

Are MDCK Cells Transfected with the Human *MDR1* Gene a Good Model of the Human Intestinal Mucosa?

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Purpose. To investigate whether Madin–Darby canine kidney cells transfected with the human *MDR1* gene (MDCK-MDR1) are a good model of the human intestinal mucosa.

Methods. P-glycoprotein (P-gp) expression in Caco-2 cells was compared with P-gp expression in MDCK wild-type (MDCK-WT) and MDCK-MDR1 cells using Western blotting methods. The polarized efflux activities of P-gp(s) in MDCK-MDR1 cells, MDCK-WT cells, and Caco-2 cells were compared using digoxin as a substrate. Apparent Michaelis–Menten constants (K_M , V_{max}) for the efflux of vinblastine in these three cell lines were determined. Apparent inhibition constants (K_I) of known substrates/inhibitors of P-gp were determined by measuring their effects on the efflux of digoxin in Caco-2 or MDCK-MDR1 cell monolayers.

Results. MDCK-MDR1 cells expressed higher levels of P-gp compared to Caco-2 and MDCK-WT cells, as estimated by Western blots. Two isoforms of P-gp were expressed in Caco-2 and MDCK cells migrating with molecular weights of 150 kDa and 170 kDa. In MDCK-MDR1 cells, the 150 kDa isoforms appeared to be overexpressed. The MDCK-MDR1 cells exhibited higher polarized efflux of [³H]-digoxin than did Caco-2 and MDCK-WT cells. K_M values of vinblastine in Caco-2, MDCK-WT, and MDCK-MDR1 cells were 89.2 ± 26.1 , 24.5 ± 1.1 , and 252.8 ± 134.7 μ M, respectively, whereas V_{max} values were 1.77 ± 0.22 , 0.42 ± 0.01 , and 2.43 ± 0.86 $\text{pmolcm}^{-2}\text{s}^{-1}$, respectively. Known P-gp substrates/inhibitors showed, in general, lower K_I values for inhibition of digoxin efflux in Caco-2 cells than in MDCK-MDR1 cells.

Conclusions. These data suggest that the MDCK-MDR1 cells overexpress the 150 kDa isoform of P-gp. MDCK-MDR1 cells are a useful model for screening the P-gp substrate activity of drugs and drug candidates. However, the apparent kinetics constants and affinities of substrates determined in the MDCK-MDR1 cell model may be different than the values obtained in Caco-2 cells. These differences in substrate activity could result from differences in the relative expression levels of total P-gp in Caco-2 and MDCK-MDR1 cells and/or differences in the partitioning of substrates into these two cell membrane bilayers.

KEY WORDS: P-glycoprotein; MDCK-MDR1; Caco-2; transporter; kinetics.

INTRODUCTION

Caco-2 cell monolayers grown on polycarbonate filters have been widely used as an *in vitro* model of the human intestinal mucosa since the early 1990s (1). This cell culture

model has been used extensively to elucidate the pathways (e.g., paracellular vs. transcellular; passive vs. transporter-mediated) by which drugs permeate the intestinal mucosa (1,2). Because the permeation of drugs across Caco-2 cell monolayers correlates with their human intestinal mucosa permeation, investigators have suggested that this cell line can be used to predict the oral absorption of drugs (3). This suggestion probably is reasonable if one assumes that the drugs permeate by passive diffusion and are not metabolized (4). However, we now realize that many drugs are substrates for transporters in the intestinal mucosa that can facilitate (e.g., peptide transporter) or restrict (efflux transporters) their permeation (5,6). We also now realize that the permeation of some drugs can be significantly restricted by their substrate activity for enzymes (e.g., cytochrome P450 3A4) present in the intestinal mucosa (7).

One of major barriers limiting oral drug delivery is the active efflux of drugs from the intestinal mucosa into the intestinal lumen by the multidrug resistance (MDR) transporter P-glycoprotein (P-gp) (8). Humans have two *MDR* genes: *MDR1* and *MDR3*. Only the P-gp encoded by *MDR1* gene (MDR1) appears to have a role in the efflux of drugs (9). P-gp was first found in cancer cells in 1976 and was correlated with the drug resistance phenomenon (10). Recently, P-gp was also found to be expressed widely in normal tissues, such as gastrointestinal mucosa, liver, pancreas, brain, and kidney (11). P-gp is localized on the apical side of polarized cell membranes, such as the human intestinal mucosa (6). This polarized expression of P-gp suggests that it functions as a secretory detoxifying system; thus, it represents a major barrier impeding drug permeation across the intestinal mucosa (7).

Caco-2 cells have been reported to express P-gp (6,12,13), and they exhibit polarized efflux of P-gp substrates (14). Caco-2 cells, grown onto Transwells®, undergo complete differentiation into monolayers and express P-gp on the apical side of membranes (6,15). One “disadvantage” of using Caco-2 cell monolayers for P-gp-related studies is that Caco-2 cells take 21–28 days to reach confluency (15) and 17–27 days for P-gp to become fully functional (12). Another problem with Caco-2 cells is that the expression levels of transporters can vary depending on how the cells are manipulated (12,16). Therefore, if the expression levels of the transporters in Caco-2 cells are not checked, the data generated in these cells may be misleading (17). Caco-2 cells have also been found to express multidrug resistance-related proteins (e.g., MRP2) (13). The expression of multiple transporters in this cell model, although representative of the human intestinal mucosa, makes it difficult to design studies to elucidate the structure–transport relationship for individual transporters.

