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RESEARCH PAPER

pH Dependent Uptake of Loperamide Across the Gastrointestinal Tract: An In Vitro Study

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ABSTRACT

Loperamide is a peripherally acting antidiarrheal opioid with some affinity for P-glycoprotein (P-gp). One of the main reasons for its lack of central nervous system (CNS) activity is a combination first-pass metabolism and P-gp-mediated efflux preventing brain penetration. It was assumed that P-gp would also have a similar effect at the intestinal tract, limiting loperamide systemic absorption. However, previous in vitro studies had not determined loperamide flux using pH gradients present in the intestinal tract. Hence, our aim was to determine the influence of pH gradient conditions on the gastrointestinal uptake of loperamide, including any changes to its P-gp-mediated efflux. *Methods.* Cellular uptake and transcellular transport were determined after exposure to various concentrations of loperamide (2–50 μM) with and without the presence of active efflux protein inhibitors. Loperamide was detected at 214 nm using high-performance liquid chromatography (HPLC) protocols. *Results.* Bidirectional transport studies of 10 μM loperamide with a pH 6.0/7.4 apical (Ap)-to-basolateral (Bas) gradient showed efflux to be 17-fold higher than influx (10 ng/cm^2/min Bas → Ap compared to 0.6 for Ap → Bas). This differential was much greater than when examined at pH 7.4/7.4 (only two-fold higher). The potent P-gp inhibitor, PSC-833, had only a moderate effect at blocking loperamide efflux under pH gradient conditions, yet could equilibrate bidirectional transport at pH 7.4. This suggested the presence of significant P-gp independent mechanisms, preventing loperamide access to the basolateral chamber. Amiloride and 5-(N-ethyl-N-isopropyl) amiloride had some effect on reducing efflux, hence the $Na^+ - H^+$ antiporter may have some involvement. Accumulation of loperamide into Caco-2 cells reduced almost 70% at pH 6.0 compared to pH 7.4, yet P-gp was always able to approximately double the equilibrium concentration in the cells within a defined pH study. This showed that P-gp was not affected by pH conditions. *Conclusions.* P-gp-mediated efflux of loperamide is supplemented under pH gradient conditions. Hence, drugs used to

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decrease acid secretion in the stomach could result in higher plasma loperamide levels based on our in vitro system reflecting the in vivo environment. The addition of a P-gp inhibitor could potentially further increase the gastrointestinal absorption of loperamide.

Key Words: Amiloride; Ca^{2+} channel blockers; PSC-833; P-gp; Diphenoxylate; H^+ antiport.

INTRODUCTION

Morphinelike opioids were considered to be an effective drug treatment for diarrhea; however, their use has been limited as in vivo studies showed a low degree of separation of unwanted central nervous system effects from their antidiarrheal properties.[1,2] Loperamide (Imodium®) has largely replaced the use of other opiate-like antidiarrheal prototype agents[3,4] because of its marked separation of central and peripheral activities. This was thought to be due to a large first-pass effect combined with poor permeability of the blood–brain barrier (BBB), partially as a result of its efflux through p-glycoprotein (P-gp) mediated transport.[2,5]

P-gp within the BBB has a direct role in effluxing many chemotherapeutic agents from the brain back to the capillary circulation. In addition, the activity of P-gp may be a factor that limits entry of drugs from the intestinal lumen into the systemic circulation.[6] Unlike other organs, the gastrointestinal tract provides a unique environment of cellular transport due to the presence of the Na^+-H^+ antiporter in the intestinal tract and the incomplete neutralization of stomach acid entering the intestinal tract, both of which are responsible for an acidic micro-climate and pH gradient conditions within the lumen of the intestine.[7,8] Therefore, the significance of P-gp mediated efflux of loperamide in the intestine may be different from systemic organs as a result of pH gradient properties unique to the gastrointestinal tract.

Current research within our laboratory has suggested that P-gp may not be very potent at preventing loperamide uptake into organs of the body when the drug is at low micromolar concentrations.[9] It is known that bioavailability of orally administered loperamide is very low,[10] and P-gp mediated efflux has been implicated in this low bioavailability.[11] However, our in vitro results at pH 7.4 suggested loperamide is able to pass through tight monolayers with overexpressed P-gp at greater rates than many other P-gp substrates. Hence, intestinal efflux of loperamide via P-gp may not be an effective barrier to initial loperamide transport into the body. This implies that coadministration with P-gp inhibitors may not have a large effect on circulating loperamide concentrations. Therefore, the aim of this study was to determine the effectiveness of both uptake and efflux mechanisms in Caco-2 cell monolayers, as an in vitro model of the gastrointestinal tract, using a pH gradient system in bidirectional transport and accumulation studies.

