Potential roles of P-gp and calcium channels in loperamide and diphenoxylate transport

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Received 10 April 2003; accepted 28 July 2003

Abstract

This study examined the accumulation and transport of two related systemic opioids used as antidiarrhoeal drugs and compared their rates of transport with known P-glycoprotein (P-gp) substrates used in our in vitro environment. Cellular uptake and efflux and transcellular transport were all determined using Caco-2 cells after exposure to loperamide or diphenoxylate, with or without a range of efflux inhibitors. Bidirectional transport studies of 5 μM loperamide showed efflux to be fivefold higher than influx (42 × 10⁻⁶ compared to 8 × 10⁻⁶ cm/s); however, this decreased to twofold at 10 μM and was abolished using 100 μM loperamide. An uptake pathway was also discovered when P-gp was inhibited which, in the presence of Ca²⁺ channel blockers, was amplified, providing a potential mechanism for central nervous system effects to be increased upon blockage of L-type calcium channels, quite separate from any P-gp inhibition. Diphenoxylate transport, however, showed little sign of P-gp-mediated efflux. Diphenoxylate accumulated readily within cells, yet transport through cells was very low. Additionally, efflux inhibitors had little impact on transport or accumulation, suggesting that diphenoxylate was not a substrate for an efflux mechanism.

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Keywords: MRP1; P-gp; Nifedipine; Verapamil; Nisoldipine; Nimedipine; PSC-833; Caco-2; BBB

Introduction

Classical opioid drugs such as morphine have powerful analgesic effects caused by agonistic action at μ-opioid receptors in the brain. Unfortunately, it is through action on these same central nervous system receptors that the euphoric respiratory depression and dependence are thought to operate. A secondary action of μ-opioid receptor agonists is to increase gastrointestinal muscle tone while reducing peristaltic motility, which can be quite effective at reducing diarrhea or even causing constipation over a long period of use. Diphenoxylate and loperamide are two examples of opiates that only cross the blood–brain barrier (BBB) to a very limited degree, thereby having much greater action in the systemic environment, and hence are useful as antidiarrhoeal agents (Dehaven-Hudkins et al., 1999) without the problems associated with central nervous system effects.

The active efflux transporters P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) are known to provide resistance to chemotherapy and immuno-suppressive treatments as well as enhancing excretion of many drugs through the kidneys and the intestine (Aszalos and Ross, 1998; Larsen et al., 2000). It is also clear that P-gp and MRP1 are important limiters of brain penetration from some circulating lipophilic drugs by preventing transport through the BBB (Schinkel, 1999).

It is already known that both loperamide and morphine are substrates for P-gp (Schinkel et al., 1996; King et al., 2001; Wandel et al., 2002), although morphine is a relatively weak substrate (Crowe, 2002) and is still able to penetrate into the brain when given as an intravenous injection. The relative extent to which loperamide is effluxed by P-gp compared to other known substrates has only been addressed recently (Wandel et al., 2002). Additionally, P-gp inhibitory activity has been suggested for loperamide, affecting the transport of other substrates, such as digoxin (Wandel et al., 2002). Many orally active antineoplastic...
agents, immunosuppressants, and HIV protease drugs cause diarrhea as a side effect. The use of loperamide or diphenoxylate is relatively common in patients attempting to overcome drug-induced diarrhea. Knowing the transport characteristics of these opioids and the importance of P-gp in their transport are important issues when coadministering them with other drugs that are likely to have some P-gp inhibitory activity.

Both of these pharmaceuticals are supplied as over the counter medications for antidiarrheal effects because of their very limited blood–brain barrier permeability. Yet abuse of these, especially diphenoxylate, can result in typical morphine-like effects, suggesting transmission through the blood–brain barrier into the brain interstitium. The aim of this paper was to examine both loperamide and diphenoxylate transport into and through Caco-2 cell monolayers, a cell line known to express endogenously high levels of P-gp, in order to determine the extent that P-gp efflux may be keeping these opiates out of the sensitive organs and to compare the rates of efflux determined here with previous work on morphine transport conducted within our laboratory (Crowe, 2002).

Materials and methods

Materials

Loperamide, nifedipine, probenecid, and triethylamine were supplied by Sigma Aldrich (Castle Hill, NSW, Australia). Verapamil hydrochloride was purchased from ICN Biomedicals (Seven Hills, NSW, Australia). Nimodipine was kindly supplied from the Department of Pharmacy at Sir Charles Gardiner Hospital in Perth, WA. All other materials were of analytical grade.

The cell culture reagents phosphate-buffered saline (PBS), Hanks’ buffered salt solution (HBSS), and high-glucose Dulbecco’s modified Eagle’s medium (DMEM) were from Gibco BRL (Melbourne, Australia); penicillin G and streptomycin were from Trace Biosciences (Castle Hill, NSW, Australia), while the fetal calf serum was obtained from the Australian Commonwealth Serum Laboratories (Parkville, Vic, Australia).