Madin–Darby canine kidney (MDCK) cells are a dog renal epithelia cell line. When grown onto Transwells®, MDCK cells differentiate into columnar epithelium and form tight junctions in a shorter period of time than do Caco-2 cells (3 days vs. 21 days) (18,19). MDCK cells and Caco-2 cells have been reported to share many common epithelial cell characteristics (19). A good correlation between the permeation of passively absorbed drugs in Caco-2 cells and MDCK cells (19) suggests that the MDCK cell model may be used in place of Caco-2 cells as a model of the intestinal mucosa.

In the late 1990s, an MDCK-MDR1 cell line was gener-

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ated by transfecting the human *MDR1* gene into MDCK cells (20). MDCK-MDR1 cells have been reported to express a high level of P-gp and, like this efflux transporter in Caco-2 cells, the overexpressed P-gp in MDCK-MDR1 cells is localized on the apical side of polarized cell monolayers (21). Therefore, MDCK-MDR1 cells have been used as a model of the human P-gp in the intestinal mucosa (22,23).

The purposes of this study were: 1) to compare the P-gp expression in Caco-2 and MDCK-MDR1 cells; 2) to investigate the polarized efflux activities of P-gp expressed in these cells; and 3) to compare the kinetics and the affinity of known substrates/inhibitors of P-gp in these cell lines.

MATERIALS AND METHODS

Materials

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Maryland). MDCK strain II (MDCK-WT) epithelial cells and MDCK cells transfected with the human *MDR1* gene (MDCK-MDR1) were gifts from Professor Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). GF120918 was donated by Dr. Kenneth Brouwer (GlaxoSmithKline, Research Triangle Park, North Carolina). Dulbecco's phosphate-buffered saline, Hanks' balanced salts (HBSS) (modified), cyclosporin A (CsA), reserpine, vinblastine sulfate, quinidine, verapamil, vincristine sulfate, etoposide, daunorubicin, phenylmethyl sulfonyl fluoride, and anti-rabbit IgG-HRP were purchased from Sigma Chemical Co. (St. Louis, Missouri). L-Glutamine 200 mM, penicillin (10,000 U/mL), streptomycin (10,000 µg/mL), and non-essential amino acids [10 mM (100×) in 85% saline] were obtained from Gibco BRL, Life Technologies (Grand Island, New York). Dulbecco's Modified Eagle Medium (DMEM) and trypsin/EDTA solution (0.25% and 0.02%, respectively, in Ca⁺²- and Mg⁺²- free HBSS) were obtained from JRH Bioscience (Lenexa, Kansas). Rat-tail collagen (type I) was obtained from Collaborative Biomedical Products (Bedford, Massachusetts). Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, Georgia). Polycarbonate Transwells® (3-µm pore size) and polyester Transwells® (0.4-µm pore size) were obtained from Corning Costar (Cambridge, Massachusetts). [¹⁴C]-Mannitol (specific activity 51 mCi/mmol) and [³H]-digoxin (specific activity 19 Ci/mmol) were purchased from NEN Life Science Products, Inc. (Boston, Massachusetts). [³H]-Vinblastine sulfate (specific activity 11.1 Ci/mmol) was obtained from Amersham Life Science (Arlington Heights, Illinois). Protease inhibitor cocktail (components: pancreas-extract, pronase, thermolysin, chymotrypsin, and papain) was obtained from Boehringer Mannheim (Germany). Molecular weight markers, 6% Tris-glycine gels, nitrocellulose membrane (0.45 µm pore size), and sodium dodecyl sulfate (SDS) sample loading buffer were purchased from Invitrogen Corporation (Carlsbad, California). The bicinchoninic acid protein assay reagent kit was obtained from Pierce Chemical Company (Rockford, Illinois). The primary antibody mdr Ab-1 and mdr peptide antigen were purchased from Oncogene Science Corporation (Cambridge, Massachusetts). Antibody C219 was obtained from Signet Pathology Systems, Inc. (Dedham, Massachusetts). Electrochemiluminescence Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

Western Blotting and Protein Detection

Western blotting was performed using a modification of the procedure described by Hosoya *et al.* (12). Confluent cell monolayers of Caco-2, MDCK-WT, or MDCK-MDR1 cells grown in 150-cm² flasks were collected and lysed in freshly prepared lysis buffer (1% TX-100 (v/v), 20 mM Tris HCL, 150 mM NaCl, 1:1000 protease inhibitor cocktail, 1 mM phenylmethyl sulfonyl fluoride, and 1 mM EDTA). The cell lysates were centrifuged and the supernatants containing the solubilized protein were collected. The total cellular protein content was determined using the bicinchoninic acid assay. Aliquots (5 µg, 10 µg) of protein were size-fractionated in a 6% Tris-glycine acrylamide gel in 0.1% SDS Tris-glycine buffer. Proteins were then transferred onto nitrocellulose membranes. P-gps were probed using the primary antibody mdr Ab-1 (1:20 dilution) and the secondary antibody anti-rabbit IgG-HRP (1:6000 dilution). Antibody detection was performed using the Electrochemiluminescence method following the protocol provided by the manufacturer.

Cell Culture

Caco-2 cells were seeded at 79,000 cells/cm² on Transwell® filter inserts (6 well) and maintained in high-glucose DMEM supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 2 mM L-glutamine solution, 100 U/mL penicillin and 100 µg/mL streptomycin as described previously (15). Cells (passages 20–40) were used on days 21–28 post-seeding.

