In Vitro Intestinal Permeability of Factor Xa Inhibitors: Influence of Chemical Structure on Passive Transport and Susceptibility to Efflux

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Received August 22, 2001; accepted September 6, 2001

Purpose. To study the *in vitro* intestinal permeability of a number of newly synthesised factor Xa inhibitors to better understand the poor oral absorption of these compounds.

Methods. The bidirectional transport of the fXa inhibitors was studied in the Caco-2 cell model and isolated rat ileal tissue. An attempt was made to characterize efflux mechanisms with the help of commonly used substrates and inhibitors of various transport proteins. In addition, the transport of the fXa inhibitors was studied in MDCK cells transfected with the human *MDR1* gene and expressing large amounts of P-glycoprotein (Pgp).

Results. The *in vitro* absorptive permeability was low for all but one of the fXa inhibitors. For compounds with non-substituted amidine, a charge (due to ionisation at neutral pH) may have resulted in poor membrane partitioning. Neutral compounds with substituted amidines were effluxed from the epithelial cells. The significance of the secretion process was illustrated by the results obtained for a neutral analogue showing high absorptive Caco-2 cell permeability that was not obviated by efflux. Transport inhibition studies in Caco-2 and permeability studies in the *MDR1*-transfected MDCK cells consistently showed that Pgp is not involved in the secretion of fXa inhibitors. Besides efflux, metabolic liability limited the permeation of the neutral lipophilic analogues with a carbamate ester.

Conclusions. Poor intestinal permeability may be an important factor in the incomplete oral absorption of the bisbenzimidazole-type fXa inhibitors. Poor permeability may be related to poor membrane partitioning for hydrophilic analogues, whereas susceptibility to efflux transports and gastro-intestinal enzymatic degradation may limit the permeability of some of the neutral less hydrophilic derivatives.

KEY WORDS: intestinal permeability; Caco-2; factor Xa inhibitors; efflux; oral absorption.

INTRODUCTION

The activated serine protease factor Xa (fXa) plays an important role in blood coagulation due to its central role in the coagulation pathways. Inhibition of fXa, as opposed to the inactivation of thrombin, interrupts thrombin formation without impairing platelet haemostatic functions.

FXa inhibitors may therefore be useful in the treatment of thrombosis without the increased risk of bleeding associated with thrombin inhibitors such as heparin (1). A number of natural polypeptides and synthetic analogues have been identified as antagonists of fXa and found to produce antithrombotic effects in experimental animals and humans (2). Recently, PNU200001 and related derivatives were developed as potent fXa inhibitors. The compounds are bisbenzimidazoles with an amidine group at the proximal benzene ring (Fig. 1). The possibility of oral administration would be an additional advantage over conventional antithrombotic therapy with heparin. However, preliminary studies have indicated that the oral bioavailability of the fXa inhibitors is low in rats. Incomplete oral absorption may result from presystemic degradation, low solubility in the gastrointestinal tact, or poor permeation through the intestinal mucosa.

The purpose of this work was to study the intestinal absorption of a number of the newly developed fXa inhibitors. Studies focused on the elucidation of the barriers limiting oral absorption. The bidirectional permeability of the fXa inhibitors was studied in monolayers of intestinal Caco-2 cells and isolated intestinal segments of rat ileum. These models have been widely used as models for drug absorption, both for predicting the extent of absorption and for identifying the mechanisms of absorption (3–6).

Specialized transport mechanisms exist in the intestinal epithelium which secrete compounds in the direction blood to lumen. The best known example is the *MDR1* gene product, P-glycoprotein (Pgp), which is a membrane-associated transporter (7,8). Others include the multidrug resistance protein family (MRP1-6) (9), which includes the MRP2, multispecific organic anion transporters (cMOAT), and the organic cation transporter (OTC) (10). The physiological role of such efflux transport systems has been suggested as a barrier to the uptake of foreign toxic substances and the facilitation of the excretion of toxic compounds and endogenous metabolites (7). The secretory transport mechanisms have implications in oral drug delivery since the secretion of drugs from the intestinal epithelium may have a considerable influence on oral absorption (11–14). In the present study an attempt was made to characterize the efflux transport mechanisms involved in the active secretion of fXa inhibitors by using more or less specific substrates and inhibitors of various transport proteins. In addition, the transport of the fXa inhibitors was studied in Madine-Darby canine kidney (MDCK) cells stably transfected with the human *MDR1* gene and expressing large amounts of Pgp on their apical membrane (15).