METHODS

Materials

Loperamide, probenecid, lansoprazole, sodium ortho-vanadate, amiloride, 5-(N-ethyl-N-isopropyl) amiloride (EIPA), nifedipine, and nimodipine were supplied by Sigma Aldrich (Castle Hill, NSW, Australia). PSC-833 and cyclosporin A were kindly donated by Novartis Pharmaceuticals (Basel, Switzerland). Verapamil hydrochloride and chlorpheniramine were purchased from ICN Biomedicals (Seven Hills, NSW, Australia). Nisoldipine was kindly donated by Dr. Livia Hool from the School of Physiology, University of Western Australia. All other materials were of analytical grade.

Cell culture reagents: Phosphate buffered saline (PBS), Hanks buffered salt solution (HBSS), 4-(2-hydroxyethyl)piperazine-1-ethansulfonic acid (HEPES), and high glucose Dulbecco's Modified Eagle 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 (FCS) was obtained from the Australian Commonwealth Serum Laboratories (Parkville, Vic, Australia).

Cell Transport Studies

Caco-2 cells were seeded onto Falcon polyethylene teraphthalate (PET) 0.9 cm^2 filter inserts in 12 well plates at 65,000 cells/cm^2. Cells were grown in growth medium (high glucose DMEM with 25 mM HEPES, 2 mM glutamine, 1 mM nonessential amino acids,

100 U/mL penicillin-streptomycin, and 10% FCS set at pH 7.4 in a 37°C incubator with 5% CO_2.

Monolayers were incubated for 21–25 days to allow full maturation of the cells, including P-gp expression and increased transepithelial electrical resistance (TEER) formation.[12,13] The TEER was measured both before and immediately after the study using an epithelial voltage/ohm meter (EVOM) and the ENDOHM 12 chamber (World Precision Instruments, Sarasota, FL) with readings between 390–800 $\Omega.cm^2$ 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 $\Omega.cm^2$ are 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 to give final concentrations of 25 and 10 mM, respectively. The pH was adjusted to either 7.4 or 6.8 using 1M NaOH. For pH 6.0 studies, 10 mM Bis-Tris (USB, Cleveland, OH) was used instead of HEPES and the pH adjusted with 1 M HCl.

Cells were incubated in prewarmed HBSS +/− an efflux inhibitor for 30 min at the correct pH, and then rinsed in the same medium. The TEER was measured and HBSS +/− inhibitors were placed in the receiver chambers. Loperamide at various concentrations between 5 and 50 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-hour 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.

P-gp and Other Transport Inhibition

In studies where inhibition of P-gp or multi drug resistance-associated protein (MRP1) were performed in conjunction with loperamide, 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. The MRP1 inhibitor 500 μM probenecid was also used. To explore any remote proton pump activity influencing transport, 50 μM lansoprazole was used, while the H_1 receptor blocker 500 μM chlorpheniramine was also trialed, as it has been shown to inhibit H^+ linked transport of other H_1 receptor blockers.[14] Amiloride can block the intestinal Na^+-H^+ transporter, among other H^+ transporters, and was included in this study at 1 mM, while its more potent analogue, EIPA with greater specificity for the Na^+-H^+ antiporter was used in this study at 100 μM. The calcium channel blockers, nifedipine, nimodipine, and nisoldipine were also used at 100, 50, and 50 μM, respectively, to determine whether pH adjustments could influence the Ca^{2+} affected transport of loperamide. During the transport study these modifying agents were also present in the donor chamber with loperamide and in the receiver chamber, at the same concentrations as stated above.

Metabolic Inhibition

In studies where the active transport of loperamide was examined, Caco-2 monolayers were subjected to a 20-min exposure of 10 mM sodium azide in HBSS with 50 mM 2-deoxy-D-glucose replacing D-glucose in order to deplete functional ATP concentrations.[15] Continuous exposure to sodium azide at this concentration would be severely damaging to the cells. Therefore, after the preincubation period, the concentration of azide and deoxy glucose was reduced to 20% of initial concentrations when coincubated with loperamide.

Cell Accumulation

Caco-2 cells were grown in 24-well culture plates using growth medium at an initial seeding density of 5000 cells/cm^2. The cells were used 21–25 days after seeding. Loperamide was added at either 5, 10, or 20 μM in HBSS for various times up to 90 min. Diphenoxylate accumulation was also determined with a 20 μM initial concentration. Triplicate cell wells were washed 3× in ice cold PBS before 500 μL of H_2O 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.

Equilibrium Study

These transport studies were conducted under two different apical pHs, each being incubated with or without 4 μM PSC-833 throughout the study. This was a modified cell transport study where an equal concentration of loperamide (2 μM) was placed into both apical and basolateral chambers after an initial 30 min pre-equilibration step using transport medium without loperamide. This step removed the gradient effect of the drug itself, removing the diffusional component of transport. Only active mechanisms within Caco-2 cells should allow greater apical or

basolateral sequestering. As with the initial transport studies, volumes were 0.5 mL in the apical chamber and 1.4 mL in the basolateral chamber, which was designed to form an equilibrated pressure differential across the membrane, ensuring that no bulk volume flux altered loperamide movement. Samples were removed from both chambers at various times up to 3 hours after initial loperamide incubation. Medium was replaced on both sides with prewarmed, fresh medium at the correct pH. All calculations to allow for dilution effect were conducted using Microsoft Excel for Windows XP.