MDCK-WT and MDCK-MDR1 cells were seeded at 50,000 cells/cm² on polyester Transwell® filter inserts (6 well) and maintained under the same conditions as described above for Caco-2 cells. Experiments were conducted on these cells after 6 days of culture. The expression of P-gp transporter in the transfected cells was checked using Western blotting and cells were split twice a week with 1:10 dilution and used within 2 months after being removed from liquid N₂.

All cell lines were maintained at 37°C in a humidified 5% CO₂, 95% air atmosphere. Because all test compounds used in this study were [³H]-labeled, it was feasible to analyze the transport of a test compound and monitor the membrane integrity using [¹⁴C]-mannitol simultaneously. The apparent permeability coefficients (P_{app}) of [¹⁴C]-mannitol across Caco-2 or MDCK cell monolayers were typically 0.1–0.6 × 10⁻⁶ cm/s.

Transport Experiments

Bidirectional transport experiments were performed as previously described (15) with some modifications. All experiments were done at 37°C in air with constant mixing in a shaking water bath (60 rpm). Briefly, both the apical (AP) and the basolateral (BL) chambers of each insert were washed twice with 37°C-HBSS for 15 min. Test drugs: [³H]-digoxin, [³H]-vinblastine, or [¹⁴C]-mannitol at a concentration of 0.011 µM were added to the donor side (1.5 mL for the AP chamber and 2.5 mL for the BL chamber) and fresh HBSS was placed in the receiver compartment. To inhibit the efflux activity of P-gp, cell monolayers were incubated with HBSS containing GF120918 (2 µM) or CsA (25 µM) for an additional 15 min. The test compounds were added to the donor side along with GF120918 (2 µM) or CsA (25 µM), and fresh HBSS containing inhibitor [i.e., GF120918 (2 µM) or CsA (25

μM) was placed in the receiver side. Aliquots (100 μL) were withdrawn from receiver side at various time intervals to 80 m. Fresh HBSS or HBSS containing inhibitor (100 μL) was replaced in the receiver side after sampling.

P_{app} values were calculated using the equation:

$$P_{\text{app}} = \Delta Q / \Delta t / (A * C_0) \quad (1)$$

where $\Delta Q / \Delta t$ is the linear appearance rate of mass in the receiver solution, A is the filter/cell surface area (4.71 cm^2), and C_0 is the initial concentration of the test compounds.

The net efflux of a test compound was assessed by calculating the ratio of P_{app} in the BL-to-AP direction vs. P_{app} in the AP-to-BL direction ($P_{\text{app, BL-to-AP}} / P_{\text{app, AP-to-BL}}$). A ratio of substantially greater than 1.0 indicates net efflux.

Determination of Apparent Michaelis–Menten Constants (K_M , V_{max}) of [^3H]-Vinblastine

The time course for the total flux rates of [^3H]-vinblastine in the BL-to-AP direction across Caco-2, MDCK-WT, and MDCK-MDR1 cell monolayers was determined by sampling at 20-min intervals. The concentration dependence of the flux rate of [^3H]-vinblastine was also determined at a concentration range of 0.01–150 μM . The passive diffusion flux rate (excluding the influence of efflux transporter) of vinblastine was estimated by conducting the transport experiment in the presence of CsA (25 μM). The active transport flux rates were then estimated by subtracting the passive diffusion flux rates from total flux rates. Michaelis-Menten parameters were estimated using Origin 6.0 (OriginLab, Northampton, Massachusetts).

Determination of the Inhibitory Effects of Drugs on the Active BL-to-AP Transport of [^3H]-Digoxin across Caco-2 and MDCK-MDR1 Cell Monolayers

Apparent inhibition constants (K_i) of known substrates/inhibitors of P-gp on the active BL-to-AP transport of [^3H]-digoxin across Caco-2 and MDCK-MDR1 cell monolayers were measured and calculated using the method previously published by Gao *et al.* (24). Working Equations 2–4 were used in these calculations.

$$K_i = [(P_1/P_0)/(1 - P_1/P_0)] * [I] \quad (2)$$

$$P_0 = P_{\text{app,1}} - P_{\text{app,3}} \quad (3)$$

$$P_1 = P_{\text{app,2}} - P_{\text{app,3}} \quad (4)$$

In Equation 2, K_i is the apparent inhibition constant of a test compound. P_1 and P_0 are P_{app} values of [^3H]-digoxin in the presence and the absence of the test compound, respectively. P_1/P_0 is a reflection of the inhibitory effect of the test compound on the active BL-to-AP transport of [^3H]-digoxin across the cell monolayers. $[I]$ is the concentration of the test compound in the donor and the receiver side. $P_{\text{app,1}}$, $P_{\text{app,2}}$, and $P_{\text{app,3}}$ were determined from the BL-to-AP transport of [^3H]-digoxin in the absence of any compound, in the presence of a test compound, and in the presence of GF120918 (2 μM), respectively. GF120918 at the concentration of 2 μM totally inhibits the polarized efflux of [^3H]-digoxin in Caco-2 and MDCK-MDR1 cells, as determined in Table I in this study. The active component of the permeation of [^3H]-digoxin in the absence of a potential inhibitor (P_0) and in the presence of a potential inhibitor (P_1) were determined by subtracting $P_{\text{app,3}}$ (the passive diffusion component) from $P_{\text{app,1}}$ (total transport in the absence of any compound), and $P_{\text{app,2}}$ (total transport in the presence of a potential inhibitor), respectively. To avoid cell-cell variations, the transport experiments for the determination of $P_{\text{app,1}}$, $P_{\text{app,2}}$, and $P_{\text{app,3}}$ were always conducted on the same day using the same batch of cells.

