Characterization of the Efflux Transporter(s) Responsible for Restricting Intestinal Mucosa Permeation of the Coumarinic Acid-Based Cyclic Prodrug of the Opioid Peptide DADLE

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Purpose. To elucidate the efflux transporter(s) responsible for restricting the permeation of a coumarinic acid-based cyclic prodrug of the opioid peptide DADLE (CD) thorough Caco-2 cell monolayers.
Methods. The cellular permeability characteristics of CD were investigated using Caco-2 cells, Madin-Darby canine kidney-wild type II cells (MDCK-WT), MDCK cells transfected with the human *MDR1* gene (MDCK-MDR1), and MDCK cells transfected with human *MRP2* gene (MDCK-MRP2). These cells were grown as monolayers onto microporous membranes. The disappearance from the donor side and appearance on the receiver side of CD were monitored by HPLC. The substrate activity of CD for P-gp was determined by using GF120918, a known P-gp specific inhibitor. The substrate activity of CD for MRP2 was determined by using cyclosporin A (CsA), a known MRP2 and P-gp inhibitor.
Results. In Caco-2 cells, the ratio of the apparent permeability coefficients (P_{app}) of CD flux in the basolateral (BL) to apical (AP) direction vs. the flux in the AP-to-BL direction ($P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$) was 71. In the presence of GF120918 (2 μM), the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was decreased to 16. In the presence of CsA (25 μM), the ratio was decreased to 5.6. In MDCK-WT, MDCK-MDR1, and MDCK-MRP2 cells, the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios of CD were 13, 35, and 22, respectively. CsA (25 μM) greatly decreased the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios in MDCK-WT and MDCK-MDR1 cells to 1.5 and 3.2, respectively. However, in MDCK-MRP2 cells, CsA (25 μM) decreased the ratio only to 11. A mixture of GF120918 (2 μM) and CsA (25 μM) decreased the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios of CD in MDCK-WT, MDCK-MDR1, and MDCK-MRP2 cells to 1.4, 2.7, and 5.4, respectively.
Conclusions. These data suggest that CD is a good substrate for both P-gp and MRP2 and that the restricted permeation of this cyclic prodrug in Caco-2 cells and in the intestinal mucosa is probably due to its substrate activities for both of these efflux transporters.

KEY WORDS: caco-2 cell monolayers; DADLE; MDR 1; MRP 1; acid-based cyclic prodrug.

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ABBREVIATIONS: AD, acyloxyalkoxy-based cyclic prodrug of the opioid peptide DADLE; AP, apical; ATCC, American Type Culture Collection; BBB, blood-brain barrier; CD, coumarinic acid-based cyclic prodrug of the opioid peptide DADLE; DMEM, Dulbecco's Modified Eagle Medium; DPBS, Dulbecco's phosphate buffered saline; HBSS, Hanks' balanced salts; MDCK, Madin Darby canine kidney

INTRODUCTION

Recently, our laboratory showed that P-glycoprotein (P-gp, MDR1 in humans) was the efflux transporter responsible for restricting the permeation of an acyloxyalkoxy-based cyclic prodrug of the opioid peptide DADLE (AD) through Caco-2 and Madin Darby canine kidney (MDCK) cells (1,2) and through the intestinal mucosa and the blood-brain barrier (BBB) (Chen et al., unpublished data). This cyclic prodrug was a weak substrate for multidrug resistance-associated protein 2 (MRP2) (2), which has also been reported to be present in Caco-2 cells (3) and the intestinal mucosa (4) and to limit the mucosal permeation of some drugs (5).

To elucidate which transporter(s) are involved in restricting the Caco-2 cell permeation of AD, we utilized MDCK cells transfected with human *MDR1* gene (MDCK-MDR1) and MDCK cells transfected with human *MRP2* gene (MDCK-MRP2) (2). MDCK-MDR1 and MDCK-MRP2 cells have been shown previously to over express MDR1 and MRP2 (6), respectively. Similar to the MDR1 and MRP2 expressed in Caco-2 cells, the human P-gp and MRP2 expressed in MDCK-MDR1 and MDCK-MRP2 cells are polarized on the apical (AP) side of these cell monolayers and these transporters efflux substrates to the AP side of the cell monolayers (6).