Cell transport studies

The human colon carcinoma cell line (Caco-2) used in our laboratory originated from University Hospital (Utrecht, NL) and was cultured in a similar fashion to that done by Artursson (1990). Caco-2 cells were seeded onto Falcon polystyrene terrathalate (PET) 0.9-cm² filter inserts in 12-well plates at 65,000 cells/cm². Cells were grown in growth medium [high-glucose DMEM with 25 mM HEPES, 2 mM glutamine, 1 mM NEAA, 100 U/ml of penicillin–streptomycin, and 10% fetal calf serum (FCS) set at pH 7.4] in a 37°C incubator with 5% CO₂.

Monolayers were incubated for 21–25 days to allow full maturation of the cells, including P-gp expression, which was confirmed using Western blotting techniques, and increased transepithelial electrical resistance (TEER) formation (Bailey et al., 1996; Hosoya et al., 1996). The TEER was measured both before and immediately after the study using an EVOM meter and the ENDÖHM 12 chamber (World Precision Instruments, Sarasota, FL, USA), with readings between 390 and 800 Ω · cm² for all cells in this study. Resistance readings at the end of each experiment were not significantly different from initial values. Cell cultures with TEER values below 300 Ω · cm² were routinely excluded from studies.

Filter inserts were transferred to fresh 12-well plates for the studies. The studies were conducted using assay medium consisting of HBSS supplemented with both glucose (Ajax Chemicals, NSW, Australia) and HEPES (Gibco BRL) to give 25 and 10 mM final concentrations, respectively. The pH was adjusted to 7.4 using 1 M NaOH. Cells were incubated in prewarmed HBSS ± an efflux inhibitor for 30 min and then rinsed in the same medium. TEER was measured and HBSS ± inhibitors was placed in the receiver chambers. Loperamide at various concentrations between 5 and 100 μM was added to the donor chamber of each well. The apical (Ap) and basolateral (Bas) chambers received 0.5 and 1.4 ml of medium, respectively. Sample was removed from the receiver chamber at various times over a 3-h period. Constant volumes were maintained by adding prewarmed medium to the receiver chambers in order to maintain an equilibrium pressure differential between the volumes in the donor and receiver chambers. Transport studies in the Ap to Bas and Bas to Ap directions were simultaneously conducted with diphenoxylate using concentrations between 10 and 20 μM. The solubility of diphenoxylate was limiting in the maximal concentration achievable, while not exceeding 0.75% DMSO in the final buffer concentration.

P-gp and MRP1 inhibition

In studies where inhibition of P-gp or MRP was performed in conjunction with loperamide or diphenoxylate transport, cells were preincubated in HBSS containing the inhibitors for 30 min before initiation of the study. The inhibitors included the following P-gp inhibitors: 4 μM PSC-833, 100 μM verapamil, and 10 μM cyclosporin A. Additionally the MRP1 inhibitors 500 μM probenecid and 1 mM sulfinpyrazone were examined. To determine the role of glutathione in loperamide transport, which would indirectly implicate MRP1, glutathione (1 mM) or DL-buthionine sulfoxamine (BSO) (40 μM) was included with the DMEM growth medium for overnight monolayer incubation prior to the loperamide studies. During the transport study these modifying agents were also present in the donor chamber with loperamide or diphenoxylate and in the receiver chamber at the same concentrations as stated above.
Metabolic inhibition

In studies examining the general role of ATP-dependent transport of our opioids, Caco-2 monolayers were subjected to a 30-min exposure of 2 mM sodium azide in HBSS with 20 mM 2-deoxy-D-glucose instead of D-glucose in order to deplete functional ATP concentrations (Dantzig and Bergin, 1990; Hidalgo and Borchardt, 1990).

Cell accumulation

Caco-2 cells were grown in 24-well culture plates using growth medium at an initial seeding density of 5000 cells/cm². The cells were used 21–25 days after seeding. Loperamide was added at 5, 10, or 20 μM in HBSS for various times up to 120 min. Diphenoxylate accumulation was also measured, using either 10 or 20 μM concentrations. Triplicate cell wells were washed 3× in ice-cold PBS before 500 μl of H2O was added, and the samples were collected in microcentrifuge tubes and disrupted in a sonicating water bath for 5 min before storing at −80°C. Diphenoxylate samples degraded (or stuck to the plastic vessel walls) relatively quickly; therefore, diphenoxylate samples were unable to be stored and had to be examined by HPLC analysis immediately after the transport or accumulation studies, with glass vessels used instead of polypropylene or polycarbonate. Efflux studies were not attempted with diphenoxylate.

Cell efflux

In parallel studies with cell accumulation, identical 24-well plates also seeded at 5000 cells/cm² were grown for 21–25 days. Initially, cellular accumulation of loperamide was allowed to progress for 60 to 90 min, in order to reach a saturation solution for the concentration used. Caco-2 monolayers were washed 4× in ice-cold PBS before incubating with 800 μl of prewarmed HBSS containing either P-gp or MRP inhibitors. Additional parameters evaluated included incubation with energy inhibitors (2 mM NaN₃ and 20 mM 2-deoxy-D-glucose) and performing efflux studies at 4°C. Samples (80 μl) were removed to NUNC polycarbonate 96-well plates at various times up to 40 min after addition of the prewarmed (or cooled for the 4°C study) HBSS and sealed with polyethylene capping membranes. Medium was not replaced to allow drug concentrations to build in the remaining volume as the amount effluxed was close to detection limits in some instances.

