Are MDCK Cells Transfected with the Human *MRP2* 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 *MRP2* gene (MDCK-MRP2) are a good model of the human intestinal mucosa.

Methods. MRP2 expression in Caco-2 cells was compared with the expression of this efflux transporter in MDCK-wild type (MDCK-WT) and MDCK-MRP2 cells using Western blotting methods. The polarized efflux activities of MRP2 in the MDCK-MRP2, MDCK-WT, MDCK cells transfected with the human *MDR1* gene (MDCK-MDR1), and Caco-2 cells were compared using vinblastine as a substrate. Apparent Michaelis-Menten constants (K_M, Vmax) for the efflux of vinblastine in Caco-2 and MDCK-MRP2 cells were determined in the presence of GF120918 (2 μM), which inhibits P-glycoprotein but does not affect MRP2. Apparent inhibitory constants (K_I) of known substrates/inhibitors of MRP2 were determined by measuring their effects on the efflux of vinblastine in these cell lines.

Results. MDCK-MRP2 cells expressed higher levels of MRP2 than MDCK-WT and Caco-2 cells as measured by Western blotting technique. Two isoforms of MRP2 expressed in Caco-2 and MDCK cells migrated at molecular weights of 150 kD and 190 kD. In MDCK-MRP2 cells, the 150 kD isoform appeared to be overexpressed. MDCK-MRP2 cell monolayers exhibited higher polarized efflux of vinblastine than Caco-2 and MDCK-WT cell monolayers. K_M values for vinblastine in Caco-2 and MDCK-MRP2 cells were determined to be 71.8 ± 11.6 and 137.3 ± 33.6 μM, respectively, and V_{max} values were determined to be 0.54 ± 0.03 and 2.45 ± 0.31 pmolcm^{-2}s^{-1}, respectively. Known substrates/inhibitors of MRP2 showed differences in their ability to inhibit vinblastine efflux in Caco-2 cells as compared to MDCK-MRP2 cells

Conclusions. These data suggest that MDCK-MRP2 cells overexpress only the 150 kD isoform of MRP2. The 190 kD isoform, which was also found in Caco-2 cells and MDCK-WT cells, was present in MDCK-MRP2 cells but not over expressed. The apparent kinetics constants and affinities of some MRP2 substrates were different in Caco-2 cells and MDCK-MRP2 cells. These differences in substrate activity could result from differences in the relative expression levels of the MRP2 isoforms present in Caco-2 cells and MDCK-MRP2 cells and/or differences in the partitioning of substrates in these two cell membrane bilayers.

KEY WORDS: MRP2; MDCK cells; efflux; transport; Caco-2 cells; affinity.

INTRODUCTION

Similar to multidrug resistance protein [P-glycoprotein (P-gp), MDR1 in human], multidrug resistance-associated protein 2 (MRP2) is apically polarized in intestinal mucosa cells (1,2). Although MRP2 was originally described as the canalicular multispecific organic anion transporter (cMOAT) in liver facilitating drug clearance (3), it has also been found to play a role in limiting drug absorption through the intestinal mucosa (4,5). Since this efflux transporter can significantly affect the biodistribution of drugs/drug candidates (4), *in vitro* cell culture models containing MRP2 would be useful tools for pharmaceutical scientists.

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 (6). 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 (6,7). Caco-2 cells were reported to express P-gp and MRP2 (8,9) and exhibit polarized efflux of substrates of P-gp and MRP2 (10). It was recently reported that the mRNA expression levels of P-gp and MRP2 in Caco-2 cells were well correlated the mRNA levels of these two efflux transporters in the human intestine (11). Therefore, Caco-2 cells have been used as a cell model to determine the structure-transport relationship (STR) for these efflux transporters (9,12,13).

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 Caco-2 cells (3 days vs. 21days) (14). A good correlation between the permeation of passively absorbed drugs in Caco-2 cells and MDCK cells has been reported (14). Therefore, this canine kidney cell line has been proposed as a model for membrane permeation screening (14).

In the late 1990s, MDCK cell lines that overexpress human P-gp (15) and MRP2 (5) were generated by transfecting the human *MDR1* and *MRP2* genes into MDCK cells. The resulting MDCK-MDR1 (15) and MDCK-MRP2 (5) cell lines have been used as models of the intestinal mucosa (4,12,16). However, to date, investigators have assumed without any definitive evidence that MRP2 overexpressed in MDCK cells was identical structurally and functionally to MRP2 expressed in intestinal mucosal cells.

