Transport of Peptidomimetic Thrombin Inhibitors with a 3-Amidino-Phenylalanine Structure: Permeability and Efflux Mechanism in Monolayers of a Human Intestinal Cell Line (Caco-2)

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Purpose. Peptidomimetic thrombin inhibitors derived from Nα-(2-naphthylsulfonyl)-3-amidino-phenylalanine with different basic and acidic substituents were investigated with respect to their intestinal transport behavior.

Methods. Intestinal permeability coefficients were studied using Caco-2 monolayers and a reversed-phase HPLC method for quantitation.

Results. Apparent permeability coefficients P_{app} of compounds with a free amidino group were in general low ($<10 \times 10^{-8}$ cm/s) and independent of the structure of the amide part (C-terminus). Polarized efflux, however, was strongly affected by substituents in the amide moiety yielding the following efflux ratios (ER): methylpiperidide **(1)** (ER 45) > piperidine carboxylic acid methylester (ER 6-11) > piperidine carboxylic acids (ER 1.9-2.9) > piperazide (ER -0.17). Efflux of **(1)** was temperature-dependent, but independent of the enantiomeric configuration, accompanied by an increase in transepithelial electrical resistance (TEER), and could be reduced by P-gp inhibitors (PSC 833, Cremophor EL) but not by indomethacin. Replacement of the amidine group of **(1)** by aminomethyl-, amino-, and oxamidine- moieties drastically increased absorptive permeability (46-68 fold) with ER < 3.4. In contrast, the oxamidine with a C-terminal nipecotic acid residue **(8)** displayed also a temperature dependent efflux- without altering TEER (ER 22). This efflux was sensitive to PSC833/Cremophor EL and indomethacin.

Conclusions. Basic and acidic residues of amidino-phenylalanine-derived thrombin inhibitors mediate affinity to intestinal efflux pumps, presumably P-gp and MRP. P-gp mediated efflux was related to a net positive charge and accompanied by an increased TEER. Among the methylpiperide **(1)** promoieties studied the oxamidino group seems to be very promising in overcoming both transport and efflux problems frequently encountered with peptidomimetics containing amidines.

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INTRODUCTION

The design of drug molecules with intestinal absorption rates allowing oral therapy of chronic diseases remains a major challenge for drug development. Peptidomimetic drugs are currently under investigation as receptor antagonists or enzyme inhibitors for various disorders offering higher stability against presystemic degradation compared to peptides. Low membrane permeability due to their hydrophilic and charged structures causes frequently low oral bioavailability which is compounded by efflux carriers abundant in the gastrointestinal tract and hepato-biliary elimination (1–3). Among these transporters are P-glycoprotein (P-gp), multiple drug resistance associated protein 1 and 2 (MRP 1 in the intestine, MRP 2 in the liver) and organic anion transporting polypeptide (OATP in the liver). For rational drug design also taking into account oral bioavailability it is necessary to understand the structural aspects of peptides/peptidomimetics on the various transport mechanisms effective in drug absorption and presystemic elimination.

Numerous substrates for intestinal P-glycoprotein have been described, e.g., β- receptor antagonists, cytostatics, vinblastine, actinomycin D and different peptides and cyclopeptides, such as cyclosporine A or pristinamycin (1–6). Structural requirements for P-gp binding seem to be high hydrophobicity, neutral or positive charge and a planar molecular geometry. But also hydrophilic peptides were reported to be potential substrates of P-glycoprotein-mediated intestinal secretion (7).

Recently, we have demonstrated that modification of the amidino group in hydrophilic $\alpha_{IIb}\beta_3$-inhibitory peptidomimetic drugs affects the affinity to a polarized efflux system, most likely P-gp, in Caco-2 monolayers (8). MRP1 complements the broad substrate specificity of P-gp, transporting preferentially negatively charged and neutral compounds such as organic anions like indomethacin, carboxyfluoresceine- derivatives and leucotriene C4 as well as phase II metabolites like glutathion or sulfate conjugates (1–3). Moreover, some anionic chemotherapeutics with peptidomimetic activity were identified as substrates of an efflux mechanism independent of P-gp, which could be inhibited by the MRP1 reversal agents probenecid (9) and indomethacin (10).

Here we investigate the effect of structural modifications in a series of peptidomimetic thrombin inhibitors on intestinal transport characteristics. The backbone consists of Nα-(2-naphthylsulfonyl)-3-amidino-phenylalanine which was modified by basic and acidic functionalities, yielding at physiological pH positively charged, negatively charged or zwitterionic molecules of different lipophilicities (Fig. 1). Using this series of compounds our goal was to identify structural elements responsible for low intestinal permeability of peptidomimetic thrombin inhibitors with strongly basic groups.

An essential requirement for designing peptidomimetics with improved pharmacokinetic properties, such as improved peroral absorption, is the establishment of a structure transport relationship. In this context we evaluated the human intestinal cell line Caco-2 as tool to assess transepithelial transport and metabolism.

