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Segmental Dependent Transport of Low Permeability Compounds along the Small Intestine Due to P-Glycoprotein: The Role of Efflux Transport in the Oral Absorption of BCS Class III Drugs

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Abstract: The purpose of this study was to investigate the role of P-gp efflux in the *in vivo* intestinal absorption process of BCS class III P-gp substrates, i.e. high-solubility low-permeability drugs. The *in vivo* permeability of two H_2-antagonists, cimetidine and famotidine, was determined by the single-pass intestinal perfusion model in different regions of the rat small intestine, in the presence or absence of the P-gp inhibitor verapamil. The apical to basolateral (AP−BL) and the BL−AP transport of the compounds in the presence or absence of various efflux transporters inhibitors (verapamil, erythromycin, quinidine, MK-571 and fumitremorgin C) was investigated across Caco-2 cell monolayers. P-gp expression levels in the different intestinal segments were confirmed by immunoblotting. Cimetidine and famotidine exhibited segmental dependent permeability through the gut wall, with decreased P_{eff} in the distal ileum in comparison to the proximal regions of the intestine. Coperfusion of verapamil with the drugs significantly increased the permeability in the ileum, while no significant change in the jejunal permeability was observed. Both drugs exhibited significantly greater BL−AP than AP−BL Caco-2 permeability, indicative of net mucosal secretion. Concentration dependent decrease of this secretion was obtained by the P-gp inhibitors verapamil, erythromycin and quinidine, while no effect was evident by the MRP2 inhibitor MK-571 and the BCRP inhibitor FTC, indicating that P-gp is the transporter mediates the intestinal efflux of cimetidine and famotidine. P-gp levels throughout the intestine were inversely related to the *in vivo* permeability of the drugs from the different segments. The data demonstrate that for these high-solubility low-permeability P-gp substrates, P-gp limits *in vivo* intestinal absorption in the distal segments of the small intestine; however P-gp plays a minimal role in the proximal intestinal segments due to significant lower P-gp expression levels in this region.

Keywords: P-glycoprotein; intestinal absorption; BCS class III drugs; segmental dependent permeability; efflux transporters

Introduction

Oral bioavailability is mainly dependent on three general processes: the fraction of dose absorbed (F_a); the fraction of drug escaped from metabolism in the gut wall (F_g); and the fraction of drug escaped from hepatic metabolism (F_h).[1−3] Hence, the oral bioavailability of a drug is mainly a function of effective permeability (P_{eff}) across the intestinal mucosa, solubility characteristics in the gastrointestinal (GI) milieu and metabolic stability.[4,5] Through the use of data obtained from solubility and permeability experiments, an active

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moiety can be classified into one of four biopharmaceutical classes: class I (high solubility, high permeability); class II (low solubility, high permeability); class III (high solubility, low permeability); class IV (low solubility, low permeability).[6] This Biopharmaceutical Classification System (BCS) is one of the most significant prognostic tools created to facilitate product development in recent years, and has been adopted by the FDA for setting bioequivalence standards for drug product approval.

P-glycoprotein (P-gp) is a 170 kDa glycosylated transmembrane efflux pump, which was first characterized as the ATP-dependent transporter responsible for efflux of chemotherapeutic agents from multidrug resistant (MDR) cancer cells. P-gp is widely expressed in many tissues, such as the membrane of endothelial cells in the intestine,[7,8] liver,[7] kidney,[9] placenta,[10] blood−brain barrier[11] and blood−testis barrier.[12] P-gp is present on the villus tip of the apical brush border membrane of gut enterocytes and actively causes efflux of drugs from gut epithelial cells back into the intestinal lumen.[7,8,13] The question as to whether or not a certain P-gp substrate is likely to show P-gp efflux dependent intestinal absorption *in vivo* is still to be addressed. While several researchers have suggested a very limited influence of P-gp on *in vivo* intestinal absorption,[14−19] other authors have found that P-gp substrates do show P-gp dependent intestinal permeability.[20−22] The BCS was designed to scientifically predict the rate-limiting step in the absorption process of drugs following oral administration; however, the BCS may be used for better prediction of the significance of efflux systems in the *in vivo* intestinal absorption process of a given drug. Recently, Cao et al. have shown that the *in vivo* intestinal absorption of BCS class I P-gp substrates, i.e. high-solubility high-permeability drugs, is dominated by their permeability properties, and that P-gp plays a minimal role in the intestinal absorption process of this class of drugs.[1]

The first purpose of the present study was to evaluate the significance of P-gp efflux in the *in vivo* intestinal absorption process of BCS class III P-gp substrates, i.e. high-solubility low-permeability drugs. Cimetidine and famotidine (Figure 1), two H_2-receptor antagonists, were chosen as the class III model drugs. To establish that these drugs are in fact substrates for intestinal efflux mediated by P-gp, we confirmed the role of efflux transporters in the epithelial permeability of these drugs across Caco-2 cell monolayers in both apical (AP) to basolateral (BL) and BL to AP directions, in the presence or absence of various P-gp, MRP2 and BCRP inhibitors. The second issue we aimed to address in the present study was the effect of the different expression levels of P-gp along the small intestine on the *in vivo* intestinal permeability of class III P-gp substrates from the different intestinal segments. The permeability of the drugs

