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ScienceDirect
International Journal of Pharmaceutics 356 (2008) 12–18
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Application and limitation of inhibitors in drug–transporter interactions studies

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Received 10 October 2007; received in revised form 14 December 2007; accepted 17 December 2007
Available online 24 December 2007

Abstract

The objective of the present study was to investigate the reliability of transporter inhibitors in the elucidation of drug–transporter interactions when multiple transporters are present in a test system. The bidirectional permeabilities of digoxin, estrone-3-sulfate (E3S), and sulfasalazine, substrates of P-gp, BCRP/MRP2 and unspecified efflux transporters, respectively, were examined in Caco-2 and MDR-MDCK cells in the absence and presence of transporter inhibitors: CsA (P-gp), FTC (BCRP) and MK571 (MRP). Digoxin showed significant efflux ratios (ER) in both Caco-2 (ER = 17) and MDR-MDCK (ER = 120), whereas E3S and sulfasalazine only showed significant efflux in Caco-2 (ER = 15 and 88, respectively) but not in MDR-MDCK cells (ER = 1.1 and 1.3, respectively). CsA at 10 μM showed complete inhibition of digoxin efflux, partial inhibition of E3S efflux and no effect on sulfasalazine efflux. FTC and MK571 had different inhibitory effects on the efflux of these compounds. The present study shows evidence of the functional expression of multiple efflux transporter systems in Caco-2 cells. Although the use of Caco-2 cells and selected inhibitors of efflux transporters can provide useful mechanistic information on drug–drug interactions involving efflux transporters, the potential cross-reaction of inhibitors with multiple transporters makes it difficult to discern the role of individual transporters in drug transport or drug–drug interactions.

Keywords: Caco-2; Transporter; Inhibition; Interaction

1. Introduction

A number of *in vitro* systems, including excised intestinal segments (Sugawara et al., 1990), intestinal tissues mounted in Ussing chambers (Lennernas et al., 1997) and various cell models (Hidalgo, 2001), have been developed to correlate the vectorial transport of drug with *in vivo* intestinal drug absorption potential. Among these systems, Caco-2 cells exhibit morphological characteristics of small intestinal cells (e.g., tight intercellular junctions and microvilli) and express a multitude of transporters, including PEPT1 (Watanabe et al., 2006), P-glycoprotein (P-gp, Guo et al., 2002), multidrug-resistance associated proteins 2 (MRP2, Guo et al., 2002), and breast-cancer resistance protein (BCRP, Xia et al., 2005). The multiple applications of Caco-2 cells has led to their wide utilization throughout the pharmaceutical industry. More importantly, *in vitro* permeability coefficient values obtained in the Caco-2 cell model generally exhibit a good correlation with *in vivo* fraction absorbed; thus leading to the widespread use of these cells for drug screening purposes.

P-gp, the best-understood efflux transporter, is involved in numerous, well-documented drug–drug interactions including digoxin and quinidine (De Lannoy and Silverman, 1992), digoxin and ritonavir (Ding et al., 2004), and fexofenadine and ketoconazole (Petri et al., 2004; Ogasawara et al., 2007). Therefore, the P-gp interaction potential of drug can-

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; NEAA, nonessential amino acids; OATP, organic anion transporting polypeptides; CsA, cyclosporin A; FTC, fumitremorgin C; MK571, 3-([(3-(2-[7-chloro-2-quinolinyl]ethyl)phenyl]-[(3-dimethylamino-3-oxoporphyl)-thio)-methyl]-thio) propanoic acid; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP, multidrug-resistance associated proteins; E3S, estrone-3-sulfate.

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doi:10.1016/j.ijpharm.2007.12.024

didates needs to be evaluated as part of the drug development program.

Observations made in several laboratories, including ours, indicate that some compounds undergo higher transport in the basolateral-to-apical direction (B-to-A) than in the apical-to-basolateral direction (A-to-B) in Caco-2 cells, whereas the same compounds did not exhibit such an asymmetric transport in MDR-MDCK cells, Madin–Darby canine kidney cells transfected with the human P-gp gene. These results suggest that non-P-gp mediated transport was involved in transport of these compounds, which has been previously reported (Stephens et al., 2001; Lowes et al., 2003). In addition to its use in drug screening, Caco-2 cells have been used in combination with inhibitors in mechanistic studies of different drug transporters. However, the use of these inhibitors often does not produce a clear outcome for certain compounds in both Caco-2 and MDR-MDCK cells, making the observations difficult to interpret (Bertelsen et al., 2006; Schrickx and Fink-Gremmels, 2007).