Therefore, the objectives of this study were: (i) to compare the MRP2 expression in MDCK-MRP2 cells and Caco-2 cells; (ii) to investigate the polarized efflux activities of MRP2 expressed in these cells; and (iii) to compare the kinetics and the affinity of known substrates/inhibitors of P-gp/MRP2 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, MDCK cells transfected with the human *MDR1* gene (MDCK-MDR1), and MDCK cells transfected with the human *MRP2* gene (MDCK-MRP2)

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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). Cyclosporin A (CsA), reserpine, vinblastine sulfate, quinidine, verapamil, vincristine sulfate, etoposide, daunorubicin, and anti-mouse IgG-HRP were purchased from Sigma Chemical Co. (St. Louis, Missouri). [^{3}H]-Vinblastine sulfate (specific activity 11.1 Ci/mmol) and [^{3}H]-CsA (specific activity 7.0 Ci/mmol) were obtained from Amersham Life Science (Arlington Heights, Illinois). Anti-human MRP2 M_2I-4 was purchased from Kamiya Biomedical Company (Seattle, Washington). Electrochemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Buckinghamshire, England).

Western Blotting and Protein Detection

Western blotting was performed using a modification of the procedure described by Hosoya *et al.* (8). Proteins were isolated from Caco-2, MDCK-WT, and MDCK-MRP2 cell monolayers and transferred onto nitrocellulose membranes as described by Tang *et al.* (17). MRP2 was probed using the primary antibody M_2I-4 (1:20 dilution) and the secondary antibody anti-mouse IgG-HRP (1:6000 dilution). Antibody detection was performed using the ECL method following the protocol provided by the manufacturer.

Cell Culture

Caco-2, MDCK-WT, MDC-MDR1 and MDCK-MRP2 cells were seeded on Transwell® filter inserts (6-well) and maintained as described by Tang *et al.* (17). The membrane integrity was checked using [^{14}C]-mannitol. The apparent permeability coefficients (P_{app}) of [^{14}C]-mannitol across Caco-2 or MDCK cell monolayers were typically in the range of $0.1 - 0.6 \times 10^{-6}$ cm/sec.

Transport Experiments

Bi-directional transport experiments of [^{3}H]-vinblastine and [^{3}H]-CsA and experiments with inhibitors [GF120918 (2 μM), CsA (25 μM), or MK571 (50 μM)] were performed as previously described (17,18)

P_{app} values were calculated using the equation:

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

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 ($P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$). A ratio of substantially greater than 1.0 indicates a net efflux of the solute.

Determination of Apparent Michaelis-Menten Constants (K_M, V_{max}) of [^{3}H]-Vinblastine in Caco-2 Cells and MDCK-MRP2 Cells

The time course over 80 min for the flux of [^{3}H]-vinblastine in the BL-to-AP direction across Caco-2 and MDCK-MRP2 cell monolayers was conducted in the presence of GF120918 (2 μM). GF120918 was used to totally inhibit efflux by P-gps expressed in Caco-2 and MDCK-MRP2 cells. The concentration-dependence of the total flux rate of [^{3}H]-vinblastine was also determined using a concentration range of 0.01–150 μM. The passive diffusion flux rate (excluding the influence of efflux transporter) of [^{3}H]-vinblastine was estimated by conducting the transport experiment in the presence of CsA (25 μM) (in Caco-2 cells) or in the presence of MK571 (50 μM) (in MDCK-MRP2 cells). The active transport flux rates were then estimated by subtracting the passive diffusion flux rates from total flux rates. Michaelis-Menten parameters (K_M, V_{max}) were estimated using Origin 6.0 (OriginLab, Northampton, Massachusetts).

Determination of the Inhibitory Effects of Drugs on the Active BL-to-AP Transport of [^{3}H]-Vinblastine

The effects of known substrates/inhibitors of P-gp/MRP2 on the active BL-to-AP transport of [^{3}H]-vinblastine across Caco-2 and MDCK-MRP2 cell monolayers were measured in the presence of GF120918 (2 μM), which inhibits P-gp in these cell lines. Apparent inhibition constants (K_I) were calculated using the method previously published by Gao *et al.* (19). Working equations 2–4 were used in these calculations.