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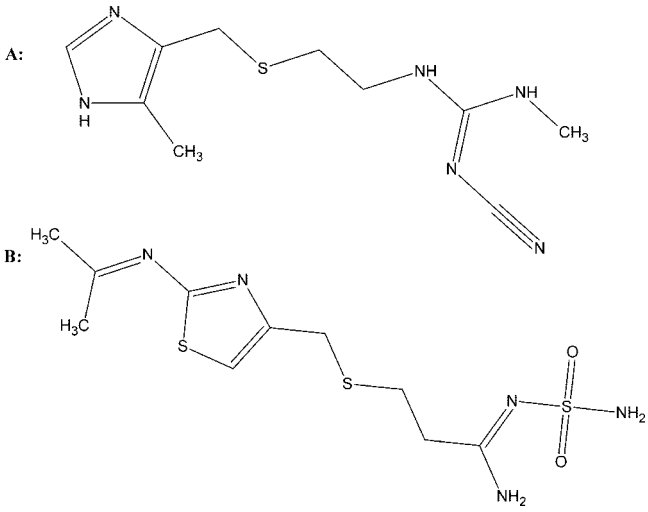

Figure 1. Molecular structures of cimetidine (A) and famotidine (B).

in different intestinal regions was measured by the *in situ* single-pass intestinal perfusion model in rats, in the presence or absence of the P-gp inhibitor verapamil. The levels of the P-gp pumps in the different small intestinal segments were measured by Western blot analysis. Overall, this setup allowed us to confirm the role of P-gp efflux in the *in vivo* permeability process of these drugs along the small intestine, and may contribute to better prediction of the relevance and the regional dependent absorption of a given P-gp substrate.

Materials and Methods

Materials. Cimetidine, famotidine, verapamil, metoprolol, erythromycin, quinidine, fumitremorgin C (FTC), phenol red, lucifer yellow, MES buffer, $CaCl_2$, $MgCl_2$, sodium dodecyl sulfate, dithiothreitol, pepstatin, leupeptin, phenylmethylsulfonyl fluoride and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium chloride and NaCl were obtained from Fisher Scientific Inc. (Pittsburgh, PA). MK-571 was purchased from Alexis Biochemicals (Lausen, Switzerland). Acetonitrile and water (Acros Organics, Geel, Belgium) were HPLC grade. Physiological saline solution was purchased from Hospira Inc. (Lake Forest, IL). All other chemicals were of analytical reagent grade.

Single-Pass Intestinal Perfusion Studies (SPIP) in Rats. All animal experiments were conducted using protocols approved by the University Committee of Use and Care of Animals (UCUCA), University of Michigan, and the animals were housed and handled according to the University of Michigan Unit for Laboratory Animal Medicine guidelines. Male albino Wistar rats (Charles River, IN) weighing 250−280 g were used for all perfusion studies. Prior to each experiment, the rats were fasted overnight (12−18 h) with free access to water. Animals were randomly assigned to the different experimental groups.

The procedure for the *in situ* single-pass intestinal perfusion followed previously published reports.[23−25] Briefly, rats were anesthetized with an intramuscular injection of 1 mL/kg of ketamine−xylazine solution (9%:1%, respectively) and placed on a heated surface maintained at 37 °C (Harvard Apparatus Inc., Holliston, MA). The abdomen was opened by a midline incision of 3−4 cm. A proximal jejunal segment (3 ± 1 cm average distance of the inlet from the ligament of Treitz), midsmall intestine (35 ± 5 cm average distance of the inlet from the ligament of Treitz), or a distal ileal segment (3 ± 1 cm average distance of the outlet from the cecum), of approximately 10 cm was carefully exposed and cannulated on two ends with flexible PVC tubing (2.29 mm i.d., inlet tube 40 cm, outlet tube 20 cm, Fisher Scientific Inc., Pittsburgh, PA). Care was taken to avoid disturbance of the circulatory system, and the exposed segment was kept moist with 37 °C normal saline solution. The perfusate was incubated in a 37 °C water bath to maintain temperature, and a perfusion solution containing 10 mM MES buffer, pH 6.5, 135 mM NaCl, 5 mM KCl, and 0.1 mg/mL phenol red with an osmolarity of 290 mosm/l was pumped through the intestinal segment (Watson Marlow Pumps 323S, Watson-Marlow Bredel Inc., Wilmington, MA). The isolated segment was rinsed with blank perfusion buffer, pH 6.5 at a flow rate of 0.5 mL/min in order to clean out any residual debris.

At the start of the study, perfusion solution containing the tested drug was perfused through the intestinal segment at a flow rate of 0.2 mL/min. Phenol red was added to the perfusion buffer as a nonabsorbable marker for measuring water flux. Metoprolol was coperfused with the other drugs as well, as a compound with known permeability that serves as a marker for the integrity of the experiment, and as a reference standard for permeability in close proximity to the low/high permeability class boundary. The concentrations of the drugs used in the perfusion studies were determined by dividing the highest prescribed oral dose by 250 mL (Table 1), the standard volume for a glass of water advised to be taken with the dose and hence the accepted minimal gastric volume, in order to represent the maximal drug concentration present in the intestinal segment, and were within their intrinsic solubility reported at pH 6.5. The perfusion buffer was first perfused for 1 h, in order to ensure steady state conditions (as also assessed by the inlet over outlet concentration ratio of phenol red which approaches 1 at steady state). Following reaching to steady state, samples were taken in 10 min intervals for 1 h (10, 20, 30, 40, 50,

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Table 1. Cimetidine, Famotidine and Metoprolol Permeability Coefficient Values (P_{eff}; cm/s) Following *in Situ* Single-Pass Rat Intestinal Perfusion to the Different Small Intestinal Regions, in the Presence or Absence of the P-gp Inhibitor Verapamil (0.1 mM)[a]