Protein Determinations

Protein concentrations for all aspects of this study were determined using a micro-Lowry method adapted for use with multiwell plates on a Sunrise 96 well plate spectrophotometer with a 750 nm filter (TECAN, Maennedorf, Switzerland) using Magellan 3 software for Windows NT 4.0.

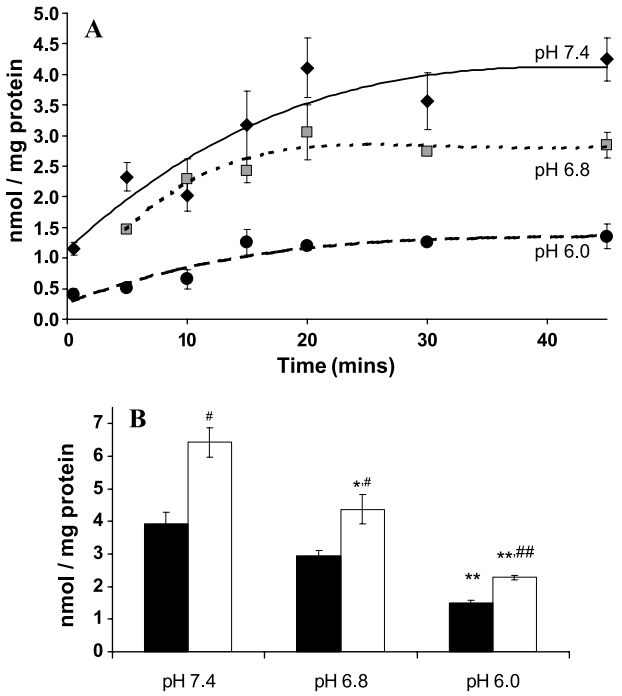

Figure 1. A) Accumulation of 5 μM loperamide into Caco-2 cell cultures over 60 min using medium of pH 7.4, 6.8, or 6.0. B) The effect of coincubation with 4 μM PSC-833 on loperamide accumulation after 60 min at each of the three pH values tested. Each data point is the mean of 3 samples ± SEM. # Significant increases in loperamide accumulation with PSC-833. * Significant reductions in loperamide accumulation as pH changed from 7.4. * or #=$p<0.05$, ** or =$p<0.005$.

HPLC Analysis

Samples collected in HBSS from efflux and transport studies were immediately collected into 450 μL wells of polypropylene 96-well plates. No extraction phases were required. The mobile phase consisted of 25 mM KH_2PO_4 (BDH-Merck, Kilsyth, Victoria) (pH 2.8): Far-UV grade acetonitrile (EM Science, Gibbstown, NJ) (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-C_{18} column, 5 μm pores, 15 cm × 4.6 mm I.D. with mated guard column (Agilent, NSW, Australia). 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 minutes, respectively.

Opioid 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 diphenoxylate from the cells. Pelleting of cellular debris for 3 min at 8500 g resulted in a visually clear solution that had clear chromatograms at typical elution times. The detection limit for loperamide was 40 nM 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 min, which would vary depending on the initial concentration used, and as an apparent permeability coefficient as calculated previously.[16]

Results in this study are presented as the mean ± standard error of the mean (SEM), standardized on individual protein concentrations. Significant differences between values were examined using Student's two-tailed unpaired t-test or one way analysis of variance (ANOVA). Results were considered significant if $p<0.05$.

RESULTS

Accumulation Studies

Accumulation studies were performed at pH 6.0, 6.8, and 7.4 using 5 μM loperamide as shown in Fig. 1A. Equilibrium was achieved between 20 and

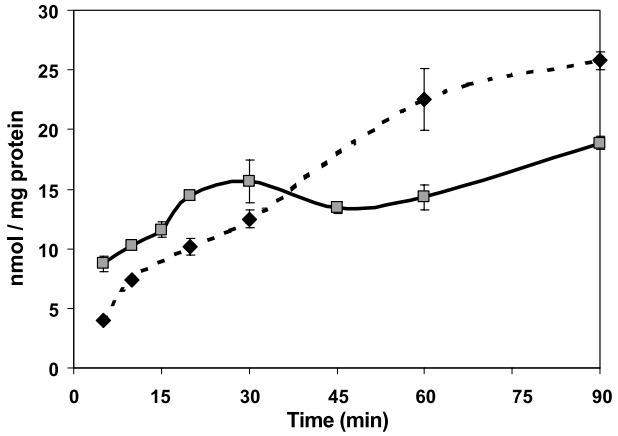

Figure 2. Accumulation of 20 μM diphenoxylate in Caco-2 cells over 90 min using medium at pH 7.4 (dotted line) or pH 6.0 (solid line). Results are the means ± SEM of triplicate samples.

