DELINEATING THE CONTRIBUTION OF SECRETORY TRANSPORTERS IN THE EFFLUX OF ETOPOSIDE USING MADIN-DARBY CANINE KIDNEY (MDCK) CELLS OVEREXpressING P-GlycOProtein (Pgp), MULTIDRUG Resistance-ASSOCIATED PROTEIN (MrP1), AND CANALICULAR MULTISPECIFIC ORGANIC ANION TRANSPORTER (cMOAT)

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ABSTRACT:

Multidrug resistance conferred to cancer cells is often mediated by the expression of efflux transporter “pumps”. It is also believed that many of the same transporters are involved in drug efflux from numerous normal endothelial and epithelial cell types in the intestine, brain, kidney, and liver. Etoposide transport kinetics were characterized in Caco-2 cells and in well established Madin-Darby canine kidney (MDCKII) cell lines that were stably-transfected with a human cDNA encoding P-glycoprotein (Pgp), human multidrug resistance protein (MrP1), or the canalicular multispecific organic anion (cMOAT) transporters to determine the roles of these transporters in etoposide efflux. Etoposide transport kinetics were concentration-dependent in the MDCKII-MDR1 and MDCKII-cMOAT cells. The apparent secretory Michaelis constant (Kapp) and carrier-mediated permeability (Papp) values for Pgp and cMOAT were 254.96 ± 94.39 μM and 5.96 ± 0.41 × 10⁻⁶ cm/s and 616.54 ± 163.15 μM and 1.87 ± 0.10 × 10⁻⁶ cm/s, respectively. The secretory permeability of etoposide decreased significantly in the basal to apical (B to A) (i.e., efflux) direction, whereas the permeability increased 2.3-fold in the apical to basal (A to B) direction in MDCKII-MDR1 cells in the presence of elacridar (GF120918). Moderate inhibition of etoposide efflux by leukotriene C₄ (LTC₄) was observed in MDCKII-cMOAT cells. Furthermore, etoposide inhibited LTC₄ efflux, confirming the involvement of cMOAT. The flux of etoposide in MDCKII-MRP1 cells was similar to that in MDCKII/wt control cells. The current results demonstrate that the secretory transport mechanism of etoposide involves multiple transporters, including Pgp and cMOAT but not MRP1. These results demonstrate that Pgp and cMOAT are involved in the intestinal secretory transport of etoposide. Since the intestinal secretion of etoposide was previously reported in the literature, it also suggests that they may be involved in the in vivo intestinal secretion of etoposide; however, mechanistic in vivo studies are required to confirm this.

Drug resistance to chemotherapeutic agents is a major obstacle in human cancer chemotherapy. Among various mechanisms of drug resistance, multidrug resistance (MDR) is an important mechanism of cellular and clinical drug resistance to chemotherapeutic agents. MDR is often associated with the expression of efflux transporter proteins, such as P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP1), and canalicular multispecific organic anion transporter (cMOAT). These transporters belong to the superfamily of ATP-binding cassette proteins and are involved in ATP-dependent drug efflux (Gottesman and Pastan, 1993; Borst et al., 1999).

The correlation between MDR phenotype and the expression of an efflux transporter was originally established by the discovery of Pgp, the product of mdr1 (Juliano and Ling, 1976). Expression of Pgp was correlated with both the degree of resistance and the reduced intracellular accumulation of drugs. MRP1 has also been suggested to be involved in MDR and the transport of glutathione, glucuronate, and sulfate conjugates (Cole et al., 1994; Ishikawa et al., 1997). Recently, cMOAT (also known as MR2) has been shown to be involved in resistance to vincristine, cisplatin, etoposide, CPT-11, and SN-38 (Chu et al., 1997; Cui et al., 1999; Kawabe et al., 1999). Expression of Pgp was found in the adrenal cortex, the brush border of proximal tubules of the kidney, the apical biliary surface of hepatocytes, the blood-brain barriers, and the apical membrane of mucosal cells of the intestine (Ford et al., 1996). Like Pgp, cMOAT is localized in the apical domains of hepatocytes and enterocytes, in the brush-border membrane of kidney, and in cMOAT-transfected polarized MDCK cells (Paulusma et al., 1997; Evers et al., 1998). MRP1 protein was...
mainly detected in the plasma membrane of resistant cells (nonpolar cells) and in the basolateral plasma membrane of polarized cells (Zaman et al., 1994; Evers et al., 1996). MRP1 is localized on the basolateral membrane in normal human and rat liver cells and in ciliated epithelial cells (Mayer et al., 1995; Brechot et al., 1998). It has been demonstrated that the overexpression of Pgp, MRP1, and cMOAT by the transfection of cells with their respective cDNAs confers MDR phenotype-enhancing resistance to a broad range of drugs (Gros et al., 1986; Cole et al., 1994; Breuninger et al., 1995; Evers et al., 1998; Cui et al., 1999).