			P_{eff} (cm/s)				
			without verapamil			with verapamil (0.1 mM)	
	highest prescribed dose (mg)	concentration in perfusate (mg/mL)	proximal jejunum	mid small intestine	distal ileum	proximal jejunum	distal ileum
cimetidine	800	3.2	7.2×10^{-6} (4.7×10^{-7})	6.2×10^{-6} (6.3×10^{-7})	4.1×10^{-6} (3.8×10^{-7})	7.3×10^{-6} (5.2×10^{-7})	7.4×10^{-6} (5.4×10^{-7})
famotidine	40	0.16	3.7×10^{-6} (8.7×10^{-7})	3.0×10^{-6} (9.9×10^{-7})	1.0×10^{-6} (3.8×10^{-7})	4.8×10^{-6} (1.0×10^{-6})	5.2×10^{-6} (7.7×10^{-7})
metoprolol	100	0.4	1.7×10^{-5} (8.4×10^{-7})	1.6×10^{-5} (9.7×10^{-7})	1.7×10^{-5} (9.0×10^{-7})	1.7×10^{-5} (8.6×10^{-7})	1.6×10^{-5} (8.9×10^{-7})

[a] Metoprolol was co-perfused with the other drugs as a reference standard for permeability in close proximity to the low/high permeability class boundary. Data presented as mean (SD); $n = 4$ for cimetidine and famotidine; $n = 8$ for metoprolol.

and 60 min). All samples, including perfusion samples at different time points, original drug solution, and inlet solution taken at the exit of the syringe, were immediately assayed by HPLC. Following the termination of the experiment, the length of each perfused intestinal segment was accurately measured, as well as the distance of the different segments from the pylorus or from the cecum.

Net Water Flux Measurement. The net water flux in the single-pass intestinal perfusion studies, resulting from both water absorption and efflux in the intestinal segment, was determined by measurement of phenol red, a nonabsorbed, nonmetabolized marker. The phenol red (0.1 mg/mL) was included in the perfusion buffer and coperfused with the tested drugs. The measured C_{out}/C_{in} ratio was corrected for water transport according to the following equation:

$$\frac{C'_{out}}{C'_{in}} = \frac{C_{out}}{C_{in}} \times \frac{C_{in\ phenol\ red}}{C_{out\ phenol\ red}} \quad (1)$$

where $C_{in\ phenol\ red}$ is equal to the concentration of phenol red in the inlet sample, and $C_{out\ phenol\ red}$ is equal to the concentration of phenol red in the outlet sample.

Cell Culture. Caco-2 cells (passage 27−31) from American Type Culture Collection (Rockville, MD) were routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% nonessential amino acids, 1 mM sodium pyruvate, and 1% L-glutamine. Cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C.

Caco-2 cells were seeded on semipermeable filter inserts (12-well Transwell plate, Corning Costar Co., Cambridge, MA). The cells on the insert were cultured for 21 days at 37 °C in a humidified incubator containing 5% CO_2 in air. The differentiation status of the formed monolayer was evaluated by measuring the transepithelial electrical resistance (TEER) (Millicell-ERS epithelial Voltohmmeter, Millipore Co., Bedford, MA). Following the 21 days in cell culture, the monolayers developed a TEER values above 300 Ω cm^2.

Caco-2 Permeability Studies. Transcellular transport studies were performed in a method described previously with minor modifications.[26] The uptake buffer contained 1 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 145 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 5 mM D-glucose, and 5 mM MES. Similar pH was used in both apical and basolateral sides (pH 6.5) in order to maintain constant degree of ionization in both AP−BL and BL−AP direction experiments, and to avoid possible influence of this factor on the permeability across the cells. Briefly, cells were seeded onto collagen-coated membranes (0.4-μm pore size, 12 mm diameter, Costar, Cambridge, MA) and were allowed to grow for 21 days. Mannitol and Lucifer yellow permeability was assayed for each batch of Caco-2 monolayers ($n = 3$) and TEER measurements were performed on all monolayers. Monolayers with apparent [^{14}C]mannitol and Lucifer yellow permeability $< 3 \times 10^{-7}$ cm/s and TEER values >300 Ω cm^2 were used for the study. 0.5 mL of drug solution (0.1 mM) in the uptake buffer (pH 6.5), with or without inhibitor, was added to the apical side of the monolayer in the AP−BL direction studies, and 1.5 mL of similar uptake buffer was added to the receiver compartment on the basolateral side of the monolayer. In the BL−AP direction studies, 1.5 mL of drug solution (0.1 mM), with or without inhibitor, was added to the basolateral side of the monolayer, and 0.5 mL of blank buffer was added to the receiver compartment on the apical side of the monolayer. Five efflux transporters inhibitors, each in 3 different concentrations, were investigated: (1) verapamil (100, 50 and 10 μM); (2) erythromycin (100, 50 and 10 μM); (3) quinidine (100, 50 and 10 μM); (4) MK-571 (100, 50 and 10 μM); and (5) fumitremorgin C (20, 10 and 5 μM). Samples were taken from the receiver side at various time points up to 120 min (100 μL from basolateral side or 20 μL from apical side), and similar volumes of blank buffer were added following each withdrawal. At the last time point (120min), sample was taken from the donor side as well, in order to confirm mass balance. Samples were immediately assayed for drug content. Caco-2

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monolayers were checked for confluence by measuring the TEER before and after the transport study.