30 min after initial uptake of loperamide, regardless of the pH examined. The equilibrium concentration of loperamide accumulated at pH 7.4 was around 4 nmol/mg protein, which represented approximately 24% of the available loperamide in the wells. Reducing the extracellular pH to 6.8 resulted in a reduction in maximal accumulation to 2.8 nmol/mg. Lowering the extracellular pH to 6.0 resulted in a 70% reduction in accumulation compared to pH 7.4, with only 1.3 nmol/mg being the maximum intake, which represented approximately 7% of the available loperamide in the wells. The pKa of loperamide is 8.66, hence, pH values used in this study would have had loperamide from 1.26 to 2.66 pH units below its pKa (94.5% to 99.8% ionized). Although the reduction in loperamide accumulation at pH 6.0 can be partially attributed to the lower percentage of unionized species at pH 6.0, the total percentages involved in cellular accumulation suggest that the ionized species are also taken up by the Caco-2 cells in significant amounts, and that mechanisms other than physical chemistry changes in loperamide are responsible for the observed effect.

Each of these accumulation studies was repeated with the addition of the P-gp inhibitor, PSC-833. At all pH values the use of PSC-833 increased the maximal accumulation of loperamide. For example, at pH 7.4, PSC-833 increased intracellular drug concentrations from 4.0 nmol/mg to 5.6 nmol/mg protein when cells were exposed to 5 μM loperamide, an increase of 40% over non-P-gp inhibited conditions. Increases of approximately 40% at pH 6.8 and 50% for pH 6.0 were also observed (Fig. 1B). Therefore, although the amount accumulated became significantly lower as the extracellular pH dropped, P-gp was still able to function. Accumulation at 4°C using medium of pH 6.0 showed no detectable accumulation of loperamide. Even after 1 hour, any accumulation within the Caco-2 cells was below detection limits (results not shown).

To illustrate the unique properties of loperamide uptake using pH gradient conditions, a related systemic opioid, diphenoxylate, was also examined for accumulation in this system. It was found in this current study that diphenoxylate was not greatly affected by a pH reduction to 6.0, even though its pKa of 7.1 would dramatically reduce the unionized component of this compound at pH 6.0 compared to pH 7.4. There was some evidence that after 45 min of continuous exposure, more diphenoxylate associated with Caco-2 cells at ph 7.4 than at pH 6.0 (Fig. 2); however, any effect was slight in comparison to that observed for loperamide.

Calcium channel blockers with minimal P-gp inhibitory action were able to increase loperamide accumulation at pH 7.4 as determined from recent studies within our laboratory,[9] hence, this finding was also explored here using pH 6.0. In this study, the calcium channel blocker nifedipine, used at 100 μM, significantly increased loperamide accumulation ($p<0.003$) when using the pH 6.0 buffered transport medium (Fig. 3), just as it had when pH 7.4 was used.

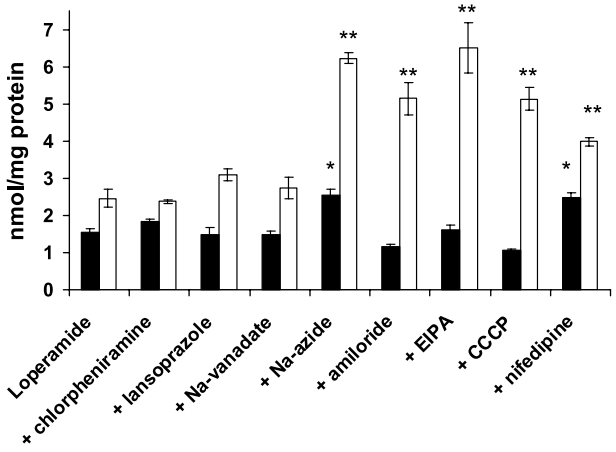

Figure 3. The accumulation of loperamide in Caco-2 cell cultures after 5 min (black columns) and 60 min (outlined columns) exposure to the drug (10 μM) in pH 6.0 medium. Cells were coincubated with inhibitors of energy metabolism, specific channels, or carriers for 30 min prior to, and during, the administration of loperamide. Each result is the mean of three to eight samples ± SEM. * Significance of coincubation of loperamide with the additional compounds. *=$p<0.05$, **=$p<0.005$.