MATERIALS AND METHODS

Materials

PNU20001, PNU200969, PNU200681, PNU200648, PNU200603 and PNU200647 were prepared by Axys Pharmaceutical Company (San Francisco, CA) (Fig. 1). ^{14}C-mannitol with a specific activity of 45–55 mCi/mmol and ^{3}H-testosterone (53.5 Ci/mmol) were obtained from Dupont Scandinavia (Kista, Sweden). ^{3}H-vinblastine sulphate with a specific activity of 5–25 Ci/mmol was from Amersham Life Science (Solna, Sweden). Verapamil hydrochloride, probenicid, guanidine hydrochloride, and tetraethylammonium chloride were from Sigma (St. Louis, MO). Tissue culture media

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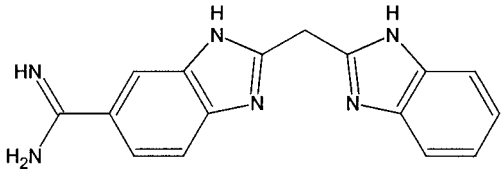

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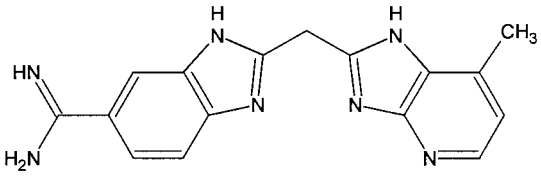

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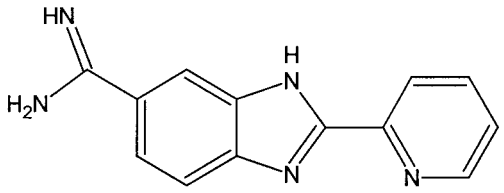

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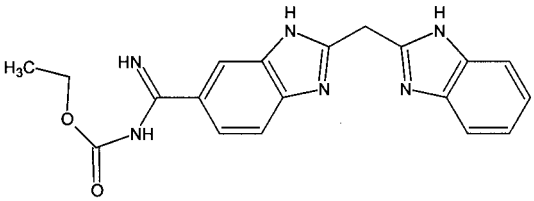

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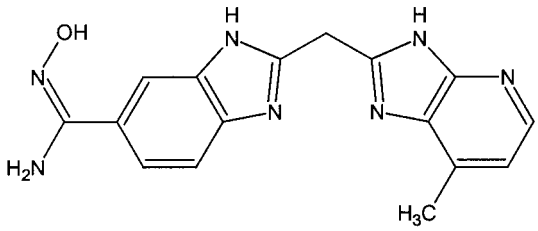
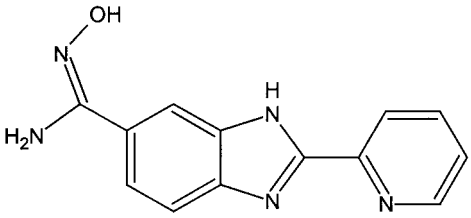


PNU200681



PNU200647

Fig. 1. Molecular structures of fXa inhibitors. PNU200001, 200969, 200648, and 200681 are covered in patent WO/99/26932 and PNU200603, 200647 in US patent 6,150,379.

were obtained from Gibco via Life Technologies (Stockholm, Sweden).

Determination of Acid Dissociation and Distribution Constants

A potentiometric (pH-metric) method based on one-phase (water) and two-phase (water and 1-octanol) titrations were used to determine pK_a and log K_D (distribution constant as defined by International Union of Pure and Applied Chemistry (16)) for the fXa inhibitors. Titrations were performed at 25°C using the Sirius PCA 101.

Cell Culture

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD). The wild-type (WT) MDCK cell line and MDCK cells transfected with the human *MDR1* gene expressing large amounts of P-glycoprotein (Pgp) were obtained from Dr. Michael M. Gottesman (NIH/NCI) under material transfer agreement CA-MTA#106085 96. Caco-2 cells and WT and MDR1-MDCK cells were maintained according to methods described by Artursson *et al.* (17). Caco-2 cells of passage numbers 36 to 46 were used. They were used for experiments 14 to 28 days after seeding.

WT and MDR1-MDCK cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 μg/ml streptomycin and 100 IU/ml benzyl penicillin. Selective pressure was kept on the MDR1-MDCK cells by including 80 ng/ml colchicine in the culture medium. Incubation was at 37°C in humidified 5% CO_2. Following trypsinisation in tissue culture flasks, the cells were plated at a density of 0.05×106 on Transwell cell culture inserts with a diameter of 24 mm. The medium was changed every other day. Cells of passage 15 were used 7 or 8 days after seeding.