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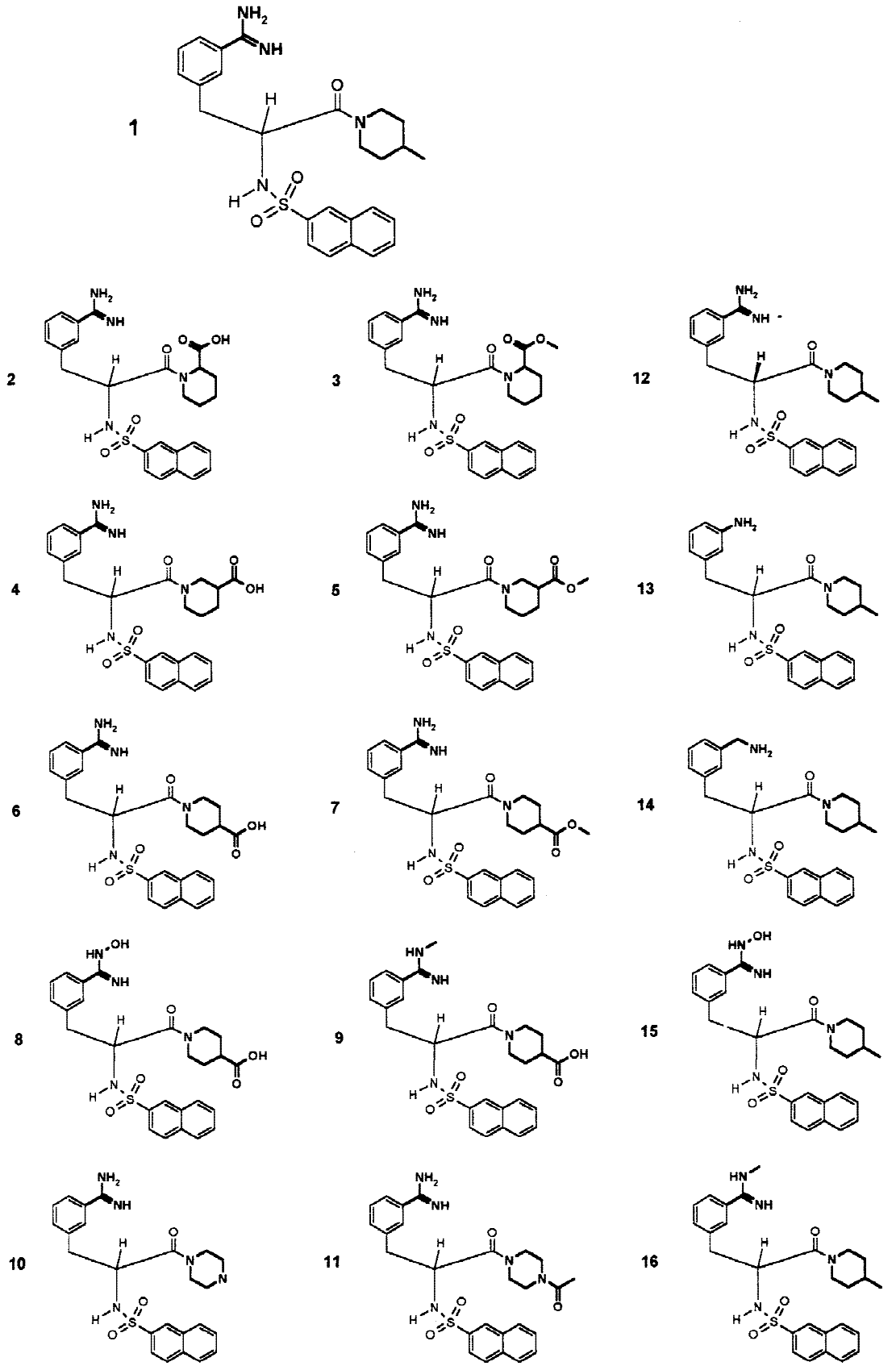

Fig. 1. Chemical structure of different thrombin inhibitors with a Nα-(2-naphthylsulfonyl)-3-amidino-phenylalanine-amide backbone used for transport studies in Caco-2 monolayers.

MATERIALS AND METHODS

Materials

Tissue culture reagents were from Gibco (Eggenstein, Germany). Tissue culture articles were purchased from Nunc (Wiesbaden, Germany) and polycarbonate membrane cell culture inserts (Transwell, Cat.No. 3412, Costar, membrane pore diameter 0.45 μm) were supplied by Integra Biosciences (Fernwald, Germany). Cremophor EL was from BASF AG (Ludwigshafen, Germany) and SDZ PSC 833 was a kind gift of Prof. Dr. Fahr (Philipps-University Marburg, Germany). All other chemicals and reagents of analytical grade were obtained from E. Merck AG (Darmstadt, Germany) and Sigma Chemicals Co., St.Louis, MO.

General Synthetic Methods

The compounds were synthesized as described previously (11).

Apparent Octanol/Buffer Distribution Coefficients (log D) and pKa-Values of the Compounds

Log D data of the compounds were calculated under transport conditions (pH 6.7) using the Prolog D V.2.1 software. pKa-values were obtained with the pKalc V.3.2. software. Both were from CompuDrug Chemistry Ltd., Budapest, Hungary.

Cell Culture

Caco-2 cells at passages 43–46 were routinely maintained in DMEM, supplemented with 4.5 g glucose/L, 10% fetal calf serum, 1% nonessential amino acids, 2 mM glutamine at 37°C without antibiotics in an atmosphere of 10% CO_2. Cells grown on 100 mm-diameter polystyrene petri-dishes were passaged every 5 days at a split ratio of 1:5 to 1:7. For transport and metabolism studies only mycoplasma screened cells were used (12).

Transport Studies

Transport studies were performed as described previously (12). Briefly, cells were seeded at a density of 6.5×10^4 cells/cm^2 on microporous polycarbonate filters (Costar Transwell, Cat. No. 3412). Transport experiments were performed between the 21st and 23rd day post seeding directly on the filter inserts in a 10% CO_2 atmosphere. The compounds (concentrations see below) and the paracellular marker FITC-dextran 4000 (FD-4, 200μg/ml) were dissolved in transport buffer. In apical (A) to basolateral (B) transport studies 1.5 mL of drug containing transport buffer (PBS, 15 mM glucose, pH 6.7) was placed on the A or luminal and 2.6 mL of transport buffer (PBS, 15 mM glucose, pH 6.7) on the basolateral (B) side. B➠A transport was studied vice versa. After predetermined time intervals up to 120 min, 1 mL samples were withdrawn from the receiver compartment and the volume was replaced by fresh buffer. The integrity of the cell monolayers was checked at the beginning and the end of all experiments by measuring the transepithelial electrical resistance (EVOM, WPI, Germany).

Inhibition Experiments

To evaluate efflux mechanisms we investigated the effects of indomethacin, verapamil as well as of the cyclosporine (CsA) derivative PSC 833 and of Cremophor EL on **1** and **8** transport. For A➠B transport and vice versa indomethacin or verapamil (only with **1**) were added each at 250 μM to the donor solution of the compound (100 μM **1**, **8**), while transport buffer was applied to the acceptor side. In PSC 833 inhibition (20 μM)- experiments addition of DMSO and Cremophor EL, each at final concentrations of 0.1% was necessary for solubilization. For studying A➠B transport the respective thrombin inhibitor was dissolved at a final concentration of 100 μM in that solution. For B➠A transport PSC 833/Cremophor EL buffer solution without drug was added to the apical receiver chamber.

Analytical Methods

Aliquots of the samples were analyzed by reversed phase HPLC using an automatic sampler (model AS-2000A), a column thermostat (model T-6300) and a fluorescence detector (model L1050), all from Merck-Hitachi (Darmstadt, Germany). Data acquisition and integration were carried out by the Millenium 2010 software (Millipore Waters, Eschborn, Germany). The compounds were separated on a reversed-phase HPLC-column (LiChrocart RP 18, 5μm, 250 × 4 mm; Merck AG, Germany) under isocratic conditions. The mobile phase (flow rate = 1.2 mL/min) consisted of 33-50% (v/v) acetonitrile in water with 0.1% trifluoroacetic acid (TFA) as the ion-pairing agent. Detection was performed using fluorimetric analysis at an excitation wavelength of λ_{Exc} = 232 nm and an emission wavelength of λ_{Emm} = 343 nm. Under these conditions retention times for the compounds were between 3 and 7 min at detection limits around 4 nM. The parameters for the reversed phase HPLC-detection of the paracellular marker FD-4 were: column RP 18, 3μM, 30 × 4mm; mobile phase 8.5% Acetonitrile/91.5% KH_2PO_4 (2.5 mM, pH 7.0), flow 1 mL/min, retention time 0.55 min, wavelengths for fluorimetric analysis were λ_{Exc} = 490nm and λ_{Emm} = 515 nm; the detection limit was ca. 2 ng/mL.