The H_1 receptor blocker, chlorpheniramine, and the proton pump inhibitor, lansoprazole, increase the pH of the stomach, which may result in higher loperamide accumulation in vivo (see Fig. 1), but were not expected to have a direct role in influencing loperamide accumulation at pH 6.0 in vitro, as these agents would not be expected to directly affect Caco-2 cells. The results here confirm this (Fig. 3). There is a belief by some authors that P-gp ATPase is not responsive to Na-azide,[17] so a more specific ATP binding cassette (ABC)-ATPase inhibitor, sodium ortho-vanadate, was also used in some studies to expand analysis of transport requiring ATP. At pH 6.0, vanadate had no significant effect on loperamide accumulation, while the more general energy inhibitor Na-azide was clearly effective at increasing loperamide concentrations. The concentration of loperamide increased from 2.4 nmol/mg protein to over 6.0 nmol/mg ($p<0.001$) when Caco-2 cells were exposed to 10 mM Na-azide and 50 mM 2-deoxy-D-glucose for 20 min prior to the accumulation study. This signified that P-gp was unlikely to be involved, yet it was evident that a different ATP dependent efflux process was transporting loperamide at this pH.

The intestinal Na^+-H^+ antiporter inhibitor, amiloride, was shown to significantly increase the accumulation of loperamide into Caco-2 cells, as was its more potent analogue that has a very high affinity for the Na^+-H^+ antiporter[18] (Fig. 3), which provided clear evidence for the involvement of an H^+ antiporter in the pharmacodynamics of loperamide flux.

Transport Studies

Transport studies examining loperamide movement through Caco-2 cells rather than just into the cells allow a closer in-vivo-like analysis of loperamide transport. The use of an apical pH of 6.0 and a basolateral pH of 7.4 emulated intestinal lumen and mesenteric vein conditions. These pH gradient bidirectional transport studies showed a basolateral-to-apical flux that was 17-fold greater than in the opposite direction (10 ng/cm^2/min compared to 0.6 ng/cm^2/min). When this pH 6.0/7.4 gradient was replaced with pH 7.4 medium on both sides, efflux rates were only 2.2-fold greater than influx (4.6 ng/cm^2/min compared with 2.1 ng/cm^2/min). Apical-to-basolateral transport and

Table 1. Transport rates of 10 and 50 μm loperamide through caco-2 cell monolayers in both apical (Ap)-to-basolateral (Bas) and Bas-to-Ap directions from cells grown on filter inserts suspended in culture using a pH 7.4 buffer on the basolateral side of the monolayer, and either a pH 7.4 or 6.0 buffer on the apical side.

Concentration (μM) ± efflux modifiers	Apical pH	Ap → Bas (pg/cm^2/min)	Bas → Ap (pg/cm^2/min)	Fold difference (net flow direction)
10	7.4	2086 ± 199	4606 ± 390[b]	2.2 (efflux)
10	6.0	578 ± 45	10023 ± 677[c]	17.4 (efflux)
10 + PSC-833	7.4	2885 ± 145[a]	3223 ± 285[a]	1.1 (no net flux)
10 + PSC-833	6.0	1630 ± 33[a]	6720 ± 399[b]	4.1 (efflux)
10 + verapamil	6.0	1172 ± 150[a]	6662 ± 155[a,c]	5.7 (efflux)
10 + nimodipine	6.0	950 ± 18[a]	5562 ± 172[a,b]	5.8 (efflux)
10 + nisoldipine	6.0	927 ± 32[a]	6226 ± 657[a,c]	6.7 (efflux)
10 + nifedipine	6.0	1009 ± 28[a]	5866 ± 368[a,c]	5.8 (efflux)
10 + amiloride	7.4	3350 ± 175[a]	2758 ± 114[a,b]	1.2 (uptake)
10 + amiloride	6.0	728 ± 27	4629 ± 138[a,c]	6.4 (efflux)
10 + EIPA	7.4	5446 ± 241	6679 ± 146[b]	1.2 (efflux)
10 + EIPA	6.0	1607 ± 27	10347 ± 596[c]	6.4 (efflux)
10 (pH 6 BASO)	6.0	2566 ± 168	8102 ± 69	3.2 (efflux)
50	7.4	14104 ± 1188	24415 ± 2703[b]	1.7 (efflux)
50	6.0	2109 ± 122	31419 ± 3414[a]	14.9 (efflux)

Results are described as picograms per cm^2 per min during the initial 180 min transport. Drugs were used at the following concentrations: PSC-833—4 μM, verapamil, nifedipine, and EIPA—100 μM, nimodipine and nisoldipine—50 μM, while amiloride was used at 1 mM.

[a]$p<0.05$. Significance of inhibitor additions in relation to controls for a particular direction.

[b]$p<0.05$. Significance of transport difference in each direction within a single group.

[c]$p<0.005$. Significance of transport difference in each direction within a single group.