Bidirectional Transport in Epithelial Cell Monolayers

Bidirectional transport experiments were performed as described by Artursson *et al.* (17). The buffer solutions used for Caco-2 and MDCK cells were Hank's balanced salt solution buffered with 25 mM Hepes to pH 7.2 (HBSS) and phosphate-buffered saline with calcium, magnesium, 1 g/l glucose and 15 mM Hepes at pH = 7.2 (PBS), respectively. Test solutions with fXa inhibitors at concentrations of 0.05 to 0.5 mM were prepared, adding specific substrates or inhibitors and ^{14}C-mannitol, when appropriate. The tightness and integrity of the cell monolayers were monitored with the paracellular marker molecule ^{14}C-mannitol. Mannitol permeability did not exceed 0.4×10^{-6} cm/sec for any of the cell monolayers under any condition.

Animals

Male Sprague-Dawley rats (Charles River, Kingston, NY) weighing 250–375 g were used. They were kept in cages with free access to food and water. The animal studies were approved by the regional ethical board in Stockholm (Sweden), with an application number for approval of N314/95.

Bidirectional Transport in Isolated Intestinal Segments

Permeation studies with rat tissue were adapted from methods described in (18,19). The buffer used throughout the experiments was Krebs Bicarbonate Ringer salt solution (KBR) with the following composition: 1.25 mM $CaCl_2$, 4.7 mM KCl, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, 95 mM NaCl, 15 mM $NaHCO_3$, 1.8 mM Na_2HPO_4, 11 mM glucose, 4.9 mM sodium glutamate, 5.4 mM sodium fumarate, 4.9 mM sodium pyruvate, and 20 mM Hepes. The KBR solution was gassed with O_2/CO_2 (95%/5% v/v) gas mixture to give a constant pH of 7.4. Permeability experiments were initiated by replacing KBR solutions with solutions containing 0.2 mM fXa inhibitor and 1.5 mL prewarmed KBR buffer in the donor and acceptor chambers, respectively. Immediately, trace amounts of ^{14}C-mannitol and ^{3}H-testosterone were added to the donor solutions, which were used as reference substances of low and high permeability, respectively.

Analysis

Factor Xa inhibitors were analysed by HPLC using isocratic elution and UV or fluorescence detection. A Symmetry C18 100 × 4.6 mm, 3.5 micron column were used. The mobile phase consisted of water containing 0.1% trifluoroacetic acid, modified with acetonitrile to obtain a retention time of about 4 min at a flow of 1 ml/min. UV-spectrophotometry was used to determine the lambdamax for each compound.

^{14}C- and ^{3}H-labelled substances were analysed by liquid scintillation counting (Tricarb, 1900CA, Packard Instrument, Downers Grove, IL). In addition, 10 mL scintillation liquid (Ultima Gold Packard, Meriden, Conn.) was added to a 20–500 μl sample solution.

Calculations and Statistical Evaluation

Apparent permeability coefficients (Papp) were calculated with Papp (cm/s) = $(dQ/dt)/(A \times C_0)$, where dQ/dt is the permeability rate (mass transfer, mol/s), C_0 is the initial concentration in the donor chamber (mol/ml) and A is the surface area of the membrane (cm^2). All data are expressed as the mean ± s.d. of 3–6 experiments, except for permeability experiments with MDCK cells, which were a mean of two experiments only.

RESULTS AND DISCUSSION

Transport of fXa Inhibitors across Caco-2 Cell Monolayers

The apparent permeability coefficients of the fXa inhibitors in the Caco-2 cell model are given in Table I. Bisbenzimidazoles with a non-substituted amidine group, such as PNU200001, PNU200648, and PNU200603, had an apparent permeability coefficient (Papp) of $<0.3 \times 10^{-6}$ cm/s in the absorptive, i.e., the apical to basolateral, direction. These permeation rates are similar to or less than those observed for mannitol, suggesting that the absorption of the fXa inhibitors through the epithelial cell layers is low. This is consistent with the low *in vivo* oral absorption observed in rats (<5%, unpublished). The octanol/water distribution coefficients and the acid dissociation constants show that the fXa inhibitors with free amidine (a weak base) are positively charged and hydrophilic at neutral pH (Table I). To increase the lipophilicity and thereby increase partitioning into the lipophilic cell membrane, substances were synthesised with an amoxidime group (PNU200681 and PNU200647) or an ester function on the amidine (PNU200969) to avoid the positive charge. The pKa values of these compounds (pKa_3 of approx. 10–12 (deprotonation) and pKa_2 of 5 (protonation)) indicate that the substituted amidines remain neutral within a wide pH

