

# Characterization of P-glycoprotein Mediated Transport of K02, a Novel Vinylsulfone Peptidomimetic Cysteine Protease Inhibitor, Across MDR1-MDCK and Caco-2 Cell Monolayers

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Received March 23, 1998; accepted July 1, 1998

**Purpose.** Here we characterized the transport properties of morpholine-urea-phenylalanine-homophenylalanine-vinylsulfone-phenyl (K02), a newly developed peptidomimetic cysteine protease inhibitor, across monolayers of P-gp-expressed MDR1 transfected MDCK cells (MDR1-MDCK) and Caco-2 cells.

**Methods.** MDR1-MDCK, MDCK and Caco-2 cells, grown to confluence on Transwell insert membranes, were used to investigate transcellular transport of [<sup>14</sup>C]-K02.

**Results.** The basolateral to apical (B-A) flux of 10 μM [<sup>14</sup>C]-K02 across MDR1-MDCK cells was markedly greater than its apical to basolateral (A-B) flux (ratio = 39). This specific B-A transport was temperature dependent and saturable, with an apparent Michaelis-Menten constant and maximum velocity of 69.1 ± 19.5 μM and 148.9 ± 16.3 pmol/min/cm<sup>2</sup>, respectively. This B-A flux was significantly inhibited by cyclosporine (IC<sub>50</sub> = 17.1 ± 0.7 μM), vinblastine (IC<sub>50</sub> = 75.9 ± 13.0 μM) and verapamil (IC<sub>50</sub> = 236 ± 63 μM). In Caco-2 cell monolayers, the B-A flux was reduced about 50% compared to that in MDR1-MDCK and the A-B flux was increased about 8-fold. The apparent Michaelis-Menten constant and maximum velocity values for the B-A transport were 71.8 ± 45.9 μM and 35.3 ± 9.0 pmol/min/cm<sup>2</sup>. This B-A flux was also significantly inhibited by P-gp substrates/inhibitors. Western blots showed that the P-gp expression in MDR1-MDCK cells was about 10-fold that in Caco-2 cells.

**Conclusions.** K02 is transported by P-gp in both MDR1-MDCK and Caco-2 cells, and the *in vitro* interactions between K02 and various P-gp substrates may provide strategies to overcome the bioavailability barrier by intestinal P-gp.

**KEY WORDS:** vinylsulfone peptidomimetic; cysteine protease inhibitor; P-glycoprotein; MDR1-MDCK; Caco-2.

## INTRODUCTION

Morpholine-urea-phenylalanine-homophenylalanine-vinylsulfone-phenyl (K02) is a member of a class of newly developed peptidomimetic cysteine protease inhibitors (1). K02 is an irreversible inhibitor to many cysteine proteases, such as cathepsins B, L, and cruzain with nanomolar K<sub>i</sub> values *in vitro*. Cathepsins B, L are overexpressed in many cancer tissues and are potential therapeutical targets for new anticancer drug design (2). Cruzain is a cathepsin L-like cysteine protease expressed in all life stages of *Trypanosoma cruzi*, a protozoan parasite, which is the etiologic agent of American trypanosomiasis or

Chagas' disease (3). Cruzain is a major proteolytic enzyme in *Trypanosoma cruzi* and thus has been selected as a new therapeutic target for antiparasite drug design.

P-glycoprotein (P-gp) is a plasma membrane glycoprotein of about 170kDa that belongs to the superfamily of ATP-binding cassette (ABC) transporters (4,5). It functions as an ATP-dependent drug efflux pump originally discovered in multidrug resistance (MDR) cancer cells (6). It is also expressed in certain normal tissues. P-gp is expressed on the apical surfaces of many epithelial cells in a number of tissues, at high levels in columnar epithelial cells of the jejunum and colon, renal proximal tubules epithelium, the luminal surface of biliary hepatocytes, and on the intraluminal surface of capillary endothelial cells of the brain and testis (7). Although P-gp's exact physiological roles are still under investigation, P-gp is generally considered to play an important detoxification role via countertransport. Studies utilizing *mdr1a* and/or *mdr1b* gene disruptions in mice (knockout mice) have helped to define the exact physiological role of mammalian P-gp and to elucidate the influence of P-gp on the pharmacokinetics of therapeutic agents which are P-gp substrates (8,9). Pharmacokinetic studies employing inhibitors of P-gp along with P-gp-substrate drugs also demonstrate the importance of P-gp in drug absorption, distribution, elimination and toxicity (10).