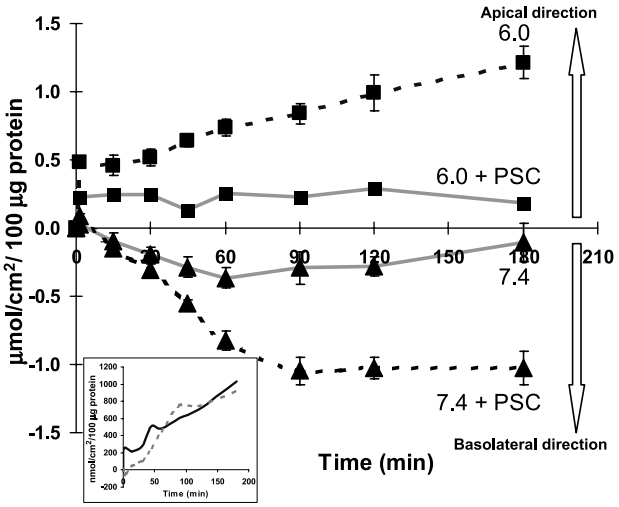

Figure 4. Net loperamide flux in the apical (positive) or basolateral direction (negative) during a 3-hour study in Caco-2 cell monolayers where 2 µM loperamide was placed in both apical and basolateral chambers simultaneously. One study was performed with both apical and basolateral chamber pH at 7.4, while another study had apical pH reduced to 6.0. Each study was performed with or without 4 µM PSC-833 present on both sides of the monolayer. The insert shows the purely P-gp-dependent effect calculated from the PSC-833 inhibition of P-gp compared to no inhibition for each pH study.

that in the reverse direction were significantly reduced and increased respectively to produce this comprehensive efflux event with an apical pH of 6.0 (Table 1). These effects signified that under a proton transcellular gradient, as would be expected at the duodenum in vivo, efflux within the gastrointestinal tract would be significantly greater than determined from pH 7.4 studies.[19] Even the use of 50 µM loperamide did not affect the efflux driven system, pushing loperamide into the apical compartment (Table 1). The transport rates increased because of the higher concentration of loperamide (50 µM rather than 10 µM), yet the 17-fold difference remained. These results suggest that the pH gradient shown in the earlier parts of the small intestine would have a significant role in preventing absorption of loperamide from the upper small intestinal tract (apical side) into the blood (basolateral side) and may be of equal importance with P-gp in keeping loperamide out of the circulation.

Using PSC-833, which was the most potent inhibitor of P-gp used in this study, the efflux ratio was reduced from 17 to around 4. The efflux ratio is a measure of the fold difference in transport in the basolateral-to-apical direction compared with that in the apical-to-basolateral direction. Hence, this P-gp inhibitor was able to increase apical-to-basolateral transport to a significant degree, but still left a sizable efflux gradient that was unlikely to be due to P-gp mediated action (Table 1). These data differed from those observed when pH 7.4 conditions were present on both sides of the membrane where PSC-833 was able to completely inhibit efflux of loperamide (Table 1).

The calcium channel blockers were less effective than PSC-833 at reducing efflux in the presence of the pH 6.0/7.4 proton gradient, retaining a 5.5- to 7-fold efflux gradient after exposure. This was vastly different to that observed when the pH was set at 7.4 on both sides in an earlier study. Under those conditions, all traditional voltage-gated calcium channel blockers, irrespective of their P-gp inhibitory activity, accelerated transport of loperamide in the Ap→ Bas direction, such that uptake became the dominant direction of transport.[9] Yet, in this study, the presence of a proton gradient was enough to eliminate uptake, relegating net transport back to the efflux direction.

The results from the equilibrium study are shown in Fig. 4, where 2 µM loperamide was placed in both chambers to eliminate any drag coefficients and to remove the diffusion component of transport. This left only carrier-based transport mechanisms evident. Using pH 6.0 in the apical chamber, enabling a proton gradient, was enough to illustrate significant basolateral-to-apical efflux as loperamide continued to accumulate

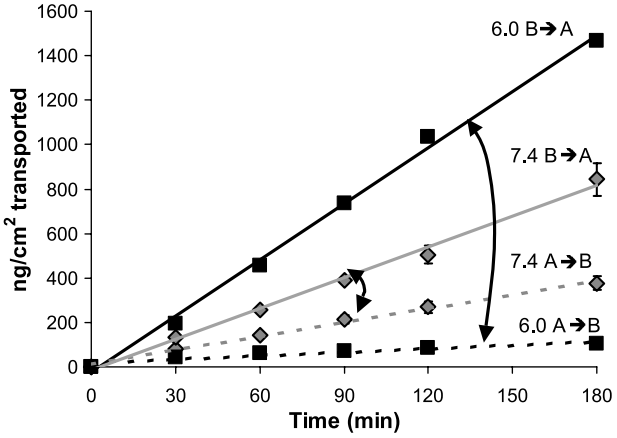

Figure 5. Bidirectional transport of 10 µM loperamide under two different apical pH conditions through 3-week-old Caco-2 cells grown on filter inserts. Transport is expressed as ng/cm^2. All studies were conducted with a basolateral pH of 7.4. Apical pH was either 7.4 (◆) or pH 6.0 (■). Apical-to-basolateral direction of both sets of data is shown as dotted lines, while the basolateral-to-apical direction is shown as solid lines. Results are the mean of three to six samples ± SEM.