Statistical Analysis

Statistical analyses were performed using Student's two-tailed t test between two mean values (Stat View 4.53, Abacus Concepts, Inc., Berkeley, California). A probability of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

RESULTS

Western Blotting

As shown in Figure 1, cell lysates of Caco-2, MDCK-WT, and MDCK-MDR1 cross-reacted with the P-gp antibody mdr Ab-1 resulting in the staining of two bands on polyacrylamide gel electrophoresis (PAGE) having apparent molecular weights of ~150 kDa and ~170 kDa. In a competition experiment using the mdr peptide antigen, the intensity of the 150 kDa and 170 kDa bands stained by the mdr Ab-1 antibody were reduced completely (data not shown). Similar results were also observed using antibody C219 (data not shown).

Table I. P_{app} Values of [^3H]-Digoxin across Caco-2, MDCK-WT, and MDCK-MDR1 Cell Monolayers

Cell type	Treatment	$P_{\text{app}} * 10^6$ (cm/s)		Ratio ($P_{\text{app, BL-to-AP}} / P_{\text{app, AP-to-BL}}$)
		AP-to-BL	BL-to-AP	
Caco-2	—	0.96 \pm 0.14	13.10 \pm 1.76	14
	GF120918 (2 μM)	6.40 \pm 0.40	6.94 \pm 0.98	1.1
	CsA (25 μM)	4.86 \pm 0.23	5.15 \pm 0.27	1.1
MDCK-WT	—	0.90 \pm 0.17	7.59 \pm 0.40	8.4
	GF120918 (2 μM)	3.10 \pm 0.13	3.23 \pm 0.70	1.0
	CsA (25 μM)	3.49 \pm 0.05	3.48 \pm 0.10	1.0
MDCK-MDR1	—	0.31 \pm 0.08	10.46 \pm 0.65	33
	GF120918 (2 μM)	2.34 \pm 0.06	2.75 \pm 0.12	1.1
	CsA (25 μM)	2.82 \pm 0.08	2.83 \pm 0.41	1.0

Note: P_{app} values of [^3H]-digoxin were measured in the absence or presence of inhibitor GF120918 (2 μM) or CsA (25 μM) in various cell lines as described in Materials and Methods. P_{app} values are presented as mean \pm SD ($n = 3$).

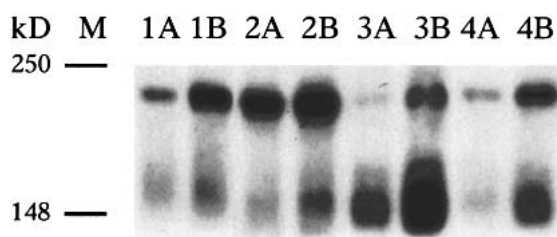


Fig. 1. Western blotting of P-gps in total cell lysates from Caco-2, MDCK-WT, and MDCK-MDR1 cell monolayers. Proteins were size fractionated in 6% Tris-glycine polyacrylamide gel in 0.1% of SDS Tris-glycine buffer. After electroblotting, P-gps were stained with mdr Ab-1, and the protein-antibody interaction was visualized using the chemiluminescence technique as described in Materials and Methods. A, 5 μ g; B, 10 μ g of the total cellular protein. 1: Caco-2 cells; 2: MDCK-WT cells; 3: MDCK-MDR1 cells with lower passage number; 4: MDCK-MDR1 cells of higher passage number. M: molecular weight marker.

Subsequently, these proteins are referred to as the 150 kDa isoform and 170 kDa isoform of P-gp. Caco-2 and MDCK-WT cells expressed higher levels of 170 kDa isoform than 150 kDa isoform (Fig. 1, Lanes 1A, 1B, 2A, 2B). MDCK-MDR1 cells appeared to overexpress the 150 kDa isoform of P-gp (Fig. 1, Lanes 3A, 3B, 4A, 4B). The enhanced expression of the 150 kDa isoform in MDCK-MDR1 cells was cell passage dependent, i.e., cells with lower passage numbers (Fig. 1, Lanes 3A, 3B) expressed greater amounts of the 150 kDa isoform than did the higher passaged cells (Fig. 1, Lanes Fig. 4A, 4B).

Transport of [3 H]-Digoxin across the Caco-2, MDCK-WT, and MDCK-MDR1 Cell Monolayers

Digoxin, a known substrate for P-gp (25–27), was chosen to compare the functional efflux activities of P-gps in Caco-2, MDCK-WT, and MDCK-MDR1 cell monolayers. GF120918, a known inhibitor of P-gp (28), was used in these studies as a specific inhibitor of this efflux transporter. To evaluate whether MRP2 contributed to the polarized efflux of [3 H]-digoxin in these cell lines, CsA, a known inhibitor of P-gp and MRP2 (29–31), was also used in these experiments. As shown in Table I, [3 H]-digoxin exhibited strong polarized efflux in Caco-2 cells (i.e., $P_{app\ BL-to-AP} \gg P_{app\ AP-to-BL}$). The ratio of $P_{app\ BL-to-AP}/P_{app\ AP-to-BL}$, which is used here as an indicator of efflux activity, was 14. In the presence of GF120918 (2 μ M), $P_{app\ AP-to-BL}$ increased and $P_{app\ BL-to-AP}$ decreased, resulting in a $P_{app\ BL-to-AP}/P_{app\ AP-to-BL}$ ratio of 1.1. The inhibition effect of CsA (25 μ M) on the polarized efflux of [3 H]-digoxin in Caco-2 cells was similar to that observed with GF120918, resulting in a $P_{app\ BL-to-AP}/P_{app\ AP-to-BL}$ ratio of 1.1.