Table I. Apparent Permeability and Physicochemical Properties of fXa Inhibitors

Compound	Papp × 10^{-6} (cm/s)[a] apical to basolateral	Papp × 10^{-6} (cm/s)[a] basolateral to apical	Ratio[b] $Papp_{(b-a)}/Papp_{(a-b)}$	Molecular weight (dalton)	#HBAn	#HBAo	#HBD	log K_D (octanol/water)	log D (pH 7.2)[c]	$PK_{a1;2;3}$[d]
PNU200001	0.20 ± 0.01	0.30 ± 0.1	1.5	290.3	6	0	5	1.27	–2.0	1.9; 4.6; 10.2
PNU200648	0.09 ± 0.03	n.d.	—	305.3	7	0	5	1.2	–1.9	4.0; 6.8; 10.0
PNU200603	0.14 ± 0.01	<0.15	<1.1	237.3	5	0	4	–0.53	–4.0	1.9; 2.6; 10.4
PNU200969	0.7 ± 0.2	39.5 ± 3.3	25.5	362.4	6	2	4	1.5	1.5	1.6; 4.8; 11.9
PNU200681	0.2 ± 0.4	4.0 ± 0.2	28.6	321.3	7	1	5	1.0	1.0	3.7; 5.0; 10.3
PNU200647	22.3 ± 3.2	11.2 ± 0.7	0.50	253.3	5	1	4	1.2	1.6	2.9; 4.9; 11.3
^{14}C-mannitol	0.17 ± 0.08	0.16 ± 0.06	0.9	182.2	—	—	—	n.d.	—	—

[a] Papp is the apparent permeability in Caco-2 cells. Donor concentrations were 0.1 mM for PNU200001, 1 mM for PNU 200648, 1 mM for PNU200603, 0.5 mM for PNU 200969, 0.25 mM for PNU200681 and 0.1 mM for PNU200647. Data are given as the mean ± s.d. of 3–4 experiments.

[b] The ratio of permeabilities in the basolateral to apical versus the apical to basolateral direction is given. Number of hydrogen bond acceptor nitrogen atoms (#HBAn), number of hydrogen bond acceptor oxygen atoms (#HBAo) and number of hydrogen bond donor atoms on nitrogen and oxygen atoms (#HBD).

[c] The octanol/water distribution ratios were calculated from the values for log K_D and pK_a.

[d] pK_{a3} is protonation for PNU200001, 200648 and 200603, and deprotonation for amidine substituted PNU200969, 200681 and 200647, all other pK_a values being protonations. n.d. = not determined.

range. Although the lipophilicities of the inhibitors were thereby increased considerably, the permeabilities of the Caco-2 cell layers for PNU200681 and PNU200969 were not increased concomitantly. The transport rates in the apical to basolateral direction of PNU200681 and the corresponding compound with free amidine PNU200648 were similar, while the transport rates of PNU200969 and the corresponding PNU200001 differed only by a factor of 3. On the other hand, the absorptive permeability of the epithelial cell layer for the amidoxime PNU200647 ws more than two magnitudes higher than for the corresponding free amidine PNU200603. The improved permeability of PNU200647 compared to the other fXa inhibitors was accompanied by a much better oral absorption of this compound *in vivo* in rats (>25%, not published).

Secretory Transport of fXa Inhibitors

Transport experiments were performed in the apical to basolateral direction and vice versa (Table I). Drugs transported across the epithelium by passive diffusion, such as mannitol, have similar permeabilities in both directions in the Caco-2 model, whereas asymmetrical transport is an indication of active transport or metabolic involvement in the absorption process. PNU200681 and PNU200969 had a pronounced asymmetrical transport, the absorption in the apical to basolateral direction being more than 25 times smaller than in the opposite direction. This is indicative of secretion of the substances from the cells into the lumen. For the bisbenzimidazoles with a free non-substituted amidine, such as PNU200001 and PNU200603, the permeabilities in absorptive and secretory directions were comparable, i.e., they were within a twofold difference. The apical to basolateral and basolateral to apical permeation rates were low. The positively charged bisbenzimidazoles are therefore either not good substrates for secretory transport proteins or they are too hydrophilic to participate in the cell membrane and do not reach the transport protein(s). Interestingly, the only amidoxime derivative with a high absorptive permeability, PNU200647, was not subject to secretory transport.

