, vinblastine, actinomycin D, colchicines, and daunorubicin are actively effluxed from tumor cells, rendering P-gp a major barrier to chemotherapy. Also, the high level of expression of this transporter on the blood−brain barrier (BBB) restricts the passage of P-gp substrates such as anti-HIV drugs ritonavir, saquinavir, nelfinavir, and various anticancer agents into the brain, imposing a major challenge in the treatment of various brain diseases.[10,11]

It has been previously reported that inhibition of P-gp by various modulators can lead to improved absorption of drugs across intestine, kidney, and BBB.[12−15] Various agents that are inhibitors of P-gp are often co-administered with P-gp substrates (therapeutic agents), resulting in increased bioavailability of the therapeutic agent.[16] However, systemic administration of P-gp inhibitors is limited by their toxicity resulting from high serum concentrations achieved with the doses required to inhibit P-gp. Although various approaches to overcoming P-gp-mediated drug efflux have been studied, P-gp still remains a major barrier to oral and CNS drug absorption.

Besides efflux transporters, such as P-gp being expressed in the inner leaflet of the apical membrane, a number of nutrient transporters are also expressed on the outer leaflet of cellular membranes. These nutrient transporters are responsible for the influx of various nutrients and drugs into various epithelial cells (enterocytes) and endothelial cells (e.g., BBB).[17−20] Recently, transporter-targeted prodrug derivatization has attracted much attention among drug delivery scientists. Prodrugs have been designed such that the modified compounds become substrates of nutrient transporters causing enhanced absorption of these drugs across various physiological barriers.[21−23]

When a substrate binds to a nutrient transporter, it triggers a configurational change in the transport protein, as a result

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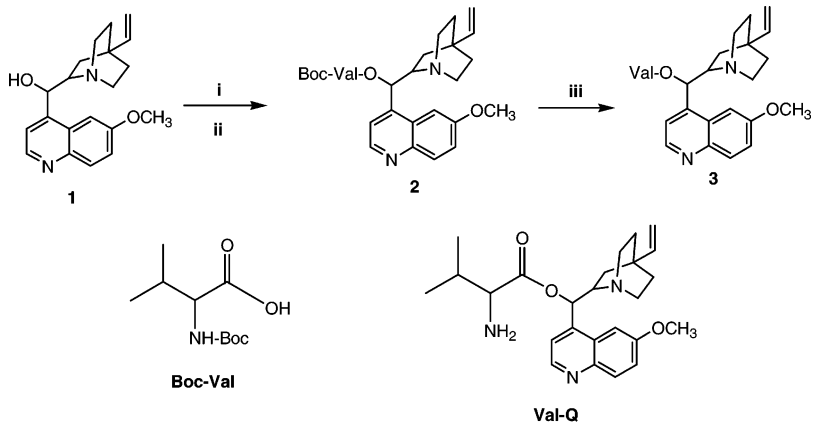
Scheme 1. Synthetic Pathway to Val-Quinidine[a]



[a] (i) Boc-Val, DCC, DMF, 0 °C, 1 h; (ii) DMAP, DMF, 24 h, room temperature; (iii) TFA, 0 °C, 60 min.

of which it is translocated across the membrane into the cell cytoplasm and released. 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. The objective of this study is to investigate whether transporter-targeted prodrug derivatization of quinidine, a model P-gp substrate, can result in the prevention of P-gp-mediated efflux. To accomplish this goal, an amino acid prodrug of quinidine, val-quinidine (Val-Q), was synthesized in our laboratory. Transport and uptake studies were carried out with the Madin-Darby canine kidney (MDCKII-MDRI) cell line. This cell line has been genetically engineered to express high levels of P-gp and is widely used in the study of P-gp-mediated efflux.[24] [^{3}H]Ritonavir and cyclosporine were selected as a model P-gp substrate and/or inhibitor to elucidate the mechanism involved in the uptake and transport of quinidine and val-quinidine.

Experimental Section

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]Arginine (41.0 Ci/mmol), [^{3}H]alanine (85.0 Ci/mmol), [^{3}H]glutamic acid (42.9 Ci/mmol), [^{14}C]glycine (112 mCi/mmol), [^{3}H]lysine (98.5 Ci/mmol), and [^{3}H]tyrosine (51.5 Ci/mmol) were purchased from Perkin-Elmer Life Sciences Inc. (Boston, MA). MDCK cells, retrovirally transfected with the human *MDR1* cDNA (MDCKII-MDR1), were a gift from P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). The growth medium Dulbecco's modified Eagle's Medium (DMEM), calf serum, minimum essential medium, and nonessential amino acids were obtained from Gibco (Invitrogen, Grand Island, NY). Penicillin, streptomycin, sodium bicarbonate, quinidine, and HEPES were purchased from Sigma Chemical (St. Louis, MO). Culture flasks (75 cm^2 growth area), polyester Transwells (pore size of 0.4 μm and 12 mm diameter), and 12-well plates were obtained from Costar (Cambridge, MA). Boc-Val-OH, buffer components, and other solvents were obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Synthesis of Val-Quinidine. Boc-Val-OH (2.00 g, 9.26 mmol) and dicyclohexylcarbodiimide (DCC, 1.90 g, 9.26 mmol) were dissolved in dry DMF (20 mL) under a nitrogen atmosphere (Scheme 1). The mixture was stirred continuously for 1 h at 0 °C. A solution of quinidine (Q, 1.50 g, 4.63 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (DMAP, 0.06 g, 0.65 mmol) in DMF (100 mL) was added dropwise to the reaction mixture at 0 °C. The reaction mixture was stirred and allowed to warm to room temperature. After 24 h, the reaction was checked by TLC (1:13 methanol/dichloromethane mixture) and LC−MS analysis, and was found to be complete. The urea derivative was removed by filtration and washed with ethyl acetate. Solvents from the combined filtrate and washing were totally removed under reduced pressure.