In the present study, four test compounds with different transport characteristics were selected: (1) digoxin, a P-gp substrate, which has an absolute fraction absorbed greater than 70% in humans (Huffman et al., 1975); (2) estrone-3-sulfate (E3S), an endogenous estrone metabolite, considered as a BCRP substrate (Xia et al., 2005); (3) sulfasalazine, a compound that has a low fraction absorbed in humans, and is a substrate of efflux proteins located in the human intestinal tract (Balimane et al., 2006). In addition, the bidirectional permeability of these compounds was also determined in MDR-MDCK cells in the present study, and the results were compared with those from Caco-2 cells.

Bidirectional transport measurements in various cell models can produce valuable information for determining whether a new molecular entity is a P-gp substrate and/or inhibitor. As a result, in the present study we measured the bidirectional permeability of four selected test compounds in Caco-2 and MDR-MDCK cells in the absence and presence of cyclosporin A (CsA), fumitremorgin C (FTC) and MK571, which are inhibitors of P-gp, BCRP and MRP, respectively. These inhibitors were selected due to the availability and proprietary restrictions.

2. Materials and methods

2.1. Materials

[^{3}H]-E3S (specific activity: 57.3 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). FTC was purchased from EMD Biosciences (San Diego, CA); MK571 was purchased from BIOLMOL (Plymouth Meeting, PA). All other test compounds were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). MDR-MDCK cells were obtained from NIH (Bethesda, MD). All cells were maintained in high glucose (4.5 g/L) DMEM supplemented with 10% FBS, 1% NEAA, 1% L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) at 37 °C in a humidified incubator with 5% CO_2. The culture media used for MDR-MDCK cells contained 80 ng/mL colchicine to maintain selective pressure. All cells were seeded at a density of 60,000 cells/cm^2 onto collagen-coated, microporous, polycarbonate membranes in 12-well Transwell® plates. Caco-2 cells were used between passages number 60 and 66; MDR-MDCK cells were used between passages number 10 and 14. The culture medium was changed 24 h after seeding to remove cell debris and dead cells; afterwards the medium was changed every other day for 3 weeks for Caco-2 cells and for 6 days for MDR-MDCK cells.

2.3. Transport studies

Prior to the transport experiments, each batch of cell monolayers was certified based on our internal criteria (Table 1) on transepithelial electrical resistance (TEER) values and permeability coefficient measurements of the control compounds: propranolol, atenolol, and digoxin.

The permeability assay buffer was Hanks' balanced salts solution containing 10 mM hydroxyethylpiperazineethane sulfonic acid (HEPES) and 15 mM glucose at pH 7.4 (HBSSg buffer). The test compounds were prepared in HBSSg buffer to a final concentration of 10 μM each for digoxin and sulfasalazine. [^{3}H]-E3S was added to HBSSg buffer at a final activity of 2 μCi/mL (concentration equal 0.035 μM). In the inhibition study, the cells were pre-incubated with 10 μM CsA, 10 μM FTC, or 30 μM MK571 for 30 min and the same concentration of inhibitors was present during the transport phase of the experiment in both the apical and basolateral chambers. The test compounds were dosed on the apical side (A-to-B transport) or basolateral side (B-to-A transport) and incubated at 37 °C with 5% CO_2 and 90% relative humidity. For A-to-B transport, a 1.5 mL aliquot of blank buffer was applied to the bottom chamber (well), and 0.5 mL of the compound-containing solution was applied to the top chamber (insert, time was set as zero). At 30, 60 and 90 min, 200 μL aliquots were taken from the well and