Immunoblot Analysis of P-gp Expression. Immunoblot analysis of P-gp was performed by a method described before with some modifications.[27] Epithelial cells from rat proximal jejunum, mid small intestine, and distal ileum were suspended in lysis buffer (PBS containing 3% sodium dodecyl sulfate, 2 mM dithiothreitol, 0.2 mM pepstatin, 0.2 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 45 min, followed by a 30 s homogenization (Polytron) and 10 s ultrasonication on ice. The homogenate was centrifuged at 12,000 *g* for 30 min at 4 °C, and the supernatant was used for protein content determination (BioRad DC protein assay, Bio-Rad Laboratories Inc., Hercules, CA) and immunoblotting. For each sample, 50 μg of protein were resolved in 8% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Invitrogen Corporation, Carlsbad, CA) followed by electrophoretic transfer onto Hybond ECL nitrocellulose membrane (Amersham, U.K.). Membranes were blocked overnight in TSB-T solution containing 3% BSA at 4 °C, followed by incubation with monoclonal anti P-gp antibody (C219, 1:200 dilution in TSB-T, Zymed Laboratories Inc., San Francisco, CA) for 2 h at room temperature. The secondary antibody was goat anti-mouse IgG (Alexa Fluor 488, Invitrogen Corporation, Carlsbad, CA) used at a dilution of 1:750. Blots were developed using Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Amersham Pharmacia Biotech, Sweden).

Data Analysis. The effective permeability (P_{eff}) through the rat gut wall in the single-pass intestinal perfusion studies was determined assuming the "plug flow" model expressed in the following equation:[28]

$$P_{eff}(\text{cm/s}) = \frac{-Q \ln(C\prime_{out}/C\prime_{in})}{2\pi RL} \quad (2)$$

where Q is the perfusion buffer flow rate, C'_{out}/C'_{in} is the ratio of the outlet concentration and the inlet or starting concentration of the tested drug that has been adjusted for water transport, R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the intestinal segment.

Permeability coefficient (P_{app}) across Caco-2 cell monolayers for each compound was calculated from the linear plot of drug accumulated in the receiver side versus time, using the following equation:

$$P_{app} = \frac{1}{C_0 A} \times \frac{dQ}{dt} \quad (3)$$

where dQ/dt is the steady-state appearance rate of the drug on the receiver (serosal in the case of AP−BL studies, or mucosal in the case of BL−AP studies) side, C_0 is the initial concentration of the drug in the donor side, and A is the monolayer growth surface area (1.12 cm^2). Linear regression was carried out to obtain the steady-state appearance rate of the drug on the receiver side ($R^2 > 0.99$ in all experimental groups).

Analytical Methods. The amount of cimetidine and famotidine, as well as the simultaneous analysis of metoprolol and phenol red with each of the compounds in the perfusion medium or in the Caco-2 studies buffer, was assayed using a high performance liquid chromatography (HPLC) system (Waters 2695 Separation Module) with a photodiode array UV detector (Waters 2996). Medium aliquots of 10 μL were injected into the HPLC system. The HPLC conditions were as follows: XTerra, RP_{18}, 3.5 μm, 4.6 × 100 mm column (Waters Co., Milford, MA); an isocratic mobile phase, 80:20% v/v aqueous/organic phase respectively for cimetidine and famotidine; the aqueous phase was 0.1% trifluoroacetic acid in water, and the organic phase was 0.1% trifluoroacetic acid in acetonitrile; flow at a rate of 1 mL/min in room temperature. Detection wavelengths were 230, 260, 275 and 265 nm for cimetidine, famotidine, metoprolol and phenol red respectively. Separate standard curves were used for each experiment ($R^2 > 0.999$). The inter- and intraday coefficients of variation were <1.0 and 0.5%, respectively.

Statistical Analysis. All single-pass intestinal perfusion model experiments were done with an n equal to 4 rats with 6 samples collected per rat. Mean permeabilities (P_{eff}) and standard deviation (SD) of the tested drugs were determined from the permeabilities obtained from 4 rats. The permeability of the tested drugs was calculated at each timed sample, 10−60 min. For each rat, then, the mean of 6 permeability values was calculated. The mean permeability value and SD of 4 rats was then determined for each experimental group.

To determine statistically significant differences among the experimental groups, the nonparametric Kruskal−Wallis test was used for multiple comparisons, and the two-tailed nonparametric Mann−Whitney U test for two-group comparison when appropriate. A p value of less than 0.05 was termed significant.

Results

***In Vivo* Permeability in the Single-Pass Intestinal Perfusion Model in Rats.** The *in vivo* permeability coefficients (P_{eff}) obtained for cimetidine and famotidine following *in situ* perfusion in the proximal jejunum, midsmall intestine, or in the distal ileum, are presented in Figure 2 and summarized in Table 1. The effect of the P-gp inhibitor verapamil on the permeability of cimetidine and famotidine in the different intestinal segments is shown in Figure 3 and summarized in Table 1. For both drugs, different P_{eff} values were obtained following the perfusion to the different segments, with decreased permeability in the distal parts of the small intestine. Coperfusion of 0.1 mM of verapamil to the different intestinal segments significantly elevated the