Protein concentrations for all aspects of this study were determined using a micro-Lowry method adapted for use with multiwell plates on a TECAN Sunrise 96-well plate spectrophotometer, using Magellan 3 software for Windows NT 4.0.

HPLC analysis

HPLC conditions were modified from those of De Luca and co-workers (1993) to suit our equipment and conditions. Samples collected in HBSS from efflux and transport studies were immediately collected into 96-well plates or 2.0-ml HPLC vials with glass 350-μl low-volume inserts. No extraction phases were required. The mobile phase consisted of 25 mM KH₂PO₄ (BDH-Merck, Kilsyth, Victoria) (pH 2.8) containing 0.1% triethylamine:far-UV grade acetoni- trile (EM Science, Gibbstown, NJ, USA) (55:45 v/v). The HPLC system consisted of an Agilent 1100 series system run though the Agilent PC package Chemstation for Windows 2000. The quaternary pump ran at 1.5 ml/min and a Perkin–Elmer Series 200 autosampler injected 70 μl of sample through a Zorbax RX-C₁₈ column or an Econosphere CN column, both 5-μm pores, 15 cm × 4.6 mm i.d., with mated guard columns (Agilent, NSW, Australia, and Alltech, NSW, Australia, respectively). The effluent was detected on an Agilent 1100 variable wavelength UV detector. Both loperamide and diphenoxylate were measured at 214 nm, with typical retention times of 2.9 and 4.7 min, respectively.

Opoid determinations from cell lysates were also performed without the need for complex extraction protocols. A 5-min sonication of cell monolayers in microcentrifuge tubes was adequate for liberation of loperamide or diphe- noxylate from the cells. Pelleting of cellular debris for 3 min at 8500g resulted in a visually clear solution that had clear chromatograms at typical elution times. The detection limit for the opioids was 40 nM for loperamide and 80 nM for diphenoxylate, each using 70-μl injections on the column.

Data analysis

Drug transport through cell monolayers was calculated both as a simple amount passing the monolayer per minute, which would vary depending on the initial concentration used, and as an apparent permeability coefficient as calculated previously (Crowe and Lemaire, 1998).

The efflux of drugs after an accumulation phase for cells grown on the base of wells followed first-order kinetics. Maximal efflux was determined through the Y intercept on a linear double reciprocal plot of amount effluxed over time. Time to the half-maximal efflux was determined by calculating the X intercept.

Results expressed in this study are presented as the mean ± SEM, standardized on individual well protein concentrations. Significant differences between values were exam- ined using Student’s two-tailed unpaired t test or one-way ANOVA. Results were considered significant if P < 0.05.

Results

Accumulation

To examine whether P-gp could affect the accumulation of our test opioids, uptake studies in Caco-2 monolayers grown on the base of 24-well plates using 5 μM loperamide
higher equilibrium levels compared to 5 μM loperamide, 7 nmol/mg of protein was detected. Additionally, 50 μM loperamide also gave the expected 32 to 34 nmol/mg of protein, which initially suggested that diffusion was the major factor contributing to loperamide cellular uptake at these concentrations. As PSC-833 increased 5 μM loperamide accumulation (Fig. 1A), it was surprising not to observe a relatively lower equilibrium concentration compared to the higher concentrations without the use of P-gp inhibitors. Further loperamide accumulation studies were conducted by incubating Caco-2 cells with 100 μM verapamil and 10 μM cyclosporin A. Co-incubation of 5 μM loperamide with these P-gp inhibitors also resulted in an increase in loperamide accumulation (Fig. 2A). Diphenoxylate accumulation studies were also repeated with other P-gp inhibitors and the MRPI inhibitor sulfinpyrazone. As with the PSC-833 study (Fig. 1B), no significant differences from the control study were observed (data not shown).

To confirm that energy-dependent processes were operating, regardless of the specific transporters involved, the ATP inhibitor sodium azide along with the nonmetabolized sugar 2-deoxy-D-glucose was incubated with Caco-2 cell cultures for 40–60 min before adding 5 μM loperamide to examine accumulation under ATP-depleted conditions. Sodium azide was able to significantly increase loperamide accumulation in these cells to over 6000 pmol/mg (Fig. 2A), which was 60% higher than loperamide accumulation alone and closely matched the increases observed using P-gp inhibitors. Yet another loperamide uptake study performed at 4°C provided evidence that loperamide required the dynamics of the semifluid nature of cell membranes in order to initiate absorption, as there was significantly reduced uptake of loperamide into Caco-2 cells when the 60-min study was conducted on ice (Fig. 2A). Although ATP function would be diminished at 4°C, which should have increased accumulation if efflux protein activity was suppressed, membrane fluidity in this case is likely to be a more important parameter based on the very low uptake results of only 1000 pmol/mg of protein shown here (Fig. 2A).