Table 1
Internal criteria of Caco-2 and MDR-MDCK cells

Batch description	Acceptance criteria for Caco-2 cells	Acceptance criteria for MDR-MDCK cells
TEER value (Ω cm^2)	450–650	>1400
Lucifer yellow P_{app} ($\times 10^{-6}$ cm/s)	<0.4	<0.4
Atenolol P_{app} ($\times 10^{-6}$ cm/s)	<0.5	<0.5
Propranolol P_{app} ($\times 10^{-6}$ cm/s)	15–30	15–30
Digoxin efflux ratio	>3	>10

replaced with 200 μL of fresh buffer with or without appropriate inhibitor. For B-to-A transport, a 0.5 mL of buffer was applied to the top chamber (insert), and 1.5 mL aliquot of the compound-containing solution was applied to the bottom chamber (well, time was set as zero). At 30, 60 and 90 min, 200-μL aliquots were taken from the insert and replaced with 200 μL of fresh buffer with or without appropriate inhibitor. All samples, except for [^{3}H]-E3S, taken from the inserts and the wells were collected into 96-deep well plates.

2.4. Sample analysis

[^{3}H]-E3S samples were placed in scintillation vials and mixed with 10 mL Ecolume scintillation liquid, and then counted in a LS6500 Scintillation Counter (Beckman, Fullerton, CA). Disintegrations per minute (dpm) were corrected for blank radioactivity measured with the assay buffer only. All other test compounds were analyzed by LC/MS/MS analysis.

2.4.1. LC/MS/MS conditions

The samples were collected into 96-well plates containing 200 μL of acetonitrile. The plates were mixed vigorously and centrifuged at 2000 × *g* for 10 min. After centrifugation, an aliquot of supernatant (150 μL) was transferred into vials for LC/MS analysis. The LC equipment consisted of PerkinElmer Series 200 autosampler and PerkinElmer Series 200 micro pump. Chromatography was conducted in the reverse phase mode using a 30 mm × 2.0 mm i.d. 3 μm Keystone Hypersil BDS C18 column with guard column. The mobile phase buffer was 25 mM ammonium formate buffer (pH 3.5); the aqueous reservoir was 90% deionized water and 10% buffer (v/v); the organic reservoir was 90% acetonitrile and 10% buffer (v/v). Typical gradients started at 0% organic and changed linearly over 1.5 min to 95% organic solvent at a flow rate of 300 μL/min. Total run times were 3.5 min. All mass spectrometry was conducted on a PE Sciex API3000 triple quadrupole mass spectrometer in the multiple reaction monitoring modes using a turbo ionspray interface. The Q1/Q3 settings are as follows: +798.6/651.5 for digoxin and +398.9/381.1 for sulfasalazine, respectively.

2.5. Data analysis

The apparent permeability coefficient, P_{app}, was calculated as follows:

$$P_{app} = \frac{dQr/dt}{A \times C_0}$$

$$\text{Efflux ratio} = \frac{P_{app}\,(\text{B-to-A})}{P_{app}\,(\text{A-to-B})}$$

dQr/dt is the cumulative amount in the receiver compartment versus time in M s^{-1}; A is the area of the cell monolayer; C_0 is the initial concentration of the dosing solution (M).

All permeability determinations were performed in quadruplicate, and the data are presented as average ± S.D. The results were verified by repeating the experiments with the same test compounds and different passages of cells with similar results. A one-way ANOVA followed by Dunnett post hoc test was performed to evaluate the statistical significance of the differences between different groups (no inhibitor group as control, $^{*}p < 0.05$, $^{**}p < 0.01$, respectively).

3. Results

3.1. Bidirectional permeability of test compounds in Caco-2 cells

In the absence of inhibitors digoxin showed significant B-to-A transport in Caco-2 cells, with an efflux ratio of 10 (Fig. 1A). The presence of CsA completely abolished the polarized transport of digoxin: A-to-B transport increased from 1.64×10^{-6} to 6.48×10^{-6} cm/s ($p < 0.01$) and B-to-A transport decreased from 17.0×10^{-6} to 5.16×10^{-6} cm/s ($p < 0.01$). Similar results were obtained in the presence of MK571, which, like CsA, inhibited the polarity of transport of digoxin. FTC also increased the A-to-B transport ($p < 0.05$) and decreased the B-to-A transport ($p < 0.01$), but in contrast to CsA and MK571, FTC did not block the efflux completely.