The resulting oil was dissolved in ethyl acetate and washed three times with a 5% $NaHCO_3$ solution. Each time, the organic layer was separated and the combined layers were dried over anhydrous magnesium sulfate. Ethyl acetate was rotary evaporated at 40 °C, and the oily residue was redissolved in ethyl acetate and kept at 4 °C for 3 h. The white precipitate was removed by filtration and washed with another small amount of ethyl acetate. The solvent was removed with a rotary evaporator at 40 °C, and the compound was completely dried under vacuum (92% yield). The product Boc-Val-Q (**2**, Scheme 1) was confirmed by LC−

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MS analysis [MS (+ESI) M + 1 = 523.8]. Boc-Val-Q (3.00 g) was dissolved in trifluoroacetic acid (TFA, 60 mL) and stirred at 0 °C for 1 h. The excess acid was removed under vacuum, and the oily residue was mixed with toluene (100 mL) and rotary evaporated at 40 °C. The product Val-Q was kept under vacuum overnight until it was completely dry. Val-Q was a highly hygroscopic yellowish solid fluffy material (yield of 3.21 g). The structure was confirmed by ^{1}H NMR and LC−MS analysis [MS (+ESI) M + 1 = 423.8]: ^{1}H NMR of val-quinidine (Me_2SO-d_6) δ 1.06 [6H, m, $(CH_3)_2CH$], 1.5, 1.8 [4H, m, $(CH_2)_2$-C], 2.00 [1H, m, CH-$(CH_3)_2$], 2.16 (2H, m, CH_2CH), 2.7, 2.9 [4H, m, $(CH_2)_2N$], 3.31 (3H, m, NH_2 and CH-N), 3.50 (1H, m, CH-NH_2), 4.01 (3H, s, CH_3O), 4.4−5.2 (3H, m, CH_2═CH), 6.19 (1H, m, CHO), 7.15, 7.49, 7.53, 7.68, 8.02, 8.30, 8.60, 8.78 (6H, aromatic). Elemental analysis indicated that Val-Q contains 2 mol of TFA.

Cell Culture. MDCK cells retrovirally transfected with the human *MDR1* cDNA (MDCKII-MDR1) (passages 4−10) were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cell confluence was assessed by light microscopy. Cells were passed at 80−90% confluence using 0.05% trypsin EDTA and were seeded at a density of 50 000 cells/cm^2 on 12-well tissue culture plates or on Transwell inserts. Cells were maintained in DMEM, supplemented with 10% calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acid, 3.7 g of sodium bicarbonate, and 10 mM HEPES (pH 7.4). Cells were allowed to grow for 5−8 days. Monolayer integrity was evaluated by monitoring transepithelial electric resistance (TEER), with an EVOM (epithelial volt ohmmeter from World Precision Instruments, Sarasota, FL). TEER values of the cell monolayer were approximately 250 Ω·cm^2.

Stability Studies in Cell Suspension 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 scraper, suspended in 2 volumes of water, and homogenized (Multipro variable-speed homogenizer from DREMEL, Racine, WI). Suitable dilutions were made to generate a final protein concentration of 0.25 mg/mL. The protein content was determined according to method of Bradford[25] with the Bio-Rad protein estimation kit.

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

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Stability in DPBS was determined by incubating 200 μL of a prodrug solution with 800 μL of DPBS with the pH adjusted to 5, 6, or 7.4. The slope of the line of the "log percentage prodrug remaining versus time" plot was employed to calculate the degradation rate constants.

Uptake Studies. Uptake studies were conducted using confluent cell monolayers 6−8 days postseeding. The medium was aspirated, and cells were washed thrice with DPBS (pH 7.4). Uptake was initiated by adding 1 mL of a drug solution (in the presence or absence of competing substrates) to the wells. Incubation was carried out for a period of 10 min. At the end of the incubation period, the drug solution was removed and the cell monolayer was washed thrice with ice-cold stop solution. Cells were lysed overnight [using 1 mL of 0.1% (w/v) Triton X-100 in 0.3 N sodium hydroxide] at room temperature. Aliquots (500 μL) were withdrawn from each well and transferred to scintillation vials containing 5 mL of scintillation cocktail. Samples were then analyzed by liquid scintillation spectrophotometry using a Beckman scintillation counter (model LS-6500, Beckman Instruments, Inc.). The rate of uptake was normalized to the protein content of each well. The amount of protein in the cell lysate was quantified by the method of Bradford utilizing the Bio-Rad protein estimation kit (Bio-Rad, Hercules, CA).