Our previous studies demonstrated that K02 is a substrate for cytochrome P450 3A (CYP3A) and that CYP3A is the principle CYP responsible for K02 oxidation in liver and intestinal microsomal incubation systems *in vitro* (11). Our preliminary data also demonstrated that, in MDR1 transfected Madin-Darby Canine Kidney cells (MDR1-MDCK, the basolateral to apical (B-A) transport of K02 was much higher than its apical to basolateral (A-B) transport and that this B-A transport was significantly inhibited by vinblastine, a P-gp substrate (11). These preliminary data indicate that K02 is a P-gp substrate. We anticipated that K02, a CYP3A and P-gp substrate, would be a low oral bioavailability compound due to its interaction with the CYP3A/P-gp synergistic barrier present at the tip of villi of the small intestine (12). A pharmacokinetic study in male Sprague-Dawley (SD) rats conducted in our laboratory confirmed that K02 is a poorly orally available peptidomimetic with an oral bioavailability around 3% (13). Oral administration of 20 mg/kg ketoconazole, a dual CYP3A and P-gp inhibitor, together with 30 mg/kg K02 resulted in a significant increase in the area under the plasma concentration time curve (AUC) of K02 (13). Here, we further characterize the transport kinetics and drug interactions of K02 in P-gp-transfected MDR1-MDCK cells (14) and Caco-2 cells, a cell line selected from human colon adenocarcinoma with endogenous P-gp expression. Caco-2 has been used as an *in vitro* model for evaluating intestinal drug absorption (15). We believe the present study will help us to define the role of P-gp in the pharmacokinetics of K02, a promising new peptidomimetic lead compound for cysteine protease inhibitors.

## MATERIALS AND METHODS

### Materials

The MDR1-MDCK and MDCK cell lines were generously provided by Dr. Ira Pastan of the National Cancer Institute (14).

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The Caco-2 cell line (HTB-37) was purchased from American Type Culture Collection (ATCC) (Rockville, MD). [ $^{14}$ C]-K02 (27.6 Ci/mol) was kindly supplied by Dr. James Palmer of Arris Pharmaceutical Corporation (South San Francisco, CA). Cyclosporine was a gift from Sandoz Pharmaceutical (Basel, Switzerland). Vinblastine and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO).  $^3$ H mannitol (27 Ci/mol) was purchased from Amersham (Arlington Heights, IL). Other chemicals were of reagent grade and also purchased from Sigma.

### Cell Cultures

MDR1-MDCK and MDCK cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For MDR1-MDCK cells, 80 ng/ml colchicine was present in the culture medium. Caco-2 cells were in culture in Minimum essential medium Eagle with 2 mM L-glutamine and Earle's BSS containing 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and with 10% fetal bovine serum. For transport experiments, MDR1-MDCK (early passages after transfection), MDCK (50–60 passages) and Caco-2 (30–40 passages) cells were grown as epithelial layers by seeding onto permeable filter matrices of Transwell inserts (4.7 cm<sup>2</sup> growth area, about 10<sup>6</sup> cells/insert, Corning Costar Corporation, Cambridge, MA) in 6-well cluster plates for 4–5 and 18–21 days, respectively. The integrity of the cell monolayers were measured by transepithelial electrical resistance (TEER) by a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA). The average values of TEER for MDR1-MDCK, MDCK and Caco-2 cell monolayers were 1800, 600 and 500  $\Omega$ /4.7 cm<sup>2</sup>, respectively.