In the absence of inhibitors, E3S showed significant B-to-A transport in Caco-2 cells, with an efflux ratio of 15 (Fig. 1B). In contrast to what occurred with digoxin, CsA did not completely block the polarized transport of E3S. B-to-A transport decreased from 31.0×10^{-6} to 9.81×10^{-6} cm/s ($p < 0.01$), but A-to-B transport did not change significantly (2.93×10^{-6} cm/s vs. 2.66×10^{-6} cm/s). The presence of FTC caused a dramatic decrease in the B-to-A transport ($p < 0.01$) and a two-fold increase in A-to-B transport (from 2.93×10^{-6} to 6.98×10^{-6} cm/s, $p < 0.05$) of E3S, effectively reversing the transport polarity of E3S. The effect of FTC on E3S transport contrast with that of CsA in that CsA decreased B-to-A transport without altering A-to-B transport whereas FTC decreased B-to-A transport and increased A-to-B transport, effectively reversing the polarity of E3S transport. MK571 abolished the efflux of E3S in Caco-2 cells by causing a large decrease in B-to-A transport with only a marginal, and non-statistically significant increase in A-to-B transport.

Sulfasalazine exhibited highly polarized transport in Caco-2 cells, with a B-to-A transport 90 times greater than the A-to-B transport (Fig. 1C). The presence of CsA did not affect either the A-to-B or B-to-A transport of sulfasalazine. FTC alone increased the A-to-B transport of sulfasalazine from 0.18×10^{-6} to 1.32×10^{-6} cm/s ($p < 0.01$) and decreased the B-to-A transport from 15.73×10^{-6} to 3.45×10^{-6} cm/s ($p < 0.01$). MK571 alone decreased the B-to-A transport and increased the A-to-B transport of sulfasalazine in Caco-2 cells. Neither FTC nor MK571 alone eliminated the net secretion of sulfasalazine completely: in each case, the residual efflux ratio was about 3. However, the combination of FTC and MK571 not only completely eliminated the net secretion of sulfasalazine, but it actually reversed the directionality of transport. A-to-B transport increased from 0.18×10^{-6} to 2.27×10^{-6} cm/s