Bidirectional transport

Transport of loperamide through Caco-2 monolayers occurred in both apical to basolateral and basolateral to apical directions; however, basolateral to apical transport was significantly greater than transport in the other direction, at least for concentrations up to 50 μM (Table 1). The use of 100 μM loperamide completely inhibited any efflux mechanisms present within the Caco-2 cell monolayers, resulting in uptake levels of 19 × 10⁻⁶ cm/s (Table 1), which equated to 45 ng/cm²/min transport. This was similar to our passive permeability results for propranolol and progesterone (results not shown) suggesting transcellular transport within the body to be rapid with high micromolar circulating concentrations of loperamide. The efflux ratio increased with decreasing concentration of loperamide. Even though a
A fivefold difference was shown here for 5 μM loperamide, this decreased to only twofold at 10 μM loperamide (Table 1, Fig. 3A), indicating that P-gp may only be able to effec-
were unable to be conducted. It would be expected that given the underlying trend for increased efflux with decreasing loperamide concentrations, that nanomolar concentrations would likely be adequately effluxed to prevent entry of the drug to organs. At 5 μM though, apical to basolateral transport was still 8.5 × 10⁻⁶ cm/s, which would result in significant organ penetration, even if it was eventually effluxed from the organ. The P-gp inhibitor PSC-833 was able to completely abolish any efflux ratio (Fig. 3B; Table 1), suggesting that P-gp was solely responsible for the efflux characteristics observed.

Diphenoxylate had very low transport rates through Caco-2 cells. In the apical to basolateral direction the rate was 0.2 × 10⁻⁶ cm/s, while the basolateral to apical rate was 1.5 × 10⁻⁶ cm/s. Differences between very low transport rates do not necessarily reflect P-gp involvement as this efflux protein would be expected to transport substrates to a much greater extent than 1.5 × 10⁻⁶ cm/s. Hence, the eightfold difference in directional transport in favor of efflux back to the apical side is unlikely to have any clear significance (Fig. 3C; Table 2). In comparison to both morphine transport from our earlier work (Crowe, 2002) and loperamide transport here, PSC-833 did little to alter the low transport rates of diphenoxylate in this cellular system (Fig. 3D; Table 2), suggesting little P-gp involvement. However, verapamil was able to equilibrate transport (Table 2).

### Calcium channel blockers

Verapamil was found to promote apical to basolateral transport of 5 μM loperamide (45.4 × 10⁻⁶ cm/s) to a significantly greater degree than in the reverse direction (18.7 × 10⁻⁶ cm/s) (Table 1). Bidirectional transport of 10 μM loperamide with verapamil also showed an increased net apical to basolateral transport, but not to the same extent as that seen with 5 μM loperamide (1.74 vs 2.43-fold uptake ratio). Overall, this was a complete reversal of the efflux-driven movements of loperamide shown here. Generally, inhibiting efflux mechanisms only results in equivalent transport in both directions if no other transport system apart from diffusion was operating. These results here implied that verapamil could stimulate uptake of loperamide through cellular barriers. Therefore, other calcium channel blockers with very low P-gp inhibitory activity (Barancik et al., 1994) were used in this study to verify Ca²⁺ channel blocking ability as a means to accelerate loperamide transport through the Caco-2 cell monolayers. Nifedipine (100 μM) also resulted in a 2.2-fold increase in

### Table 1

<table>
<thead>
<tr>
<th>Concentration (μM) ± efflux modifiers</th>
<th>Ap → Bas (cm/s)</th>
<th>Bas → Ap (cm/s)</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(× 10⁻⁶)</td>
<td>(× 10⁻⁶)</td>
<td>(net flow direction)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + 2 mM Na-azide</td>
<td>8.4 ± 1.5</td>
<td>42.4 ± 2.2**</td>
<td>5.02 (Efflux)</td>
</tr>
<tr>
<td>5 + 2 mM Na-azide (4°C)</td>
<td>3.8 ± 2.5*</td>
<td>4.7 ± 0.6*</td>
<td>1.25 (No net flux)</td>
</tr>
<tr>
<td>5 + 4 μM PSC-833</td>
<td>15.8 ± 3.5*</td>
<td>9.5 ± 0.5*</td>
<td>1.66 (No net flux)</td>
</tr>
<tr>
<td>5 + 100 μM verapamil</td>
<td>45.4 ± 3.7*</td>
<td>18.7 ± 0.4***</td>
<td>2.43 (Uptake)</td>
</tr>
<tr>
<td>5 + 500 μM probenecid</td>
<td>7.8 ± 1.0</td>
<td>40.1 ± 0.6***</td>
<td>5.16 (Efflux)</td>
</tr>
<tr>
<td>5 + 10 μM cyclosporin A</td>
<td>26.4 ± 2.5*</td>
<td>14.9 ± 1.1***</td>
<td>1.77 (Uptake)</td>
</tr>
<tr>
<td>5 + 100 μM genistein</td>
<td>9.5 ± 2.9</td>
<td>26.9 ± 1.9***</td>
<td>2.83 (Efflux)</td>
</tr>
<tr>
<td>5 + 4 mM calcium</td>
<td>11.8 ± 0.8</td>
<td>41.1 ± 1.6***</td>
<td>3.5 (Efflux)</td>
</tr>
<tr>
<td>10</td>
<td>10.5 ± 1.2</td>
<td>21.2 ± 2.0**</td>
<td>2.01 (Efflux)</td>
</tr>
<tr>
<td>10 + 4 μM PSC-833</td>
<td>17.0 ± 1.3*</td>
<td>15.0 ± 1.8*</td>
<td>1.13 (No net flux)</td>
</tr>
<tr>
<td>10 + 100 μM genistein</td>
<td>8.6 ± 0.7</td>
<td>13.9 ± 0.8***</td>
<td>1.61 (Efflux)</td>
</tr>
<tr>
<td>10 + 100 μM kempferol</td>
<td>12.9 ± 0.8</td>
<td>20.4 ± 0.6**</td>
<td>1.93 (Efflux)</td>
</tr>
<tr>
<td>10 + 50 μM indomethacin</td>
<td>13.5 ± 0.9</td>
<td>20.2 ± 0.4**</td>
<td>1.49 (Efflux)</td>
</tr>
<tr>
<td>10 + 1 mM glutathione</td>
<td>7.0 ± 0.8</td>
<td>9.5 ± 0.6</td>
<td>1.36 (No net flux)</td>
</tr>
<tr>
<td>10 + 40 μM BSO</td>
<td>4.1 ± 0.6*</td>
<td>14.6 ± 1.1***</td>
<td>3.59 (Efflux)</td>
</tr>
</tbody>
</table>