$$K_I = [(P_I/P_0)/(1 - P_I/P_0)]*[I] \quad (2)$$

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

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

In Equation (2), K_I is the apparent inhibition constant of a test compound. P_I and P_0 are P_{app} values of [^{3}H]-vinblastine in the presence and the absence of the test compound, respectively. P_I/P_0 is a reflection of the inhibitory effect of the test compound on the active BL-to-AP transport of [^{3}H]-vinblastine across the cell monolayers. [I] is the concentration of the test compound in the donor and the receiver side. $P_{app,\ 1}$, $P_{app,\ 2}$, and $P_{app,\ 3}$ were determined from the BL-to-AP transport of [^{3}H]- vinblastine in the absence of a test compound, in the presence of a test compound, and in the presence of CsA (25 μM) (in Caco-2 cells) or MK571 (50 μM) (in MDCK-MRP2 cells), respectively. The active component of the permeation of [^{3}H]-vinblastine in the absence of a potentialinhibitor (P_0) and in the presence of a potential inhibitor (P_I) was determined by subtracting $P_{app,\ 3}$ (the passive diffusion component) from $P_{app,\ 1}$ (total transport in the absence of any compound), and $P_{app,\ 2}$ (total transport in the presence of a potential inhibitor), respectively. To avoid cell-cell variations, the transport experiments for the determination of $P_{app,\ 1}$, $P_{app,\ 2}$, and $P_{app,\ 3}$ 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 Fig. 1, cell lysates of Caco-2 (Panel 1A-B), MDCK-MRP2 (Panel 2A-B), and MDCK-WT (Panel 3A-B) cells cross-reacted with the MRP2 antibody resulting in the staining of two bands on polyacrylamide gel electrophoresis (PAGE) having apparent molecular weights of ~150 kD and ~190 kD. Subsequently, these proteins are referred to as the150 kD isoform and the190 kD isoform of MRP2. Inter-

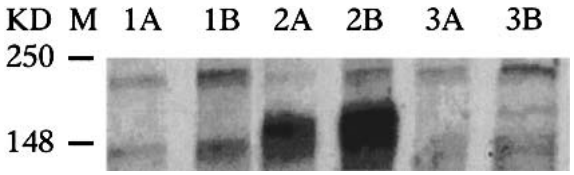

Fig. 1. Western blotting of MRP2 in the total cell lysates from Caco-2, MDCK-MRP2, and MDCK-WT cell monolayers. Proteins were size fractionated in 6% Tris-glycine polyacrylamide gel in 0.1% of SDS Tris-glycine buffer. After electro blotting, MRP2 was stained with M_2I-4 and the protein-antibody interaction was visualized using the chemiluminescence technique as described in Materials and Methods. A: 10 μg, B: 20 μg of total cellular protein. 1: Caco-2 cells; 2: MDCK-MRP2 cells; 3: MDCK-WT cells. M: Molecular weight marker.

estingly, the MDCK-MRP2 cells appeared to over express only the 150 kD isoform.

Transport of [^{3}H]-Vinblastine across the Caco-2, MDCK-WT, MDCK-MDR1 and MRP2 Cell Monolayers

Vinblastine, a substrate for P-gp and MRP2 (5,20), was chosen to compare the functional efflux activities of the transporters in Caco-2, MDCK-WT, MDCK-MDR1, and MDCK-MRP2 cell monolayers. GF120918 (21,22) was used in these experiments to specifically inhibit P-gp, allowing us to dissect the substrate kinetics parameters for MRP2. To evaluate MRP2 activities in these cell lines, CsA, a known inhibitor for P-gp and MRP2 (23–25), was also used in these experiments. As shown in Table I, [^{3}H]-vinblastine exhibited strong polarized efflux in Caco-2 cells (i.e., $P_{app\ BL\text{-}to\text{-}AP} >> P_{app\ AP\text{-}to\text{-}BL}$). The ratio of $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$, which is used here, as an indicator of efflux activity, was 18. In the presence of GF120918 (2 μM), $P_{app\ AP\text{-}to\text{-}BL}$ increased and $P_{app\ BL\text{-}to\text{-}AP}$ decreased, resulting in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 1.9. CsA (25 μM) completely inhibited the polarized efflux of [^{3}H]-vinblastine in Caco-2 cells resulting in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 1.0.

MDCK-WT cells also showed polarized efflux of [^{3}H]-vinblastine, with a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 15. GF120918 (2 μM) partially inhibited the efflux of [^{3}H]-vinblastine in MDCK-WT cells, resulting in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 1.7. CsA (25 μM) completely inhibited the polarized efflux of [^{3}H]-vinblastine in MDCK-WT cells, resulting in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 1.1.