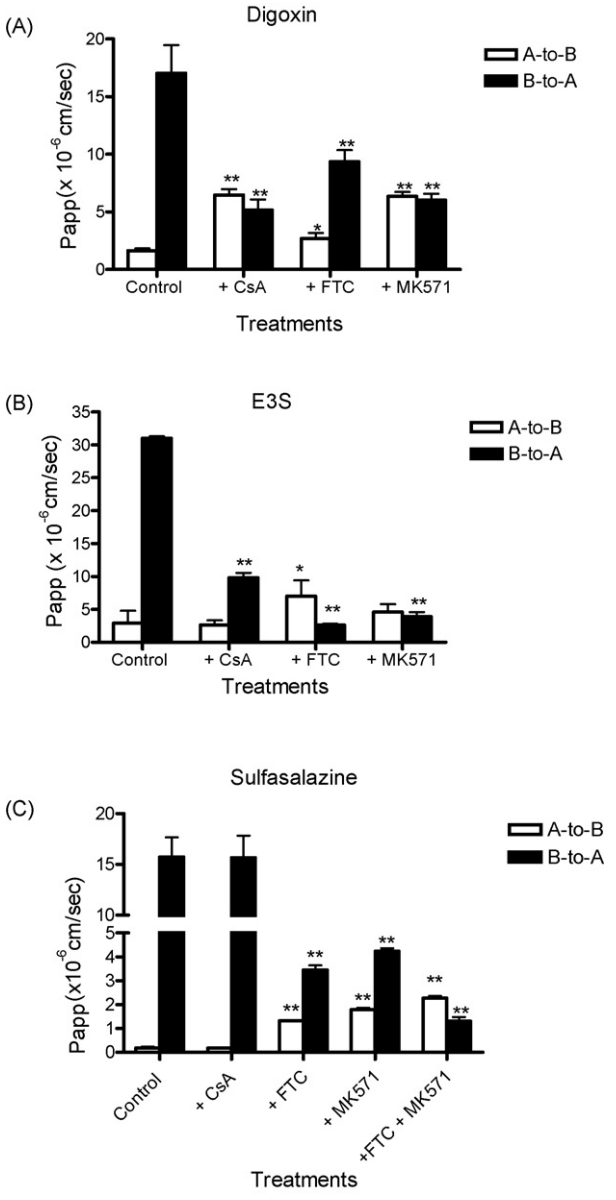

Fig. 1. The effect of CsA, FTC and MK571 on the bidirectional permeability of selected test compounds in Caco-2 cells. (A) Digoxin in the absence and presence of 10 μM CsA, 10 μM FTC and 30 μM MK571. (B) Estron-3-sulfate in the absence and presence of 10 μM CsA, 10 μM FTC and 30 μM MK571. (C) Sulfasalazine in the absence and presence of 10 μM CsA, 10 μM FTC, 30 μM MK571 and in the combination of 10 μM FTC and 30 μM MK571. Each data point represents the average ± STD ($n = 4$). Statistical significance, $^{*}p < 0.05$, $^{**}p < 0.01$, in comparison with no inhibitor groups.

($p < 0.01$) and B-to-A transport decreased from 15.73×10^{-6} to 1.30×10^{-6} cm/s ($p < 0.01$).

3.2. Bidirectional permeability of test compounds in MDR-MDCK cells

The bidirectional permeability of digoxin in MDR-MDCK cells is illustrated in Fig. 2. Similar to Caco-2 cells, digoxin showed high net secretion in the absence of CsA. In the presence of CsA, A-to-B transport increased from 0.06×10^{-6} to

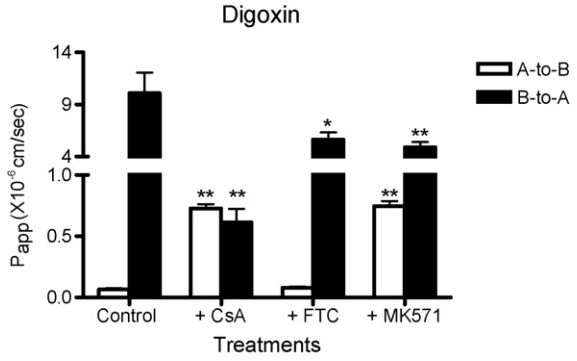

Fig. 2. The effect of CsA, FTC and MK571 on the bidirectional permeability of digoxin in MDR-MDCK cells. The bidirectional permeability of digoxin was determined in the absence and presence of 10 μM CsA, 10 μM FTC and 30 μM MK571. Each data point represents the average ± STD ($n = 4$). Statistical significance, $^{*}p < 0.05$, $^{**}p < 0.01$, in comparison with no inhibitor groups.

0.70×10^{-6} ($p < 0.01$) and B-to-A transport decreased from 10.1×10^{-6} to 0.61×10^{-6} cm/s ($p < 0.01$). FTC significantly decreased the B-to-A transport of digoxin from 10.1×10^{-6} to 5.64×10^{-6} cm/s ($p < 0.01$), but it did not increase the A-to-B transport, whereas MK571 increased the A-to-B transport and lowered the B-to-A transport in MDR-MDCK cells.

The results of bidirectional permeability of E3S and Sulfasalazine in MDR-MDCK cells are presented in Table 2. No efflux was observed for any of these compounds; thus the bidirectional permeability of these compounds in the presence of inhibitors was not determined.

4. Discussion

The results presented in this manuscript illustrate not only the complexity of the interactions between substrates, inhibitors, and efflux proteins, but also indicate that cross-reactions, which are common in experiments with transporters make it difficult to identify the role of individual transporter that mediates the transport of test compounds. The bidirectional permeability of digoxin, E3S and sulfasalazine in the absence and presence of different chemical inhibitors are summarized in Table 3. The observation and its possible explanation of each individual test compound are as follows.

Digoxin is widely recognized as a typical P-gp substrate (De Lannoy and Silverman, 1992), whose P-gp-mediated B-to-A transport is inhibited by the well-known P-gp inhibitor, CsA (Hunter et al., 1993). In the current study, digoxin exhibited polarized transport in Caco-2 and MDR-MDCK cells, and this efflux was completely blocked by CsA. In addition, treating

Table 2
Bidirectional permeability of selected test compounds in MDR-MDCK cells

Compounds	P_{app} ($\times 10^{-6}$ cm/s)	
	A-to-B	B-to-A
Estrone-3-sulfate	1.37 ± 0.14	1.11 ± 0.044
Sulfasalazine	0.11 ± 0.07	0.14 ± 0.05

Table 3
Summary results of bidirectional permeability of selected test compounds in Caco-2 and MDR-MDCK cells