Transport Studies. Transport studies were conducted with MDCKII-MDRI cell monolayers grown on 12-well Transwell inserts (12 mm diameter). The medium was aspirated, and cell monolayers were washed three times (10 min per 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. Aliquots (200 μL) were withdrawn at predetermined time intervals, i.e., 15, 30, 45, 60, 90, 120, 150, and 180 min, and replaced with fresh DPBS (pH 7.4) to maintain sink conditions. Samples were stored at −80 °C until further analysis. Experiments designed to investigate the effect of inhibitors involved preincubation of the cells with the inhibitor for a period of 30 min (inhibitor solution was added to both donor and receiver chambers) prior to the addition of the drug solution (also containing the inhibitor) to initiate the transport study. All transport experiments were performed at 37 °C.

Analytical Procedure. Quinidine and val-quinidine samples were assayed by reversed phase high-performance liquid chromatography (HPLC). The HPLC system comprised an HP 1050 pump, an Agilent 1100 series fluorescence detector, and an Alcott autosampler (model 718AL HPLC). A C(8) Luna column (250 mm × 4.6 mm; Phenomenex, Torrance, CA) was employed in the analysis. The mobile phase consisted of 20 mM phosphate buffer (pH adjusted to 2.5) and 12% acetonitrile as the organic modifier. The parent drug and the prodrug were detected with a fluorescence detector (λ_{ex} = 240 nm, λ_{em} = 450 nm). Val-quinidine eluted at approximately 6 min, while quinidine eluted at 7 min.

Data Analysis. Cumulative amounts of val-quinidine and quinidine transported across the cell monolayers were plotted

Table 1. Buffer Hydrolysis and Enzymatic Degradation in the MDCK Cell Homogenate (protein content diluted to 0.2 mg/mL with water) of Val-Quinidine ($n = 4$)

	degradation rate constant (min^{-1})		% val-quinidine remaining after 3 h		degradation $t_{1/2}$ (h)	
	mean	SD	mean	SD	mean	SD
DPBS (pH 5)	1.06×10^{-4}	0.33×10^{-4}	96.66	1.86	116.5	38
DPBS (pH 6)	10.78×10^{-4}	0.25×10^{-4}	82.69	0.26	10.71	0.25
DPBS (pH 7.4)	20.44×10^{-4}	0.97×10^{-4}	70.27	2.61	5.65	0.26
MDCK homogenate	20.13×10^{-4}	0.513×10^{-4}	65.97	1.01	5.74	0.14

as a function of time to determine the permeability coefficient. Linear regression of the amounts transported as a function of time yielded the rate of transport across the cell monolayer (dM/dt). The rate divided by the cross-sectional area available for transport (A) generated the steady-state flux as shown in eq 1.

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

Permeability was calculated by normalizing the steady-state flux to the donor concentration (C_d) of the drug according to eq 2.

$$\text{permeability} = \text{flux}/C_d \quad (2)$$

Statistical Analysis. All experiments were conducted at least in quadruplicate ($n = 4$), and results are expressed as means ± the standard deviation (SD). Statistical comparison of mean values was performed using one-way analysis of variance (ANOVA) or a Student's t test (Graph Pad INSTAT, version 3.1). A P of <0.05 was considered to be statistically significant.

Results

Effect of Val-Quinidine on the Uptake of [^{3}H]Ritonavir by MDCKII-MDR1 Cells. [^{3}H]Ritonavir (0.5 μCi/mL) uptake studies were conducted in the presence of equimolar concentrations of quinidine and val-quinidine on MDCKII-MDR1 cells. Uptake studies were initiated by adding 1 mL of a [^{3}H]ritonavir solution (0.5 μCi/mL) in the presence or absence of quinidine and val-quinidine (75 μM). Solutions were prepared in DPBS (pH 7.4), and the studies were conducted for 10 min. The rate of [^{3}H]ritonavir uptake dramatically accelerated from 2.5 to 5.3 pmol min^{-1} mg^{-1} (a 2-fold increase) in the presence of quinidine (75 μM). However, uptake of [^{3}H]ritonavir in the presence of val-quinidine (75 μM) was unaltered (Figure 1). Cellular uptake rates of [^{3}H]ritonavir in the presence of 100 and 200 μM val-quinidine were 2.8 and 3.1 pmol min^{-1} mg^{-1}, respectively (Figure 1).

Stability of Val-Quinidine in Transport Buffers and MDCKII-MDR1 Cell Homogenates. The stability of val-quinidine was determined in MDCKII-MDR1 cell homogenates with DPBS at pH 5, 6, or 7.4 for 3 h. The percentage of val-quinidine remaining in the solution after 3 h ranged from 65% in the MDCK cell homogenate to 97% in DPBS at pH 5 (Table 1). As a control, degradation studies were carried out in water for 3 h. No appreciable degradation was seen in water. The degradation rate constant was highest in

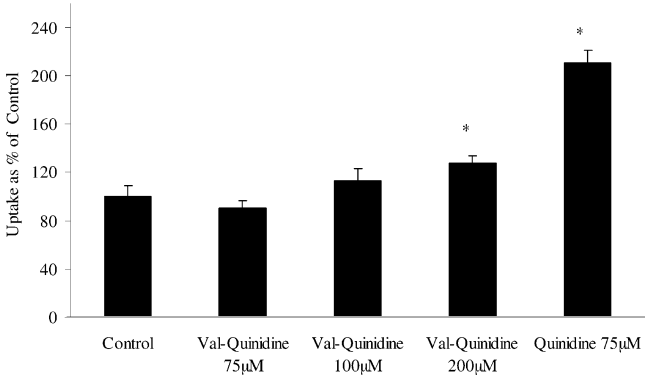

Figure 1. Cellular uptake of [^{3}H]ritonavir (0.5 μCi/mL, 1.0 Ci/mmol) by MDCKII-MDR1 cell monolayers in the absence (control) and presence of quinidine and val-quinidine. Significant inhibition of P-gp-mediated efflux of [^{3}H]ritonavir was observed with 75 μM quinidine and 200 μM val-quinidine (asterisks indicate $p < 0.05$). Values are means ± SD ($n = 4$).