MDCK-WT cells also showed polarized efflux of [3 H]-digoxin with a $P_{app\ BL-to-AP}/P_{app\ AP-to-BL}$ ratio of 8.4 (Table I). GF120918 and CsA completely inhibited the efflux of [3 H]-digoxin in MDCK-WT cells, resulting in $P_{app\ BL-to-AP}/P_{app\ AP-to-BL}$ ratios of 1.0 (Table I). As expected, the polarized efflux of [3 H]-digoxin, as measured by the $P_{app\ BL-to-AP}/P_{app\ AP-to-BL}$ ratio, was significantly higher in MDCK-MDR1 cell monolayers than in MDCK-WT cell monolayers (i.e., 33 vs. 8.4, respectively; Table I). As expected, this polarized efflux of [3 H]-digoxin in MDCK-MDR1 could be totally inhibited

by adding CsA (25 μ M) or GF120918 (2 μ M) to the incubation mixture (Table I).

Michaelis–Menten Constants (K_M , V_{max}) for Active BL-to-AP Transport of [3 H]-Vinblastine across Cell Monolayers

The apparent Michaelis–Menten constants (K_M , V_{max}) for [3 H]-vinblastine were determined using Caco-2, MDCK-WT, and MDCK-MDR1 cell monolayers. [3 H]-Vinblastine was used in these experiments because this P-gp substrate has good water solubility (10 mg/mL) (32,33). In contrast, digoxin has very low solubility (<10 μ M) and thus could not be used in these kinetic experiments. As shown in Figure 2, the active BL-to-AP flux of [3 H]-vinblastine in Caco-2 and MDCK-MDR1 monolayers was concentration-dependent and saturable. Similar results were observed in MDCK-WT (data not shown). Using these data, K_M and V_{max} values for the active BL-to-AP flux rates of [3 H]-vinblastine

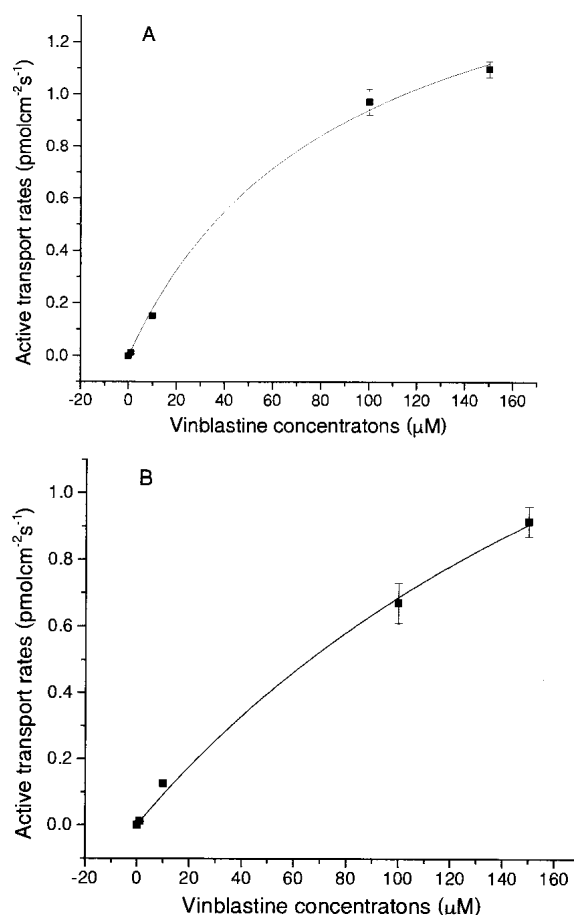


Fig. 2. Concentration dependence of the active BL-to-AP flux of [3 H]-vinblastine across A: Caco-2 and B: MDCK-MDR1 cell monolayers. The total transport flux rates of [3 H]-vinblastine were determined over a concentration range from 0.01–150 μ M. The passive diffusion flux rates were determined in the presence of CsA (25 μ M), which totally inhibits the active transport of [3 H]-vinblastine. The active transport flux rates were determined by subtracting the passive diffusion flux rates from total transport flux rates. Michaelis–Menten parameters (K_M , V_{max}) were estimated using Origin 6.0 as described in Materials and Methods. Data points are means \pm SD ($n = 3$).

in Caco-2, MDCK-WT, and MDCK-MDR1 cells were determined (Table II).