Substrates and inhibitors	In Caco-2 cells				In MDR-MDCK cells			
	P_{app} ($\times 10^{-6}$ cm/s)		Efflux ratio	Inhibition	P_{app} ($\times 10^{-6}$ cm/s)		Efflux ratio	Inhibition
	A-to-B	B-to-A			A-to-B	B-to-A		
Digoxin	1.64	17.0	10	–	0.064	10.1	158	–
+CsA (10 μM)	6.48 ↑	5.16 ↓	~1	Complete	0.73 ↑	0.61 ↓	~1	Complete
+FTC (10 μM)	2.69 ↑	9.36 ↓	3.5	Partial	0.08 ↔	5.64 ↓	70	Partial
+MK571 (30 μM)	6.33 ↑	6.01 ↓	~1	Complete	0.75 ↑	4.89 ↓	6	Partial
Estrone-3-sulfate	2.91	31.0	11	–	1.37	1.11	~1	–
+CsA (10 μM)	2.62 ↔	9.83 ↓	3.8	Partial	N.T.	N.T.	–	–
+FTC (10 μM)	7.03 ↑	2.69 ↓	<1	Complete	N.T.	N.T.	–	–
+MK571 (30 μM)	4.55 ↔	3.94 ↓	~1	Complete	N.T.	N.T.	–	–
Sulfasalazine	0.18	15.73	87	–	0.11	0.14	~1	–
+CsA (10 μM)	0.18 ↔	15.65 ↓	87	–	N.T.	N.T.	–	–
+FTC (10 μM)	1.32 ↑	3.45 ↓	2.6	Partial	N.T.	N.T.	–	–
+MK571 (30 μM)	1.79 ↑	4.24 ↓	2.4	Partial	N.T.	N.T.	–	–
+MRK571 and FTC	2.27 ↑	1.30 ↓	<1	Complete	N.T.	N.T.	–	–

Symbols stand for: ↑ increase, ↓ decrease, ↔ unchanged; N.T. stands for 'not tested'.

the cells with MK571, a known MRP inhibitor, significantly decreased the B-to-A and increased the A-to-B transport of digoxin in Caco-2 cells (Fig. 1A), leading to an efflux ratio close to one. The inhibition of digoxin efflux by both CsA and MK571 observed in this study is in agreement with a previous study that also found that MK571 inhibited the efflux of digoxin in Caco-2 cells (Lowes et al., 2003). This observation is in contrast with a lack of inhibitory effect of MK571 on digoxin efflux in MDR-MDCK cells thus leading to the suggestion that there might be an MK571-sensitive efflux component(s), in addition to P-gp, involved in the B-to-A transport of digoxin in Caco-2 cells (Lowes et al., 2003).

Our current findings that MK571 inhibited the B-to-A transport of digoxin in MDR-MDCK cells contradicts the observations reported in Lowes' study. As shown in Fig. 2, MK571 did not abolish the digoxin efflux completely in MDR-MDCK cells. Although it increased the A-to-B transport 9 times and decreased the B-to-A transport 2.5 times, the remaining efflux ratio was still 7. This partial inhibition of digoxin efflux in MDR-MDCK cells, despite a complete inhibition in Caco-2 cells, appears to suggest that the concentration of MK571 was insufficient to block the interaction of digoxin with P-gp. This hypothesis is supported by an early study showing that the level of P-gp protein expression in MDR-MDCK cells is higher than that in Caco-2 cells based on Western blot analysis (Troutman and Thakker, 2003). Unfortunately, this hypothesis could not be tested because we observed that MK571 concentrations above 30 μM caused a loss of cell monolayer integrity, as indicated by elevated atenolol and lucifer yellow permeability coefficients (data not shown). Previous studies have demonstrated that digoxin is a specific substrate for P-gp (De Lannoy and Silverman, 1992; Hunter et al., 1993), and other studies have pointed out that it is not an MRP2 substrate (Lowes et al., 2003; Taipalensuu et al., 2004); thus one may postulate that the inhibition of digoxin efflux in both Caco-2 and MDR-MDCK cells reflects the ability of MK571 to interact with P-gp.