### Western Blot

The relative P-glycoprotein expression level in MDR1-MDCK, MDCK and Caco-2 was measured by Western blot. MDR1-MDCK, MDCK and Caco-2 cells were grown in culture for the same time periods as those used in transport experiments. The cells were removed from the flask by scraping in the phosphate-buffered saline. The cells were pelleted at 10,000  $\times$  g and resuspended in storage buffer (100 mM potassium phosphate, pH 7.4, 1.0 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 20  $\mu$ M butylated hydroxytoluene and 0.5 mM phenylmethylsulfonyl fluoride), and lysates were generated by sonication. The protein contents of cell lysates were determined by the method of Bradford using a Biorad Protein Assay Kit with albumin protein standard (Biorad, Richmond, CA). Lysates (50  $\mu$ g) were resuspended in Laemmli sample buffer, loaded onto 4–15% polyacrylamide gel (Biorad, Richmond, CA), and electrophoresed. Proteins were transferred to nitrocellulose and then incubated sequentially with P-gp mouse monoclonal C219 (Signet Laboratories, Inc., Dedham, MA) and peroxidase conjugated anti-mouse IgG (Gibco BRL, Gaithersburg, MD) and developed with the Amersham ECL detection system (Arlington Heights, IL). Densitometric readings of integrated gel band (molecular weight about 190 KDa) density were made using a Pharmacia LKB UltraScan XL densitometer (Pharmacia LKB, Alameda, CA).

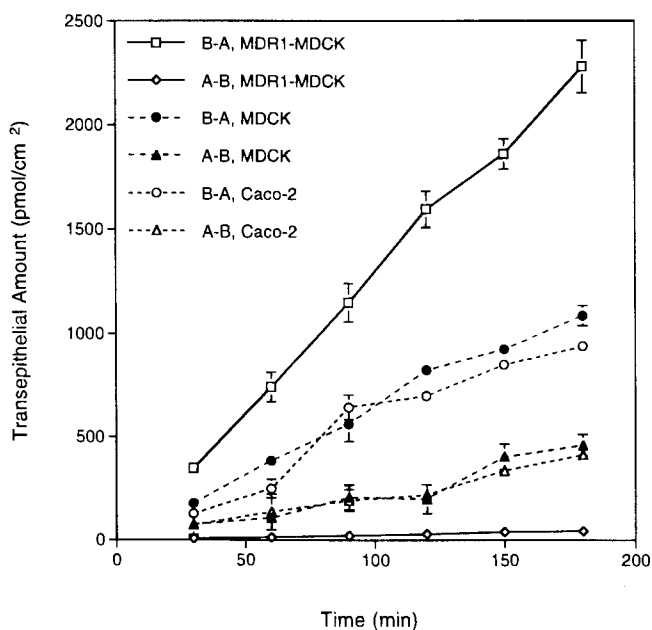
### Measurement of Bidirectional Transepithelial K02 Fluxes

The detailed experimental procedure adapted from Hunter (16) was reported previously (11). Briefly: 1.5 ml and 2.5 ml serum-free medium were pipeted into insert cups (apical solution) and 6-well plates (basolateral solution), respectively. The medium on either the apical or basolateral side of the monolayers contained 10  $\mu$ M [ $^{14}$ C] K02. The monolayers were then incubated at 37°C for up to 3 hours. For the temperature dependence study, the monolayers were also incubated at 4°C for up to 3 hours. 50  $\mu$ l of solution was taken from the receiving side every 30 minutes and subjected to liquid scintillation counting (Beckman LS1801 scintillation counter, Beckman Instruments, Inc., Palo Alto, CA). All P-gp substrates/inhibitors used to assess the inhibitory effects on K02 bidirectional transport were all applied to the basolateral side at various concentrations.

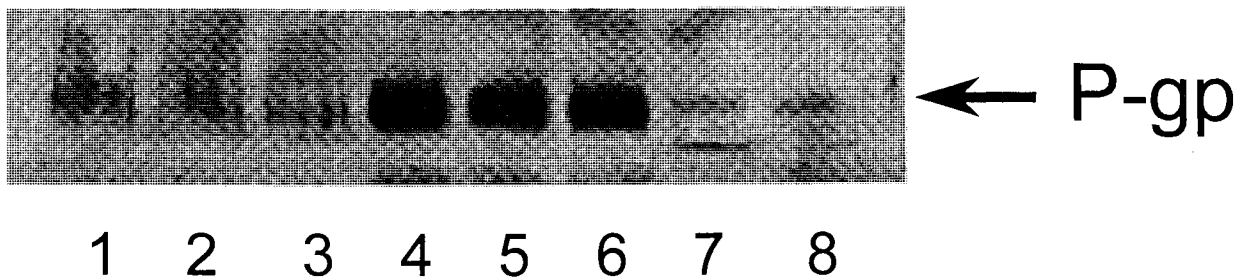
## RESULTS

### K02 Bidirectional Transepithelial Fluxes

As depicted in Figure 1, the basolateral to apical (B-A) flux of 10  $\mu$ M [ $^{14}$ C]-K02 across MDR1-MDCK cell monolayers was about 2-fold that across MDCK cell monolayers. The B-A flux of 10  $\mu$ M [ $^{14}$ C]-K02 across MDR1-MDCK cell monolayers (solid lines) was markedly greater than its apical to basolateral (A-B) flux (ratio = 39). In MDCK monolayers (closed circles and triangles), the B-A to A-B flux ratio is 2.4. In Caco-2