The efflux transporters may be clinically important since the oral bioavailability of drug substrates may be reduced in the presence of active efflux pumps (Benet et al., 1999). Pgp, MRP1, and cMOAT are likely to be involved in the direct elimination of drugs since they are expressed in intestinal tissues. Recently, Perdaems et al. (1999) showed that intestinal excretion of etoposide largely accounts for its elimination in vivo. Studies from our laboratory using rat and human intestinal tissue and Caco-2 cells in side-by-side diffusion chambers demonstrated that the efflux of etoposide and other putative efflux substrates was regionally dependent and potentially mediated by multiple transporters (Makhey et al., 1998; Kunta et al., 2000). Once again, the specific mechanisms of transport could not be delineated since it is difficult to evaluate the specific interactions between a given drug and transporter in a system expressing multiple transporters, such as intestinal tissue or Caco-2 cells. The availability of engineered cells overexpressing the Pgp, MRP1, or cMOAT protein has enabled the characterization of interactions between etoposide and individual transporters. However, there is still a paucity of in vitro mechanistic studies, controlled in vivo studies, and the correlation between the two.

Etoposide is an anticancer drug used in the treatment of small cell lung cancer, lymphomas, and leukemia (O’Dwyer et al., 1985). It was also evaluated for use in the acquired immunodeficiency syndrome-related disease Kaposi’s sarcoma (Sprinz et al., 2001). Numerous contradictory studies have been published describing the roles of Pgp, MRP1, and cMOAT as efflux transporters for etoposide (Koike et al., 1997; Sharom 1997; Cui et al., 1999; Kawabe et al., 1999). Those results, however, were based on cell resistance (viability) studies or on the intracellular accumulation of etoposide. A mechanistic transport characterization of the interactions of etoposide with Pgp, MRP1, and cMOAT is currently lacking. In the present study, mechanistic kinetic interactions were characterized between etoposide and these efflux transporters using various MDCKII cell lines overexpressing Pgp, MRP1, or cMOAT. The current results suggest that Pgp and cMOAT, but not MRP1, are involved in the intestinal secretion of etoposide. However, since in vivo mechanistic studies are lacking, the link to the reported in vivo intestinal secretion of etoposide cannot be formally established at this time.

**Experimental Procedures**

**Materials.** [3H]Etoposide was purchased from Moravek Biochemicals (Brea, CA). [3H]Mannitol and [3H]leukotriene (LTc) were obtained from PerkinElmer Life Sciences (Boston, MA). Medium, fetal bovine serum, non-essential amino acids, and trypsin were purchased from Fisher Scientific (Fair Lawn, NJ). Pgp and the MRP monoclonal antibodies (mAb) C219 and MRPr1 were obtained from Signet Laboratories (Dedham, MA). Transwell plates were purchased from Costar Corporation (Cambridge, MA). Superscript II reverse transcriptase, Taq DNA polymerase, RNA isolation reagent, and Hanks’ balanced salt solution were purchased from Invitrogen (Carlsbad, CA). GF120918, a specific Pgp inhibitor (Yu et al., 1999), was kindly provided by Glaxo Wellcome, Inc. (Research Triangle Park, NC). All other chemicals were obtained from Fisher Scientific or Sigma Chemical Co. (St. Louis, MO).

**Cell Culture.** MDCKII/wt, MDCKII-MDR1, MDCKII-MRP1, and MDCKII-cMOAT cells were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands) (Evers et al., 1997; 1998; Bakos et al., 1998). Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) at passage 22. Caco-2 cells at passage number 28 to 40 were used in the studies. All cells were grown in Dulbecco’s modified Eagle’s medium containing 90% Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 1% nonessential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin. All cells were grown at 37°C in a humidified atmosphere of 5% CO2. Culture medium was changed every other day, and cells were passed every 3 to 5 days by trypsinization with 0.05% trypsin and 0.5 mM EDTA at 37°C for 10 min.