FTC also inhibited digoxin transport differently in Caco-2 versus MDR-MDCK cells. FTC increased the A-to-B transport ($p < 0.05$) and decreased the B-to-A transport of digoxin in Caco-2 cells ($p < 0.01$), whereas in MDR-MDCK cells only decreased the B-to-A transport of digoxin ($p < 0.05$) without increasing the A-to-B transport. To the best of our knowledge, this is the first report showing that FTC, a presumed specific BCRP inhibitor (Rabindran et al., 2000), inhibited the P-gp-mediated transport of digoxin. Although the nature of this inhibition is not known, an interaction between FTC and P-gp seems possible given the documented substrate promiscuity of P-gp (De Lannoy and Silverman, 1992; Shapiro and Ling, 1998). One can hypothesize that FTC interacts with P-gp, but the elucidation of the specific mechanism by which FTC inhibits digoxin transport (e.g., competitive or non-competitive) cannot be determined from this study. High-resolution structural analysis of P-gp in the presence of FTC might prove valuable in the characterization of the interaction between P-gp and FTC.

The bidirectional permeability properties of E3S were different from those of digoxin in both cell lines tested. E3S is a sulfate metabolite of estrone and its physiological function is to carry estrogen in plasma and tissues. Given that MDR-MDCK cells express higher levels of functional P-gp protein compared to Caco-2 cells, results from this study showing polarized transport of E3S in Caco-2 cells but not in MDR-MDCK cells indicate that E3S is not likely a P-gp substrate. Previous studies have stated that E3S is a BCRP substrate (Imai et al., 2003; Xia et al., 2005). In the presence of CsA the B-to-A transport of E3S in Caco-2 cells decreased more than 50%, but the A-to-B transport was not affected. This result is consistent with previous findings that indicated that CsA interacts with multiple drug transporters, including P-gp (Hunter et al., 1993), BCRP (Pawarode et al., 2007) and MRP2 (Tang and Borchardt, 2002; Arimori et al., 2003). During the preparation of this manuscript, Xia et al. (2007) reported that CsA might be a weak inhibitor of BCRP-mediated E3S transport in Caco-2 cells. The inhibition

of E3S efflux by CsA in Caco-2 cell, together with the known interaction of CsA with other transporters suggest that E3S transport in Caco-2 cells might be mediated by MRP2 and/or BCRP. Unlike CsA and MK571, both of which caused a reduction of the B-to-A transport of E3S without enhancing the A-to-B transport, FTC not only decreased the B-to-A transport of E3S, but also increased its A-to-B transport in Caco-2 cells (Fig. 1B, $p < 0.05$). This resulted in a P_{app} (A-to-B) value greater than the P_{app} (B-to-A) value. However, the net absorption of E3S transport in Caco-2 cells caused by FTC cannot be explained in terms of interaction with BCRP only.

It has been demonstrated that organic anion transporting polypeptide (OATP) is responsible for the active apical influx of E3S in Caco-2 cells (Sai et al., 2006). Based on limited data, it has been suggested that FTC is a specific inhibitor of BCRP (Rabindran et al., 2000), but there is no evidence that FTC interacts with OATP. In contrast to FTC, CsA has been shown to inhibit OATP-mediated pitavastatin influx in human hepatocytes (Hirano et al., 2006). Taken together, it would appear that the apparent bidirectional transport of E3S across Caco-2 cells is the net result of active influx and efflux processes, with the efflux process playing the major role and the influx process the minor role. In addition, the fact that OATP is expressed in Caco-2 cells (Sai et al., 2006), but not in wild type MDCK cells (Goh et al., 2002) is consistent with the absence of active influx of E3S in MDR-MDCK cells (Table 1).

It is generally acknowledged that co-administration of drugs that interact with efflux transporters (i.e. P-gp) can lead to drug–drug interactions, which could lead to either toxicity or lack of therapeutic effect. In addition, a few studies have shown that commonly used efflux transporter inhibitors also inhibit influx transporters. For example, CsA not only inhibited Pgp and MRPs, but it also inhibited OATPs in human hepatocytes and OATP-transfected MDCK cells, respectively (Hirano et al., 2006; Letschert et al., 2006). Therefore, for many compounds, interpretation of transport results is frequently difficult due to the interaction of substrates and/or inhibitors with multiple transporters. Although P-gp is expressed in Caco-2 and MDR-MDCK cells and P-gp inhibition by FTC could explain the reduction in the B-to-A transport of digoxin in both cell lines (Figs. 1A and 2), it is possible that FTC might inhibit an unknown influx process on the basolateral membrane, resulting in a reduction of digoxin influx and consequent decreased B-to-A flux. Similarly, the reduction in the B-to-A transport of E3S by MK571 could also be explained by inhibition of an influx system on the basolateral membrane (Fig. 1B). Further studies to verify these hypotheses would be facilitated by the use of truly specific compounds and/or cell lines with complementary transporter expression.