In the course of conducting the experiments with these MDCK cell lines, we decided to use a coumarinic acid-based cyclic prodrug of this opioid peptide (CD) (Fig. 1) as a "non-efflux transporter substrate" control. The choice of CD as a control was based on earlier studies from our laboratory that showed that this prodrug had an AP-to-BL permeability coefficient ($P_{app\ AP\text{-}to\text{-}BL}$) in Caco-2 cells that was 31 times greater than that of DADLE (7). Unlike AD, CD appeared not to be a substrate for an efflux transporter in Caco-2 cells since the $P_{app\ BL\text{-}to\text{-}AP}$ value for CD was almost identical to its $P_{app\ AP\text{-}to\text{-}BL}$ value (7). However, when the transport of CD (the "non-efflux transporter substrate") was studied in MDCK-MDR1 and MDCK-MRP2 cells, we were surprised to find that it exhibited polarized efflux similar to that observed with AD (the "known efflux transporter substrate"). These observations led us to repeat the CD transport experiments using Caco-2 cells. It should be noted that the Caco-2 cells used in the current study were taken from a new lot of cells obtained from American Type Culture Collection (ATCC). Further, it should be noted that this new lot of Caco-2 cells is different than the lot of Caco-2 cells used to generate the original CD transport data (7). To our surprise, CD exhibited significant polarized efflux in the current study.

Therefore, the objectives of this study were: (i) to determine the possible reasons for the contradictory transport data observed with CD when different lots of Caco-2 cells were used; and (ii) to elucidate which efflux transporter(s) are responsible for restricting CD permeation through Caco-2 cell monolayers.

MATERIALS AND METHODS

Materials

CD was synthesized using methods previously described by our laboratory (8). Caco-2 cells were obtained from ATCC

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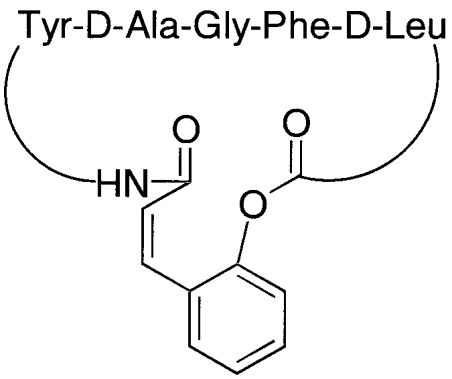

Fig. 1. Chemical structure of CD.

(Rockville, Maryland). MDCK strain II (MDCK-WT), MDCK-MDR1, and MDCK-MRP2 cells were a gift from Professor Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). GF120918 was a gift from Dr. Kenneth Brouwer (GlaxoSmithKline, Research Triangle Park, North Carolina). Dulbecco's phosphate buffered saline (DPBS), Hanks' balanced salts (HBSS) (modified), cyclosporin A (CsA), acetonitrile, phosphoric acid, and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, Missouri). L-Glutamine (200 mM), penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and nonessential 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 (FBS) 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). [^{14}C]-Mannitol (specific activity 51 mCi/mmol) and [^{3}H]-digoxin (specific activity 19 Ci/mmol) were purchased from NEN Life Science Products, Inc. (Boston, Massachusetts). [^{3}H]-Vinblastine sulfate (specific activity 11.1 Ci/mmol) was obtained from Amersham Life Science (Arlington Heights, Illinois).

Cell Culture

Caco-2 cells were seeded at 79,000 cells/cm^2 on Transwell® filter inserts (6-well) and maintained in high-glucose DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 2 mM L-glutamine solution, 100 U/ml penicillin and 100 μg/ml streptomycin as described previously (9). Cells (passages 20–40) were used on days 21–28 post-seeding.

MDCK-WT, MDCK-MDR1, and MDCK-MRP2 cells were seeded at 50,000 cells/cm^2 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-gps and MRP2s in the transfected MDCK cells were confirmed by Western blotting and measuring the efflux of known substrates of these transporters (10,11). The cells were split twice a week with 1:10 dilution and used within two months after being removed from liquid N_2.

All cell lines were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere. The membrane integrity was checked using [^{14}C]-mannitol. The apparent permeability coefficients (P_{app}) of [^{14}C]-mannitol across Caco-2 and MDCK cell monolayers were typically in the range of 0.1–0.6 × 10^{-6} cm/sec.