**Directional Transport Assays.** Directional transport assays were performed essentially as described by Irvine et al. (1999). Briefly, MDCKII and Caco-2 cells were seeded in Transwell plates at a density of 6.65 × 103 and 6.25 × 103 cells/cm2, respectively. MDCKII cells were incubated for 3 to 4 days at 37°C in a humidified atmosphere of 5% CO2 and medium was changed every day. Caco-2 cells were fed with fresh medium every other day and cultured for 21 to 25 days. Before the assay, cells were rinsed with transport medium Hanks’ balanced salt solution, pH 7.4, containing 10 mM Hepes. Cells were equilibrated in transport medium at 37°C for 30 min. The transepithelial electrical resistance (TEER) of cell monolayers was measured using “chop-stick” electrodes (World Precision Instruments, Sarasota, FL). The TEER values were determined and corrected by subtracting the resistance of blank filters. Various concentrations of [3H]etoposide (1 to 500 µM) were applied to the donor side, and cells were incubated at 37°C with 40 rpm shaking. Samples were taken from the receiver side at 30 to 105 min at 15-min intervals. Samples were added to 5 ml of scintillation fluid, and the activity was determined by scintillation counting.

**Inhibition Studies.** Cells were preincubated with inhibitors for 20 min before the transport experiment. The concentration of inhibitors used was 200 nM except when etoposide was used as an inhibitor (250 µM). Etoposide transport studies were carried out in the presence of inhibitors in both apical and basolateral chambers. Studies were carried out as described for the directional transport assays.

**Immunoblotting Analysis.** MDCKII cells and Caco-2 cells were cultured in T-75 flasks for 4 and 21 days at densities of 2.7 × 103 and 5.3 × 103 cells/cm2, respectively. Cells were harvested by treatment with 0.05% trypsin and 0.53 mM EDTA, as described previously (Makhey et al., 1998). The cells were rinsed twice in phosphate-buffered saline, pH 7.4, and centrifuged at 300g for 10 min. Cells were lysed in lysis buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM Tris-Cl, pH 7.4, and 0.5% SDS). Ten micrograms of protein from each cell line were size fractionated in a polyacrylamide gel containing 7% SDS and transferred to a nitrocellulose membrane. The blots were incubated with antibodies C219 or MRPr1, and subsequently incubated with anti-mouse (for Pgp) or anti-rat (for MRP1) immunoglobulin (1:10,000) (Makhey et al., 1998). Pgp and MRP were detected with an enhanced chemiluminescence system (Amersham Biosciences, Downers Grove, IL).

**Analysis of Gene Expression by RT-PCR.** MDCKII cells and Caco-2 cells were seeded in six-well (3.5-cm diameter) plates at a density of 6.65 × 105 cells/cm2. After the cells were rinsed twice with phosphate-buffered saline solution, total RNA was isolated from cells by directly adding 1 ml of TRIzol reagent according to the manufacturer’s instructions (Invitrogen). First-strand cDNA was synthesized in a 20-µl reaction volume containing 3 µg of total RNA, 2 pmol of reverse primer for cMOAT, 10 mM dithiothreitol, 0.5 mM dATP, dCTP, dGTP, and dTTP, and 200 units of Superscript II reverse transcriptase. Two primers were synthesized based on the cDNA sequence of human cMOAT. The sequences for forward and reverse primers are 5’-TTTGGCTTACCGGAGCTCTGGG-3’ and 5’-TGCTGGTCTCACAAGGC-ACGG-3’, respectively. PCR was performed in a 50 µl of reaction volume containing 10 ng of cDNA, 0.2 mM MgCl2, 0.5 µM primers, and 2.5 units of Taq DNA polymerase.