In the absence of inhibitors, sulfasalazine and E3S did not exhibit efflux in MDR-MDCK cells, but showed high efflux in Caco-2 cells; however, unlike E3S, the efflux of sulfasalazine was not affected by CsA treatment. Both FTC and MK571 significantly decreased the B-to-A transport of sulfasalazine in Caco-2 cells, suggesting that this compound is subject to non-P-gp mediated efflux, possibly by BCRP and/or MRP2. These results are not surprising because previous studies found that sulfasalazine appears to be a substrate for MRP, organic anion transporter (OAT), and the anion exchange transporter (AET), but not for P-gp (Liang et al., 2000; Mols et al., 2005). However, information on the specific efflux protein(s) responsible for sulfasalazine transport is unclear. For example, one study argued that MRP proteins play a major role in the efflux of sulfasalazine in Caco-2 cells (Liang et al., 2000) and a second study, based on results with different MRP inhibitors, reported that the efflux of sulfasalazine is mediated by MRP proteins (Mols et al., 2005). In contrast, a more recent pharmacokinetic study using BCRP(−/−) and wild type mice concluded that sulfasalazine is a BCRP substrate (Zaher et al., 2006). In this study, after oral administration of 20 mg/kg sulfasalazine, the area under the plasma concentration (AUC) time profile in BCRP(−/−) mice was approximately 111-fold higher than that in FVB wild-type (WT) mice. After intravenous administration of 5 mg/kg sulfasalazine, the AUC in BCRP(−/−) mice was approximately 13-fold higher than that in WT mice (Zaher et al., 2006).

Although treatment with FTC or MK571 alone did not eliminate the efflux of sulfasalazine completely (residual efflux ratio was approximately 3), when the two inhibitors were added together the efflux of sulfasalazine was eliminated, as shown in Fig. 1C. This seems to indicate that both MRP2 and BCRP might be involved in sulfasalazine efflux in Caco-2 cells. However, the involvement of MRP2 appears less likely because the presence of CsA, an inhibitor of P-gp and MRP2, did not alter the net secretion of sulfasalazine in Caco-2 cells. Thus, it appears that neither P-gp nor MRP2 are involved in sulfasalazine transport. In fact, it seems that BCRP is the efflux protein primarily responsible for mediating the efflux of sulfasalazine. Consequently, it is likely that, presumably specific transporter inhibitors such as MK571 cross-react with both MRP2 and BCRP. Multiple transporter cross-reactivities were observed in the present study: as summarized in Table 3, FTC and MK571 inhibited the B-to-A transport of digoxin in Caco-2 cells, MK571 and CsA inhibited the B-to-A transport of digoxin in MDR-MDCK cells, and CsA, FTC and MK571 inhibited the efflux of E3S in Caco-2 cells.

In conclusion, the current study has highlighted the danger of relying excessively on so-called specific transporter inhibitors to elucidate the role of individual transporters on the transcellular transport of drugs. The presence of a large complement of influx and efflux systems in Caco-2 cells makes this cell line a relevant model system to study interactions between drug candidates and intestinal transporters. However, the lack of truly specific substrates and inhibitors presents a formidable challenge in reaching reliable conclusions about drug–transporter interactions based only on transport data obtained in culture cells that express multiple transporters such as Caco-2. This limitation complicates the ability to predict potential drug–drug interaction associated with a given transporter and drug. Together, bidirectional permeability experiments using not only multiple substrates and chemical inhibitors, but also genetically modified cell lines will be necessary to achieve the degree of insight on transporter-mediated intestinal drug absorption/efflux that would permit to predict the role these transporters will play on the pharmacokinetics performance of NMEs and potential transporter-mediated drug–drug interactions.

Acknowledgement

No source of fund is provided for the present study.

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