Transport Experiments

Bi-directional transport experiments were performed as previously described (9) 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 AP and the BL chambers of each insert were washed twice with 37°C-HBSS for 15 min. Test drugs [CD (40 μM), [^{3}H]-digoxin (0.011 μM), [^{3}H]-vinblastine (0.011 μM), or [^{14}C]-mannitol (0.011 μM)] were added to the donor side (1.5 ml for AP chamber and 2.5 ml for BL chamber) and fresh HBSS was placed in the receiver compartment. To inhibit the activity of efflux transporters, cell monolayers were incubated with HBSS containing GF120918 (2 μM), CsA (25 μM), or a cocktail of GF120918 (2 μM) and CsA (25 μM) for an additional 15 min. The test compounds were then added to the donor side along with GF120918 (2 μM) and/or CsA (25 μM), and fresh HBSS containing inhibitor [i.e., GF120918 (2 μM) and/or CsA (25 μM)] was placed in the receiver side. Aliquots (100 μl) were withdrawn from the receiver side at various time intervals up to 80 min. Fresh HBSS or HBSS containing inhibitor (100 μl) was replaced in the receiver side after sampling. When radioactive substrates were used, radioactivity in aliquots from the donor and receiver sides of the monolayers was measured in a scintillation counter LS6001C (Beckman Instruments, Inc., Fullerton, California). In experiments using CD, aliquots were stabilized by the addition of aliquots of acetonitrile [10% (v/v)] and diluted phosphoric acid [0.1% (v/v)]. The acidic mixture was immediately analyzed at 4°C using HPLC or frozen and kept at –80°C until HPLC analysis.

[^{3}H]-Digoxin, a substrate for P-gp (10), and [^{3}H]-vinblastine, an substrate for both P-gp and MRP2 (11) were used to check the functional efflux activity of P-gp and MRP2 in these cell models (10–11). The ratios of $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ of [^{3}H]-digoxin across Caco-2, MDCK-WT, and MDCK-MDR1 cell monolayers were 14, 8.4, and 33, respectively. These ratios were decreased to 1.1 in the presence of P-gp specific inhibitor GF102918 (2 μM) (12). The ratios of $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ of [^{3}H]-vinblastine across Caco-2, MDCK-WT, and MDCK-MRP2 cell monolayers were 18, 15, and 36, respectively. These ratios were decreased to 1.9, 1.7, and 16, respectively, when the P-gp inhibitor GF 120918 (2 μM) was included in the incubation media.

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

The effects CD (40 μM) 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. The inhibition experiments were carried out as described in the Transport Experiment section.

HPLC Analysis

Chromatographic analyses were carried out on a Shimadzu LC-10A gradient system (Shimadzu, Inc, Tokyo,

Japan) consisting of LC-10 AD pumps, a SCL-10A controller, a SPD-10A UV detector, a SIL-10 A autoinjector equipped with a sample cooler, and two FCV-2A high pressure switching valves. Aliquots of 100 μl from the sample tray were injected into a C-18 reversed-phase column (Vydac #218TP54). Gradient elution was performed at a flow rate of 1 ml/min from 20–50% (v/v) acetonitrile in water using trifluoroacetic acid (0.1%, v/v) as an ion-pairing agent. The eluents were monitored at a wavelength of 214 nm using a UV detector. Data were collected and analyzed using Star Chromatography Workstation and Varian Software (Varian Associates, Inc., Walnut, California). The low limit of quantification (LLQ) of the peptide prodrug was 10 nM. The recovery of the peptide prodrug in the transport experiments was consistently >95%.

Data Analysis

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

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

Transport Characteristics of CD across Caco-2 Cell Monolayers

As shown in Fig. 2 and Table I, CD exhibited strong polarized efflux in the new lot of Caco-2 cells used in these studies (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 71. In the presence of GF120918 (2 μM), a known inhibitor of P-gp (12), $P_{app\ AP\text{-}to\text{-}BL}$ 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 16 (Table I). The inhibitory effect of CsA (25 μM), a known inhibitor of P-gp and MRP2 (13,14), on the polarized efflux of CD in Caco-2 cells was greater than that observed with GF120918 (2 μM), resulting in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 5.6 (Table I). The cocktail of GF120918 (2 μM) and CsA (25 μM) further decreased the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio to 3.1 (Fig. 2, Table I).