For the substituted amidines, such as PNU200681, PNU200969, and PNU200647, the presence or absence of a pronounced asymmetrical transport coincides with a low or high absorptive flux, respectively. This suggests that besides poor membrane diffusion, as observed for the hydrophilic positively charged free amidines, active secretory transport processes play a significant role in the poor permeation of the neutral fXa inhibitors.

In order to investigate the involvement of Pgp in the secretory transport process, permeability studies with fXa inhibitors were performed using verapamil as an inhibitor of Pgp activity (21–23). Verapamil did not influence the absorptive flux of PNU200681 and PNU200969. The Papp values were 0.2 ± 0.4 and 0.7 ± 0.2 without verapamil and 0.1 ± 0.1 and 1.0 ± 0.2 × 10^{-6} cm/sec with verapamil for PNU200681 and PNU200969, respectively. The secretory flux, on the other hand, was decreased in the presence of verapamil for PNU200681 and PNU200969 from 4.0 ± 0.2 to 2 ± 0.1 and from 39.5 ± 3.3 to 22.6 ± 9.1 × 10^{-6} cm/sec, respectively, (Fig. 2). Although the transport process of the fXa inhibitors was thus influenced by the Pgp modulator, the inhibitors did not behave like typical Pgp substrates. Such compounds show

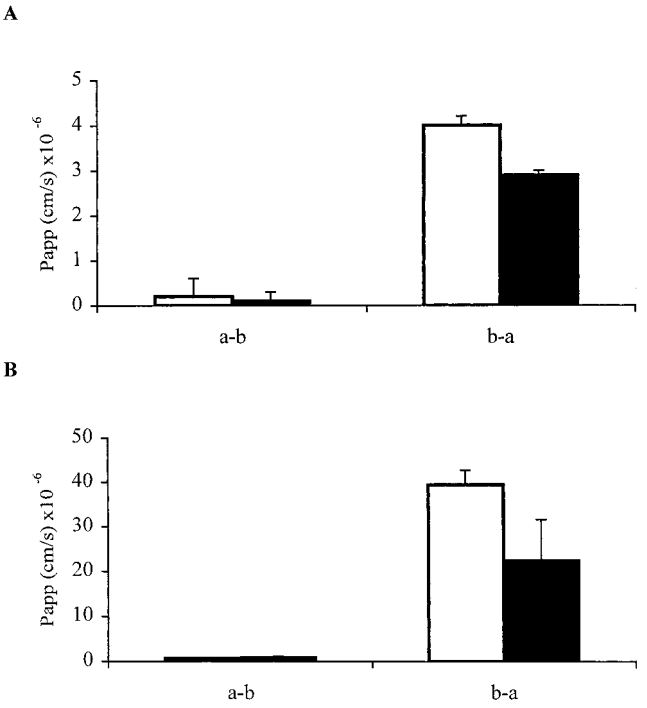


Fig. 2. Effects of verapamil on the bidirectional permeability of fXa inhibitors across Caco-2 cell monolayers. Verapamil was added to the drug solutions and the buffer solutions at the acceptor side of the cells. (A) 0.25 mM PNU200681, in the absence (□) and the presence (■) of 0.2 mM verapamil. (B) 0.05 mM PNU200969, in the absence (□) and the presence (■) of 0.5 mM verapamil.

increased absorptive and decreased secretory transport on inhibition by verapamil (21,22).

In Fig. 3 the effects of guanidine, tetraethylammonium, and probenicid on the permeability of the fXa inhibitor PNU200969 are shown. Guanidine and tetraethylammonium were used because they are organic cations that are transported in kidney epithelium by two distinct organic ion-transporting proteins (24). For both cations, asymmetrical secretory transport has been demonstrated in intestinal epithelium (25,26). Probenicid is a common inhibitor of the multispecific organic anion transporter (27). Probenicid, gua-

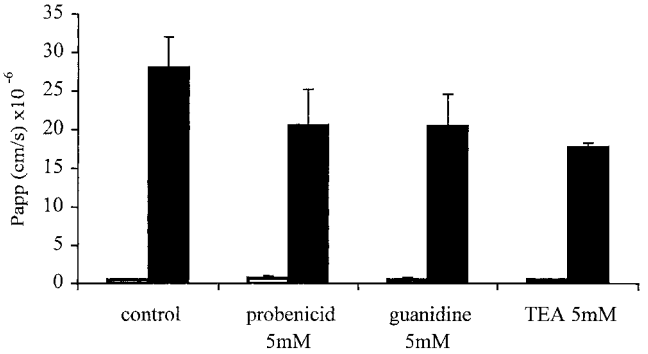


Fig. 3. Effects of various inhibitors of active secretion on the bidirectional permeability, Ap-B1 (□) and B1-Ap (■), 0.5 mM PNU200969 across Caco-2 cell monolayers. The inhibitors were added to the PNU200969 solutions to a concentration of 5 mM. Buffer solutions at the acceptor side of the cells were without inhibitor.