**Note.** The ratio of the highest transport rate over the lowest transport rate is given under Fold difference. The net flow direction refers to either Ap to Bas (upake) or Bas to Ap (efflux) or that transport is the same in both directions, equivalent to diffusion such that no direction is preferred (no net flux). All modifying agents were added to the cell cultures for 30 min prior to initiation of and during transport studies using loperamide. Transport results are calculated from all measurements between 0 and 180 min as all tests showed linear transport during this time. Results show the mean of three to six samples ± SEM.

* Significance of inhibitor additions in relation to controls for a particular direction (P < 0.05).

** Significance of transport difference in each direction within a single group (P < 0.05).
the apical to basolateral transport over that in the reverse direction, although the absolute values were both lower than that of verapamil (Table 1). Nimodipine was used at 3 μM, and although it did not show a clear advantage in apical to basolateral transport over 10 μM loperamide, it did equilibrate transport in both directions (Table 1), which was remarkable for such a low concentration of a very weak P-gp inhibitor. Use of the more potent nisoldipine at 50 μM showed similar efficacy to both verapamil and nifedipine at increasing loperamide transport through Caco-2 monolayers in the apical to basolateral direction (Table 1).

Cellular uptake was readdressed with 50 μM nisoldipine and 100 μM nifedipine in combination with loperamide. It was shown that both calcium channel blockers significantly increased the accumulation of loperamide into Caco-2 cells grown on the base of 24-well plates when cells were exposed to 10 μM loperamide (Fig. 2B). Nifedipine induced a significant increase in uptake of loperamide within 5 min of uptake (results not shown), which resulted in a 57% increase over control cells at 60 min. The increased uptake induced with nisoldipine was greater than that with nifedipine, with a significant 90% increase in loperamide concentration to 12,800 pmol/mg of protein (Fig. 2B) after 1 hr.

**Drug efflux**

Efflux studies similar to those done previously (Crowe, 2002) compliment the bidirectional transport studies, as efflux is driven by only those concentrations inside the cells rather than the bulk concentrations outside the cells. This should allow P-gp or other transporters to operate with less risk of saturation. It was shown that the P-gp inhibitors all

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**Fig. 3.** Transport of 10 μM loperamide (A, B) and 20 μM diphenoxylate (C, D) though 3-week-old Caco-2 cells grown on polyethylene terephthalate filter inserts. Transport with opioids alone is shown in A and C, while results from 4 μM PSC-833 preincubated for 30 min and co-incubated with Caco-2 cell monolayers during loperamide transport are shown in B and D. Transport is expressed as a concentration-independent value (cleared volume). Apical (Ap) to basolateral (Bas) directed transport is shown with squares and dotted lines, while Bas to Ap transport is shown with diamonds and solid lines. Results are the mean of three to five samples ± SEM.
Transport rates of 20 and 100 µM diphenoxylate through Caco-2 cell monolayers in both apical (Ap) to basolateral (Bas) and Bas to Ap directions

<table>
<thead>
<tr>
<th>Concentration (µM) ± efflux modifiers</th>
<th>Ap → Bas (cm/s) ($\times 10^{-6}$)</th>
<th>Bas → Ap (cm/s) ($\times 10^{-6}$)</th>
<th>Fold difference (net flow direction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.2 ± 0.1</td>
<td>1.5 ± 0.3**</td>
<td>9.37 (Efflux)</td>
</tr>
<tr>
<td>20 + 4 µM PSC-833</td>
<td>0.1 ± 0.3</td>
<td>0.9 ± 0.0**</td>
<td>8.09 (Efflux)</td>
</tr>
<tr>
<td>20 + 100 µM verapamil</td>
<td>0.9 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>1.18 (Net flow)</td>
</tr>
<tr>
<td>100</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>1.15 (Net flow)</td>
</tr>
<tr>
<td>100 + 4 µM PSC-833</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.1*</td>
<td>1.09 (Net flow)</td>
</tr>
</tbody>
</table>

Note. The ratio of the highest transport rate over the lowest transport rate is given under Fold difference. The net flow direction refers to either Ap to Bas (uptake) or Bas to Ap (efflux) or that transport is the same in both directions, equivalent to diffusion such that no direction is preferred (no net flow). All modifying agents were added to the cell cultures for 30 min prior to initiation of and during transport studies using diphenoxylate. Transport results are calculated from all measurements between 0 and 180 min as all tests showed linear transport during this time. Results show the mean of three to five samples ± SEM.