Data Treatment

Apparent permeability coefficients (P_{app}) were calculated as described previously (12). All results are expressed as the mean of at least 3 experiments ± standard deviation. Statistical analyses were performed using two sided independent *t*-test.

Net secretory fluxes [pmol/(cm^2 × min)] were calculated from the summed amounts of the compound in the acceptor compartments according to:

$$\text{NetSecretoryFlux} = \frac{\text{pmol(B}\rightarrow\text{A)} - \text{pmol(A}\rightarrow\text{B)}}{Ar \times t}$$

where Ar is the monolayer surface (4.71 cm^2) and t is the transport time (120 min).

RESULTS

Transport of Compounds 1–11 Across Caco-2 Monolayers

As shown in Table I large differences in transport parameters, especially with regard to the efflux behavior, were found. In all experiments a linear flux was observed from

Table I. Physico-Chemical Data, Permeability Through Caco-2 Monolayers, Changes in Transepithelial Electrical Resistance (TEER) of the Thrombin Inhibitor **1** and Related Compounds. Concentration of the Compounds Each 250 μM

Compound	log D pH 6.7	pKa acidic	pKa basic	Transport direction	P_{app} [$\times 10^{-8}$ cm/s][a]	Secretory Net flux [pmol/(cm^2 × min)]	Efflux ratio B → A/A → B	Relative Teer 120 min [%][b]	K_i-Thrombin [μMol]
1	0.47	9.6	11.62	A → B	4.87 ± 0.95	32.3	45.0	170 ± 5	0.0025
				B → A	219 ± 6.10			251 ± 11	
2	–2.48	3.8	11.62	A → B	4.14 ± 0.60	1.15	2.8	110 ± 3	0.19
				B → A	11.6 ± 0.50			118 ± 2	
3	1.2	9.6	11.62	A → B	7.66 ± 0.99	10.6	10.5	143 ± 2	0.048
				B → A	80.1 ± 2.89			183 ± 6	
4	–1.76	4.3	11.62	A → B	4.13 ± 0.14	1.01	2.7	119 ± 1	1.6
		9.6		B → A	11.1 ± 0.49			129 ± 5	
5	0.63	9.6	11.62	A → B	3.91 ± 0.51	6.05	11.1	132 ± 2	0.15
				B → A	43.4 ± 2.00			161 ± 4	
6	–1.69	4.5	11.62	A → B	4.59 ± 0.56	0.66	1.9	108 ± 2	0.19
		9.6		B → A	8.62 ± 0.41			117 ± 2	
7	0.63	9.6	11.62	A → B	8.08 ± 1.58	6.70	6.0	129 ± 6	0.010
				B → A	48.7 ± 3.35			174 ± 9	
8	–0.86	4.5	10.46	A → B	4.31 ± 0.16	12.4	22.1	105 ± 1	160
		9.6		B → A	95.4 ± 4.27			111 ± 2	
9	–1.6	4.5	12.98	A → B	7.12 ± 1.69	0.05	1.1	98 ± 4	0.21
		9.6		B → A	7.84 ± 0.49			104 ± 4	
10	–3.05	9.6	11.62	A → B	9.28 ± 2.00	–0.17	0.8	122 ± 8	0.012
			8.97	B → A	7.69 ± 1.90			119 ± 6	
11	–1.44	9.6	11.62	A → B	4.31 ± 0.64	0.96	2.4	101 ± 3	0.17
				B → A	10.5 ± 0.67			124 ± 4	

[a] Mean ± S.D. (n = 3).
[b] In relation to the initial TEER-values. Mean ± S.D. (n = 3).

donor to acceptor compartment after a short lag time (< 5 min), independent of whether the drug was initially applied to the apical (A) or basolateral (B) chamber (not shown). The recovery of intact drug substance (mass balance) in each experiment was higher than 90%.

Due to the amidino group the methylpiperidide structure **1** carries a strong positive charge at pH = 6.7 (pKa_{bas} = 11.62) and is also quite hydrophobic (log D = 0.47). For **1** we observed an apparent permeability coefficient P_{app} = 4.87 ± 0.95 × 10^{-8} cm/s in A➠B direction and a secretory permeability coefficient of 219 ± 6.1 × 10^{-8} cm/s, yielding an efflux ratio (ER), defined as the ratio of the permeability coefficients B➠A/A➠B, of about 45. No significant differences between absorptive and secretory flux (P_{app} A➠B = 0.61 ± 0.09 × 10^{-8} cm/s, P_{app} B➠A = 0.75 ± 0.06 × 10^{-8} cm/s) were noted for FD-4, a commonly used paracellular marker, suggesting a transcellular efflux process for **1**.

Replacement of the methyl group in the piperidide residue of **1** by a carboxylic group yields—the compounds **2** (2-position), **4** (3-position) and **6** (4-position). Absorptive permeability coefficients of these zwitterionic hydrophilic molecules were between 4 and 5 × 10^{-8} cm/s, independent of the position of the carboxyl group. An efflux was still observed, most pronounced with **2** and **4** (about 11-12 × 10^{-8} cm/s with ER ca. 2.7) and significantly smaller with the 4-derivative **6** (8.62 × 10^{-8} cm/s, ER 1.9). In this case the efflux was drastically reduced compared to **1**.

Esterification of the carboxyl group leads to the more lipophilic (log D = 0.63 - 1.2) positively charged methylesters and increased the absorptive permeability of the 2- (**3**) and 4-derivatives (**7**) almost 2 fold, whereas the 3-derivative (**5**) was unaffected. Compared to the zwitterionic parent compounds efflux increased drastically up to 80.1 ± 2.89 × 10^{-8} cm/s (**3**, ER = 10.5), 43.4 ± 1.97 × 10^{-8} cm/s (**5**, ER = 11.1) and 48.7 ± 3.35 × 10^{-8} cm/s (**7**, ER = 6.0), pointing to efflux mechanism(s) relying on positive net charge and lipophilicity as structural elements. Cell-mediated cleavage of the methylesters to the free acids was low yielding recoveries of the intact ester after transport experiments > 95 %.