**Fig. 1.** Bidirectional transepithelial transport of K02 across MDR1-MDCK and MDCK and Caco-2 cell monolayers. [ $^{14}$ C]-K02 is 10  $\mu$ M. Solid lines depict basolateral to apical (connecting open squares) and apical to basolateral (connecting open diamonds) transport in MDR1-MDCK cells. Dashed lines depict basolateral to apical (connecting closed circles) and apical to basolateral (connecting closed triangles) transport in MDCK cells. Dashed lines depict basolateral to apical (connecting open circles) and apical to basolateral (connecting open triangles) transport in Caco-2 cells. Values are means of three measurements  $\pm$  standard deviation.

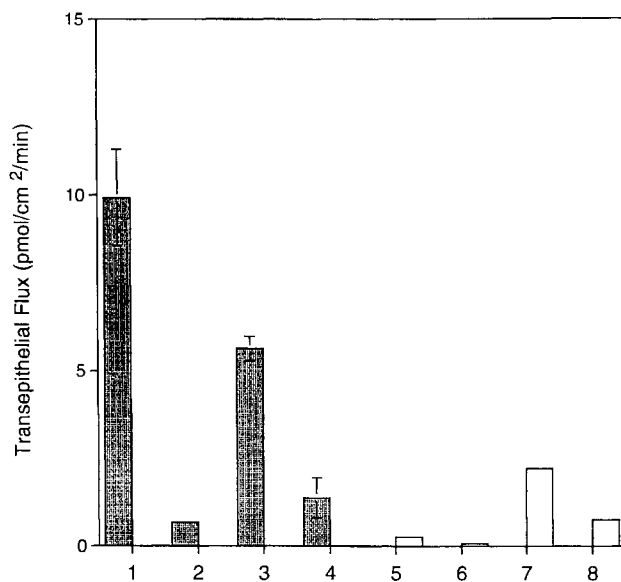


**Fig. 2.** Western blots of whole cell lysates of MDR1-MDCK, MDCK and Caco-2 cells using P-gp mouse monoclonal antibody, C219. Lanes 1-3: Caco-2, lanes 4-6; MDR1-MDCK, lanes 7-8: MDCK.

monolayers (open circles and triangles), the B-A flux was reduced about 50% compared to that of MDR1-MDCK and the A-B flux was increased about 8-fold. The corresponding B-A to A-B flux ratio in Caco-2 cells is 2.5. The densitometric reading of the Western blots (Figure 2) showed that the P-gp expression level in MDR1-MDCK cells was about 25-fold that in MDCK cells and 10-fold that in Caco-2 cells. There was no significant non-specific cross reactivity of C219 in our experiment, since we observed a significant difference between MDR1 transfected and non-transfected MDCK cells by Western blotting.

#### Temperature Dependent of K02 Bidirectional Transepithelial Fluxes

This specific B-A transport was temperature dependent as demonstrated in Figure 3. The ratio of B-A flux at 37°C to that at 4°C is about 15 in MDR1-MDCK cells and 4 in Caco-2 cells.



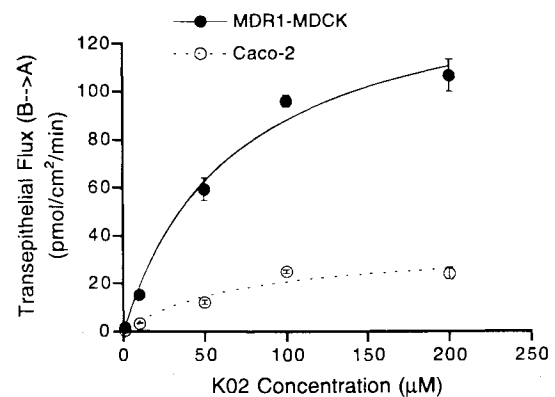
**Fig. 3.** Temperature dependence of bidirectional transepithelial flux of K02 in MDR1-MDCK and Caco-2 cell monolayers. [<sup>14</sup>C] K02 is 10  $\mu$ M. 1, MDR1-MDCK, B-A, 37°C; 2, MDR1-MDCK, B-A, 4°C; 3, Caco-2, B-A, 37°C; 4, Caco-2, B-A, 4°C; 5, MDR1-MDCK, A-B, 37°C; 6, MDR1-MDCK, A-B, 4°C; 7, Caco-2, A-B, 37°C; 8, Caco-2, A-B, 4°C. Values are mean of three measurements  $\pm$  standard deviation.