in higher quantities in the apical compartment. Using pH 7.4 on both sides of the membrane resulted in an initially very small basolateral directed transport, followed by diffusion as the major contributor to transport. This was surprising, as loperamide placed on only one side of the membrane clearly showed greater basolateral-to-apical transport than apical-to-basolateral transport in traditional transport studies (Table 1; Fig. 5). PSC-833 significantly reduced efflux using pH 6.0/7.4, leaving a reduced efflux potential into the apical compartment, while at pH 7.4/7.4 the small basolateral transport became a significant basolateral movement of loperamide, which suggested a transport mechanism for the accumulation of this drug rather than its efflux. It was clear that P-gp activity was not affected by pH changes, as was evident from Fig. 5 (insert), which shows only the effect of P-gp, by subtracting the PSC-833 affected transport from uninhibited transport under the same pH conditions. Hence, P-gp was working to efflux loperamide to the same degree at each pH, with the main overall difference in direction of transport being the pH conditions themselves.

DISCUSSION

It has been shown in this study that loperamide transport through, and into, Caco-2 cell monolayers was reliant on the extracellular pH to determine the extent of its transport. Slightly acidic conditions present in the upper gastrointestinal tract are likely to result in little loperamide entering the blood circulation based on the limited accumulation and transport evident from this in vitro study. It is likely that loperamide is not only a substrate for P-glycoprotein, but also for a proton-based efflux pump. It is known that some drugs such as diphenhydramine, enalapril, and captopril, and peptide based drugs such as cephalosporin, utilize proton dependent uptake or symport mechanisms to cross the gastrointestinal tract,[14,20,21] and that accelerated uptake occurs due to the proton gradient established when stomach acid is not being completely neutralized in the upper small intestine. Hydrogen ions are pumped into the apical lumen, mainly by Na^+-H^+ antiporters to establish an acidic microclimate that is maintained along the gastrointestinal tract by the presence of microvilli on enterocytes.[7] This ensures that when luminal pH increases above 7.0 in the lower intestine, there is still a pH gradient for transport systems to utilize in molecular transport at the transfer surface on enterocytes. It is this principle that allows many amino acids and di/tri peptides to be absorbed in the direction of the pH gradient.[22] It is proposed from this study that the opposite is occurring for loperamide. It would enter the cell readily due to its high lipophilicity, yet would be effluxed through an H^+ antiport system as well as through P-glycoprotein to prevent it from reaching the blood side of the enterocyte. Caco-2 cells have already been shown to exhibit active Na^+-H^+ antiporters,[20] located on the basolateral side of differentiated cells;[23] however, it is currently unknown what other H^+ antiporters may be present in this cell line.

P-glycoprotein was clearly not the only contributor to loperamide efflux in this study. Results from the equilibrium study showing P-gp to be equally effective regardless of the pH conditions, and the lack of response to the selective ATPase inhibitor, sodium vanadate, reinforce the limited effect that potent P-gp inhibitors had on loperamide flux under pH gradient conditions. Additionally, as the more generic metabolic inhibitor, sodium azide, was able to increase loperamide absorption into Caco-2 cells, the likelihood of a separate energy-dependent process causing the efflux of loperamide is evident. Amiloride, an inhibitor of the Na^+-H^+ antiporter, was effective at increasing loperamide accumulation and at reducing efflux in the transport studies. However, at the concentrations used in this study, it was unknown how specific amiloride was for the Na^+-H^+ antiporter, or whether other H^+ antiporters were also affected. Yet, when the $200\times$ more potent amiloride analog (EIPA) that is more selective for the Na^+-H^+ antiporter was used[18] at a $10\times$ lower concentration, the fold difference between $Ap \rightarrow Bas$ and $Bas \rightarrow Ap$ transport was exactly the same as amiloride. Even if there was a different H^+ antiporter being blocked by these amiloride agents, it was clear from this study that a proton antiport-based efflux system existed for loperamide contributing heavily to the reduced bioavailability of this drug.

A previous study had tentatively linked P-gp to Na^+-H^+ antiporter activity, by showing that EIPA was able to increase the intracellular accumulation and improve the cytotoxicity of doxorubicin in resistant LoVo intestinal carcinoma cells.[18] However, other work stated that P-gp activity is not affected by extracellular pH,[24] just as our own equilibrium study showed equivalent P-gp activity regardless of the pH changes made. This clearly implied that a different transporter was responsible for our efflux data rather than modifications to P-gp activity under these conditions.