Transport Characteristics of CD across MDCK-WT Cell Monolayers

MDCK-WT cells also showed polarized efflux of CD with a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 13 (Table II). The polarized efflux of CD was significantly less in MDCK-WT cells than in Caco-2 cells as measured by the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios (i.e., 13 *vs.* 77). GF120918 (2 μM) decreased the $P_{app\ BL\text{-}to\text{-}AP}$ value, resulting in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 9.0. CsA (25 μM) exhibited greater inhibitory effects, which almost completely collapsed the polarized efflux, and the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was decreased to 1.5. The cocktail of GF120918 (2 μM) and CsA (25 μM) had inhibitory effects on the polarized efflux of CD similar to that observed with CsA (25 μM), which resulted in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 1.4.

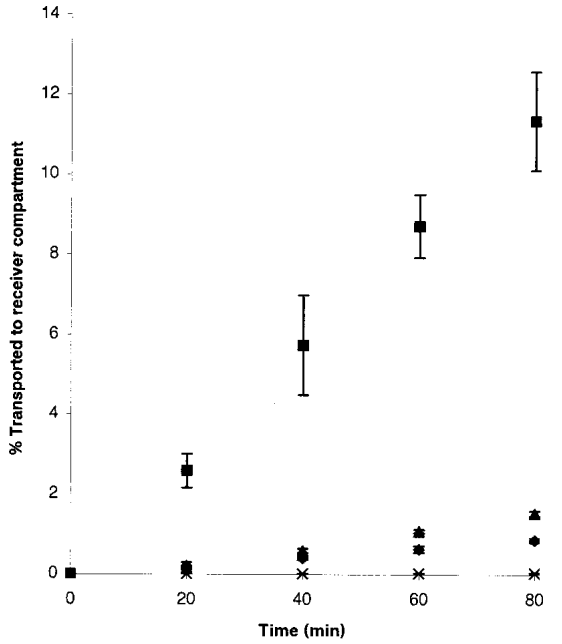

Fig. 2. Bi-directional transport of CD across Caco-2 cell monolayers in the absence and presence of inhibitors. <: BL-to-AP, no inhibitor; 5: BL-to-AP + GF120918 (2 μM)+CsA (25 μM); ♦: AP-to-BL + GF120918 (2 μM)+CsA (25 μM); r: AP-to-BL, no inhibitor. Data are presented as mean ± SD (n = 3).

Transport Characteristics of CD across MDCK-MDR1 Cell Monolayers

As shown in Table III, the polarized efflux of CD was higher in MDCK-MDR1 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 were 35 vs. 13, respectively). This polarized efflux of CD in

Table I. P_{app} Values of CD across Caco-2 Cell Monolayers in the Presence and Absence of Known Inhibitors of P-gp and MRP2

Treatment	Papp*10^7 (cm/s)		Ratio ($P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$)
	AP-to-BL	BL-to-AP	
—	1.81 ± 0.22	129.0 ± 13.8	71
GF120918 (2 μM)	2.19 ± 0.12	35.57 ± 0.95	16
CsA (25 μM)	3.98 ± 0.22	22.27 ± 1.81	5.6
GF120918 (2 μM) + CsA (25 μM)	6.62 ± 0.45	21.02 ± 0.58	3.1

Note: P_{app} values of CD were measured in the absence or the presence of inhibitors [GF120918 (2 μM), CsA (25 μM), or GF120918 (2 μM) and CsA (25 μM)] in Caco-2 cells as described in Materials and Methods. P_{app} values are presented as mean ± S.D. (n = 3).

Table II. P_{app} Values of CD across MDCK-WT Cell Monolayers in the Presence and Absence of Known Inhibitors of P-gp and MRP2

Treatment	P_{app}*10^7 (cm/s)		Ratio ($P_{app\ BL\text{-}to\text{-}AP}$/ $P_{app\ AP\text{-}to\text{-}BL}$)
	AP-to-BL	BL-to-AP	
—	1.93 ± 0.52	27.25 ± 4.76	13
GF120918 (2 μM)	1.69 ± 0.42	15.26 ± 0.51	9.0
CsA (25 μM)	2.48 ± 0.37	3.81 ± 0.03	1.5
GF120918 (2 μM) + CsA (25 μM)	2.66 ± 0.44	3.74 ± 0.26	1.4

Note: P_{app} values of CD were measured in the absence or the presence of inhibitors [GF120918 (2 μM), CsA (25 μM), or GF120918 (2 μM) and CsA (25 μM)] in MDCK-WT cells as described in Materials and Methods. P_{app} values are presented as mean ± S.D. (n = 3).