#### Basolateral to Apical (B-A) Transport of K02 Is Concentration Dependent and Saturable

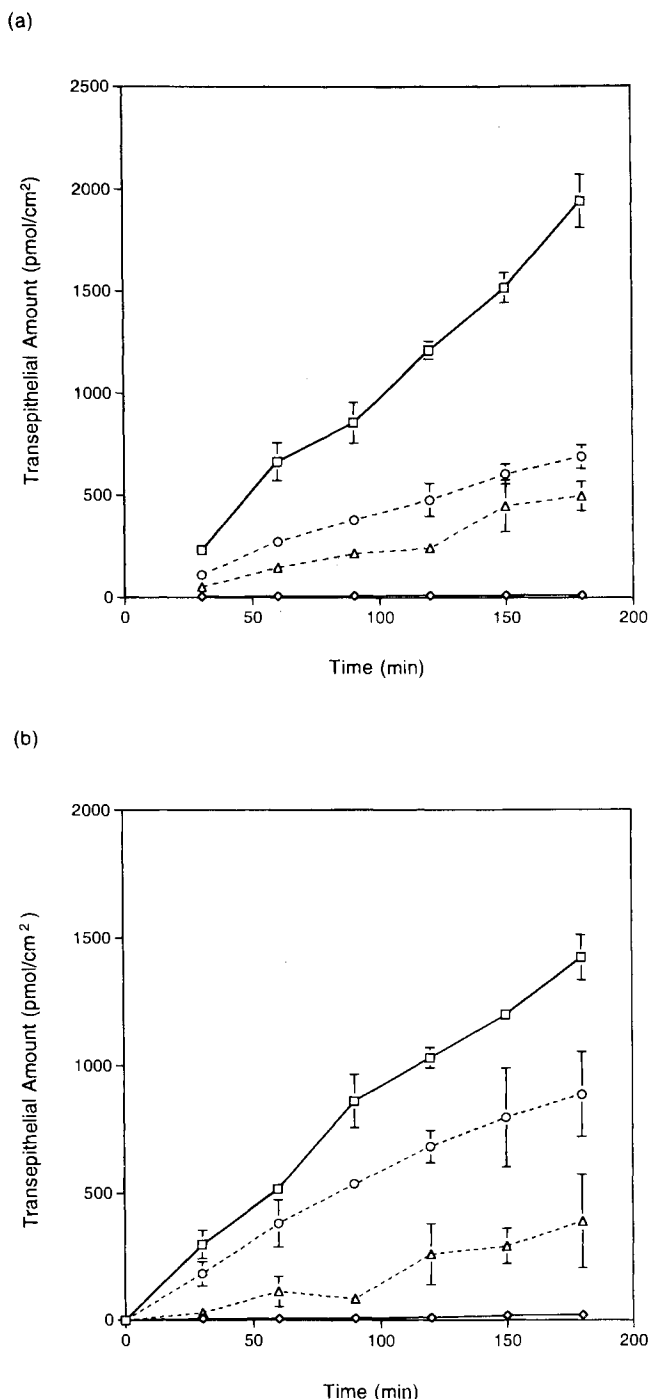
The B-A transport of K02 in either MDR1-MDCK or Caco-2 cell monolayers were concentration dependent, saturable and followed simple Michaelis-Menten type kinetics (Figure 4). The apparent Michaelis-Menten constant and maximum velocity values for this B-A transport were  $69.1 \pm 19.5 \mu$ M and  $148.9 \pm 16.3$  pmol/min/cm<sup>2</sup> in MDR1-MDCK and  $71.8 \pm 45.9 \mu$ M and  $35.3 \pm 9.0$  pmol/min/cm<sup>2</sup> in Caco-2, respectively.