As expected, the polarized efflux of [^{3}H]-vinblastine, as measured by the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio, was significantly higher in MDCK-MDR1 cell monolayers than in MDCK-WT cell monolayers (i.e., 60 vs. 15, respectively). This polarized efflux of [^{3}H]-vinblastine in MDCK-MDR1 could be significantly inhibited by inclusion of CsA (25 μM) or GF120918 (2 μM) in the incubation mixture (Table I).

The polarized efflux of [^{3}H]-vinblastine was significantly higher in MDCK-MRP2 cell monolayers than in MDCK-WT cell monolayers (i.e., the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios are 36 vs. 15, respectively). Inclusion of GF120918 (2 μM) in the incubation media only decreased the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio to 16. CsA (25 μM) significantly inhibited the polarized efflux of [^{3}H]-vinblastine in MDCK-MRP2, but could not completely inhibit this efflux (i.e., the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was 5.1). MK571 (50 μM), an MRP2 inhibitor (9), which has a higher solubility than CsA, completely inhibited the polarized efflux of [^{3}H]-vinblastine in MDCK-MRP2 cells (Table I).

Michaelis-Menten Constants (K_M,V_{max}) for Active BL-to-AP Transport of [^{3}H]-Vinblastine across Caco-2 and MDCK-MRP2 Cell Monolayers

The apparent Michaelis-Menten constants (K_M, V_{max}) for MRP2 using [^{3}H]-vinblastine as a substrate were determined using Caco-2 and MDCK-MRP2 cell monolayers in the presence of GF120918 (2 μM). As shown in Fig. 2, the active BL-to-AP flux (mediated by MRP2) of [^{3}H]-vinblastine in Caco-2 and MDCK-MRP2 monolayers was concentration-dependent and saturable. Similar results were observed in MDCK-MRP2 cell monolayers. Using these data, K_M and V_{max} values for the active BL-to-AP flux of [^{3}H]-vinblastine

Table I. P_{app} Values of [^{3}H]-Vinblastine across Caco-2, MDCK-WT, MDCK-MDR1 and MDCK-MRP2 Cell Monolayers

Cell type	Treatment	Papp*10^6 (cm/s)		Ratio ($P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$)
		AP-to-BL	BL-to-AP	
Caco-2	—	1.07 ± 0.04	19.18 ± 0.49	18
	GF120918 (2 μM)	4.08 ± 0.16	7.84 ± 0.45	1.9
	CsA (25 μM)	4.12 ± 0.04	4.05 ± 0.06	1.0
MDCK-WT	—	0.80 ± 0.34	12.18 ± 2.05	15
	GF120918 (2 μM)	1.50 ± 0.15	2.59 ± 0.37	1.7
	CsA (25 μM)	1.50 ± 0.20	1.59 ± 0.03	1.1
MDCK-MDR1	—	0.20 ± 0.11	11.96 ± 1.31	60
	GF120918 (2 μM)	0.83 ± 0.08	2.60 ± 0.08	3.0
	CsA (25 μM)	1.11 ± 0.05	1.64 ± 0.11	1.5
MDCK-MRP2	—	0.37 ± 0.03	13.40 ± 0.21	36
	GF120918 (2 μM)	0.82 ± 0.01	12.98 ± 1.03	16
	CsA (25 μM)	1.08 ± 0.20	5.56 ± 0.76	5.1
	MK571 (50 μM)	1.60 ± 0.05	1.70 ± 0.12	1.1

Note: P_{app} values of [^{3}H]-vinblastine were measured in the absence or the presence of inhibitor GF120918 (2 μM), CsA (25 μM), or MK571 (50 μM) in various cell lines as described in Materials and Methods. P_{app} values are presented as mean ± S.D. (n = 3).