Table IV. P_{app} Values of CD across MDCK-MRP2 Cell Monolayers in the Presence and Absence of Known Inhibitors of P-gp and MRP2

Treatment	P_{app}*10^7 (cm/s)		Ratio ($P_{app\ BL\text{-}to\text{-}AP}$/ $P_{app\ AP\text{-}to\text{-}BL}$)
	AP-to-BL	BL-to-AP	
—	1.05 ± 0.19	24.10 ± 1.39	23
GF120918 (2 μM)	1.68 ± 0.11	19.07 ± 1.99	11
CsA (25 μM)	1.50 ± 0.55	16.76 ± 0.55	11
GF120918 (2 μM) + CsA (25 μM)	1.91 ± 0.18	10.33 ± 0.26	5.4

Note: P_{app} values of CD were measured in the absence or the presence of inhibitors [GF120918 (2 μM), CsA (25 μM), or GF120918 (2 μM) and CsA (25 μM)] in MDCK-MRP2 cells as described in Materials and Methods. P_{app} values are presented as mean ± S.D. (n = 3).

MDCK-MDR1 could be significantly inhibited by inclusion of GF120918 (2 μM) in the incubation mixture, resulting in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 16 (Table III). CsA had a much greater inhibition effect on the active BL-to-AP transport of CD than GF120918. In the presence of CsA (25 μM), the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was decreased to 3.2. The mixture of GF120918 (2 μM) and CsA (25 μM) had inhibition effects on the polarized efflux of CD similar to that observed with CsA (25 μM), resulting in a $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio of 2.7.

Transport Characteristics of CD across MDCK-MRP2 Cell Monolayers

As shown in Table IV, MDCK-MRP2 cells exhibited strong polarized efflux of CD (i.e., the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was 23) (Table IV). In the presence of GF120918 (2 μM) or CsA (25 μM), the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratios were decreased to 11. The mixture of GF120918 (2 μM) and CsA (25 μM) increased $P_{app\ AP\text{-}to\text{-}BL}$ and decreased $P_{app\ BL\text{-}to\text{-}AP}$. The $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was decreased to 5.4.

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

To determine the inhibitory effects of CD on MRP2, experiments were conducted in the presence of GF120918 (2 μM), which totally inhibits P-gp activity in these cell lines (11). As shown in Fig. 3, inclusion of CD (40 μM) and GF120918 (2 μM) in the transport media decreased the $P_{app\ BL\text{-}to\text{-}AP}$ value of [^{3}H]-vinblastine across Caco-2 and MDCK-MRP2 cell monolayers as compared to the control that only had GF120918 (2 μM) in the transport media. In Caco-2 cells, the $P_{app\ BL\text{-}to\text{-}AP}$ value of [^{3}H]-vinblastine was decreased from 3.5×10^{-6} cm/s to 2.4×10^{-6} cm/s (Fig. 3A). In MDCK-MRP2 cells, the $P_{app\ BL\text{-}to\text{-}AP}$ value was decreased from 9.0×10^{-6} cm/s to 6.9×10^{-6} cm/s (Fig. 3B). The inhibitory effects of CD on the efflux activities of P-gp were also tested in Caco-2 and MDCK-MDR1 cells. However, CD (40 μM) did not inhibit the polarized efflux of known P-gp substrates ([^{3}H]-digoxin, [^{3}H]-taxol, Hoechst 33342, and rhodamine 123)

Table III. P_{app} Values of CD across MDCK-MDR1 Cell Monolayers in the Presence and Absence of Known Inhibitors of P-gp and MRP2

Treatment	P_{app}*10^7 (cm/s)		Ratio ($P_{app\ BL\text{-}to\text{-}AP}$/ $P_{app\ AP\text{-}to\text{-}BL}$)
	AP-to-BL	BL-to-AP	
—	0.92 ± 0.29	32.12 ± 3.13	35
GF120918 (2 μM)	1.46 ± 0.02	23.25 ± 2.65	16
CsA (25 μM)	1.62 ± 0.31	5.12 ± 0.26	3.2
GF120918 (2 μM) + CsA (25 μM)	1.93 ± 0.32	5.39 ± 0.24	2.7

Note: P_{app} values of CD were measured in the absence or the presence of inhibitors [GF120918 (2 μM), CsA (25 μM), or GF120918 (2 μM) and CsA (25 μM)] in MDCK-MDR1 cells as described in Materials and Methods. P_{app} values are presented as mean ± S.D. (n = 3).