Derivative **6** with the lowest secretory flux was then further modified at the amidino group namely, the oxamidine (**8**) and the methylamidine (**9**). The oxamidino group in **8** increased the lipophilicity but did not enhance the absorptive permeability (4.31 ± 0.16 × 10^{-8} cm/s), while the efflux permeability was drastically increased (95.4 ± 4.27 × 10^{-8} cm/s vs. 8.62 ± 0.41 × 10^{-8} cm/s for **6**) yielding an ER = 22. Methylation of the amidino group increased the basicity of **9** (pKa_{bas} = 12.98) and equalized absorptive and efflux permeabilities (absorptive P_{app} = 7.12 ± 1.69 × 10^{-8} cm/s, secretory P_{app} = 7.84 ± 0.49 × 10^{-8} cm/s).

Replacement of the piperidide of **1** by a piperazide residue (**10**) generated a second basic functionality (pKa_{bas} = 8.97) of low lipophilicity (log D = –3.05). Apparent permeability coefficients of 9.28 ± 2.0 × 10^{-8} cm/s (absorption) and 7.69 ± 2.0 × 10^{-8} cm/s (efflux) demonstrate that efflux was effectively suppressed compared with **1**. Masking this second basic group by N-acetylation (**11**) decreased the absorptive P_{app} to 4.31 ± 0.64 × 10^{-8} cm/s and a small but statistically significant efflux could be observed again (10.5 ± 0.67 × 10^{-8} cm/s), indicating that an additional positive charge minimized efflux.

Changes in Transepithelial Resistance

As shown in Table I the compounds displayed different effects on the transepithelial electrical resistance (TEER). At 250 μM a significantly higher increase in TEER was observed

for **1** and the positively charged lipophilic esters **3**, **5**, and **7**, compared with the compounds having a free carboxylic group (**2**, **4**, **6**, or **8**). In all cases increase of basolateral to apical TEER was more pronounced (Table I). For the derivatives **9** and **10**, which do not alter TEER, efflux was negligible, while the efflux of **11** (amid of **10**) was coincident with a TEER increase again.

Characterization of the Secretory Transport of Compounds 1 and 8

To characterize the transport mechanism for the compounds **1** and **8** structurally differing in the amidine- and the amide part we examined the temperature dependence of the polarized efflux and the effect of coadministration of inhibitors of intestinal efflux systems, i.e., verapamil and the P-gp blocking agent SDZ-PSC833 solubilized in Cremophor EL and indomethacin as MRP1 inhibitor. Both, P-gp and MRP1, affecting the absorption of a wide variety of drugs, were recently shown to be expressed in human epithelial Caco-2 cells (13,14). Transport concentration of **1** and **8** was adjusted to 100 μM to minimize amounts of the inhibitors.

Temperature Dependence

As outlined in Fig. 2 permeability coefficients as well as ER were markedly dependent on temperature. In the case of **1** (Fig. 2A) absorptive P_{app} was lowered from 16.7×10^{-8} cm/s (37°C) to 2×10^{-8} cm/s (4°C) and secretory P_{app} from 273×10^{-8} cm/s to 9.4×10^{-8} cm/s, the corresponding ER decreased from 16.3 (37°C) to 4.7 (+4°C), compatible with a temperature dependent efflux process. Similar observations were made for **8** (Fig. 2B). At +4° C the secretory P_{app} decreased from 135×10^{-8} cm/s to 7.7×10^{-8} cm/s significantly stronger than those observed in the opposite direction (10.1×10^{-8} cm/s vs. 1.89×10^{-8} cm/s) yielding ERs of 13.4 (37°C) and 4.1 (+4°C), suggestive of an active transport process. In each experiment the temperature dependent decrease of absorptive and secretory FD 4 permeabilities was similar.

Inhibition Experiments

As shown in Fig. 2A verapamil did not influence the efflux of **1** with respect to ER. Secretory permeability was lowered from 273 to 191×10^{-8} cm/s, but also the absorptive permeability from 16.7 to 3.52×10^{-8} cm/s, yielding efflux ratios larger than the control (54.3 vs. 16.3). A similar pattern was seen for the paracellular marker FD-4, the transport of which was also reduced in both directions in the presence of verapamil.

Inhibition experiments using indomethacin, a well known inhibitor of MRP1 yielded both a slight increase in absorptive (20.1 vs. 16.7×10^{-8} cm/s) as well as in secretory permeability (424 vs. 273×10^{-8} cm/s) without reduction of the efflux ratio, indicating that MRP1 seems not to be involved in transport of **1**. PSC 833/Cremophor massively reduced efflux permeability to 24.4×10^{-8} cm/s, whereas absorption was only increased to 17.8×10^{-8} cm/s, resulting in an ER = 1.4. With the exception of verapamil, the FD-4 transport was not affected by the inhibitors used in this study.

Indomethacin significantly reduced the secretory permeability of **8** from $135 \pm 3.3 \times 10^{-8}$ cm/s to $43.1 \pm 2.9 \times 10^{-8}$ cm/s (Fig. 2B). Simultaneously absorptive permeability increased from $10.1 \pm 1.0 \times 10^{-8}$ cm/s to $14.7 \pm 1.2 \times 10^{-8}$ cm/s, yielding