In terms of opioid analgesic transport, a recent publication showed pentazocine, a structurally unrelated opioid-antagonist, to exhibit decreased transport

into the brain of rats when the pH of carotid artery perfusate was reduced to 6.5 and further to 5.5.[25] The increase in excretory action shown in this current study for loperamide may be related to this efflux system shown in situ for pentazocine, especially as these authors also showed a two fold reduction in brain transport at pH 7.4 when verapamil was present in the perfusate.[25]

It has been shown in animal studies that loperamide in the circulation can be encouraged to cross the BBB and cause central nervous system (CNS) effects, and that blockage or bypass of P-gp-mediated efflux is a key factor allowing significantly greater intracellular concentrations of loperamide to initiate this effect.[11,26] However, with regard to gastrointestinal uptake of loperamide being influenced by P-gp inhibitors, this would be much less efficient than inhibition within the circulation, as the efflux generated by the pH gradient system would equal that of any P-gp-mediated efflux. Thus, only moderate increases in plasma loperamide concentration would occur from the gastrointestinal tract when only using a P-gp inhibitor to promote absorption. For example, only a doubling of peak plasma levels has been seen in both mdr1a knockout mice and in human trials with ritonavir inhibition of intestinal P-gp.[11,27] An increased risk of CNS effects even with moderate increases in plasma loperamide concentrations may still be possible, especially as some patients have shown CNS effects from loperamide intoxication.[28–31] In one clinical trial, the coadministration of quinidine with loperamide in healthy male volunteers resulted in impaired centrally mediated respiratory response to carbon dioxide.[32]

From our current study, it was shown that transport in the uptake direction through Caco-2 cells with a H^+ gradient was improved when P-gp was blocked, which conformed with these animal- and human-based trials. However, as there appeared to be additional pH-mediated efflux mechanisms, it is possible that removal of the acidic gradient would vastly increase the peak levels in these studies beyond the doubling observed in vivo.[11,27] Hence, there is potential for agents that increase the pH in the stomach to increase the absorption of loperamide, which may allow greater concentrations within the body to cause any observed CNS effects.

There are few studies showing efflux of drugs through Caco-2 cells against the H^+ ion gradient. One such molecule is diphenhydramine and it has been shown to have relatively greater efflux as apical pH is dropped from 7.4 to 6.0. It was suggested that an organic cation/H^+ antiport system in the Caco-2 cells explained this data.[33] As loperamide is also an organic cation with pH-related efflux properties similar to those of diphenhydramine, it was initially speculated that similar mechanisms could have been in use for these two drugs. However, chlorpheniramine was able to inhibit the pH-dependent efflux of diphenhydramine,[14] yet was not able to alter accumulation of loperamide in this current study (Fig. 3), which indicated that a different transporter was involved in this case. Another study showed celiprolol to have greater basolateral-to-apical flux through Caco-2 cells with decreasing apical pH.[34] However, this study did not examine apical-to-basolateral transport, making it impossible to evaluate net transport rate alterations under acidic apical pH conditions. Regardless, amiloride increased the basolateral-to-apical flux of celiprolol,[34] which was opposite to that shown here for loperamide. Hence, no studies to our knowledge have shown transport characteristics comparable to those shown here for loperamide. Further studies will be necessary to isolate the transporter responsible for loperamide efflux.

A study from the late 1980s by Balkovetz et al. showed that loperamide was able to inhibit Na^+ uptake through the Na^+-H^+ antiporter using placental brush border vesicles.[35] It was concluded that loperamide bound to multiple sites on the Na^+-H^+ antiporter.[35] Our current study suggests that transport of loperamide may also be associated with this transmembrane antiporter, and not just that of sodium ions.

In conclusion, as mature differentiated Caco-2 cells display properties similar to epithelial cells from the small intestine, it is possible that decreasing the pH gradient across the upper gastrointestinal tract could result in a greater proportion of loperamide being absorbed from the lumen. Additionally, as loperamide is a P-glycoprotein substrate, using both acid lowering agents in combination with P-gp inhibitors could significantly increase the systemic availability of loperamide. Proton pump inhibitors like lansoprazole and H_2 receptor antagonists like cimetidine and ranitidine, are also known to be P-glycoprotein substrates themselves[36] and hence, may have additional direct effects on increasing loperamide absorption besides the increase in pH in the small intestine caused by their pharmacological action.

ABBREVIATIONS

BBB	Blood–brain barrier
CNS	Central nervous system
DMEM	Dulbecco's modified eagle medium
EIPA	5-(N-ethyl-N-isopropyl) amiloride

EVOM	Epithelial voltage/ohm meter
FCS	Fetal calf serum
HBSS	Hanks balanced salt solution
PET	Polyethylene teraphthalate
P-gp	P-glycoprotein
TEER	Transepithelial electrical resistance

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