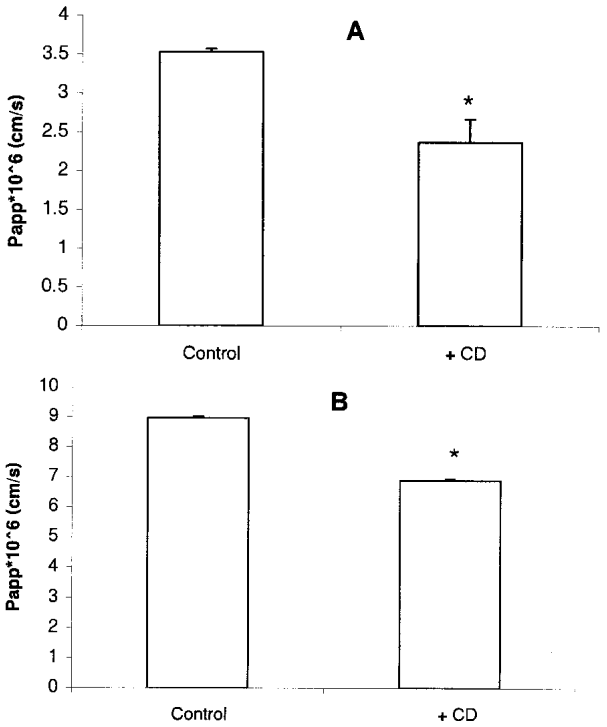

Fig. 3. Inhibitory effects of CD on the active BL-to-AP transport of [^{3}H]-vinblastine across Caco-2 cell monolayers (Panel A) and MDCK-MRP2 cell monolayers (Panel B). The BL-to-AP transport of [^{3}H]-vinblastine was carried out in the presence of GF120918 (2 μM) and in the absence of CD (control) and presence of CD (40 μM) in the transport media as described in Material and Methods. Data are mean ± SD (n = 3).
* **Significant level:** $p < 0.05$, when compared with P_{app} value of control.

in Caco-2 cells and MDCK-MDR1 cells (data not shown). It is worth noting that the solubility of CD is low ~40 μM in HBSS); therefore, higher concentrations of this prodrug could not be employed in these experiments.

DISCUSSION

In recent years, Caco-2 cells grown onto microporous membranes have become a popular model of the intestinal mucosa (15). This cell culture model is particularly useful for studying the permeation characteristics of drugs that traverse the intestinal mucosa by passive diffusion (15). However, because these cells have a colonic origin (16), they also express transporters normally found in the intestinal mucosa. In many cases, these transporters facilitate the absorption of drugs (e.g., oligopeptide transporter, bile acid transporter) (17,18). However, other transporters are known to restrict the absorption of drugs (P-gp and MRP2) in the intestinal mucosa (4,19).

Since these transporters normally found in the intestinal mucosa are also expressed in Caco-2 cells, scientists, including us, have begun to use this cell line to study transporter-facilitated and/or restricted permeation of drugs and drug candidates (1,20,21). In many cases, these experiments have been done using Caco-2 cells that have not been properly characterized for the levels of expression of the different possible transporters (e.g., P-gp, MRP2). The literature clearly shows that the levels of expression of transporters can vary depending on how the cells are manipulated prior to seeding on the microporous membrane, the culture medium, the feeding schedule, and the days in culture (22,23). However, these variables can normally be controlled within a laboratory by developing standard cell culture protocols and by monitoring the level of transporters using known substrates (24).

An issue that has not been adequately addressed is the laboratory-to-laboratory differences in the expression of transporters. These differences, which are often discussed by scientists, have not been well documented in the scientific literature. A related issue is the lot-to-lot differences often observed in the expression of transporters, i.e., different lots of Caco-2 cells obtained from ATCC exhibit different levels of expression of transporters. We believe that the experimental results described in this manuscript compared to the results reported earlier by our laboratory (7) are an illustration of lot-to-lot variability in the levels of efflux transporters expressed in Caco-2 cells.