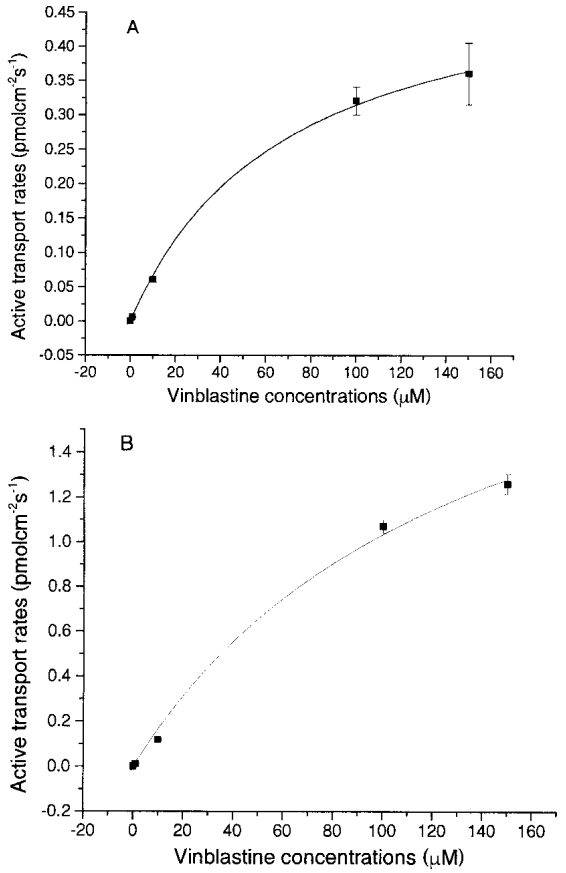

Fig. 2. Concentration dependency of the active BL-to-AP flux of [^{3}H]-vinblastine across (A) Caco-2 and (B) MDCK-MRP2 cell monolayers. The total transport flux rates of [^{3}H]-vinblastine were determined over a concentration range from 0.01–150 μM in the presence of GF120918 (2 μM) as described in Materials and Methods. The passive diffusion flux rates were determined in the presence of CsA (25 μM) or MK571 (50 μM), which totally inhibits the active transport of [^{3}H]-vinblastine in Caco-2 or MDCK-MRP2 cells. 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 ± SD (n = 3).

by MRP2 in Caco-2 and MDCK-MRP2 cells were calculated (Table II).

Inhibition Constants (K_I) of Known Substrates/Inhibitors of P-gp/MRP2 in Caco-2 and MDCK-MRP2 Cell Monolayers

To determine the inhibitory specificity of MRP2, experiments were conducted in the presence of GF120918 (2 μM), which totally inhibits P-gp activity in these cell lines (Tables I and III). As shown in Fig. 3A, inclusion of CsA (5 μM) in the transport medium containing GF120918 (2 μM) significantly inhibited the BL-to-AP transport of [^{3}H]-vinblastine across Caco-2 cell monolayers (i.e., after 80 m, the total transport of [^{3}H]-vinblastine was decreased from ~5.6% to ~4.2%). A higher concentration of CsA (25 μM) resulted in even more significant inhibition of the BL-to-AP flux of [^{3}H]-vinblastine (Figure 3A). Using the method described by Gao *et al.* (19), the apparent K_I value for CsA was calculated to be 2.69 ± 0.15 μM (Table III). In contrast, the apparent K_I value for CsA inhibition of MRP2 in MDCK-MRP2 cells was 8.11 ± 0.21 μM, which was about 3 times greater than that in Caco-2 cells (Table III). To explore this observation further, we determined the K_I values of other known substrates/inhibitors of P-gp/MRP2 on the active BL-to-AP transport (mediated by MRP2) of [^{3}H]-vinblastine in Caco-2 and MDCK-MRP2 cell monolayers; these results are presented in Table III. The phenomenon observed with CsA (higher K_I value in MDCK-MRP2 cells compared to Caco-2 cells) was also observed for MK571, vincristine, and etoposide (Table III). The K_I values for these substrates/inhibitors in MDCK-MRP2 cells were about three to seven times greater than those in Caco-2 cells. In contrast, daunorubicin had a lower K_I value in MDCK-MRP2 than in Caco-2 cells (i.e., 49.4 ± 1.49 μM *vs.* 104 ± 6.94 μM). As expected, known P-gp substrates/inhibitors (quinidine and verapamil) did not exhibit inhibitory effects on the polarized efflux of [^{3}H]-vinblastine in Caco-2 and MDCK-MRP2 cells at the concentrations tested. Reserpine did not show an inhibitory effect on the polarized efflux of [^{3}H]-vinblastine in Caco-2 cells. However, it did inhibit the efflux of [^{3}H]-vinblastine in MDCK-MRP2 cells (i.e., the K_I value was 295 ± 17.6 μM).