DPBS at 7.4 and MDCKII-MDR1 cell homogenates. Degradation half-lives in DPBS buffer at pH 5, 6, and 7.4 were found to be 116, 10.75, and 5.65 h, respectively, at 37 °C. The degradation half-life at 37 °C in the MDCKII-MDR1 cell homogenate was 5.74 h.

Degradation studies suggest that some percentage of quinidine will be generated from val-quinidine during the uptake and transport experiments.

Transport of Quinidine. Transepithelial bidirectional transport of quinidine across MDCKII-MDR1 cells reveals that the transepithelial transport of quinidine in the absorptive direction [apical (AP) to basolateral (BL)] was significantly ($p < 0.05$) slower than that in the secretory (BL to AP) direction (Figure 2A). The apparent permeability of 10 μM quinidine (P_{app}) in the basolateral to apical direction was $(18.3 \pm 1.25) \times 10^{-6}$ cm s^{-1} and in the apical to basolateral direction was $(6.5 \pm 0.66) \times 10^{-6}$ cm s^{-1}, a 3-fold difference. In the presence of 10 μM cyclosporine, the secretory permeability (P_{app}) of quinidine decreased from $(18.3 \pm 1.25) \times 10^{-6}$ to $(11.2 \pm 0.8) \times 10^{-6}$ cm s^{-1} (Figure 2B) which is statistically significant ($p < 0.05$). The metabolism of quinidine during the transport process was not observed.

Transport of Val-Quinidine. Transport of val-quinidine was carried out at the same molar concentration as that of quinidine. The transepithelial permeability of val-quinidine in the BL to AP and AP to BL direction was very similar. The val-quinidine permeability in the AP to BL direction was $(5.13 \pm 0.49) \times 10^{-6}$ cm s^{-1} and in the BL to AP

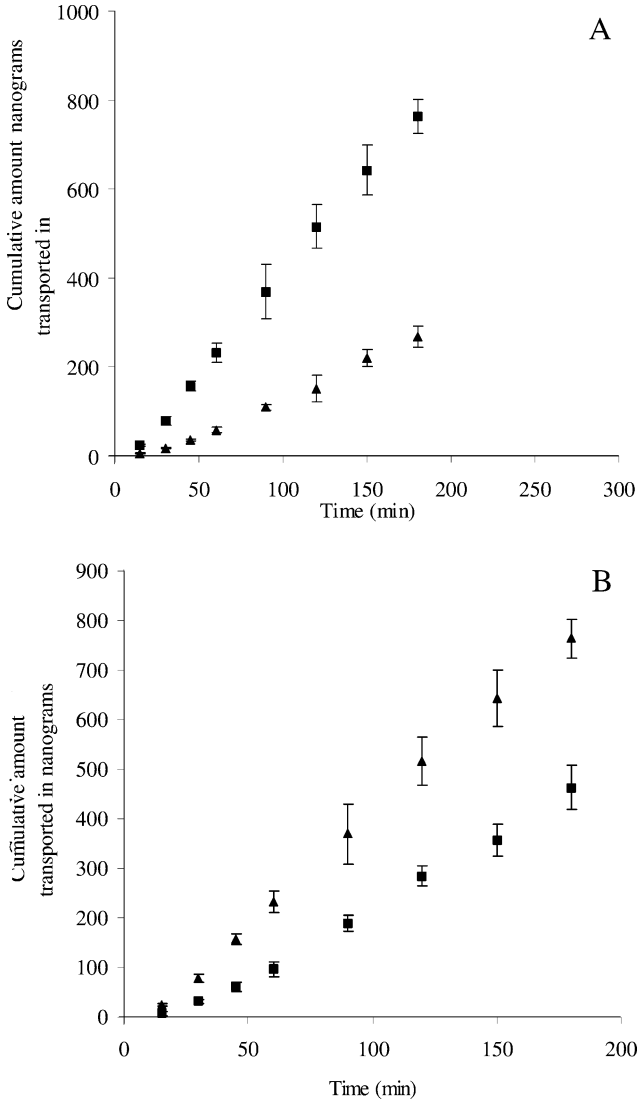

Figure 2. (A) Bidirectional transepithelial transport of quinidine (10 μM) across MDCKII-MDR1 cell monolayers: (▲) apical to basolateral (AP−BL) direction and (■) basolateral to apical (BL−AP) direction. A significant difference ($p < 0.05$) between AP−BL and BL−AP transport rates was observed. Values are expressed as means ± SD ($n = 4$). (B) Basolateral to apical (BL−AP) transport of quinidine (10 μM) across MDCKII-MDR1 cell monolayers in the absence (▲) and presence (■) of 10 μM cyclosporine. A significant inhibition ($p < 0.05$) of transport was seen in the presence of 10 μM cyclosporine. Values are expressed as means ± SD ($n = 4$).