As described in the Introduction, our laboratory (7) reported in 1999 that CD, a coumarinic acid-based cyclic prodrug of the opioid peptide DADLE, exhibited excellent Caco-2 cell permeation characteristics and did not exhibit polarized efflux. However, more recently, we have repeated these transport experiments using a new lot of Caco-2 cells obtained from ATCC. To our surprise, we observed that CD exhibited significant polarized efflux in this new lot of Caco-2 cells (Fig. 2, Table I). This raised the question of: which sets of data are correct?

In an attempt to understand this lot-to-lot difference in the permeation characteristics of CD and to elucidate the transporter(s) involved in the limiting the permeation of CD, we determined the permeation characteristics of this cyclic prodrug in Caco-2, MDCK-WT, MDCK-MDR1 and MDCK-MRP2 cells.

The bi-directional transport data for CD generated in the new lot of Caco-2 cells indicate that it is a good substrate of one or more of the efflux transporters present in Caco-2 cells (Fig. 2, Table I). GF120918 (2 μM) significantly inhibited this polarized efflux of CD in Caco-2 cells, i.e., the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was decreased from 71 to 16. GF120918 is a specific inhibitor for P-gp (12). These data indicate that the polarized efflux of CD in Caco-2 cells is probably due at least in part to the presence of P-gp. CsA, which is an inhibitor for both P-gp and MRP2 (14), exhibited greater inhibitory effects than that observed with GF120918 on the polarized efflux of CD in these Caco-2 cells, i.e., the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was decreased to 5.6 (Table I). These results suggest that CD is probably also effluxed by MRP2 expressed in Caco-2 cells. It is interesting to note that the cocktail of GF120918 and CsA could not totally inhibit the polarized efflux of CD [the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio was 3.1 (Fig. 2 and Table I)]. This effect is different from that observed by our laboratory when digoxin, a known substrate for P-gp (11), and vinblastine, a known substrate of P-gp and MRP2 (25), were studied (11). One possibility is that CD is a substrate for a yet unknown efflux transporter in Caco-2 cells.

Based on our earlier data with MDCK-WT cells (10,11), it was not surprising that MDCK-WT showed polarized efflux of CD. However, this polarized efflux was significantly less than that observed in Caco-2 cells (Tables I, II). The observations that CsA (an inhibitor for P-gp and MRP2) and GF120918 (a P-gp specific inhibitor) (Table II) could inhibit CD efflux supports the hypothesis that CD is a substrate for both P-gp and MRP2. However, the fact that GF120918 is a relatively weak inhibitor in MDCK-WT cells and CsA is a quite potent inhibitor suggests that these cells may express higher levels of functional canine MRP2 than canine P-gp. These results were consistent with our data that MDCK-WT cells not only express P-gp and MRP2 as measured by Western blotting, but also exhibit functional polarized efflux of P-gp and/or MRP2 substrates (e.g., digoxin and vinblastine) (10,11).

MDCK-MDR1 cells overexpress human P-gp and exhibit greater polarized efflux of P-gp substrates than MDCK-WT cells (26). The greater polarized efflux of CD in MDCK-MDR1 cells than in MDCK-WT cells further confirms that CD is a substrate of P-gp (Tables II, III). MDCK-MRP2 cells over express MRP2 and exhibit greater polarized efflux of MRP2 substrates than MDCK-WT cells (25). The greater polarized efflux of CD in MDCK-MRP2 cells than in MDCK-WT cells (Tables II, IV) suggests that CD is also a substrate for MRP2. It is important to note that the polarized efflux of CD was less inhibited by CsA (a P-gp and MRP2 inhibitor) in MDCK-MRP2 cells than in the other three cell lines (Caco-2, MDCK-WT, and MDCK-MDR1) [i.e., the $P_{app\ BL\text{-}to\text{-}AP}/P_{app\ AP\text{-}to\text{-}BL}$ ratio is 11 vs. 5.6, 1.5, 3.2 (Tables I–IV)]. These results are consistent with our earlier data with CsA and MDCK-MRP2 cells (11). Based on our earlier observation using Caco-2 cells, CsA (25 μM) was shown to be a potent MRP2 inhibitor in this cell line (11). However, it only partially inhibited the polarized efflux of a good MRP2 substrate (e.g., vinblastine) in MDCK-MRP2 cells (11). As suggested earlier, these differences may be due to the difference in the total MRP2 expression levels in these cells and/or differences in the membrane characteristics of these cells (11).