DISCUSSIONS

In this study, the MRP2 expression in Caco-2 cells (human colonic carcinoma), MDCK-WT cells (a canine kidney derived cell line), and MDCK-MRP2 cells (a canine kidney derived cell line transfected with the human *MRP2* gene) were compared using Western blotting methods. The observations that bands staining for MRP2 in Caco-2 cells and MDCK cells (both MDCK-WT and MDCK-MRP2 cells) migrating with apparent molecular weights of ~190 kD are consistent with the published data for this efflux transporter (9,26,27). The band staining for MRP2 at an apparent molecular weight of ~150 kD on PAGE observed in Caco-2 cell lysates was also observed by other investigators (9). It is interesting to note that MDCK-MRP2 cells appear to over express this 150 kD isoform of MRP2. A similar phenomenon (i.e., animal cells transfected with a human gene express a protein migrating at a low molecular weight) was reported previously by Evers *et al.* (28), i.e., 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 kD (28). The structural differences between the isoforms of MRP2 expressed in Caco-2 cells and MDCK cells are unknown at this time. Since MRP2 is a glycoprotein, one possible difference may be that of glycosyla-

Table II. Apparent K_M and V_{max} Values for the Active BL-to-AP Transport of [^{3}H]-Vinblastine across Caco-2 and MDCK-MRP2 Cell Monolayers

Michaelis-Menten Constants[a]	Caco-2	MDCK-MRP2
K_M (μM)	71.8 ± 11.6	137.3 ± 33.6[b]
V_{max} (pmolcm^{-2}s^{-1})	0.54 ± 0.03	2.45 ± 0.31[b]

[a] BL-to-AP transport experiments of [^{3}H]-vinblastine were conducted in Caco-2 and MDCK-MRP2 cell monolayers as described in Materials and Methods. GF120918 (2 μM) was included in the incubation medium to inhibit P-gp. K_M and V_{max} values were calculated as described in Materials and Methods.

[b] Significance level: $p < 0.05$, when compared with the value in Caco-2 cells.

Table III. K_I Values of Known P-gp/MRP2 Substrates/Inhibitors on the Active BL-to-AP Transport of [^{3}H]-Vinblastine across Caco-2 or MDCK-MRP2 Cell Monolayers

Compounds (concentration used)	K_I (μM) in Caco-2	K_I (μM) in MDCK-MRP2
GF120918 (12 μM)	No inhibition	No inhibition
CsA (5 μM)	2.69 ± 0.15	8.11 ± 0.21[a]
Vinblastine (250 μM)	479 ± 90.1	380 ± 5.16
MK571 (10 μM)	11.3 ± 1.54	26.4 ± 0.90[a]
Quinidine (15 μM)	No inhibition	No inhibition
Verapamil (50 μM)	No inhibition	No inhibition
Vincristine (200 μM)	115 ± 4.15	802 ± 21.3[a]
Etoposide (300 μM)	181 ± 22.2	756 ± 14.2[a]
Daunorubicin (30 μM)	104 ± 6.94	49.4 ± 1.49[a]
Reserpine (90 μM)	No inhibition	295 ± 17.6

Note. The inhibitory effects of P-gp/MRP2 substrates/inhibitors on the BL-to-AP transport of [^{3}H]-vinblastine were measured in Caco-2 and MDCK-MRP2 cell monolayers as described in Materials and Methods. GF120918 (2 μM) was included in the incubation media to inhibit P-gp. K_I values were calculated as described in Materials and Methods. Data are presented as mean ± S.D. (n = 3).

[a] Significance level: $p < 0.05$, when compared with K_I value in Caco-2 cells.

tion, i.e., the 150 kD isoform is less glycosylated than the 190 kD isoform of MRP2. Evidence in support of this hypothesis includes the observations by Ling *et al.* (29) that structural mutants of P-gps with greatly reduced surface carbohydrates migrated with the molecular weights of ~150 kD on Western blots.