**Parameter Calculations and Data Analysis.** The effective permeabilities (Peff) were calculated using the following equation:

\[ P_{eff} = \frac{dC}{dt} \frac{V}{C_A} \]  

where \( dC/dt \) is the permeability rate (the slope of plot of concentration versus time), \( V \) is the volume of the receiver chamber, \( C_A \) is the initial drug concent-
transportation in the donor chamber, and $A$ is the surface area of the filter. The transport parameters were calculated using nonlinear regression (Scientist v2.01; MicroMath, Inc., Salt Lake City, UT) and the following equation:

$$P_c = \frac{P_m K_m}{K_m + C} + P_m$$

(2)

where $P_c$ is the carrier permeability ($= J_{\text{max}}/K_m$), $K_m$ is the apparent Michaelis constant, and $P_m$ is the nonsaturable membrane permeability.

Each data set was generated from triplicate samples ($n = 3$) within one experiment, and each experiment was done at least twice. Statistical tests were performed using Jandel Sigma Stat version 2.0 (Jandel Scientific, San Raphael, CA). A one-way analysis of variance test was used for all data analysis, and a minimum $P$ value of 0.05 was used as the significance level for all tests.

**Results**

**Immunoblotting of Pgp, MRP1, and RT-PCR Analysis of cMOAT.** Expression of Pgp, MRP1, and cMOAT was examined in all MDCKII and Caco-2 cells. Pgp was expressed in mdr1-transfected MDCKII-MDR1 and Caco-2 cells (Fig. 1A). A faint band was also detected in the MDCKII, MDCKII-cMOAT, and MDCKII-MRP1 cells. This band was probably a canine Pgp because the human mAb C219 recognizes an epitope that is conserved in all mammalian Pgp isoforms (Georges et al., 1990). Using mAb C219, Pgp expression was detected in wild-type MDCK cells (Ito et al., 1999). Using the mAb MRPr1, MRP1 expression was detected only in MDCKII-MRP1 and Caco-2 cells and not in the other cell lines (Fig. 1A), indicating that either MRPI was not expressed in MDCKII cells or the anti-human MRPI mAb did not cross-react with the canine MRPI transporter. This result is consistent with a study in MDCKII cells by Ito et al. (1999). Since antibodies to cMOAT were not commercially available at that time, expression of cMOAT was examined using the RT-PCR technique. A single amplified DNA fragment (468 base pairs) was obtained from MDCKII-cMOAT and Caco-2 cells but not from other cell lines (Fig. 1B). Sequence analysis indicated that the amplified DNA band was a fragment of the human cMOAT gene. Results showed that Pgp, MRPI, and cMOAT are overexpressed in MDCKII-MDR1, MDCKII-MRP1, and MDCKII-cMOAT cells, respectively. Expression of Pgp, MRPI, and cMOAT was also observed in Caco-2 cells.

**Characterization of the Barrier Properties MDCKII Cell Monolayers.** During cell culture, small differences in growth rates were noticed for these MDCKII cell lines. To obtain the optimal conditions for the transport studies and compare transport kinetics among the MDCKII cell lines, the barrier properties of MDCKII cells were initially validated by monitoring the transport of mannitol, a model compound for paracellular diffusion. Mannitol transport was measured in the apical to basal (A to B) and basal to apical (B to A) directions at 3, 4 and 5 days after seeding. Results showed that the MDCKII-cMOAT cell line was somewhat distinct from the other three cell lines (Fig. 2). Diffusion of mannitol was significantly higher in MDCKII-cMOAT cells than in other cells, except for the B to A direction at 3 days after seeding. Relatively lower TEER values were obtained in MDCKII-cMOAT cells compared with the TEER values from other cells. For example, at 4 days after seeding, TEER values for MDCKII, MDCKII-MDR1, MDCKII-MRP1, and MDCKII-cMOAT cells were 133 ± 3.3, 133 ± 3.9, 140 ± 3.4, and 121 ± 3.9 Ω cm², respectively. The high permeability of mannitol correlated well with the low TEER values in MDCKII-cMOAT cells. Higher levels of mannitol permeability were observed in some of the treatments in the A to B direction rather than in the B to A direction, especially for the MDCKII-cMOAT cell line. Based on these results, day 3 and 4 were chosen for the efflux studies for MDCKII-cMOAT cells and other MDCKII cell lines, respectively.