#### Inhibitory Effects of P-Glycoprotein Substrates/Inhibitors on K02 Bidirectional Transepithelial Fluxes

The inhibitory effects of P-glycoprotein substrates/inhibitors on K02 bidirectional transepithelial fluxes in both MDR1-MDCK and Caco-2 cells were investigated. As illustrated in Figures 5a and 5b, this specific B-A transport in MDR1-MDCK was significantly inhibited by the avid P-gp substrates cyclosporine (47.0% inhibition at 10  $\mu$ M) and verapamil (42.3% inhibition at 100  $\mu$ M), while the K02 A-B transport increased. Similar results were observed in Caco-2 cells, the K02 B-A transport was inhibited by cyclosporine (11.2% inhibition at 10  $\mu$ M) and verapamil (31.4% inhibition at 100  $\mu$ M), while the K02 A-B transport increased. Typical representative IC<sub>50</sub> curves are presented in Figure 6. Table 1 summarizes the IC<sub>50</sub> values of various P-gp substrates/inhibitors for K02 B-A transport in MDR1-MDCK and Caco-2 cells.



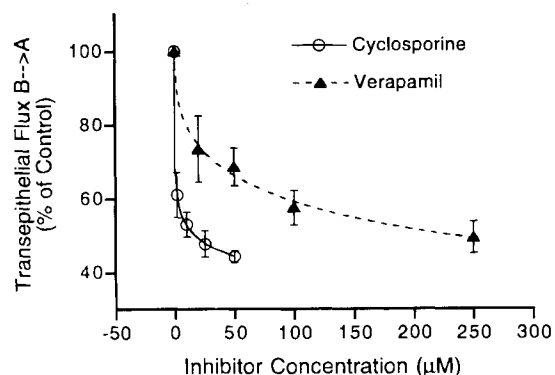
**Fig. 4.** Kinetic analysis of K02 basolateral to apical flux in MDR1-MDCK and Caco-2 cell monolayers. MDR1-MDCK (solid line connecting closed circles) and Caco-2 (dashed line connecting open circles). Values are mean of three measurements  $\pm$  standard deviation. Curve fitted assuming Michaelis-Menten kinetics and using B-A data directly.



**Fig. 5.** Effects of cyclosporine and verapamil on bidirectional transport of K02 in MDR1-MDCK cell monolayers. [<sup>14</sup>C] K02 is 10 μM. (a) 10 μM cyclosporine, (b) 100 μM verapamil. Solid lines depict basolateral to apical (connecting squares) and apical to basolateral (connecting diamonds) transport in the absence of cyclosporine and verapamil. Dashed lines depict basolateral to apical (connecting circles) and apical to basolateral (connecting triangles) transport in the presence of 10 μM cyclosporine or 100 μM verapamil in the basolateral compartment. Values are means of three measurements ± standard deviation.

**DISCUSSION**

P-glycoprotein expressed in MDR1-MDCK and Caco-2 cells has been shown to be localized on the apical side of the



**Fig. 6.** Concentration dependence of cyclosporine and verapamil on K02 basolateral to apical flux in MDR1-MDCK cell monolayers. [<sup>14</sup>C] K02 is 10 μM. Values are mean of two measurements ± standard deviation.

epithelia by immunohistochemistry and confocal laser scanning microscopy, respectively (14,16). This polarized P-gp expression in epithelial cell monolayers provides an essential tool for characterization of P-gp mediated transport properties of new potential therapeutic agents under drug development. MDR1-MDCK cell monolayers provide a unique tool for characterization of P-gp-mediated drug transport properties since the transfected-P-gp (MDR1) expression level is much higher than that of any endogenous transporter present in MDCK cells. The apparent similarity between the MDCK and the Caco-2 cells observed here is of interest, since many investigators prefer to utilize the Caco-2 system to approximate *in vivo* intestinal membrane characteristics. Since the use of MDCK cells is much simpler than the prolonged cell culture procedures with the Caco-2 system, further investigation of the non-transfected MDCK cell system may be of use to investigators evaluating intestinal P-gp effects. K02 basolateral to apical (B-A) transepithelial flux is significantly greater than its apical to basolateral (A-B) flux both in MDR1-MDCK, MDCK and Caco-2 cells (Fig. 1). K02 B-A flux in MDR1-MDCK is greater than that observed in MDCK and Caco-2, while K02 A-B flux in MDR1-MDCK is much less than that in MDCK and Caco-2. These results are qualitatively consistent with our observation obtained from Western blots (Fig. 2) that there is markedly higher expression of P-gp in MDR1-MDCK than in MDCK and Caco-2, but did not quantitatively reflect the relative P-gp expression levels in these three cell lines. Since we used whole cell lysates for Western blot analysis, this discrepancy may be explained by the fact that P-gp is expressed not only on the apical side of the cell membrane, but also intracellularly in MDR1-MDCK cells, e.g., during protein synthesis, membrane trafficking and sorting. Both K02 B-A and A-B transport show tempera-