nidine, and tetraethylammonium at concentrations of 5 mM did not affect the apical to basolateral transport of the fXa inhibitor. The absorptive permeabilities were all between 0.5 ± 0.1 (control) and 0.7 ± 0.3 (probenicid) × 10^{-6} cm/sec. The basolateral to apical transport, on the other hand was decreased from 28 ± 0.3 × 10^{-6} cm/sec for control to 21 ± 5, 20 ± 3, and 18 ± 1 × 10^{-6} cm/sec in presence of 5 mM probenicid, guanidine, and TEA, respectively.

Based on the pharmacological inhibition studies, it thus appears difficult to identify a specific efflux mechanism for the secretion of the fXa inhibitors. The apical to basolateral transport of the fXa inhibitors is not affected by any of the substrates, whereas basolateral to apical transport is decreased by Pgp, organic cation and organic anion substrates. The functional pharmacological competitive inhibition studies are complicated by the fact that the transporters usually have a broad substrate specificity, and many of the specific substrates have an affinity for more than one transporter. Apart from being a Pgp modulator, verapamil, for example, has been shown to be a very good inhibitor of the organic cation carrier (24). To demonstrate unequivocally that the fXa inhibitors were or were not substrates for the Pgp transporter, studies were performed with a MDCK cell line that was transfected with the multidrug resistance gene *MDR1*. These cells express high amounts of functionally active Pgp, whereas the wild-type cells do not (15). In the WT-MDCK and MDR1-MDCK cells permeabilities of PNU200001 were somewhat higher in the basolateral to apical direction compared to permeability in the apical to basolateral direction (1.2 vs. 0.3, and 0.6 vs. 0.0 × 10^{-6} cm/sec, respectively, see Fig. 4). The permeability in both directions, however, was low and comparable to mannitol flux (within a threefold difference).

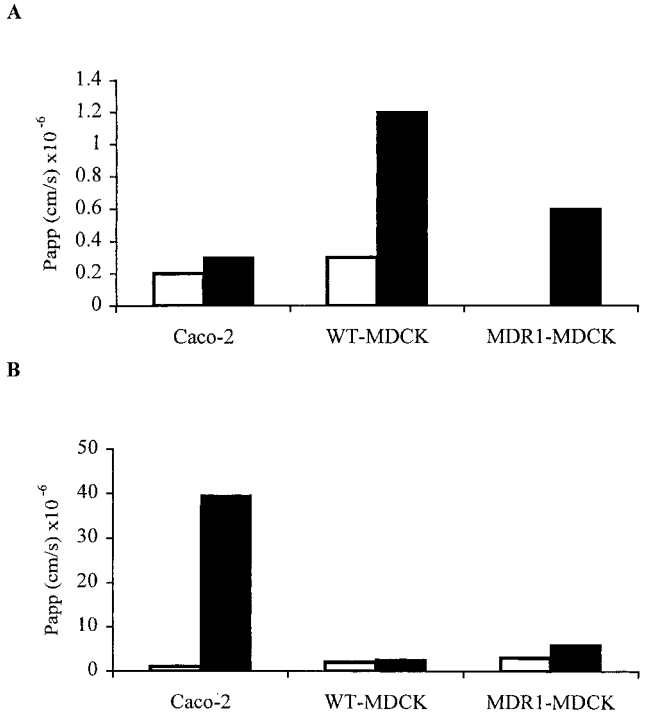


Fig. 4. Bidirectional permeabilities, Ap-B1 (□) and B1-Ap (■), of fXa inhibitors across WT-MDCK, and MDR1-MDCK cell monolayers. A: 0.05 mM PNU200001, B: 0.05 mM PNU200969. Caco-2 permeabilities are shown for reasons of comparison.

In the WT-MDCK cells absorptive and secretory fluxes for PNU200969 were 2.4 and 2.6 × 10^{-6} cm/sec. From these observations it can be concluded that PNU200969 is transported by passive diffusion through the WT-MDCK cell monolayers and that the efflux system responsible for the asymmetrical transport in the Caco-2 cells is not present in the WT-MDCK cell line. In the MDR1-MDCK cells the transport of PNU200969 was 3.1 × 10^{-6} cm/sec in the absorptive and 6.1 × 10^{-6} cm/sec in the secretory direction (Fig. 4). For comparison, a basolateral to apical versus apical to basolateral transport ratio of 8 and 157 was found for the Pgp substrate vinblastine in the WT-MDCK cells and the MDR1-MDCK cells respectively (not shown). As evidenced by the small net secretory efflux in the MDR1-MDCK cell line, PNU200969 did not seem to be a good substrate for Pgp. This is consistent with the transport inhibition studies in Caco-2 cells using Pgp modulators.