**Transport of Etoposide in MDCKII and Caco-2 Cells.** Etoposide flux was characterized by time course studies in all MDCKII and Caco-2 cells in both the absorptive and secretory directions. Slightly higher etoposide flux rates were observed in wild-type MDCKII and MDCKII-MRP1 cells (Fig. 3). It is likely that the net secretion of etoposide in MDCKII cells was mediated by endogenous canine Pgp (Fig. 1A; Horio et al., 1989; Ito et al., 1999). The secretory flux rates in the B to A direction were significantly higher than the absorptive rates in the A to B direction in MDCKII-MDR1, MDCKII-cMOAT, and Caco-2 cells. Since MRP1 is expressed in the basolateral plasma membrane (Evers et al., 1996), the flux of etoposide should be higher in the A to B direction than that in the B to A direction. Since the directional fluxes of etoposide in MDCKII-MRP1 cells were nearly identical to that in MDCKII/wt cells (Fig. 3, A and C), it may be concluded that MRP1 is probably not involved in its transport. LTC₄ inhibition studies confirm this in spite of the possible confounding interactions of etoposide with endogenous canine Pgp when LTC₄ is not present.

**Concentration Dependence of Etoposide Transport in MDCKII cells.** Permeation studies of etoposide were performed using MDCKII cells overexpressing Pgp, MRPI, or cMOAT transporters and wild-type MDCKII cells. The permeability was $2.42 ± 0.08 \times 10^{-6}$ and $3.19 ± 0.04 \times 10^{-6}$ cm/s for MDCKII and MDCKII-MRP1 cells, respectively. The permeability values were almost constant over a concentration range of etoposide from 1 to 500 μM in MDCKII cells and MDCKII-MRP1 cells (Fig. 4, A and C). On the other hand, etoposide efflux (B to A) was concentration-dependent in MDCKII-MDR1 and MDCKII-cMOAT cells (Fig. 4, B and D). Because of the limited solubility of etoposide, saturation of permeability could not be achieved in MDCKII-MDR1 and MDCKII-cMOAT cells. The best-fit lines for Pgp and cMOAT were determined using the $P_m$ value from the wild-type MDCKII cell ($2.42 ± 6$ cm/s), assuming that the $P_m$ value from the parental MDCKII cell (wild-type) represents the non-

![Fig. 1. A, Western blot analysis of Pgp and MRPI in four MDCKII cell lines and Caco-2 cells; B, detection of cMOAT expression from MDCKII and Caco-2 cells by RT-PCR.](image-url)
saturable membrane permeability of the MDCKII cell lines. The apparent Michaelis constant \((K_m)\) and carrier-mediated permeability \((P_c)\) values for MDCKII-MDR1 cells are \(254.96 \pm 94.39\) \(\mu M\) and \(5.96 \pm 0.41 \times 10^{-10}\) \(cm/s\), respectively, and \(616.54 \pm 163.15\) \(\mu M\), \(1.87 \pm 0.10 \times 10^{-10}\) \(cm/s\), respectively, for MDCKII-cMOAT cells.

Inhibition of Etoposide Transport by GF120918 and LTC4. In the presence of a Pgp inhibitor (GF120918), etoposide transport was significantly inhibited in the B to A direction and increased 2.3-fold in the A to B direction in MDCKII-MDR1 cells (Fig. 5). Effective inhibition of etoposide efflux by GF120918 was observed in Caco-2 cells. Less effective inhibition was observed by a cMOAT substrate (LTC4) in MDCKII-cMOAT cells. Flux increased 14% in the A to B direction and decreased only 16% in the B to A direction (Fig. 6). The instability of LTC4 during the 2-h incubation (a 15-min preincubation and 105-min incubation) could possibly diminish its ability as an

FIG. 2. Paracellular permeabilities of mannitol from four MDCKII cell lines.

Apical to basal and basal to apical transport of mannitol was measured using Transwell-grown MDCKII cell monolayers on day 3, 4, and 5 after seeding. Values are the mean of three measurements ± standard deviation. Asterisks indicate a significant difference between populations \((p < 0.05; t\ test)\).

FIG. 3. Transport of \(^{3}H\) etoposide in MDCKII (A), MDCKII-MDR1 (B), MDCKII-MRP1 (C), MDCKII-cMOAT (D), and Caco-2 cells (E).