**Table 1.** IC<sub>50</sub>s of P-gp Inhibitors for K02 Transepithelial Flux (B-A) Across MDR1-MDCK and Caco-2 Cell Monolayers

| Inhibitors (μM) | MDR1-MDCK   | Caco-2      |
|-----------------|-------------|-------------|
| Cyclosporine    | 17.1 ± 0.7  | 94.0 ± 15.7 |
| Vinblastine     | 75.9 ± 13.0 | 162 ± 50    |
| Ketoconazole    | 119 ± 10    | 237 ± 28    |
| Verapamil       | 236 ± 63    | 339 ± 19    |

ture dependence in MDR1-MDCK and Caco-2 cells (Fig. 3). Temperature dependence is a characteristic of carrier-mediated transport. K02 B-A flux is shown to be concentration dependent, saturable and to follow Michaelis-Menten kinetics (Fig. 4). The apparent  $K_m$  values are almost identical in MDR1-MDCK and Caco-2 indicating that K02 is mainly transported by P-gp endogenously expressed in Caco-2 cells. The apparent  $V_{max}$  value in MDR1-MDCK is about 4 times greater than that in Caco-2 which again corresponds with our Western blot results. K02 B-A flux in both MDR1-MDCK and Caco-2 cells is inhibited by various P-gp substrates and inhibitors (Figs. 5a, 5b and Table 1). Although the  $IC_{50}$  values for each P-gp substrate applied to MDR1-MDCK and Caco-2 cells differ (Table 1), the relative potency remains constant, cyclosporine > vinblastine > ketoconazole > verapamil (Table 1). The residual B-A transepithelial flux of K02 which could not be reduced by addition of higher concentration of cyclosporine or verapamil (Fig. 6) may be due to the poor solubility of these hydrophobic inhibitors in aqueous cell medium, passive diffusion or involvement of other transporters.

In Caco-2 cells, the K02 A-B flux is 2.19 pmol/min/cm<sup>2</sup>. The corresponding apparent permeability<sup>3</sup> is  $3.6 \cdot 10^{-6}$  cm/s. This permeability value is very low and we predict that K02 will exhibit poor oral bioavailability in humans. Applying P-gp inhibitors, such as cyclosporine, vinblastine, verapamil and ketoconazole to the system, increases the K02 A-B transport, e.g., cyclosporine (66% increase at 10  $\mu$ M) and verapamil (64% increase at 100  $\mu$ M). These results suggest the potential clinical importance of modulation of P-gp in the small intestine so as to increase K02 oral bioavailability.

Comparing K02 transport properties in MDR1-MDCK and Caco-2 cells, we can conclude that MDR1-MDCK cells provide an excellent tool for characterization of P-gp-mediated transport for new chemical entities which are potential therapeutic agents under drug development, while Caco-2 cells may better provide information to predict *in vivo* drug absorption (17,18). We can perceive that transfected cell lines with a wide spectra of physiologically important transporters, such as ATP-dependent transporters, P-gp, MRP (19) and cMOAT (20); dipeptide transporters (21); and organic cation transporters (22), will become standard tools for biochemical characterization of transport properties of a desired specific transporter with drug candidates.

## ACKNOWLEDGMENTS

This work was supported in part by NIH grant CA72006.

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<sup>3</sup>  $P_a = \text{flux}/(A \cdot C_0)$ .  $P_a$ , apparent permeability (cm/s); A, area of cell insert (cm<sup>2</sup>);  $C_0$ , initial concentration in donor compartment ( $\mu$ M).