* Significance of inhibitor additions in relation to controls for a particular direction ($P < 0.05$).

**Significance of transport difference in each direction within a single group ($P < 0.05$).

significantly reduced the percentages of accumulated drug leaving the cells over a 35-min period from 88% in control cells to 78, 74, and 57% for cyclosporin A, PSC-833, and verapamil, respectively (Fig. 4). Compounds used to alter MRP activity had no significant effect on efflux of 10 µM loperamide from Caco-2 cells (Fig. 4). Efflux studies were repeated at 4°C. No significant difference was seen between any of the inhibitors at this temperature (results not shown), suggesting that active transport mechanisms were responsible for the significant changes that P-gp inhibitors caused at 37°C (Fig. 4). However, although there was no difference between the efflux of any of the tested inhibitors at 4°C, efflux reduced across the board to 35% of uptake, compared to the 57–88% shown at 37°C, which, in combination with the 4°C accumulation study (Fig. 2A), indicates that membrane fluidity plays a major role in loperamide transport in these cells.

Efflux of our opioid from Caco-2 cells followed first-order kinetics, making it possible to calculate the maximum amount of loperamide effluxed in these cells. This was around 12 nmol/mg of protein for 10 µM loperamide and 9 nmol/mg of protein when energy inhibitors were used, although this reduction in efflux was not statistically significant (Table 3). The maximal amount of loperamide effluxed after PSC-833, cyclosporin A, or verapamil administration was also not significantly different than control efflux values (Table 3). Surprisingly, BSO and probenecid showed significant reductions in maximal efflux concentrations compared to controls (Table 3). However, the percentage of released loperamide was similar to that of control samples, indicating that less loperamide was able to accumulate within these cells during the uptake phase compared to control cells, but that a similar proportion of the available drug was removed from the cells in the time allowed. These efflux results reaffirm the accumulation studies (Fig. 2A), which also showed a reduced loading of loperamide when exposed to probenecid. Hence, there was a possible MRP-like action on reducing accumulation within the cells, but it was only uptake related as efflux rates and bidirectional

Table 3
Rates of efflux into the extracellular medium for 10 µM loperamide after a 90-min initial incubation to build intracellular concentrations of loperamide followed by incubation with a variety of efflux protein modifying agents

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Max amount effluxed (nmol/mg protein)</th>
<th>Time to half-maximal efflux (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>12.2 ± 0.8</td>
<td>27.0 ± 4.0</td>
</tr>
<tr>
<td>4 µM PSC 833</td>
<td>9.9 ± 1.9</td>
<td>41.6 ± 8.7*</td>
</tr>
<tr>
<td>100 µM Verapamil</td>
<td>9.2 ± 1.6</td>
<td>29.0 ± 5.6</td>
</tr>
<tr>
<td>500 µM Glutathione</td>
<td>11.1 ± 0.6</td>
<td>30.1 ± 1.9</td>
</tr>
<tr>
<td>40 µM BSO</td>
<td>8.1 ± 0.9*</td>
<td>22.4 ± 5.5</td>
</tr>
<tr>
<td>500 µM Probenecid</td>
<td>9.3 ± 0.3*</td>
<td>31.6 ± 3.4</td>
</tr>
<tr>
<td>10 µM Cyclosporin A</td>
<td>11.7 ± 0.5</td>
<td>42.9 ± 3.1*</td>
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Note. Efflux parameters were calculated over a 40-minute period. Inhibitors were present during both the uptake and efflux stages of this study. Results show the mean of three to six samples ± SEM.

* Significance of inhibitor additions in relation to controls ($P < 0.05$).
These differences in opioid accumulation are likely extracellular drug for the equivalent concentration of loperamide. This is significant since the time taken to remove the intracellular loperamide was 3.9 times the equivalent morphine concentration at equilibrium in Caco-2 cells (Table 3). These results clearly showed the inhibition of an efflux transporter resulting in such a delayed rate of transport. It was interesting to note that the other P-gp inhibitor used here, verapamil, did not affect any parameter in the efflux study. It is possible that differences occur between the way that verapamil and the cyclosporins function in their P-gp-mediated responses. Probenecid hastened the efflux of loperamide from Caco-2 cells, but equally, less loperamide was effluxed. Repeating these efflux studies with the addition of sodium azide removed all significant differences between inhibitors and normal efflux results, further indicating that active mechanisms were involved.