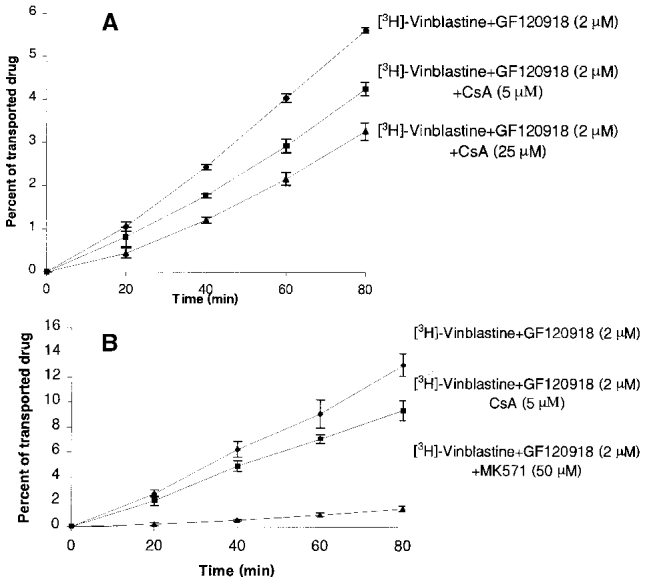

Fig. 3. Data used to calculate the K_I values of CsA on the active BL-to-AP transport of [^{3}H]-vinblastine across (A) Caco-2 and (B) MDCK-MRP2 cell monolayers. $P_{app,\ 1}$, $P_{app,\ 2}$, and $P_{app,\ 3}$ were determined from the BL-to-AP transport of [^{3}H]-vinblastine (in the presence of GF120918) in the absence of CsA (♦), in the presence of 5 μM of CsA (■), and in the presence of (A) 25 μM CsA or (B) 50 μM MK571 (▲), respectively. The active transport component of the flux rate of [^{3}H]-vinblastine in the absence a test compound (P_0) and in the presence of 5 μM of CsA (P_I) 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 ± SD (n = 3).

To investigate the efflux activities of MRP2 in Caco-2, MDCK-WT, and MDCK-MRP2 cells, we determined the polarized efflux of [^{3}H]-vinblastine [a substrate for P-gp and MRP2 (5,20)] in these cells. As shown in Table I, the polarized efflux of [^{3}H]-vinblastine in Caco-2, MDCK-WT, and MDCK-MDR1 cells was significantly inhibited by GF120918 (2 μM), a known specific P-gp inhibitor (21,22). CsA (25 μM), a known inhibitor for P-gp and MRP2 (23–25), completely inhibited the polarized efflux of [^{3}H]-vinblastine in Caco-2 and MDCK-WT cells (Table I). These data suggest that the polarized efflux of [^{3}H]-vinblastine in Caco-2 cells be mainly mediated by P-gp; however, the data on Figure 3 clearly show that these cells retain some functional MRP2 activity. In contrast, GF120918 (2 μM) exhibited much less inhibition effect on the polarized efflux of [^{3}H]-vinblastine (i.e., it only decreased the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio to 16) in MDCK-MRP2 cells. CsA (25 μM) could only partially inhibit this polarized efflux of [^{3}H]-vinblastine (i.e., the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was still 5.1). However, it should be noted that higher concentrations of CsA could not be employed in these studies because of its limited solubility. Therefore, we used MK571, an MRP2 inhibitor with good solubility (37 mM) (9). MK571 at a concentration of 50 μM completely inhibited this polarized efflux in MDCK-MRP2 (Table I). These data indicate that MRP2 expressed in MDCK-MRP2 cells retains the ability to efflux a known MRP2 substrate (vinblastine) and is inhibited by known MRP2 inhibitors (CsA and MK571).

Based on our observations with the human P-gp expressed in MDCK-MDR1 cells (17), the differences in lipid composition between the AP cell membranes of Caco-2 and MDCK cells may lead to: (i) differences in the way that MRP2s orient themselves in the membranes and (ii) differences in the partitioning characteristics of drugs into these cell membranes. These cell membrane differences could be translated into differences in the substrates/inhibitors specificity (drug binding) of MRP2s and differences in the kinetics of substrate efflux by MRP2s (17,30–32). Furthermore, the difference in the expression levels of the isoforms of MRP2 in Caco-2 cells and MDCK-MRP2 cells may also affect the cellular-associated concentration of substrates/inhibitors for this transporter; thus, affecting their apparent K_M/K_I values (17,33). Therefore, we felt that it was important to compare the kinetics of substrate efflux and the affinities of known substrates/inhibitors of MRP2 in Caco-2 and MDCK-MRP2 cell lines.