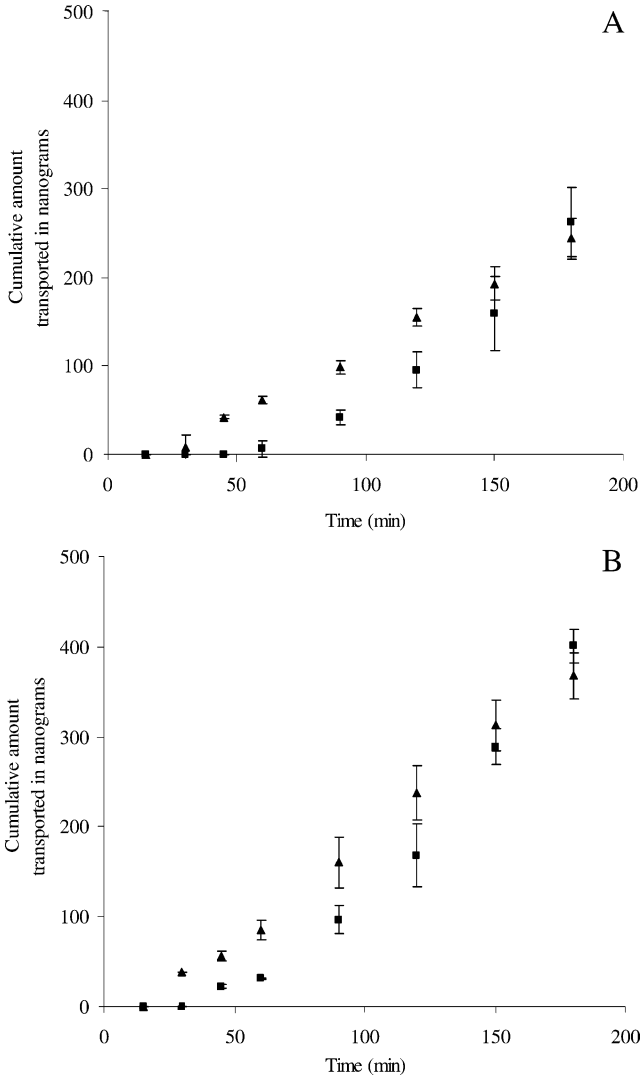

Figure 3. (A) Bidirectional transport of val-quinidine (10 μM) across MDCKII-MDR1 cell monolayers: (▲) apical to basolateral (AP−BL) direction and (■) basolateral to apical (BL−AP) direction. No significant difference ($p < 0.05$) between AP−BL and BL−AP was observed. Values are means ± SD ($n = 4$). (B) Bidirectional transport of 10 μM val-quinidine in the presence of 10 μM cyclosporine across MDCKII-MDR1 cell monolayers: (▲) apical to basolateral (AP−BL) direction and (■) basolateral to apical (BL−AP) direction. No significant difference ($p < 0.05$) between AP−BL and BL−AP was observed. Values are expressed as means ± SD ($n = 4$).

direction was $(6.17 \pm 1.28) \times 10^{-6}$ cm s^{-1} (Figure 3A), which was not a statistically significant difference ($p < 0.05$). Further, there was no change in the ratio of flux of val-quinidine across MDCKII-MDR1 cells in the presence of 10 μM cyclosporine (Figure 3B), a potent P-gp inhibitor. The ratio of flux in the BL to AP direction and in the AP to BL direction was 1.2. Hydrolysis and enzyme-mediated bioreversion of val-quinidine may generate a small amount of quinidine during an experiment. Figure 4A demonstrates that quinidine, generated as a result of hydrolysis of val-quinidine during transport, is a substrate for P-gp-mediated efflux. Transport of quinidine, generated from val-quinidine, in the AP to BL and BL to AP direction across the MDCKII-MDR1 cell line differs by almost 3-fold (Figure 4A). However, in the presence of 10 μM cyclosporine, this difference was abolished and the ratio of AP to BL flux to BL to AP flux of quinidine becomes 1 (Figure 4B).

Effect of Val-Quinidine on the Uptake of [3H]Gly-Sar by MDCKII-MDR1 Cells. Cells were incubated with solutions containing [^{3}H]Gly-Sar, a model peptide transporter substrate, in the presence or absence of unlabeled Gly-Sar and val-quinidine for a period of 10 min, and the level of