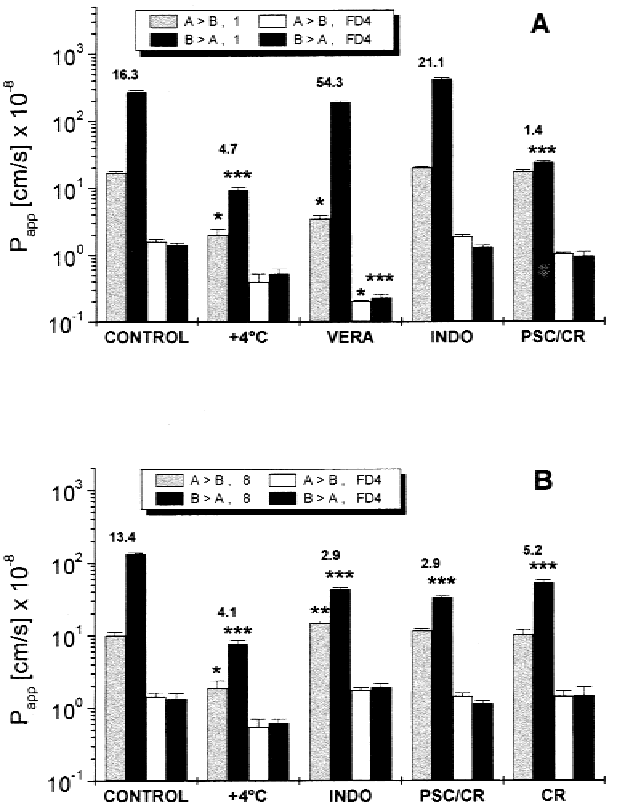

Fig. 2. Effect of temperature and different efflux inhibitors on the secretory transport of **1** (Fig. 2A) and **8** (Fig. 2B) (concentrations 100μM each) in comparison with FITC-Dextran 4 kDa. **Control**: transport without modulator at 37°C; **+4°C**: transport without modulator at +4°C; **Vera**: transport with verapamil (250 μM); **Indo**: transport with indomethacin (250 μM); **PSC/CR**: transport with PSC833 (20 μM)/Cremophor EL (0.1%); **CR**: transport with Cremophor (0.1%). B➠A / A➠B transport ratios (efflux ratios) are shown above the columns. *significantly different ($p < 0.001$) from A➠B transport of the control, **significantly different ($p < 0.01$) from A➠B transport of the control, ***significantly different ($p < 0.001$) from B➠A transport of the control; two sided independent *t*-test, mean ± SD (n = 3).

an efflux ratio of 2.9 vs. 13.4 (control). PSC 833/Cremophor EL also significantly affected the efflux of **8**. The apparent permeabilities through the monolayers were $11.7 \pm 0.61 \times 10^{-8}$ cm/s for absorption and $33.8 \pm 1.1 \times 10^{-8}$ cm/s for secretion, respectively, with the efflux ratio lowered from 13.4 to 2.9. Cremophor EL alone reduced efflux permeability to $53.5 \pm 4.2 \times 10^{-8}$ cm/s, while absorption ($P_{app} = 10.3 \pm 1.2 \times 10^{-8}$ cm/s) was not increased (efflux ratio = 5.2). FD4 -permeabilities in the inhibition experiments were not different from the control.

Further Modifications of the Benzamidino Structure in Compound 1

To further characterize the secretory permeability of **1**, whose efflux seemed to be related to P-gp, we investigated the permeabilities of **1** in comparison to derivatives **12** (D- enantiomer) and **13–16** modified at the amidino moiety (Fig. 1, Table II). Due to the limited aqueous solubility of these more lipophilic compounds only concentrations of 100 μM could be tested. All experiments displayed after a short lag time of

Table II. Influence of Variations of the Amidino Moiety of Compound **1** on Physicochemical Data, Permeability Through Caco-2 Monolayers, Changes in Transepithelial Electrical Resistances (TEER). Concentration of the Compounds Each 100 μM

Compound	log D pH 6.7	pKa acidic	pKa basic	Transport direction	P_{app} [$\times 10^{-8}$ cm/s][a]	Secretory Net flux [pmol/(cm^2 × min)]	Efflux-ratio B → A/A → B	Relative TEER 120 min [%][b]	Ki-Thrombin [μMol]
1	0.47	9.6	11.62	A → B	16.7 ± 1.1	15.5	16.3	116 ± 2	0.0025
				B → A	273 ± 17			159 ± 1	
12	0.49	9.6	11.62	A → B	13.4 ± 1.8	19.2	24.9	120 ± 7	0.19
				B → A	333 ± 0.9			135 ± 3	
13	3.38	9.51	4.38	A → B	1010 ± 62	8.4	1.14	102 ± 17	8.9
				B → A	1150 ± 17			102 ± 11	
14	0.66	9.53	9.32	A → B	773 ± 14	113	3.43	107 ± 10	1.9
				B → A	2650 ± 52			104 ± 3	
15	1.2	9.6	10.46	A → B	1140 ± 23	21	1.31	88 ± 9	2.8
				B → A	1490 ± 82			83 ± 6	
16	1.93	9.6	12.98	A → B	14.1 ± 2.0	10.1	13.0	108 ± 9	5.7
				B → A	183 ± 8.0			105 ± 19	

[a] Mean ± S.D. (n = 3).
[b] In relation to the initial TEER-values. Mean ± S.D. (n = 3).

<5–7 min linear transport kinetics. The calculated mass balances were between 92 and 103%.

Efflux behavior was independent of chiral configuration as shown by data of the D isomer **12**. Reducing the basicicity of the amidino group in **1** by introduction of a hydroxylic group (oxamidino derivatization, **15**, pKa_{bas} = 10.46, log D = 1.2), increased the absorptive P_{app} ca. 68 fold. A secretory component (ER = 1.3) disappeared almost completely compared to **1** and **8** (carboxy- analogue of oxamidine **15**).

Substitution of the amidino group either by an amino (**13**) or aminomethyl (**14**) moiety also significantly increased permeation. In the case of **13** absorption was almost 60 fold higher with an ER = 1. Efflux was still significant for **14** with an ER = 3.4, the absorptive P_{app} increased 46x. Compound **16** (methylamidino derivative) showed quite identical transport properties as **1**.

DISCUSSION

The peroral bioavailability of peptidomimetics is limited by low intestinal absorption, intestinal secretion and hepatic uptake with biliary excretion. In this context efflux transporters such as intestinal P-gp and MRP1 and/or hepatic cMOAT (MRP2), OATP and P-gp need to be considered (1–3). Peptidomimetic thrombin inhibitors with strongly basic amidino groups are notorious for their low oral bioavailability due to limited intestinal membrane permeability and/or hepatobiliary first-pass elimination (12,15–20). For amidinophenylalanine-derived thrombin inhibitors hepato-biliary clearance via OATP-mediated uptake by hepatocytes/ liver has been demonstrated (16,17). So far no information on potential intestinal efflux mechanisms of thrombin inhibitors was available.

Our data show that the absorptive permeability in Caco-2 cells of thrombin inhibitors with charged groups, a free amidino group and/or an additional free carboxyl group, is in general low due to their charged character but also due to the involvement of efflux carriers. Among the model compounds tested the amidino derivatives **1** through **7**, **10**, **11**, the oxamidino derivative **8** and the methylamidino derivatives **9** and **16** showed low A➠B P_{app} values. These findings are in accordance with literature data on Caco-2 cell permeability of thrombin inhibitors with strongly basic amidino or guanidino groups (12,18–20). Interestingly, most compounds showed considerably higher apparent permeability coefficients in the direction B➠A.

In the series of amidino derivatives with zwitterionic structure, methyl-esterification of the carboxyl group in the amide moiety, yielding positively charged molecules, increased net secretory flux (**3**, **5**, and **7** vs. **2**, **4**, and **6**). The gain in lipophilicity promoted drug efflux more effectively than absorption. Similar findings (P-gp involvement) were found with a benzyl-ester of a zwitterionic fibrinogen receptor antagonist containing a piperazine ring as the basic motif (21).