Inhibition Constants (K_i) of Known Substrates/Inhibitors of P-gp in Caco-2 and MDCK-MDR1 Cell Monolayers

As shown in Figure 3A, the inclusion of CsA (2.5 μ M) in the transport medium significantly inhibited the active BL-to-AP transport of [3 H]-digoxin across Caco-2 cell monolayers (i.e., after 80 min, the total transport of [3 H]-digoxin was decreased from ~10% to ~4.8%). Using the method described by Gao *et al.* (24), the apparent K_i value for CsA was calculated to be 0.46 ± 0.01 μ M (Table III). However, the apparent K_i value for CsA in MDCK-MDR1 cells was 2.18 ± 0.31 μ M, which was about 4 times greater than that in Caco-2 cells. To explore this observation further, we determined the K_i values of known substrates/inhibitors of P-gp on the active BL-to-AP transport of [3 H]-digoxin in Caco-2 and MDCK-MDR1 cell monolayers; these results are presented in Table III. The phenomenon observed with CsA (higher K_i value in MDCK-MDR1 cells compared to Caco-2 cells) was also observed for reserpine, vinblastine, quinidine, verapamil, vincristine, etoposide, and daunorubicin (Table III). The K_i values for these substrates/inhibitors in MDCK-MDR1 cells were approximately 2 to 15 times greater than those in Caco-2 cells. One exception was GF120918, which had the lowest K_i values of 0.39 ± 0.05 μ M in Caco-2 cells and 0.44 ± 0.03 μ M in MDCK-MDR1 cells. As expected, probenecid (1 mM) had no inhibitory effect on the polarized efflux of [3 H]-digoxin. Probenecid is a known inhibitor of MRP and not an inhibitor for P-gp (24).

DISCUSSION

In this study, the P-gp expression in Caco-2 cells (a human colonic carcinoma cell line), MDCK-WT cells (a canine kidney-derived cell line), and MDCK-MDR1 cells (a canine kidney-derived cell line transfected with the human *MDR1* gene) were compared using Western blotting methods. The observations that bands staining for P-gp in these three cells migrating with an apparent molecular weights of ~170 kDa are consistent with the published data for this efflux transporter (6,12,34). It is important to note that MDCK-WT cells also expressed significant levels of P-gp (Fig. 1, Lanes 2A, 2B) even though the functional P-gp activity is lower than that in Caco-2 cells based on the $P_{app, BL-to-AP}/P_{app, AP-to-BL}$ ratio (i.e., 8.4 vs. 14) for [3 H]-digoxin. Because MDCK-WT cells have been used as a model of the intestinal mucosa for permeability screening (19), one should consider this factor when the drugs/drug candidates being tested are potential P-gp substrates.

Table II. Apparent K_M and V_{max} Values for the Active BL-to-AP Transport of [3 H]-Vinblastine across Caco-2, MDCK-WT, and MDCK-MDR1 Cell Monolayers

	Cell line		
	Caco-2	MDCK-WT	MDCK-MDR1
K_M (μ M)	89.2 ± 26.1	24.5 ± 1.1^a	252.8 ± 134.7^a
V_{max} (pmolcm $^{-2}$ s $^{-1}$)	1.77 ± 0.22	0.42 ± 0.01^a	2.43 ± 0.86

^a $P < 0.05$, when compared with the value in Caco-2 cells.

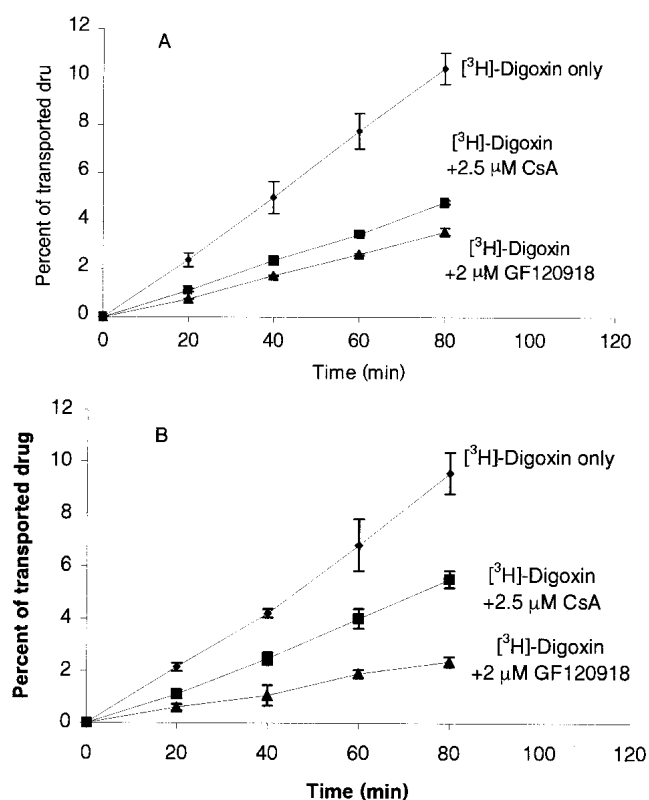


Fig. 3. Data used to calculate the K_i values of CsA on the active BL-to-AP transport of [3 H]-digoxin across (A) Caco-2 and (B) MDCK-MDR1 cell monolayers. $P_{app, 1}$, $P_{app, 2}$, and $P_{app, 3}$ were determined from the BL-to-AP transport of [3 H]-digoxin in the absence of CsA (\diamond), in the presence of 2.5 μ M of CsA (\blacksquare), and in the presence of 2 μ M of GF120918 (\blacktriangle), respectively. The active transport component of the flux rate of [3 H]-digoxin in the absence of a test compound (P_0) and in the presence of 2.5 μ M of CsA (P_1) were determined by subtracting $P_{app, 3}$ from $P_{app, 1}$ and $P_{app, 2}$ respectively. Using these data, the K_i value for CsA was calculated using equation (2) as described in Materials and Methods. Data points are mean \pm SD ($n = 3$).