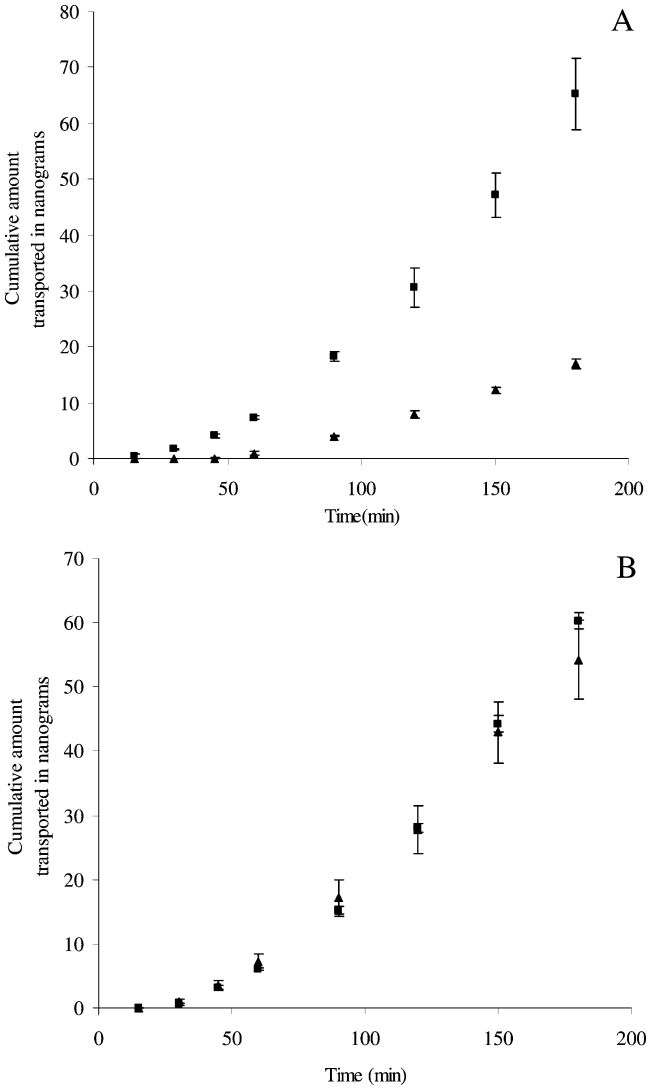

Figure 4. (A) Transport of quinidine that is generated during transport of val-quinidine across MDCKII-MDR1 cell monolayers in the apical to basolateral (AP−BL) direction (▲) and basolateral to apical (BL−AP) direction (■). A significant difference ($p < 0.05$) between AP−BL and BL−AP was observed. Values are expressed as means ± SD ($n = 4$). (B) Transport of quinidine (generated during val-quinidine transport) in the presence of a P-gp inhibitor cyclosporine (10 μM) across MDCKII-MDR1 cell monolayers in the apical to basolateral (AP−BL) direction (▲) and basolateral to apical (BL−AP) direction (■). No significant difference ($p < 0.05$) between AP−BL and BL−AP was observed. Values are expressed as means ± SD ($n = 4$).

intracellular [^{3}H]Gly-Sar accumulation was estimated. 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 the extent of cellular accumulation of [^{3}H]Gly-Sar. Gly-Sar at concentrations of 1 and 2 mM reduced the rate of uptake of [^{3}H]Gly-Sar from 0.033 to 0.026 pmol min^{-1} mg^{-1} (20%) and 0.019 pmol min^{-1} mg^{-1} (41%), respectively (Figure 5A). The rate of uptake of [^{3}H]Gly-Sar was also reduced by 21% in the

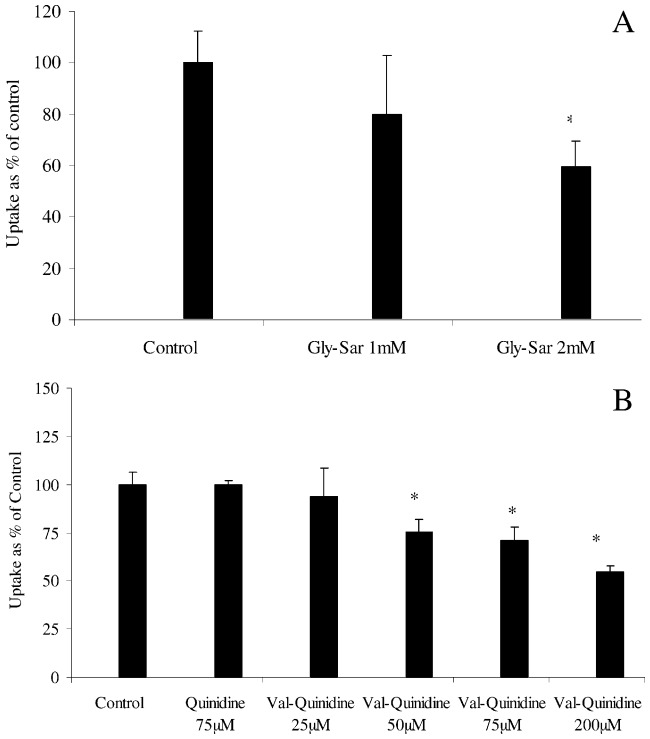

Figure 5. (A) Uptake of [^{3}H]Gly-Sar (0.5 μCi/mL) by MDCKII-MDR1 cells in the absence (control) or presence of 1 and 2 mM unlabeled Gly-Sar ($p < 0.05$). Data are expressed as means ± SD ($n = 4$). (B) Uptake of [^{3}H]Gly-Sar (0.5 μCi/mL) across MDCKII-MDR1 cell monolayers in the absence (control) and presence of quinidine and val-quinidine ($p < 0.05$). Data are expressed as means ± SD ($n = 4$).

presence of 75 μM val-quinidine, whereas an equimolar concentration of quinidine did not demonstrate any inhibition on [^{3}H]Gly-Sar uptake (Figure 5B). Val-quinidine exhibited concentration-dependent inhibition of [^{3}H]Gly-Sar uptake (Figure 5B). The rate of uptake of [^{3}H]Gly-Sar in the presence of 200 μM val-quinidine decreased to approximately 45% of the control which is equivalent to the extent of inhibition produced by 2 mM unlabeled Gly-Sar.