Apart from lipophilicity, charged functionalities affect the affinity to efflux transporters. The hydrophilic oxamidino derivative **8** with a less basic group and a carboxylic group shows drastically higher efflux than the corresponding amidino derivative **6**, whereas, the corresponding methylamidino derivative **9** with a more basic group shows almost no efflux. This is also true of the hydrophilic amidines **10** and **11**.

The substitution of the amidino- by other groups in the lipophilic compounds **13** through **16** resulted in a marked (46–68fold) increase in absorptive permeability compared to the amidine **1** together with a modest increase (4.2–9.7fold) in secretory P_{app} values.

The characterization of the efflux mechanism(s) can be summarized as follows: For derivatives with a positive net charge (**1**, **3**, **5**, **7**), a marked efflux was observed which could be completely inhibited by PSC 833/Cremophor EL. The cyclosporine derivative PSC 833 is an effective P-gp-inhibitor for reversal of multidrug resistance in cancer cells (22) and increases the intestinal permeability of P-gp-substrates, such as vinblastine, paclitaxel or cyclosporin (23). Cremophor EL, necessary to solubilize PSC 833, itself induces inhibitory effects on the apically localized efflux pump P-gp (24). The P-gp inhibitor verapamil affected transport of **1** in a complex manner due to an additional effect on paracellular permeability. Verapamil decreased the paracellular permeability of FD4 in both directions A➠B and B➠A to a similar extent (7.8fold and 6.2fold, respectively, Fig. 2). Absorptive permeability of **1** in the presence of verapamil decreased 4.7fold from 16.7 to 3.52×10^{-8} cm/s and 1.4fold from 273 to 191×10^{-8} cm/s in the opposite direction. Due to the observation that paracellular permeability of the drug in each direction is similar and low

(supported by the data of FD4) the absolute decrease of efflux permeability of about 82×10^{-8} cm/s suggests an influence of verapamil on P-gp mediated efflux of **1.** Indomethacin, a specific modulator of the MRP1 protein had no effect on the transport characteristics of **1**.

The efflux mechanism of **8** (oxamidino derivative with carboxylic group) seems to be different from that of **1** in the light of following observations: (i) **8** did not cause an increase in transepithelial electrical resistance, while lipophilic compounds with positive net charge (**1**, **3**, **5**, **7**) distinctly increased TEER; (ii) significant decrease of ER after indomethacin coadministration, while efflux of **1** was insensitive to indomethacin treatment. Recently several authors reported an inhibition of a "non P-gp" efflux mechanism by indomethacin (10). Draper *et al.* demonstrated that MRP1 was inhibited by indomethacin (25). Similar to **1,** PSC 833 and Cremophor EL significantly reduced the secretory transport of **8**, but did not completely equalize absorptive and secretory components. Using P-gp and MRP overexpressing cancer cells PSC833 as well as Cremophor EL caused a complete inhibition of P-gp, while MRP- blockade was less pronounced (27). The mechanism of inhibition seems to be a conformational change of the apically localized protein domains by interaction with the amphiphilic molecules similar to that seen for P-gp inhibition (28). Therefore, the involvement of MRP1 in the transport of the oxamidino-derivative **8** seems to be very likely.

The effect of structural variations of thrombin inhibitors on their efflux characteristics can be summarized as follows:

1. Efflux was increased by strongly basic functionalities (**1**) and increasing lipophilicity (**3**, **5**, **7**).
2. Efflux decreased drastically compared to **1** when the amide moiety contained an anionic functionality yielding a zwitterionic molecule (**2**, **4**, **6**).
3. Secretory and absorptive permeabilities coefficients of similar magnitude were observed when the amide moiety contained a second cationic structure (**10**).

Figure 3 demonstrates the relationship between log D values and observed net secretory P_{app} , indicating that both lipophilicity and the free amidino group mediate the affinity to a P-gp efflux mechanism. In accordance with the literature these data suggest that hydrophobicity seems to be a key factor determining affinity to efflux carriers, such as P-gp (3,4).

Structural variations of **1** leading to compounds with higher log D values (**12–16**) demonstrate that positive net charge seems to be an essential feature for substrate recognition by P-gp. Indeed the strongly basic amidino group is responsible for both, the low absorptive permeability and the affinity to a polarized intestinal efflux system. When the amidino group was replaced by less basic residues or substituted yielding an oxamidino derivative, the absorptive permeability increased drastically and efflux ratios were low. The oxamidine derivative **15** could be a promising candidate for effective oral delivery. Such amidine prodrug structures have been used for masking highly charged amidino groups to promote intestinal transport of RGD mimetic drugs (29,30). Also effects of some thrombin inhibitors on the TEER during transport experiments were noted. This increase in TEER especially for B➠A transport, seems to correlate with the observed B➠A efflux for compounds with a free amidine moiety (Fig. 4, Table I). From these **1** was shown to be transported presumably via P-gp. Interestingly the significant efflux of **8**, which was not transported via P-gp but MRP had no effect on TEER. Verapamil (≤300μM), a P-gp inhibitor, also increased transepithelial electrical resistance in Caco-2 cells (31). Recently a drastic increase in transepithelial resistance brought about by H_2- receptor antagonists like ranitidine and cimetidine was reported , which were also shown to be transported by P-gp (32–34). We also observed that an increase in drug concentrations of **1** (250μM vs. 100μM) increased TEER (Table I, Table II) and lowered absorptive paracellular FD 4 permeability (0.61×10^{-8} cm/s for 250μM vs. 1.6×10^{-8} cm/s for 100μM). Possible explanation for this TEER behavior could be the blockade of paracellular anionic bindings sites by cationic drug molecules as proposed by Lee *et al.* (33) in the case of H_2 receptor antagonists. Another hypothesis is that the P-gp transported drugs inhibit outwardly rectifying chloride channels as reported by (35), thereby increasing both the intracellular chloride concentration and the transepithelial resistance. Electrophysiological studies are necessary to verify

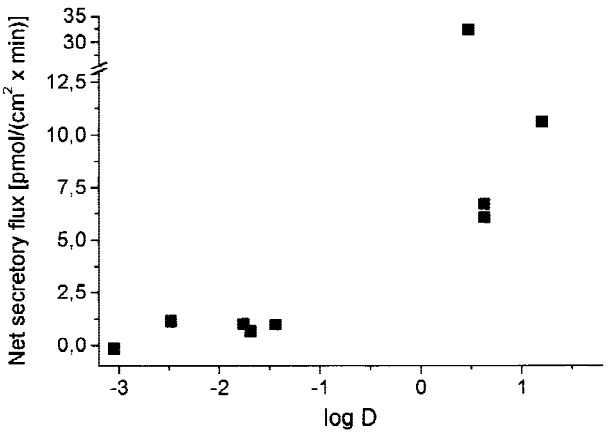

Fig. 3. Relationship between lipophilicity (log D) and net secretory flux of different Amidino-phenylalanine- derivatives with an unsubstituted amidino group in Caco-2 monolayers.