It is interesting to note that MDCK-MDR1 cells appear to overexpress the 150 kDa isoform of P-gp (Fig. 1, Lanes 3A, 3B, 4A, 4B) and the expression levels of this isoform of P-gp were cell passage number dependent (i.e., higher passaged

Table III. K_i Values of Known P-gp Substrates/Inhibitors on the Active BL-to-AP Transport of [3 H]-Digoxin across Caco-2 or MDCK-MDR1 Cell Monolayers

Compounds (Concentration used)	K_i (μ M) in Caco-2	K_i (μ M) in MDCK-MDR1
GF918 (0.2 μ M)	0.39 ± 0.05	0.44 ± 0.03
CsA (2.5 μ M)	0.46 ± 0.01	2.18 ± 0.31^a
Reserpine (10 μ M)	1.38 ± 0.09	11.5 ± 0.45^a
Vinblastine (35 μ M)	8.92 ± 0.94	140 ± 55.7^a
Probenecid (1 mM)	No inhibition	No inhibition
Quinidine (5 μ M)	3.23 ± 0.27	8.59 ± 1.68^a
Verapamil (30 μ M)	8.11 ± 0.88	15.1 ± 0.41^a
Vincristine (50 μ M)	71.1 ± 6.10	213 ± 69.4^a
Etoposide (300 μ M)	294 ± 27.0	768 ± 210^a
Daunorubicin (80 μ M)	78.1 ± 16.7	111 ± 13.6^a

Note: Data are presented as mean \pm S.D. ($n = 3$).

^a $P < 0.05$, when compared with K_i value in Caco-2 cells.

cells had lower levels of 150 kDa isoform of P-gp expression than lower passaged cells). When cells divide, some new generation cells may carry over the chromosomes without the transfected gene, thus "diluting" the transfected gene in the whole cell population (35). Therefore, to keep the high levels of P-gp expression, it was recommended that this transfected cell line be used within 2 months after being removed from liquid N₂ (R. Evers, personal communication). To investigate whether the 150 kDa isoform of P-gp in MDCK-MDR1 cells resulted from protein degradation when the samples were boiled for denaturation, Western blotting was also performed by denaturing samples at room temperature. Results similar to those shown in Figure 1 were observed (data not shown). The phenomenon that animal cells transfected with the human *MDR1* gene express protein migrating at a low molecular weight was also reported by Hammerle *et al.* (22) and Evers *et al.* (36), e.g., pig kidney epithelial cell line (LLC-PK1) transfected with the human *MDR1* gene expressed protein migrating on PAGE with an apparent molecular weight of ~120 kDa. The structural differences between the 150 kDa isoform and the 170 kDa isoform of P-gp are unknown. One possible difference may be that the 150 kDa isoform is less glycosylated than the 170 kDa isoform. Ling *et al.* (34) previously reported that a class of structural mutants of P-gps with greatly reduced surface carbohydrates migrated with a molecular weight of ~150 kDa in the Western blots. The presence of this less glycosylated isoform may also explain the weakly stained bands of ~150 kDa in Caco-2 and MDCK-WT cell lysates observed in our studies (Fig. 1, Lanes 1A, 1B, 2A, 2B).

To investigate the efflux activity of the P-gp in these cells, the polarized efflux of [³H]-digoxin was compared. MDCK-MDR1 cells exhibited higher polarized efflux of [³H]-digoxin than Caco-2 and MDCK-WT cells (Table I). The increased polarized efflux of [³H]-digoxin in MDCK-MDR1 cells was totally inhibited by the P-gp inhibitor GF120918. GF120918 is a potent and specific inhibitor for P-gp at low concentration (< ~1 μM) (37,38). It has also been reported to inhibit breast cancer resistance protein (MXR) at a concentration of 10 μM (38). These data indicate that the 150 kDa isoform expressed in MDCK-MDR1 cells retains the ability to efflux a known P-gp substrate (digoxin) and is inhibited by a known P-gp inhibitor (GF120918).

Caco-2 cells and MDCK cells are derived from different species (human vs. dog) and different tissues (colonic carcinoma vs. kidney) and they serve different biologic roles. These different biologic roles may lead to differences in the lipid composition of their membrane bilayers. The phenomena of different sorting of glycosphingolipids between Caco-2 cell and MDCK cells have been reported previously (39,40). For example, Van't Hof *et al.* observed that, in MDCK and Caco-2 cells, glucosylceramide and sphingomyelin synthesized from the short-chain sphingolipid analog *N*-6-[7-nitro-2,1,3-benzoxadiazol-4-yl] aminodecanoyl-ceramide were delivered to the cell surface with different AP/BL ratios of 2–4 and 0.6–0.9, respectively (40). Furthermore, different strains of cells from the same tissue may have different lipid composition as well (41). For example, of the total glycosphingolipid content, MDCK strain I cells were reported to express 56% glucosylceramide and 6% galactosylceramide, whereas MDCK strain II cells express 28% glucosylceramide and 16% galactosylceramide (41).

P-gp is closely connected to its membrane environment (42). During a P-gp purification process, Sharom *et al.* (42) observed that a few tightly bound lipids were retained on P-gp and Callaghan *et al.* (43) reported that the depletion of these lipids resulted in complete loss of activity, as measured by the protein's ability to hydrolyze ATP. Therefore, differences in lipid composition between the AP cell membranes of Caco-2 and MDCK cells may lead to differences in the way that P-gps orientate in the membranes; this may result in differences in substrates/inhibitors specificity (drug binding) and differences in kinetics of substrate efflux (44). Therefore, we felt that it was important to compare the kinetics and affinity of substrates/inhibitors of P-gp in Caco-2 and MDCK-MDR1 cell lines.