MRP2 was initially characterized as the canalicular multispecific organic anion transporter (cMOAT) before it was cloned (27,28). Substrates for MRP2 have been reported as glutathione conjugates, glucuronide conjugates, and non-conjugated organic anions (29). Only recently has MRP2 been reported to efflux non-conjugated and non-negatively charged drugs (e.g., *vinca* alkaloids) (25). It is interesting to note that although CD is a neutral cyclic-peptide prodrug, it exhibits good substrate activity for MRP2. The MRP2 substrate activity of CD supports Evers et al.'s hypothesis that MRP2 is an ABC-transporter that can be involved in affecting the disposition of xenbiotics (e.g., cancer drugs) (25).

The inhibitory effects of CD (40 μM) on the BL-to-AP transport of [^{3}H]-vinblastine provide further evidence that CD has high affinity for MRP2 (Fig. 3). To test the affinity of CD for P-gp, the inhibitory effects of CD (40 μM) on the polarized efflux of the P-gp substrate [^{3}H]-digoxin were determined in Caco-2 and MDCK-MDR1 cells. No inhibitory effect of CD on the efflux of [^{3}H]-digoxin was observed in these experiments (data not shown). These data suggest that while CD can serve as substrate for P-gp in Caco-2 and MDCK-MDR1 cells, based on the inhibitory effects of GF120918, its affinity for this efflux transporter must be low. An alternative explanation would be that CD binds to P-gp at a different site than digoxin (24).

The differences that we have observed in the Caco-2 cell permeation characteristics of CD in these studies vs. the studies previously published by our laboratory can only be explained by the differences in the lots of Caco-2 cells, which were obtained from ATCC, used in these transport studies. After profiling the efflux substrate specificity of CD, our hypothesis is that the new lot of Caco-2 cells used in this study has a much higher level of MRP2 expression than the old lot used in our previous studies (7). In fact, it is not unreasonable to suggest that our "old" Caco-2 cells were devoid of MRP2 and they expressed very low levels of P-gp. This efflux transporter profile for our "old" lot of cells would have been expected to generate the type of transport characteristic for AD and CD that were observed in our earlier studies (1,7). Another possibility is that the new lot of Caco-2 cells used in this study expresses a yet unknown efflux transporter that was not present in the old lot of cells. The presence of this "yet unknown efflux transporter" is suggested by the fact that a combination of GF120918 and CsA could not produce total inhibition of the polarized efflux of this cyclic prodrug (Table I).

P-gp is highly expressed in the apical surface of intestinal epithelia cells, the biliary surface of hepatocytes, and in capillary endothelial cells of the brain (19). MRP2 is highly expressed in the biliary surface of hepatocytes and in intestinal mucosa cells (4). Based on their tissue distributions, it has been proposed that P-gp and MRP2 function as secretory detoxifying systems, which could significantly influence the disposition of drugs and drug candidates (4,30). The observations reported here concerning the substrate activity of CD for P-gp and MRP2, therefore, would suggest that this cyclic prodrug would have low intestinal mucosal and BBB permeation and potentially be cleared rapidly by the liver. Unpublished data from our laboratory, using *in situ* perfused rat ileum and *in situ* perfused rat brain models confirmed these predictions, i.e., CD exhibited intestinal mucosal and brain permeation characteristics similar to those of DADLE. The permeation of CD in the *in situ* model of BBB was increased significantly by inclusion of a P-gp inhibitor (e.g., GF120918) in the perfusates (Chen et al, unpublished data).

In summary, these data raise concerns about the lot-to-lot variability that is present in the expression of transporters in Caco-2 cells. Scientists using this cell line to characterize the efflux characteristics of a drug or drug candidate need to carefully characterize their cell levels for the expression of these proteins. The data generated in this study showing that CD is a substrate for both P-gp and MRP2 are consistent with the data generated using an *in situ* perfused rat ileum model and an *in situ* perfused rat brain model. Therefore, we feel that the cell permeation data and efflux transporter profile for CD described in this manuscript more accurately reflect the permeation characteristics of this cyclic prodrug than that previously published by our laboratory (7).

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