Inspection of Table I reveals that the compounds with the highest secretion, i.e., PNU200969 and PNU 200681, are the ones with the largest number of hydrogen bond acceptor oxygen and nitrogen atoms as well as the highest molecular weight. This indicates that the protein responsible for secretion shows a substrate overlap with Pgp, as a previously published model of drug-modulated Pgp ATPase activity showed that properties related to molecular size and hydrogen bonding are important for high Pgp ATPase activity (28).

In Vitro Permeability of Rat Intestinal Epithelium for fXa Inhibitors

The permeability of fXa inhibitors was also investigated using isolated rat ileal tissue. The *in vitro* permeation of PNU200001 through the ileal epithelium was low. The absorptive permeability (mucosa to serosa) was 1.2 ± 0.4 × 10^{-6} cm/s, which is about five times less than mannitol (Table II). In contrast to results obtained in the Caco-2 cell model, secretory transport of PNU20001 in the blood to lumen direction (from serosa to mucosa) exceeded absorptive transport tenfold. The asymmetry in drug flux suggests the presence of an active secretory transport mechanism for PNU200001.

In the cell culture models, all fXa inhibitors were stable during the course of the permeability experiments, as demonstrated by mass balance values of more than 85%. The carbamate ester of PNU200969, however, was extensively hydrolysed to the non-substituted amidine (PNU200001) during exposure to rat ileal tissue. Approximately 95% of the initial donor concentration of PNU200969 was metabolised to PNU200001 after 90 min exposure to the mucosal side of the

Table II. Bidirectional Permeabilities of fXa Inhibitors and Reference Compounds across Rat Ileal Tissue

Compound	Papp × 10^{-6} (cm/s) mucosa to serosa[a]	Papp × 10^{-6} (cm/s) serosa to mucosa[a]	Ratio[b]
^{14}C-mannitol	6.6 ± 2.4	9.1 ± 2.7	1.4
^{3}H-testosterone	19.5 ± 5.2	24.3 ± 10.7	1.2
PNU200001	1.2 ± 0.4	13.7 ± 0.2	11.4
PNU200681	2.3 ± 0.9	15.8 ± 0.2	6.9

[a] Papp is the apparent permeability. Donor concentrations were 0.2 mM for the fXa inhibitors.

[b] The ratio of permeabilities in the basolateral to apical versus the apical to basolateral direction is given.

intestinal tissue (Fig. 5). Approx. 4% of the added donor substance was already in the non-substituted form at the beginning of the experiment. For PNU200969 added to the serosal side of the tissue, only 7% was metabolised to PNU200001 after 90 min exposure. The responsible hydrolytic enzymes were thus located primarily at the mucosal side of the epithelium. Following the addition of PNU200969 to Caco-2 cells at either side of the epithelium, only a minor amount was transformed to PNU200001 (Fig. 5). This shows that the apically located hydrolytic enzymes that metabolized PNU200969 in the rat ileum were not functionally present in Caco-2 cells under the conditions used. Such differences in hydrolytic activity are not uncommon (29).

Owing to the fact that PNU200969 was metabolised during the transport experiments, a permeability coefficient could not be calculated for this compound. Instead, the cumulative amount of PNU200969 and the cumulative amount of PNU200969 plus the metabolite PNU200001 transported across the tissue were plotted against time (Fig. 6). PNU200969 was found in only very small quantities in the receiver chamber for transport experiments in the mucosa to serosa and in the serosa to mucosa directions. Large amounts of PNU200001, however, appeared in the receiver chamber in the serosa to mucosa experiment but not for the experiment in the opposite direction. Because PNU200969 was not metabolized at the serosal site of the tissue, PNU200969 was taken up at the basolateral membrane of the intestinal cells in the non-hydrolysed form and was subsequently metabolized intracellularly during passage or at the mucosal membrane. This also implies that PNU200969 added to the mucosal chamber had a poor ability to cross the mucosal tissue. Before entering the epithelium, PNU200969 may have been converted into PNU200001, which has low membrane permeability. In addition, PNU200969 may have been subject to a secretory transport. In contrast to PNU200969, PNU200681 was not converted to any significant extent to the corresponding non-substituted amidine derivative, PNU200648. For PNU200681, it was therefore possible to calculate a permeability coefficient. As with the results obtained with the Caco-2 cell model, the permeability in the serosa to mucosa direction was much larger than the permeability in the mucosa to serosa direction (Table II).