Uptake of Amino Acids by MDCKII-MDR1 Cells in the Presence and Absence of Val-Quinidine. We carried out studies with various model amino acid transporter substrates to investigate the involvement of any amino acid transporter in val-quinidine uptake. Rates of uptake of [^{3}H]lysine and [^{3}H]arginine (basic amino acid) were reduced to 57 and 47% of control, respectively, by val-quinidine (200 μM). Rates of [^{3}H]tyrosine, [^{3}H]glycine, and [^{3}H]alanine (neutral amino acid) uptake were reduced to 41, 39, and 37% of control, respectively. No inhibition of the uptake of [^{3}H]glutamic acid (acidic amino acid transporter substrate) was seen with val-quinidine (200 μM). The rank order of inhibition by val-quinidine (200 μM) is as follows: lysine > arginine > tyrosine > glycine > alanine (Figure 6).

Discussion

Recently, considerable attention has been directed toward developing strategies for overcoming the permeability barrier

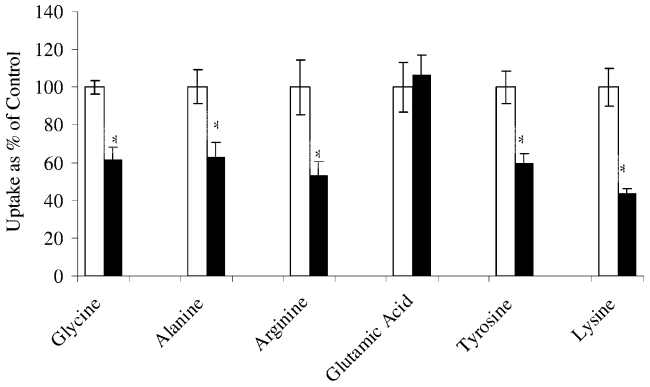

Figure 6. Uptake of [^{3}H]glycine, [^{3}H]alanine, [^{3}H]arginine, [^{3}H]glutamic acid, [^{3}H]tyrosine, and [^{3}H]lysine (0.5 μCi/mL) across MDCKII-MDR1 cell monolayers in the absence (empty columns) and presence of val-quinidine (filled columns) ($p < 0.05$). Data are expressed as means ± SD ($n = 4$).

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 also lead to increased drug permeability across various other biological barriers such as the blood−brain barrier (BBB). In this study, we have demonstrated that modification of quinidine, which is a substrate for P-gp, to val-quinidine results in circumvention of P-gp-mediated efflux of quinidine.

Cellular uptake of [^{3}H]ritonavir was enhanced 2-fold in the presence of quinidine, whereas an equimolar concentration of val-quinidine did not demonstrate any significant effect on [^{3}H]ritonavir uptake (Figure 1). This 2-fold increase in the rate of ritonavir uptake, by the MDCKII-MDR1 cell line in the presence of quinidine, confirmed that MDCKII-MDR1 cells express P-gp and that quinidine acts a substrate and/or inhibitor for the efflux pump. Val-quinidine, however, did not demonstrate any effect on [^{3}H]ritonavir uptake by the MDCKII-MDR1 cells, at equimolar concentrations (75 μM), indicating that this quinidine prodrug is not recognized by P-gp. There was a slight increase in the rates of cellular uptake of [^{3}H]ritonavir in the presence of 100 and 200 μM val-quinidine (Figure 1) which is probably due to the inhibition of P-gp efflux produced by a small percentage of quinidine generated during the experiment.

Degradation studies were carried out to delineate hydrolysis and enzyme-mediated bioreversion of val-quinidine. Degradation studies suggest that some percentage of quinidine will be generated from val-quinidine during the uptake and transport experiments (Table 1). These values support our earlier observation that a slight increase in the rate of [^{3}H]ritonavir uptake was observed in the presence of 200 and 100 μM val-quinidine (but not 75 μM) since a greater amount of quinidine will be generated from 200 or 100 μM val-quinidine than from 75 μM val-quinidine.

A classical indication of the involvement of P-gp in transport kinetics is the difference in permeation rates of P-gp substrates in the apical to basolateral and basolateral to apical directions.[26] If a compound is a substrate for P-gp, the apical to basolateral transport (AP−BL) is considerably slower than the basolateral to apical transport (BL−AP). Transepithelial bidirectional transport of quinidine across MDCKII-MDR1 cells reveals that the transepithelial transport of quinidine in the absorptive direction (AP to BL) was significantly slower than that in the secretory direction (BL to AP) (Figure 2A). This asymmetric permeation is due to the apically polarized P-gp efflux transporter, for which quinidine is a very good substrate. In the presence of 10 μM cyclosporine, the apparent permeability (P_{app}) of quinidine decreased significantly ($p < 0.05$) from $(18.3 \pm 1.25) \times 10^{-6}$ to $(11.2 \pm 0.8) \times 10^{-6}$ cm s^{-1} (Figure 2B). This decrease in the rate of secretory transport is due to the inhibitory effect of cyclosporine on P-gp-mediated efflux of quinidine.

In contrast to quinidine, the transepithelial permeability of val-quinidine in the basolateral to apical and apical to basolateral direction was very similar when studies were carried out using an equimolar concentration of val-quinidine. Further, there was no change in the ratio of flux of val-quinidine across MDCKII-MDR1 cells in the presence of 10 μM cyclosporine (Figure 3B), a potent P-gp inhibitor. These results further confirm our earlier observation that val-quinidine is not being recognized by P-gp as a substrate.