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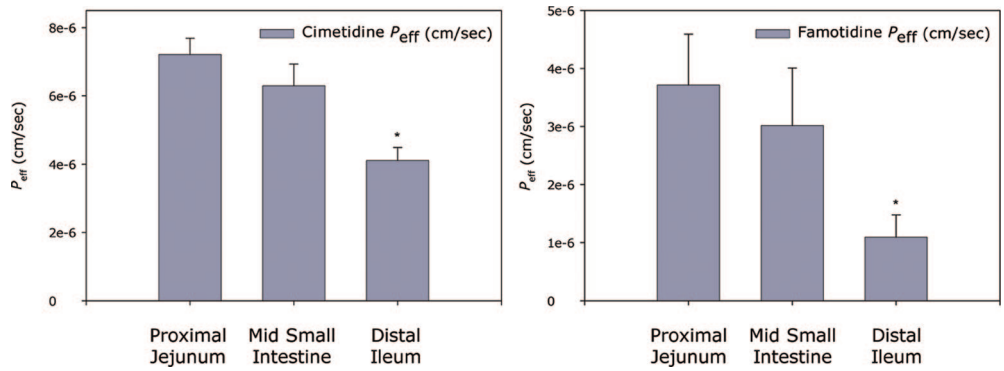

Figure 2. The permeability coefficients (P_{eff}; cm/s) obtained for cimetidine (left) and famotidine (right) following *in situ* single-pass intestinal perfusion to the proximal jejunum, mid small intestine, and distal ileum of the rat. Data presented as mean ± SD; $n = 4$ in each group.

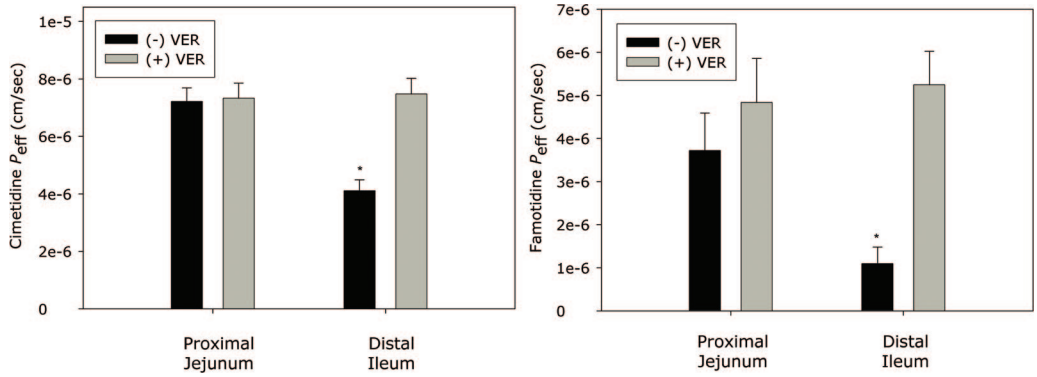

Figure 3. The effect of coperfusion of the P-gp inhibitor verapamil (0.1 mM) on the permeability of cimetidine (left) and famotidine (right) in the proximal jejunum and in the distal ileum of the rat. Data presented as mean ± SD; $n = 4$ in each group.

permeability of both drugs in the distal ileum, while no effect was observed on the jejunal permeability of the drugs. In each perfusion study, metoprolol was coperfused with the tested drug, as a compound with known permeability that served as a marker for the integrity of the experiment, and as a reference standard for permeability in close proximity to the low/high permeability class boundary. The *in vivo* permeability coefficients (P_{eff}) obtained for metoprolol are presented in Figure 4 and summarized in Table 1. It can be seen that for metoprolol, similar P_{eff} values were obtained, regardless of the intestinal segment being perfused, and the presence or absence of verapamil.

Cimetidine and Famotidine Transport across Caco-2 Monolayers. The flux of cimetidine and famotidine across Caco-2 cell monolayers in the AP to BL and in the BL−AP directions, in the presence or absence of different concentrations of verapamil is presented in Figure 5. The corresponding P_{app} values and the effect of various efflux transporters inhibitor on the permeability of cimetidine and famotidine across Caco-2 cell monolayers are presented in Figures 6 and 7 respectively. Both drugs exhibited significantly higher permeability in the BL to AP direction in comparison to the AP to BL direction, with 4 and 9 efflux ratio (ER; $P_{app\ BL-AP}/P_{app\ AP-BL}$) for cimetidine and famotidine, respectively. It can be seen that the mucosal secretion of both drugs was significantly reduced by all of the investigated P-gp inhibitors, verapamil, erythromycin and quinidine, in a concentration-dependent manner, with ER approaching to 1 in the presence of high inhibitor concentrations. Both the specific MRP2 inhibitor MK-571 and the specific BCRP inhibitor FTC showed no effect on the permeability of cimetidine and

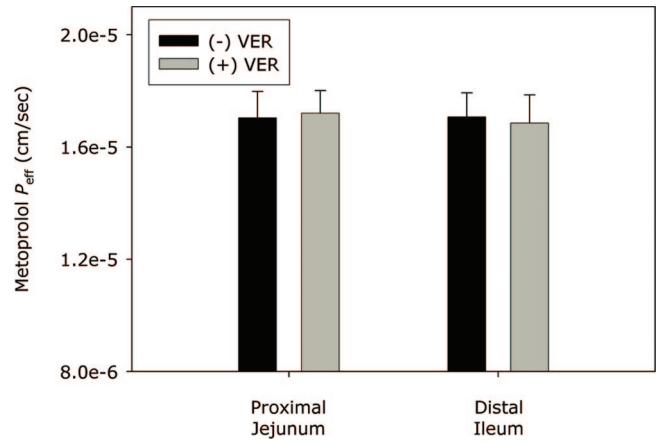

Figure 4. The permeability coefficients (P_{eff}; cm/s) obtained for metoprolol following *in situ* single-pass intestinal perfusion to the proximal jejunum and to the distal ileum of the rat, in the presence or absence of verapamil (0.1 mM). Data presented as mean ± SD; $n = 8$ in each group.