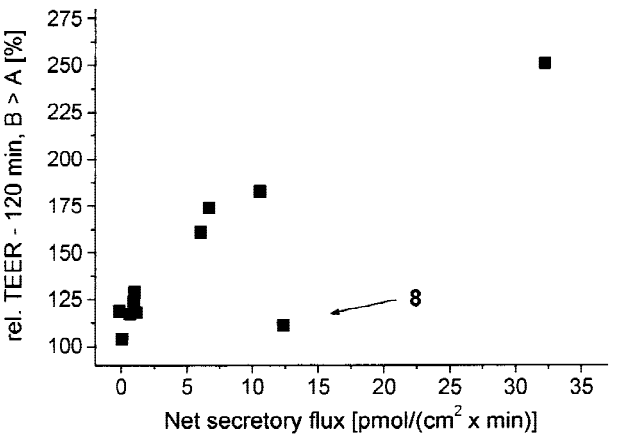

Fig. 4. Relationship between net secretory fluxes and increase in TEER (B➠A) in transport experiments with **1** through **11** (cf. Table I). The data indicate that an increase in TEER is accompanied also by an increase in net secretory flux suggesting the interaction of an activated efflux system, probably P-gp, with tight junction regulation. Note compound **8**, which is a substrate for a different efflux system than **1**.

this hypothesis. In summary, binding of drugs to P-gp may not only reduce transcellular transport but also paracellular permeability, which could be an additional principle reducing xenobiotic uptake through absorptive epithelia

One structural aspect commonly taken into account in the design of orally bioavailable thrombin inhibitors is to replace strongly basic amidino or guanidino groups by less basic groups. This approach led to increased Caco-2 cell permeability in case of analogues of the clinically used arginine amide-type thrombin inhibitor argatroban (18,19), of NAPAP and of NAPAP-type compounds (15,20). The poor membrane permeability of argatroban has been attributed to the zwitterionic character of the molecule (18,19). Esterification of the carboxyl group without amidine modification did not improve permeability (19) also seen with RGD-peptidomimetics (8). To our knowledge the results presented here demonstrate for the first time that the barriers to peroral bioavailability of peptidomimetic thrombin inhibitors may not only be limited intestinal absorption due to high hydrophilicity and hepatic first-pass but also intestinal secretion mediated by mechanisms effective also for a variety of other drugs.

CONCLUSION

The apparent permeability coefficients (A➠B) of strongly basic 3-amidino-phenylalanine-type peptidomimetic thrombin inhibitors in the Caco-2 cell line are comparatively low. Efflux mechanisms (B➠A transport), most probably mediated by MRP1 and P-gp, are responsible for a net secretory flux of these compounds limiting their absorption. Interaction with P-gp, but not MRP1, results in a significant increase in transepithelial electrical resistance.

The basic amidino group controls, depending on the overall lipophilicity of the drug candidate, the affinity to P-gp. Reducing the basicity of the amidino moiety drastically enhanced the transepithelial transport and minimized the efflux of the respective derivatives.

Among the compounds studied an oxamidino prodrug seems to be an interesting drug candidate circumventing intestinal efflux. Since oxamidines are known to be rapidly converted to the active drug after intestinal absorption this concept merits further investigations.