Hydrolysis and enzyme-mediated bioreversion of val-quinidine generate a small amount of quinidine during an experiment. The quinidine generated from val-quinidine remains a substrate for P-gp and was translocated across the cell membrane asymmetrically in the AP to BL and BL to AP direction (Figure 4A). The ratio of flux of quinidine generated in the BL−AP and AP−BL directions was observed to be 3. However, in the presence of 10 μM cyclosporine, this difference was abolished and the ratio of AP to BL and BL to AP flux of quinidine became 1 (Figure 4B). These results thus clearly suggest that quinidine remains a substrate for P-gp, whereas val-quinidine is not recognized by the efflux pump.

L-Valine is a good substrate of the neutral amino acid transporter system LAT1.[27] However, once linked to quinidine, val-quinidine may become a substrate of another transport system, e.g., hPepT1. Multiple reports indicate that when valine ester prodrugs are designed the prodrug becomes a substrate of the peptide transporter, e.g., valine esters of ganciclovir[28] and acyclovir.[29] Uptake of [^{3}H]Gly-Sar (0.5 μCi/mL), in the presence of unlabeled Gly-Sar, at concen-

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trations of 1 and 2 mM, demonstrated a concentration-dependent decrease in the level of cellular accumulation of [^{3}H]Gly-Sar. The decrease in the rate of cellular uptake in the presence of unlabeled Gly-Sar (Figure 5A) suggests expression of peptide transporters on the apical membrane of MDCKII-MDR1 cells. This is consistent with earlier reports about the expression of peptide transporters on MDCK cells.[30] The rate of uptake of [^{3}H]Gly-Sar was also reduced by 21% in the presence of 75 μM val-quinidine, whereas an equimolar concentration of quinidine did not demonstrate any inhibition of [^{3}H]Gly-Sar uptake. Moreover, val-quinidine demonstrated concentration-dependent inhibition of [^{3}H]Gly-Sar uptake (Figure 5B). A greater percentage of inhibition of [^{3}H]Gly-Sar uptake by val-quinidine, compared to equimolar concentrations of unlabeled Gly-Sar, indicates that val-quinidine may have a higher affinity for the peptide transporter than Gly-Sar. Our results thus suggest that val-quinidine may be an excellent substrate for a peptide transporter expressed on an apical membrane of MDCKII-MDR1 cells, whereas quinidine is not.

Further studies were carried out to elucidate whether val-quinidine translocation across MDCKII-MDR1 cell line involves any other transporter such as amino acid transporters. The MDCK cell line has been reported to express various amino acid transport systems[30] which plays an important role in the transepithelial transport of neutral, basic, and acidic amino acids. Amino acid transporters can be classified according to their substrate selectivity and Na^+ dependence. Na^+-dependent amino acid transporters utilize free energy stored as an Na^+ electrochemical potential gradient across the plasma membrane for uphill transport of an amino acid. Sodium-dependent amino acid transporters include system ASC, system A, the B^0,B^{0+},X^-_{AG},N,β system, and the y^+L system. The sodium-independent amino acid transport system includes broad-scope neutral amino acid system L,[31] small neutral amino acid system asc,[32] aromatic amino acid selective T,[33] basic amino selective system y^+,[34] basic and neutral amino acid transport systems $b^{0,+}$ and y^+L, and cystine and glutamate selective system x^-_c.[35−37] It has been reported that MDCK cells express various neutral and cationic amino acid transporters on the apical membrane.[38] However, expression of these transporters by the MDCKII-MDR1 cell line has not yet been confirmed.

We carried out uptake studies with various model amino acid transporter substrates to investigate the involvement of amino acid transporters in val-quinidine uptake. Our studies indicate that val-quinidine (200 μM) inhibits the uptake of various neutral and basic amino acid substrates (Figure 6), indicating the possible involvement of multiple amino acid transport systems in the permeation of val-quinidine across MDCKII-MDR1 cells. However, further studies are needed to elucidate and characterize the exact mechanism involved in the translocation of val-quinidine across the cell monolayer.

In this study, we established that the L-valine ester quinidine prodrug, val-quinidine, is not recognized by P-gp as a substrate. Transport studies conducted using MDCKII-MDR1 cell monolayers clearly indicate that quinidine is a substrate of P-gp whereas val-quinidine is not. Uptake studies suggest that val-quinidine is recognized by peptide and amino acid transporters. Translocation of val-quinidine across the apical membrane of the cell layers by transporters could be one of the mechanisms by which val-quinidine escapes efflux by P-gp. Although val-quinidine is not a substrate for P-gp, the net influx of quinidine from val-quinidine and the net influx from quinidine in the apical to basolateral direction were found to be similar. Concentrations of quinidine, the level of expression of the peptide and amino acid transporters, and the fraction of quinidine generated during val-quinidine transport could be some of the factors responsible for this observation. Further studies are currently being conducted in our laboratory to characterize and delineate the contribution of the amino acid and peptide transport systems to val-quinidine transport across the MDCKII-MDR1 cell monolayers.

In conclusion, we report for the first time that val-quinidine, an L-valine ester prodrug of quinidine, a P-gp substrate, circumvents P-gp-mediated efflux. We also present preliminary evidence of the expression of amino acid and

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peptide transport systems on the apical membrane of the MDCKII-MDR1 cell line and of the involvement of these transport systems in the translocation of val-quinidine. Transporter-targeted prodrug modification of P-gp substrates could lead to shielding from P-gp-mediated efflux. This strategy may thus lead to enhanced drug delivery across biological membranes expressing efflux pumps such as P-gp and also in chemotherapy of drug resistant tumors.

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