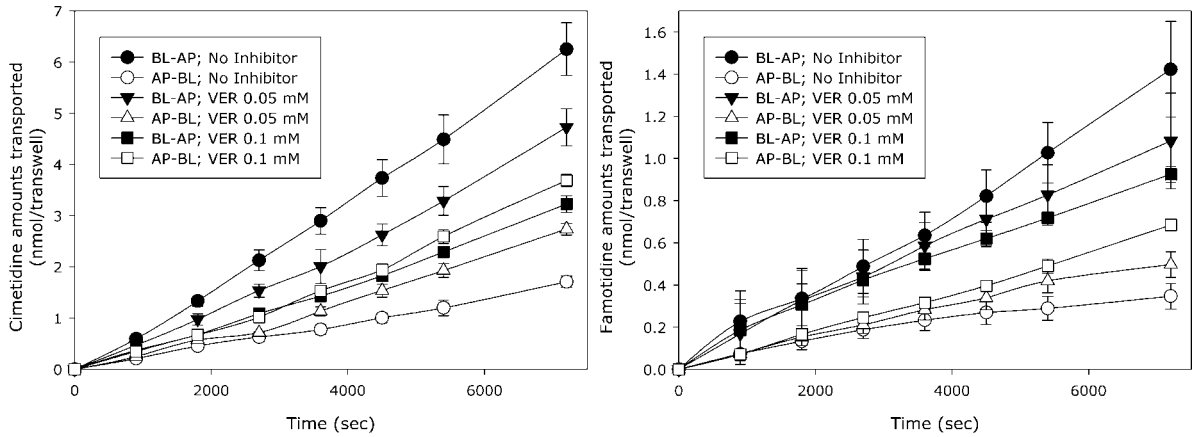

Figure 5. The flux of cimetidine (left) and famotidine (right) across Caco-2 cell monolayers in the AP to BL (empty symbols) and in the BL to AP (filled symbols) direction, in the presence or absence of various concentrations of the P-gp inhibitor verapamil. Data presented as mean ± SD; $n = 3$ in each group.

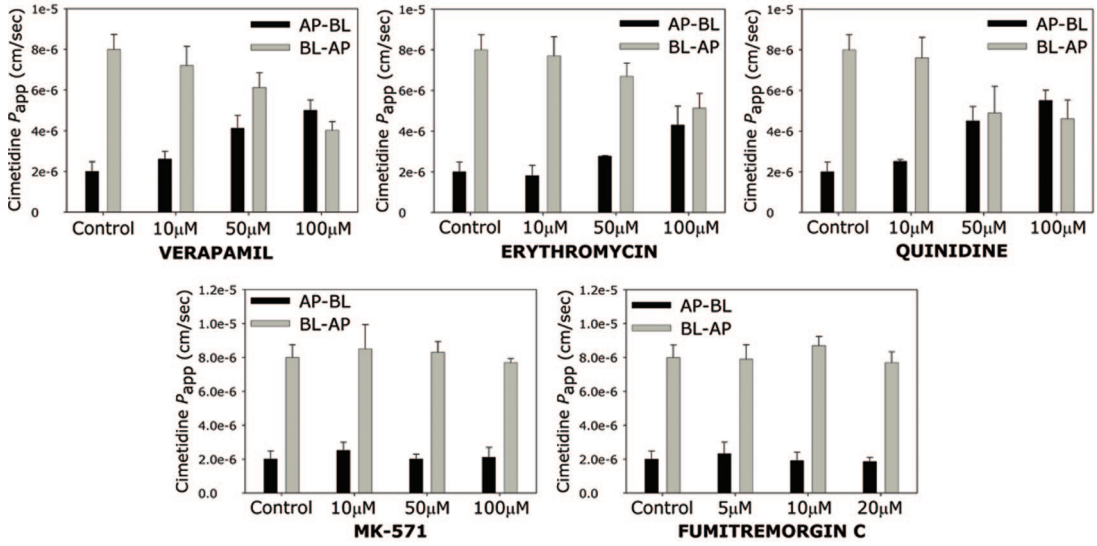

Figure 6. The apparent permeability (P_{app}) of cimetidine (0.1 mM) in the absorptive (AP−BL) and the secretory (BL−AP) directions, in the presence of different concentrations of various efflux transporters inhibitors. P-gp inhibitors: verapamil, erythromycin and quinidine (100, 50 and 10 μM); MRP2 inhibitor, MK-571 (100, 50 and 10 μM); BCRP inhibitor, FTC (20, 10 and 5 μM). Data presented as mean ± SD; $n = 3$ in each experimental group.

famotidine across Caco-2 cell monolayers, in all of the investigated concentrations and in both AP−BL and BL−AP directions.

Immunoblotting. The measurement of the molecular expression of P-gp protein in epithelial cell preparations from the different segments of the rat small intestine by Western blotting is presented in Figure 8. Significant regional dependent P-gp expression levels were found throughout the rat small intestine, showing a gradient, increasing from the proximal to the distal small intestinal segments.