The transport was measured over time from both apical to basal (●) and basal to apical (O) directions. Values are the mean of three measurements ± standard deviation.
when MDCKII-cMOAT cells were preincubated with 250 μM etoposide was concentration-dependent in MDCKII-MDR1 and MRP1, are involved in etoposide efflux transport. The permeation of etoposide, whereas MRP1 is probably not. Results confirmed that Pgp and cMOAT are involved in efflux transport of etoposide, whereas MRP1 is probably not.

Discussion

The present results clearly suggest that Pgp and cMOAT, but not MRP1, are involved in etoposide efflux transport. The permeation of etoposide was concentration-dependent in MDCKII-MDR1 and MDCKII-cMOAT cells. The apparent $K_m$ and $P_c$ values for Pgp are 254.96 ± 94.39 μM and 5.96 ± 0.41 $\times$ 10^{-6} cm/s, respectively. The apparent $K_m$ and $P_c$ values for cMOAT are 616.54 ± 163.15 μM and 1.87 ± 0.10 $\times$ 10^{-6} cm/s, respectively (Fig. 4). Etoposide efflux by Pgp was significantly inhibited by the Pgp inhibitor GF120918 (Fig. 5). Etoposide efflux by cMOAT was moderately inhibited by LTC4; conversely, LTC4 efflux was also inhibited by etoposide (Fig. 6). Concentration-dependent permeation of etoposide was not observed in MDCKII-MRP1 cells (Fig. 4).

Differences in monolayer barrier properties were observed among MDCKII cell lines by measuring the TEER values and permeability of mannitol. Interestingly, the permeability of mannitol was greater in the A to B (i.e., absorptive) direction than that from B to A (i.e., secretory) direction (Fig. 2). The asymmetry of mannitol flux has been also reported in MDCK (Thwaites et al., 1993) and MDCKII cells (Ito et al., 1999). However, the mechanism of the asymmetry feature for mannitol in MDCK cells is unknown. Compared with other cell lines, MDCKII-cMOAT cells had lower TEER values and correspondingly greater permeability to mannitol, suggesting a reduction in the barrier properties of the monolayer (Fig. 2). It is not clear whether this variation is caused by the insertion of cDNA into a particular region of genomic DNA or whether it is caused by the selection of subpopulations of cells with slightly different characteristics than the parental MDCKII cells during the stable transfection procedure. Although the permeability of mannitol, a paracellular diffusion marker, was greater in MDCKII-cMOAT cells than MDCKII-MDR1 cells, the higher permeation of etoposide efflux for cMOAT than that for Pgp is probably not due to the paracellular passive diffusion because etoposide is not a hydrophilic compound. Comparing etoposide transport in MDCKII cells, Caco-2 cells have a higher efflux rate than in MDCKII-MDR1 and MDCKII-cMOAT cells. This is probably because multiple transporters, such as Pgp, cMOAT, and possibly other unknown transporters, coexist in Caco-2 cells resulting in a synergistic, or at least an additive, effect. A synergistic effect was observed between MRP1 and Pgp in adult acute myeloid leukemia during a recent study with daunorubicin (Legrand et al., 1999).

Numerous studies have implicated Pgp, MRP1, and cMOAT as etoposide efflux transporters in MDR. Recently, Cui et al. (1999) found that expression of human and rat cMOAT in MDCK cells enhanced the resistance to etoposide 5.0- and 3.8-fold, respectively.
However, contradictory results concerning the expression of cMOAT and the resistance to etoposide have also been reported. Introduction of the human cMOAT antisense cDNA into human hepatic cancer cells, HepG2, overexpressing human cMOAT resulted in increased sensitivity to cisplatin and vincristine but not to etoposide (Koike et al., 1997). When cMOAT cDNA was transfected into polarized pig kidney epithelial cells, LLC-PK1, sensitivity to vincristine and cisplatin decreased but not to etoposide (Kawabe et al., 1999). Results from the current study show that etoposide transport by Pgp and cMOAT is higher in the secretory direction and is concentration-dependent, consistent with their involvement in an apically based efflux process.