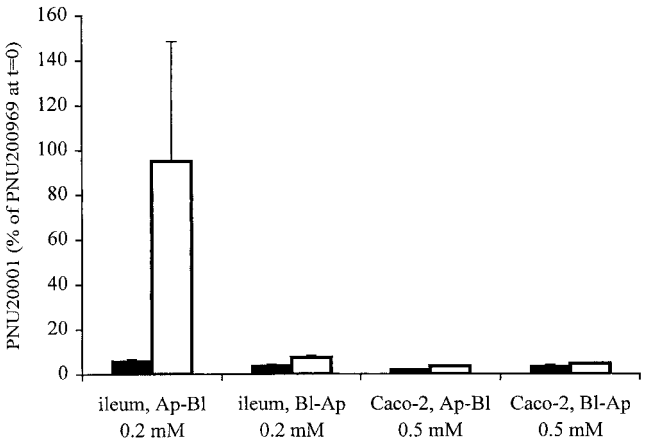


Fig. 5. Conversion of 0.2 mM PNU200969 to PNU200001 in donor solutions, start (■) and end of experiment (□), during transport experiments using excised rat ileal epithelium. The amount of the hydrolysis product PNU200001 is given relative to the donor concentration of the carbamate ester (PNU2000969) at the beginning of the transport experiment.

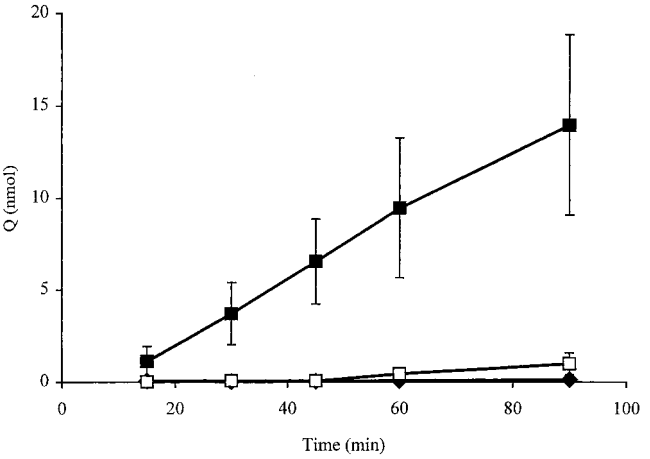


Fig. 6. Bidirectional transport of the bisbenzimidazole carbamate ester PNU200969 (Ap-B1 (◇) and B1-Ap (◆)) plus the corresponding hydrolysed product PNU200001 (Ap-B1 (□)) and B1-Ap (■)) across rat ileal tissue. Q is the cumulative amount absorbed. The donor concentration of PNU200969 was 0.2 mM.

The present study has shown that poor intestinal permeability may be important for the incomplete oral absorption of the fXa inhibitors. For the non-substituted amidines, hydrophilicity due to ionisation at neutral pH may result in poor membrane partitioning. For the neutral compounds with substituted amidines, susceptibility to active secretion may have limited the *in vitro* permeability. The significance of the secretion process was nicely illustrated by the results obtained for the neutral analogue PNU200647. Permeability of this compound through Caco-2 cell monolayers was not obviated by a secretory transport mechanism, making the absorptive permeation concomitantly high.

In addition to poor intestinal permeability limiting absorption, the carbamate ester derivative, PNU200969, was subject to metabolic hydrolysis in rat intestine. The secretory protein(s) involved in the transport of the fXa inhibitors could not be identified in this work but was most likely not the *MDR1* protein, Pgp. The transporter was, however, shown to be a significant barrier to the absorption of the fXa inhibitor.

ACKNOWLEDGMENTS

The MDCK-MDR1 cell line was kindly provided by M. M. Gottesman and the NIH through a Material Transfer Agreement. We are most grateful to J. Bourdage, C. Cole, and B. Pfund for providing access to the MDCK cell permeability studies and their skillful technical assistance and to Ardeshir Amirkhani for skillful technical assistance with the pK_a and $logK_D$ determinations. We gratefully acknowledge the help of Danny McGee and Bob Day in making the substances. We wish to thank Dr. T. J. Raub for helpful discussions and critical evaluation of the manuscript.

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