Discussion

Loperamide is an important systemically acting opioid drug that is known to be a P-gp substrate. It is thought that the activity of P-gp at the blood–brain barrier helps keep loperamide from reaching any central nervous system effects (Dehaven-Hudkins et al., 1999; Thompson et al., 2000). Diphenoxylate is also a systemically acting opioid similar to loperamide, yet in regard to potential P-gp-mediated efflux nothing was known about this opioid. Hence, one of the main goals of this study was to examine the properties of loperamide and diphenoxylate accumulation, transport, and efflux in Caco-2 cells and compare the results to those of morphine, recently published from our laboratory using the same cell line (Crowe, 2002). Although the Caco-2 cell line does not originate from brain endothelial tissue, its tight junctions and high P-gp expression are similar to those of the human brain. Additionally, efflux activity from in vitro Caco-2 monolayers correlates well with relative brain penetration in mdr1-knockout mouse studies (Fromm, 2000; Adachi et al., 2001). Therefore, historically, efflux-related activity in vitro has shown good relative correlations with in vivo efflux.

Morphine accumulation into our Caco-2 cell cultures was much lower than that for loperamide in these current studies (Crowe, 2002). In this study 5 µM loperamide had 13 times the equivalent morphine concentration at equilibrium in Caco-2 cells. Showing even greater accumulative ability than loperamide was diphenoxylate. Accumulation of 10 µM diphenoxylate led to a 50% increase in intracellular drug for the equivalent concentration of loperamide. These differences in opioid accumulation are likely explained by the differences in log P values of these opioids. Diphenoxylate has a log P of 5.0, which is higher than that of loperamide, with 3.9, and morphine, at only 0.98 (Drayton, 1990; Avdeen et al., 1996). The higher the log P, the greater the ability to traverse a lipid bilayer. However, bidirectional transport levels did not follow the same pattern, as loperamide had considerably greater flux in both directions compared to morphine, and diphenoxylate transport was very low. It is possible that diphenoxylate, with its very high log P, would have a reduced ability to traverse the aqueous cytoplasmic regions to the basolateral side and then out into the basolateral compartment, as would be predicted by Lipinski’s rule of five (Lipinski et al., 1997).

In our previous accumulation studies with morphine and paclitaxel, only paclitaxel showed clearly significant increases in accumulation when P-gp was inhibited by PSC-833 (Crowe, 2002). It was clear from this current study that loperamide accumulation was increased to a limited degree at low micromolar concentrations when PSC833 was used as a putative P-gp inhibitor, which was in keeping with the current literature suggesting that loperamide is a more efficient substrate for P-gp than morphine (Kusuhara and Sugiyama, 2001; Wandel et al., 2002). However, no concentration-dependent accumulation was observed in this study, indicating that loperamide could saturate its efflux transport mechanisms at low micromolar concentrations. It is thought that drugs saturating P-gp and other related transporters at low concentrations could also be competitive inhibitors of these efflux proteins. One recent study showed 20 µM loperamide to indeed have P-gp inhibitory activity on digoxin transport using Caco-2 cells (Wandel et al., 2002). In rats, it has been shown that circulating loperamide levels must reach 100 ng/ml before central nervous system effects are observed (Niemegeers et al., 1979), suggesting that this concentration is required to saturate the efflux transporters at the blood–brain barrier. At normal physiological concentrations, where systemic plasma levels of loperamide reach only 3 ng/ml in humans (Doser et al., 1995), loperamide will exhibit P-gp substrate rather than P-gp inhibitory activity.

To our knowledge, no previous studies had examined diphenoxylate transport in vitro with the aim of evaluating active efflux involvement. It was clear from this study that the P-gp inhibitor PSC-833 had no impact on diphenoxylate accumulation in these cells, which matched the low basolateral to apical transport observed in the bidirectional transport studies. The accumulation studies were repeated with 100 µM verapamil and similar results were observed (results not shown), suggesting that diphenoxylate was a very poor P-gp substrate, or at least it was unlikely that any P-gp action shown in vitro would be translated to the in vivo environment, especially with a very low net efflux of only 1 cm/s.

Bidirectional transport studies were better able to differentiate P-gp-mediated efflux between the opioids examined here and those studied earlier (Crowe and Lemaire, 1998; Crowe, 2002). Both loperamide and diphenoxylate showed
significantly higher basolateral to apical fluxes than those in the reverse direction, which was consistent with transport in an outward direction, i.e., efflux being dominant. In a recent study, loperamide transport had been examined using a transfected MDCK cell line and showed similar net flux results to those here (Wandel et al., 2002). However, unlike their results, it was shown here that 5 μM loperamide transport in the apical direction alone was at least twice the rate of morphine from our earlier work (Crowe, 2002), which we expect to be due to the increased log P of loperamide over morphine (Lipinski et al., 1997).