MRP1 has been postulated to cause MDR by exporting cytotoxic drugs or drug conjugates out of cells (Zaman et al., 1994; Ishikawa et al., 1997). Transfection of HeLa cells and SW-1573 cells with the MRP1 cDNA resulted in multidrug resistance to doxorubicin, vincristine, and etoposide (Cole et al., 1994; Zaman et al., 1994). In general, drug resistance results were determined by measuring the correlation between the expression of a transporter protein and the sensitivity to the drugs (i.e., cell viability) or by measuring the reduction in intracellular drug accumulation. However, a correlation between expression of an efflux transporter and cell resistance does not necessarily result from enhanced drug efflux. For example, Gaj et al. (1998) reported that KB/7D cells, an MRP-expressing cell line, displayed resistance to etoposide and doxorubicin. Accumulation of etoposide was dramatically reduced in KB/7D cells compared with the parental KB cells. However, there was no significant difference in etoposide efflux between the resistant cell line KB/7D and the sensitive cell line KB. Cole et al. (1992) also reported that H69/AR cells in which the MRP1 gene was identified were more resistant to etoposide than parental H69 cells; however, there was no difference in accumulation and drug efflux patterns in these two cell lines. The current results demonstrate that the directional flux patterns (i.e., both A to B and B to A) in MDCKII-MRP1 cells were very similar to those in control MDCKII cells (Fig. 3, A and C), and the transport kinetics of etoposide were not concentration-dependent (Fig. 4C), indicating a lack of polarized etoposide transport in MDCKII-MRP1 cells. This observation is consistent with a study of MRP1 in the polarized cell line LLC-PK1 (Evers et al., 1996). The authors showed that MRP1 increased the transport of daunorubicin to the basal side of LLC-MRP cells but failed to demonstrate MRP1-mediated polarized transport of vincristine and etoposide in LLC-MRP cells. Lack of active secretion to the basal side in the MDCKII-MRP1 cells indicates that either MRP1 had little or no role in etoposide transport or the transport of etoposide by MRP1 was so weak that it was masked by the transport of etoposide to the apical side by the endogenous Pgp. However, the mechanism of action of MRP1 remains elusive.

MRP1 and cMOAT are known to have glutathione-S-conjugate export pump activity (Ishikawa et al., 1997). The glutathione-S-conjugate export pump plays an important physiological role in the detoxification of xenobiotics and tumor drug resistance. Characterization of etoposide efflux kinetics in this study indicates that etoposide is not actively secreted by MRP1. However, we cannot yet exclude the possibility that MRP1 can mediate the conjugated form of etoposide. In membrane vesicles prepared from MRP-transfected HeLa cells or MRP-overexpressing KB/VP-4 cells, only glucuronosyl-etoposide, but not etoposide, was transported by MRP1 (Jedlitschky et al., 1996; Sakamoto et al., 1999). In contrast buthionine sulfoximine, a glutathione-depleting agent, significantly reduced glutathione levels in MRP-overexpressing cells; however, depletion of glutathione was not associated with any changes in etoposide accumulation (Grech et al., 1998). High-pressure liquid chromatography analysis has shown that the daunorubicin- and vincristine-exported form MRPI-transfected cells could be recovered in their unmodified forms (Zaman et al., 1995). Furthermore, the elimination of unchanged etoposide and conjugated etoposide was 32.9 and 5.5% of the administered dose from urine and 5.6 and 0.8% of the administered dose from bile, respectively (Perdaens et al., 1999). Recently, Borst et al. (1999) developed a minimal working model for MRP1 and cMOAT. Based on their model, MRP1 and cMOAT have two drug binding sites, one with a relatively high affinity for GSH and a low affinity for drug, the other with a relatively high affinity for drug and a low affinity for GSH. Both sites are bound by GSH in the absence of drugs. At low drug concentrations, drug replaces GSH at one site resulting in co-transport of both compounds.

Pgp, MRP1, and cMOAT have been shown to confer resistance upon cells to the anticancer drug etoposide. Unfortunately, etoposide efflux is usually described indirectly through cellular resistance or reduced intracellular accumulation studies. Interpretation of efflux function of a transporter should also be supported by quantitative kinetic studies. In the present study, the kinetics of etoposide were characterized in polarized MDCKII cells overexpressing Pgp, MRP1, or cMOAT. The results clearly suggest that Pgp and cMOAT, but not MRP1, are etoposide efflux transporters. Although etoposide efflux is operational in the current in vitro models and a preliminary causal link to the in vivo situation exists, the true role of efflux in the reduced oral bioavailability of drugs, including etoposide, remains to be determined.

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References


INTERACTION OF ETOPOSIDE WITH Pgp, cMOAT, AND MRP1