Michaelis–Menten constants (K_M , V_{max}) values for efflux of [³H]-vinblastine were different in Caco-2, MDCK-WT, and MDCK-MDR1 cells, and the results are shown in Table II. K_M values of Caco-2 cells and MDCK-WT were different (Table I) even though the major isoform of P-gps in these two cells migrate on PAGE with similar molecular weight (i.e., ~170 kDa; Fig. 1). This difference may result from the difference in the lipid composition between these two cell lines (45,46) or the difference in the substrate specificity of the canine vs. human P-gps. MDCK-MDR1 cells exhibited higher K_M values for [³H]-vinblastine than did Caco-2 and MDCK-WT cells (Table I). The differences in the expression levels of P-gp isoforms and differences in lipid composition between Caco-2 cells and MDCK cells could lead to these differences in the K_M values for [³H]-vinblastine. Soldner *et al.* (23) also observed that K_M and V_{max} values were greater in MDCK-MDR1 cells than in Caco-2 cells. They reported that the values for K_M and V_{max} of losartan in MDCK-MDR1 cells were about 2 times higher than those in Caco-2 cells (23). K_M and V_{max} data are very valuable for understanding substrate–transporter relationships. However, the low solubilities of most P-gp substrates limit the range of concentrations that can be used to determine accurate Michaelis–Menten constants.

Therefore, we decided to determine inhibition constants for various P-gp substrates/inhibitors using a method recently developed by our laboratory (24). The advantage of this "two-concentration" method is that it makes it possible to calculate the apparent K_I values for P-gp substrates/inhibitors having low water solubility. The limitation of this method is that because only the extracellular concentration of a test drug is used in the calculation, this method does not distinguish the drug concentration in cytosol with that in lipid bilayer. Therefore, caution should be used in interpretation of these data with respect to substrate and/or inhibitory activity of molecules. Using this method, the K_I values of known P-gp substrates/inhibitors were determined in Caco-2 and MDCK-MDR1 cells (Table III). In general, the K_I value for an individual compound determined in MDCK-MDR1 cells was significantly higher than the K_I value determined in Caco-2 cells (Table III). The exception was the P-gp inhibitor GF120918. Because a K_I value is the dissociation constant of the test compound from P-gp, the inverse of the K_I value reflects the compound's apparent affinity for P-gp. Therefore, the greater the value of K_I , the lower the apparent affinity of a substrate/inhibitor for this efflux transporter.

The environment (e.g., lipid phase vs. cytosol) from which P-gp extracts its substrates is unknown. Although sev-

eral lines of experimental evidence have shown that P-gp recognizes its substrates directly from the lipid phase (47,48), other experimental evidence suggests that P-gp extracts substrates directly from cytosol (49) or from both lipid phase and cytosol (50). This evidence indicates that it is difficult to assess the "effective" drug concentration upon which P-gp acts. The difference in lipid composition between Caco-2 cells and MDCK-MDR1 cells may result in differences in partitioning of drugs into the two cell membrane bilayers, thus leading to differences in apparent kinetics constants and affinity constants. On the other hand, the "effective" concentration of a substrate/inhibitor may also depend on the expression levels of P-gp in the cells. Tanaka *et al.* (51) reported that the intracellular accumulation of CsA in LLC-PK1 cells and LLC-PK1 cells transfected with human *MDR1* gene was correlated with P-gp expression levels in these cells (i.e., lower accumulation of CsA in cells expressing higher levels of P-gp). Therefore, the difference in the expression levels of P-gps between Caco-2 cells and MDCK-MDR1 cells may affect the cellular-associated concentration of a substrate/inhibitor in these cells and affect its apparent K_M/K_I values. However, the expression levels of total P-gp in cells may not be the only determinant for K_I value. For example, the Michaelis-Menten constants and K_I values of P-gp substrates obtained using Caco-2 subclones, which have higher P-gp expression levels measured by Western blotting and greater polarized efflux of known P-gp substrates (e.g., vinblastine) than their parental Caco-2 cell line, are not different from those values obtained from the parental Caco-2 cell line (H. Kazutoshi, F. Tang, R. T. Borchardt, unpublished data). In addition, insertion of a drug into a membrane can lead to modifications of the membrane bilayer (e.g., increasing the surface area, deforming vesicular shape) (52), and these modifications may also lead to a change in drug binding or a change in P-gp activity (44).

In summary, MDCK-MDR1 cells overexpressed significant amounts of P-gp, which migrated at an apparent molecular weight of 150 kDa. The MDCK-MDR1 cells exhibited increased polarized efflux of known substrates of P-gp compared to wild-type MDCK cells. Therefore, this transfected cell line may be a useful model for qualitatively screening P-gp substrate activity of drugs/drug candidates. However, the apparent kinetics constants (K_M , V_{max}) and affinity constants (K_I) of substrates/inhibitors determined in MDCK-MDR1 cells may be different from the values obtained when experiments are conducted in Caco-2 cells. These differences may result from the different levels of total P-gp expressed in Caco-2 vs. MDCK-MDR1 cells, different orientations of P-gp in the Caco-2 vs. MDCK-MDR1 cell membranes, or different partitioning of substrates/inhibitors into these two cell membrane bilayers.

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