To dissect the MPR2 activity of Caco-2 and MDCK-MRP2 cells from the P-gp activity (8,9), GF120918 (2 μM) was included in the incubation media during transport experiments which were designed to compare the kinetics of substrate efflux and the affinities of known substrates/inhibitors of MRP2 in these cell lines. GF120918 has been reported to be a potent and specific P-gp inhibitor (21,22,34). To verify the P-gp inhibitory specificity of GF120918 under our experimental conditions, the effects of several concentrations of GF120918 on [^{3}H]-vinblastine were tested. GF120918 at 2 μM (Table I), 12 μM (Table III), and 20 μM (data not shown) produced the same extent of inhibition on the polarized efflux of [^{3}H]-vinblastine in Caco-2 and MDCK-MRP2 cells (i.e., the $P_{app\ BL\text{-}to\text{-}AP}$ values did not decrease with the increase

of concentrations of GF120918). However, the $P_{app\ BL\text{-}to\text{-}AP}$ values for [^{3}H]-vinblastine decreased further if an MRP2 inhibitor was included in the incubation mixture [e.g., as shown in Table I, in the presence of GF120918 (2 μM), the $P_{app\ BL\text{-}to\text{-}AP}$ value of [^{3}H]-vinblastine across MDCK-MRP2 cell monolayers was 12.98 ± 1.03 × 10^-6 cm/s. This value was decreased to 5.56 ± 1.03 × 10^-6 cm/s when the MRP2 inhibitor CsA (25 μM) was included in the incubation mixture]. These results suggest that a concentration of 2 μM of GF120918 is sufficient to inhibit P-gp in Caco-2 and MDCK-MRP2 cells and, very importantly, GF120918 does not inhibit MRP2 in these cells.

Michaelis-Menten constants (K_M, V_{max}) for efflux (mediated by MRP2) of [^{3}H]-vinblastine were different in Caco-2 and MDCK-MRP2 cells (Table II). The K_M and V_{max} values in MDCK-MRP2 cells were higher than those in Caco-2 cells (Table II). These results are consistent with the observation for the Michaelis-Menten constant values for efflux of losartan (11) and [^{3}H]-vinblastine (16) in Caco-2 and MDCK-MDR1 cells. The differences in the expression levels of the two isoforms of MRP2 in these cells, and/or differences in lipid composition between Caco-2 cells and MDCK cells could lead to these differences in substrate kinetics constants.

The interpretation of the K_I values of substrates/inhibitors of P-gp/MRP2 in Caco-2 and MDCK-MRP2 cells is very complex; i.e., a simple trend could not be derived from the data shown in Table III,. GF120918, quinidine, and verapamil are potent P-gp inhibitors and have relatively low K_I values ranging from 0.39 to 15 μM in Caco-2 and MDCK-MDR1 cells (17). These three compounds did not inhibit MRP2 at the concentrations used in our experiments (Table III). Therefore, these drugs may be used as P-gp specific inhibitors in drug-transport relationship studies. CsA, MK571, vincristine, and etoposide exhibited greater K_I values in MDCK-MRP2 cells than in Caco-2 cells (Table III). Since a K_I value is the dissociation constant of the test compound from MRP2, the inverse of the K_I value reflects the apparent affinity of the compound for this transporter. Therefore, the greater the value of K_I, the lower the apparent affinity of a substrate/inhibitor for MRP2. These results indicate that the apparent affinities of CsA, MK571, vincristine, and etoposide in MDCK-MRP2 cells were lower than in Caco-2 cells. However, vinblastine, daunorubicin, and reserpine did not follow this trend. In contrast, daunorubicin had a higher K_I in Caco-2 cells than in MDCK-MRP2 cells. Reserpine showed no inhibition of MRP2 in Caco-2 cells, and had a K_I value of ~300 μM in MDCK-MRP2 cells. These data suggest that the apparent affinity of substrates/inhibitors for MRP2 may be different in Caco-2 and MDCK-MRP2 cells. In summary, MDCK-MRP2 cells expressed significant amounts of the isoform of MRP2, which migrated at an apparent molecular weight of ~150 kD. The MDCK-MRP2 cells exhibited increased polarized efflux of known substrates of MRP2 compared to wild-type MDCK cells. Therefore, this transfected cell line may be a useful model for qualitatively screening the MRP2 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-MRP2 cells may be different from the values obtained when experiments are conducted in Caco-2 cells. These differences may result from different levels of the two isoforms of MRP2 expressed in Caco-2 *vs*. MDCK-MRP2 cells, different orientations of MRP2 in the Caco-2 vs. MDCK-MRP2 cell membranes, or different partitioning of substrates/inhibitors into these two cell membrane bilayers.

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