Discussion

The data accumulated in the literature regarding the significance of P-gp efflux in the *in vivo* intestinal absorption of different substrates is rather confusing, as indications from substantial importance to very limited effect are available. In this study we sought to address this confusion, by exploiting the Biopharmaceutical Classification System (BCS). It has been shown before that the *in vivo* intestinal absorption of BCS class I P-gp substrates, i.e. high-solubility high-permeability drugs, is dominated by their high permeability, and that P-gp plays a minimal role in the intestinal absorption of these compounds.[1] Other attempts to correlate the significance of P-gp with the permeability of the substrate through the GI wall are also reported.[29−31] In the present research, we investigated the role of P-gp efflux in the *in vivo* intestinal absorption process of class III P-gp substrates, i.e. high-solubility low-permeability drugs. In addition, we

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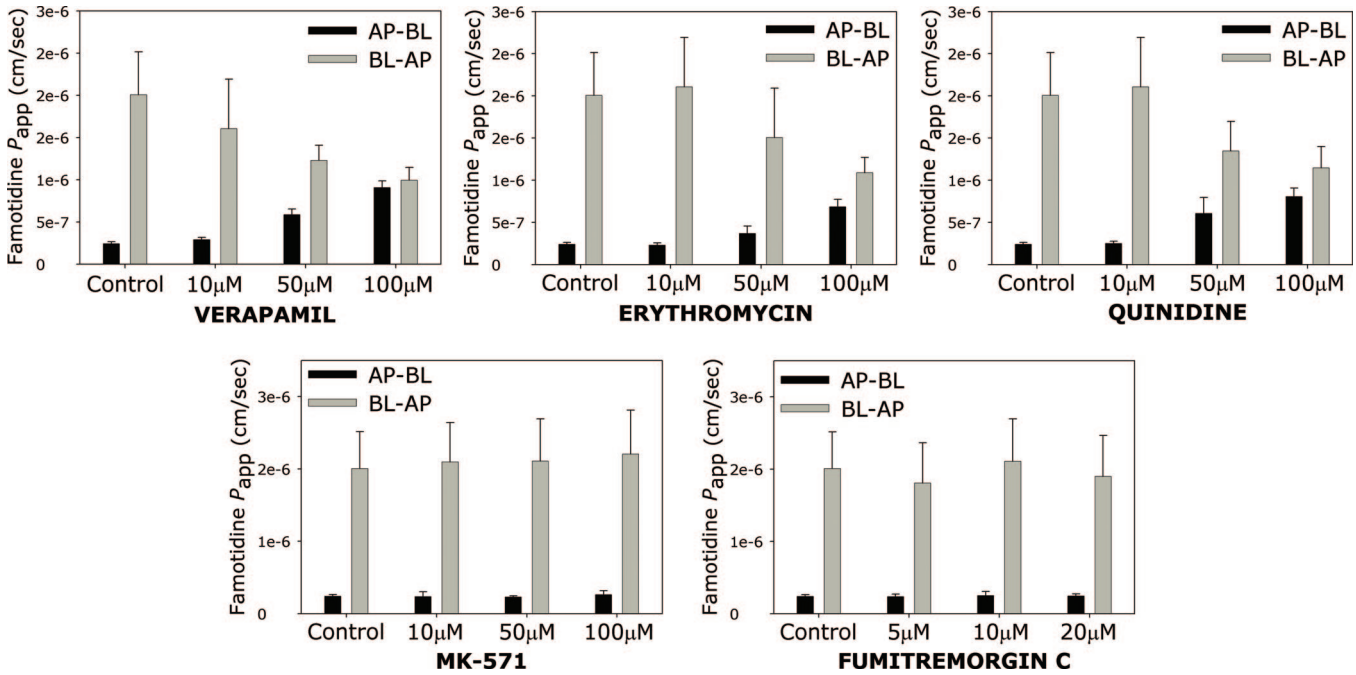

Figure 7. The apparent permeability (P_{app}) of famotidine (0.1 mM) in the absorptive (AP−BL) and the secretory (BL−AP) directions, in the presence of different concentrations of various efflux transporters inhibitors. P-gp inhibitors: verapamil, erythromycin and quinidine (100, 50 and 10 μM); MRP2 inhibitor, MK-571 (100, 50 and 10 μM); BCRP inhibitor, FTC (20, 10 and 5 μM). Data presented as mean ± SD; $n = 3$ in each experimental group.

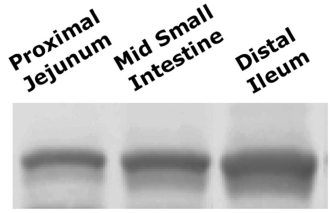

Figure 8. Analysis of P-gp levels in different segments along the rat small intestine by Western immunoblotting of purified intestinal epithelium. P-gp was probed with the monoclonal anti P-gp antibody C219. Data shown are representative of epithelial preparations from four animals.

investigated the effect of the different P-gp expression levels along the small intestine on the *in vivo* intestinal permeability of these drugs from the different intestinal segments.

Both cimetidine and famotidine exhibited asymmetric permeability across Caco-2 cell monolayers, with permeability in the BL to AP direction 4 and 9-fold higher than the AP−BL direction permeability, respectively (Table 1), indicative of a net mucosal secretion of the drugs. Consistent with previous reports,[32,33] this mucosal secretion was shown to be significantly reduced by all of the investigated P-gp inhibitors, verapamil, erythromycin and quinidine, in a concentration-dependent manner. No effect was observed neither in the presence of the specific MRP2 inhibitor MK-571 nor the specific BCRP inhibitor FTC in comparison to control. Taken together, these data indicate the involvement of P-gp in the secretory transport of these H_2-antagonists, and establish that these drugs mucosal efflux is mediated by P-gp, and not by other transporter. These two hydrophilic drugs are believed to cross the GI wall with a significant paracellular component,[34,35] and hence are not thought to be typical substrates for P-gp efflux. A transcellular component, as well as an active transport into the enterocyte across the serosal membrane may contribute to the P-gp efflux of these drugs.