REFERENCES

1. K. Arimori and M. Nakano. Drug exsorption from blood into the gastrointestinal tract. *Pharm. Res.* **15**:371–376 (1998).
2. H. Kusuhara, H. Suzuki, and Y. Sugiyama. The role of P-glycoprotein and canalicular multispecific organic anion transporter in the hepato-biliary excretion of drugs. *J. Pharm. Sci.* **87**:1025–1040 (1998).
3. J. Hunter and B. H. Hirst. Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv. Drug Deliv. Rev.* **25**:129–157 (1997).
4. P. S. Burton, R. A. Conradi, A. R. Hilgers, and N. F. Ho. Evidence for a polarized efflux system for peptides in the apical membrane of Caco-2 cells. *Biochem. Biophys. Res. Commun.* **190**: 760–766 (1993).
5. V. B. Lang, P. Langguth, C. Ottiger, H. Wunderli-Allenspach, D. Rognan, B. Rothen-Rutishauser, J. C. Perriard, S. Lang, J. Biber, and H. P. Merkle. Structure-permeation relations of metenkephalin peptide analogues on absorption and secretion mechanisms in Caco-2 monolayers. *J. Pharm. Sci.* **86**:846–853 (1997).
6. J. Karlsson, S. M. Kuo, J. Ziemniak, and P. Artursson. Transport of celiprolol across human intestinal epithelial (Caco-2) cells: Mediation of secretion by multiple transporters including P-glycoprotein. *Br. J. Pharmacol.* **110**:1009–1016 (1993).
7. B. J. Aungst and H. Saitoh. Intestinal absorption barriers and transport mechanisms, including secretory transport, for a cyclic peptide, fibrinogen antagonist. *Pharm. Res.* **13**:114–119 (1996).
8. W. Kamm, J. Gante, P. Raddatz, and T. Kissel. Prodrug approach for $\alpha_{IIb}\beta_3$-peptidomimetic antagonists to enhance their transport in monolayers of a human intestinal cell line (Caco-2): Comparison of *in vitro* and *in vivo* data. *Pharm. Res.* **17**:1527–1533 (1999).
9. H. Saitoh, C. Gerard, and B. J. Aungst. The secretory intestinal transport of some beta-lactam antibiotics and anionic compounds: a mechanism contributing to poor oral absorption. *J. Pharmacol. Exp. Ther.* **278**:205–211 (1996).
10. P. Annaert, J. van Gelder, L. Naesens, E. De Clercq, G. Van den Mooter, R. Kinget, and P. Augustijns. Carrier mechanisms involved in the transepithelial transport of bis(POM)-PMEA and its metabolites across Caco-2 monolayers. *Pharm. Res.* **15**:1168–1173 (1998).
11. J. Stürzebecher, D. Prasa, J. Hauptmann, H. Vieweg, and P. Wikström. Synthesis and structure-activity relationships of potent thrombin inhibitors: Piperazides of 3-amidinophenylalanine. *J. Med. Chem.* **40**:3091–3099 (1997).
12. E. Walter, T. Kissel, M. Reers, G. Dickneite, D. Hoffmann, and W. Stüber. Transepithelial transport properties of peptidomimetic thrombin inhibitors in monolayers of a human intestinal cell line (Caco-2) and their correlation to *in vivo* data. *Pharm. Res.* **12**:360–365 (1995).
13. J. Hunter, M. A. Jepson, T. Tsuruo, N. L. Simmons, and B. H. Hirst. Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. *J. Biol. Chem.* **268**: 14991–14997 (1993).
14. H. Gutmann, G. Fricker, M. Török, S. Michael, C. Beglinger, and J. Drewe. Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake. *Pharm. Res.* **16**:402–407 (1999).
15. J. B. Rewinkel, H. Lucas, M. J. Smit, A. B. Noach, T. G. van Dinther, A. M. Rood, A. J. Jenneboer, and C. A. van Boeckel. Design, synthesis and testing of amino-bicycloaryl based orally bioavailable thrombin inhibitors. *Bioorg. Med. Chem. Lett.* **9**: 2837–2842 (1999).
16. U. Eckhardt, W. Stüber, G. Dickneite, M. Reers, and E. Petzinger. First-pass elimination of a peptidomimetic thrombin inhibitor is due to carrier-mediated uptake by the liver. Interaction with bile acid transport systems. *Biochem. Pharmacol.* **52**:85–96 (1996).
17. J. Hauptmann and J. Stürzebecher. Influence of indocyanine green on plasma disappearance and biliary excretion of a synthetic thrombin inhibitor of the 3-amidinophenyl-alanine piperazide-type in rats. *Pharm. Res.* **15**:751–754 (1998).
18. R. N. Misra, Y. F. Kelly, B. R. Brown, D. G. M. Roberts, S. Chong, and S. M. Seiler. Argatroban analogs: Synthesis, thrombin inhibitory activity and cell permeability of aminoheterocyclic guanidine surrogates. *Bioorg. Med. Chem. Lett.* **4**:2165–2170 (1994).
19. K. S. Kim, R. V. Moquin, L. G. Quian, R. A. Morrison, S. M. Seiler, D. G. M. Roberts, M. L. Ogletree, S. Youssef, and S. Chong. Preparation of argatroban analog thrombin inhibitors with reduced basic guanidine moiety, and studies of their permeability and antithrombotic activities. *Med. Chem. Res.* **6**:377–383 (1996).
20. K. Lee, W. H. Jung, C. W. Park, C. Y. Hong, I. C. Kim, S. Kim, Y. S. Oh, O. H. Kwon, S. H. Lee, H. D. Park, S. W. Kim, Y. H. Lee, and Y. J. Yoo. Benzylamine-based selective and orally bioavailable inhibitors of thrombin. *Bioorg. Med. Chem. Lett.* **8**: 2563–2568 (1998).
21. T. Prueksaritanont, P. Deluna, L. M. Gorham, B. Ma, D. Cohn, D., J. Pang, X. Xu, K. Leung, and J. H. Lin. *In vitro* and *in vivo* evaluations of intestinal barriers for the zwitterion L-767,679 and its carboxyl ester prodrug L-775,318. Roles of efflux and metabolism. *Drug Metab.Dispos.* **26**:520–527 (1998).
22. D. Boesch, C. Gavériaux, B. Jachez, A. Pourtier Manzanedo, P. Bollinger, and F. Loor. *In vivo* circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. *Cancer Res.* **51**:4226–4233 (1991).

23. G. Fricker, J. Drewe, J. Huwyler, H. Gutmann, and C. Beglinger. Relevance of P-glycoprotein for the enteral absorption of cyclosporin A: *In vitro-in vivo* correlation. *Br. J. Pharmacol.* **118**:1841–1847 (1996).
24. M. M. Nerurkar, P. S. Burton, and R. T. Borchardt. The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. *Pharm. Res.* **13**:528–534 (1996).
25. M. P. Draper, R. L. Martell, and S. B. Levy. Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein. *Br. J. Cancer* **75**:810–815 (1997).
26. S. Döppenschmitt, H. Spahn Langguth, C. G. RegÁrdh, and P. Langguth. Radioligand-binding assay employing P-glycoprotein-overexpressing cells: Testing drug affinities to the secretory intestinal multidrug transporter. *Pharm. Res.* **15**:1001–1006 (1998).
27. A. Aszalos, K. Thompson, J. J. Yin, and D. D. Ross. Combinations of P-glycoprotein blockers, verapamil, PSC833, and Cremophor act differently on the multidrug resistance associated protein (MRP) and on P-glycoprotein (P-gp). *Anticancer Res*. **19**: 1053–1064 (1999).
28. D. W. Miller, E. V. Batrakova, and A. V. Kabanov. Inhibition of multidrug resistance-associated protein (MRP) functional activity with Pluronic block copolymers. *Pharm. Res.* **16**:396–401 (1999).
29. T. Weller, L. Alig, M. Beresini, B. Blackburn, S. Bunting, P. Hadváry, M. H. Müller, D. Knopp, B. Levet Trafit, M. T. Lipari, N. B. Modi, M. Müller, C. J. Refino, M. Schmitt, P. Schönholzer, S. Weiss, and B. Steiner. Orally active fibrinogen receptor antagonists. 2. Amidoximes as prodrugs of amidines. *J. Med. Chem.* **39**:3139–3147 (1996).
30. J. Hauptmann, M. Paintz, B. Kaiser, and F. Markwardt. Reduction of a benzamidoxime derivative to the corresponding amidine *in vivo* and *in vitro*. *Pharmazie* **43**:559–560 (1988).
31. M. Sakai, A. B. Noach, M. C. Blom Roosemalen, A. G. de Boer, and D. D. Breimer. Absorption enhancement of hydrophilic compounds by verapamil in Caco-2 cell monolayers. *Biochem. Pharmacol.* **48**:1199–1210 (1994).
32. L. S. Gan, S. Yanni, and D. R. Thakker. Modulation of the tight junctions of the Caco-2 cell monolayers by H_2-antagonists. *Pharm. Res.* **15**:53–57 (1998).
33. K. Lee and D. R. Thakker. Saturable transport of H_2-antagonists ranitidine and famotidine across Caco-2 cell monolayers. *J. Pharm. Sci.* **88**:680–687 (1999).
34. A. Collett, N. B. Higgs, E. Sims, M. Rowland, and G. Warhurst. Modulation of the permeability of H_2 receptor antagonists Cimetidine and Ranitidine by P-glycoprotein in rat intestine and the human colonic cell line Caco-2. *J. Pharmacol. Exp. Ther.* **288**:171–178 (1999).
35. C. E. Bear. Drugs transported by P-glycoprotein inhibit a 40 pS outwardly rectifying chloride channel. *Biochem. Biophys. Res. Commun*. **200**:513–521 (1997).