Although loperamide is clearly a P-gp substrate, at low micromolar concentrations it is only effluxed to twice the extent of morphine. A recent clinical trial determined that an HIV protease inhibitor could interact with P-gp and CYP3A to such an extent that loperamide levels would increase in the brain, causing central nervous system opioid effects. The significance of this study was that protease inhibitors commonly cause diarrhea, resulting in loperamide being used to correct this side effect. As loperamide is an over the counter medication, it would be difficult for medical practitioners to control for central nervous system morphine-like effects if they occurred. Their study did show dramatic increases in plasma levels of loperamide, increasing the AUC by greater than 2.5-fold, although no central nervous system effects were noted (Tayrouz et al., 2001). However, other studies have shown central nervous system effects from loperamide intoxication (Ericsson and Johnson, 1990; Motala et al., 1990; Litovitz et al., 1997; McCowat et al., 1997). In one report a child given therapeutic doses of loperamide experienced lethargy and delirium (Motala et al., 1990). Additionally, the coadministration of quinidine with loperamide in healthy male volunteers resulted in impaired centrally mediated respiratory response to carbon dioxide (Sadeque et al., 2000). The reason that quinidine may have been able to initiate central nervous system action, whereas HIV protease inhibitors, although increasing plasma levels of loperamide; were not, could be due to action on Ca²⁺ channels and cytoplasmic Ca²⁺ concentrations. Quinidine at high doses can block Ca²⁺ entry into cells in addition to P-gp inhibitory activities (Christie et al., 1992).

True calcium channel blockers, such as verapamil, nifedipine, and nisoldipine, were all able to increase the transport of loperamide through the cells in the apical to basolateral direction to a level significantly higher than the reverse direction, which implies increasing loperamide concentrations into organs that are normally protected by transport mechanisms used to extract loperamide. It has been known for over a decade that Ca²⁺ transport inhibition and P-gp function are unrelated processes (Barancik et al., 1994), and although verapamil is known to be a strong P-gp inhibitor, this is not true of the other calcium channel blockers used in this study (Barancik et al., 1994). This suggests an action other than P-gp inhibition is responsible.

If P-gp was the only active transporter involved in loperamide flux, then when P-gp is completely blocked, transport would equilibrate to the same rate in both directions, reflecting pure diffusion permeability. These events occurred when PSC-833 was used to inhibit P-gp, yet calcium channel blockers appeared to initiate a new transport process for loperamide, allowing significantly greater apical to basolateral transport. Cyclosporin A, a classic P-gp substrate and competitive inhibitor, also alters cytoplasmic Ca²⁺ levels (Park et al., 1999; Frapier et al., 2001), and this agent also allowed an increased net apical to basolateral movement of loperamide, rather than just an equivalent flux in both directions. Additionally, EGTA when used to chelate extracellular Ca²⁺ resulted in a significantly greater concentration of loperamide within Caco-2 cells. Cellular uptake was not decreased by Ca²⁺ overload though, suggesting that only low Ca²⁺ levels induce an event that leads to greater loperamide throughput.

Loperamide has been shown to have nonopioid type effects on cellular metabolism. These include functional inhibition of calmodulin and calcium channel blockades (Zavecz et al., 1982; Burleigh, 1988), so it is not surprising that an uptake mechanism involving calcium could exist for this compound. An interesting study by Harper and colleagues described loperamide activating specialized receptor-mediated calcium channels, also known as store-operated calcium (SOC) channels (Harper et al., 1997). These channels are activated when intracellular levels become low and are a separate class of Ca²⁺ channel compared to traditional voltage-gated or potential-mediated channels. The use of potent Ca²⁺ channel blockers prior to exposure of cell monolayers to loperamide may have allowed the activation of these channels in the Caco-2 cells. As loperamide is thought to increase the inflow of Ca²⁺ through these channels, it is possible that loperamide cotransported with calcium into the cell through these same channels. Additionally, Harper and his group showed that diphenoxylate and other derivatives had no effect on increasing Ca²⁺ uptake through these channels, so it is unlikely that they would be transported through this means (Harper et al., 1997). We have also found that diphenoxylate does not display the transport properties shown here for loperamide. Another study in 1995 also showed loperamide to increase Ca²⁺ entry through these channels once the channels had become activated by lowering intracellular free Ca²⁺ (Daly et al., 1995). Based on this previous evidence, it would be feasible that loperamide cotransported with Ca²⁺ through SOC channels activated through cytoplasmic Ca²⁺ stresses and that this was responsible for enhancing loperamide uptake, even in the presence of P-gp.

In conclusion, this study has shown that diphenoxylate has very little affinity for P-gp, unlike its analogue loperamide, which exhibited significant efflux parameters consistent with being a P-gp substrate, although with only moderate affinity. Of greater significance, however, was the identification of a transport system in the forward or uptake direction that appears to be related to reductions in Ca²⁺, or
interference with potential-mediated calcium channels. Further work is ongoing in the laboratory to understand this mechanism of loperamide transport.

Acknowledgments

The authors thank Dr. Simon Fox for his critical evaluation of the manuscript and Jean Wong for her technical assistance. PSC-833 and cyclosporin A were kindly donated by Novartis Pharmaceuticals (Basel, Switzerland), while nisoldipine was kindly donated by Dr. Livia Hool from the School of Physiology, University of Western Australia. This work was partially funded by a Curtin University Small Discovery Grant.

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Dantzig, A., Bergin, L., 1990. Interference with potential-mediated calcium channels. Further work is ongoing in the laboratory to understand this mechanism of loperamide transport.