To determine the *in vivo* intestinal absorption of the drugs from the different segments, we utilized the single-pass intestinal perfusion (SPIP) method in rats. This approach has been shown to provide a precise method to predict *in vivo*

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oral absorption in humans.[24,25,28,36] Both cimetidine and famotidine exhibited segmental dependent permeability through the GI wall, with significantly decreased P_{eff} in the distal ileum in comparison to the proximal segments. A trend toward decreased permeability in the midsmall intestine in comparison to the proximal jejunum was obtained as well (Figure 2), however with no statistical significance. Hence, we evaluated the impact of coperfusion of the P-gp inhibitor verapamil on the permeability of the drugs in the significantly distinct regions, i.e. the proximal jejunum and the distal ileum. Whereas the presence of verapamil had no effect on the drugs permeability in the proximal jejunum, the P-gp inhibition significantly increased the intestinal absorption in the distal ileum for both cimetidine and famotidine. The increased permeability in the presence of verapamil was similar to the permeability from the proximal regions of the small intestine, leading to constant permeability along the small intestine under P-gp inhibition conditions. Thus, it was revealed that the segmental dependent intestinal transport of both cimetidine and famotidine is due to P-gp efflux.

Membrane transport of drugs in different regions of the intestinal tract is very important in understanding the oral absorption process of drugs. Segmental dependent intestinal absorption may contribute to high variability in drug blood levels following oral administration. In addition, it is particularly crucial throughout the development of controlled release products.[37−39] To a large extent, the regional permeability is determined by the interplay between the physicochemical properties of the drug and the function of the membrane.[40−42] The different segments of the intestine differ from each other in many aspects, as the main factors affecting the permeability are total surface area of the membrane, tight junctional resistance, enzyme activities, amount and capacity of carriers, water flux, unstirred water layer, and capillary blood flow. While the factors regulating permeability and absorption from the GI tract have been shown to be significantly distinct between the small intestine and the colon, the different small intestine regions hold much less difference. However, variations were reported between the different small intestinal segments (duodenum, jejunum and ileum) in the expression profiles of transporters and metabolizing enzymes, including the P-gp efflux pumps.[1,20,43−46] In corroboration with these reports, we have found significant differences in P-gp levels along the small intestinal segments. P-gp protein expression followed a gradient pattern, increasing from the proximal regions to the distal small intestinal segments. This pattern was inversely related to the *in vivo* permeability of cimetidine and famotidine from the different intestinal segments. Hence, the results of the *in vivo* animal studies and the *in vitro* cell culture experiments were confirmed at the protein level, correlating the functional studies with the molecular expression of the P-gp efflux pump protein.

By definition, class III drugs are characterized by a dose number (D_0) equal to or less than 1:

$$D_0 = \frac{M/V_0}{C_S} \tag{4}$$

where C_S is the solubility, M is the dose, and V_0 is the volume of water taken with the dose, which is generally set to be 250 mL. As such, high concentration of the drug is available at the site of absorption. However, the intrinsic low permeability of this class of drugs essentially leads to limited amounts of drug inside the enterocyte, with potentially subsaturated P-gp levels. The next factors to determine whether or not P-gp efflux may dominate the *in vivo* intestinal absorption of the substrate would be the expression level of the P-gp pump protein in the GI wall and the affinity of the drug to the pump, i.e. whether the drug is a strong or weak P-gp substrate. The data presented in this paper demonstrate that the regional differences in GI P-gp expression levels may play a crucial role in the *in vivo* intestinal absorption of BCS class III P-gp substrates.

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In a previous publication, it has been shown that the *in vivo* intestinal absorption of both verapamil and propranolol, two BCS class I, high solubility high permeability P-gp substrates, is constant along the intestine despite significant (up to 6-fold) changes in P-gp pumps expression in the different small intestinal segments.[1] The absorption process of this class of drugs may be dominated by the passive diffusion due to their high permeability, and hence do not tend to be affected by efflux transport, regardless the expression level in the different intestinal segments. A compound with high solubility and high membrane permeability will have 100% of the fraction of drug absorbed from the intestine no matter whether the P-gp is present or not. However, for low permeability compounds, the data presented in this paper clearly demonstrate that whereas in the proximal segments of the small intestine P-gp efflux still does not affect the *in vivo* intestinal absorption, in the distal parts of the small intestine the efflux of the drug molecules back to the intestinal lumen by P-gp plays a significant role in the intestinal absorption of these drugs, and in fact dominates the permeability of the drug through the GI wall in this segment of the intestine.

Conclusions

In conclusion, the data presented in this paper demonstrate segmental dependent *in vivo* intestinal absorption of BCS class III P-gp substrates cimetidine and famotidine. The permeability of these drugs across the GI wall was dominated by P-gp in the distal small intestine, whereas efflux transport was insignificant in the proximal intestinal segments. The utilization of the BCS for understanding the *in vivo* functional role of P-gp may contribute to better prediction of the efflux *in vivo* relevance and the regional dependent absorption of a given P-gp substrate.

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