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

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Purpose. Different lipophilic derivatives of a potent $\alpha_{IIb}\beta_3$- antagonist with benzamidino-oxazolidinone structure were investigated with respect to transport and metabolism properties to evaluate their potential as prodrugs with improved absorption behavior.

Methods. Intestinal transport and metabolism of the compounds were studied in Caco-2 monolayers under in vitro conditions and quantitated by a reversed-phase HPLC- method. Peroral bioavailability in cynomolgus monkeys and inhibition of platelet aggregation (guinea pig) were compared to in vitro permeability coefficients.

Results. N-alkoxycarbonyl- and N-benzoyl- derivatization of the benzamidine-parent drug increased the apparent permeabilities across Caco-2 monolayers by a factor of 25-100 fold. Most prodrugs were transported mainly by passive diffusion, whereas the methoxycarbonyl-derivative EMD 122347 displayed directional transport from basolateral (BL) to apical (AP). This polarized efflux was concentration dependent (saturable kinetics with K_m = 207 μM, V_{max} = 0.275 nmol cm^{-2} min^{-1}) and could be reduced in the presence of verapamil (300 μM), an inhibitor of p-glycoprotein. Cell mediated cleavage of the prodrugs was low and showed only slight differences to hydrolysis in buffer solution, indicating a predominantly non enzymatic cleavage. Both peroral bioavailability (monkey) and the inhibition of ex-vivo platelet aggregation (guinea pig) gave the same rank order as the permeability coefficients obtained in Caco-2 monolayers.

Conclusions. Alkoxycarbonylamidine- and benzoylamidine promoieties of a RGD mimetic $\alpha_{IIb}\beta_3$- antagonist considerably increased both effect bioavailabilities in animal experiments as well as in-vitro permeability in cell monolayers, demonstrating the potential of this approach to enhance transport of peptidomimetic drugs.

KEY WORDS: prodrugs; $\alpha_{IIb}\beta_3$- antagonists; Caco-2 cells; peptidomimetic transport and metabolism; efflux.

INTRODUCTION

Highly potent antagonists to fibrinogen receptor GPIIb/IIIa ($\alpha_{IIb}\beta_3$) at the surface of thrombocytes are currently under investigation as novel anti-platelet drugs for the treatment and prophylaxis of various cardiovascular and cerebrovascular thromboembolic diseases (1). Responsible for fibrinogen-platelet interaction is the amino acid sequence RGD (arginine, glycine, aspartic acid) within the fibrinogen molecule (2). To antagonize fibrinogen-platelet binding in the past, several RGD-analogues had been studied, such as snake venom peptides and linear/cyclic RGD analogues. The search for more stable active compounds has led to structures which mimic the RGD-motif. These promising peptidomimetic candidates are more stable than RGD-peptides, but their low peroral bioavailability often remains problematic due to their hydrophilic, charged character (3).

We study here several prodrugs of a highly potent RGD-peptidomimetic antagonist with oxazolidinone-methyl-building block with respect to their transport properties in gastrointestinal cell monolayers to evaluate their potential for absorption enhancement (Fig. 1). The parent drug EMD 80200 with unmodified amidino- and carboxyl-groups was substituted systematically. Esterification at the carboxylic-group yielded the ethylester EMD 85900. Both compounds are positively charged under physiological conditions due to their charged amidino group. Lipophilic derivatization of the amidino group by methoxycarbonyl- (EMD 122347), benzyloxycarbonyl- (EMD 87314) and benzoyl- (EMD 87313) groups respectively led to dual prodrug structures, which are less charged and whose transepithelial transport rates should be significantly increased compared to the hydrophilic parent molecule.

Transport and metabolism of the compounds were assessed using Caco-2 cell-monolayer, a widely accepted human cell culture model for the investigation of intestinal drug absorption and metabolism (4).

MATERIALS AND METHODS

Materials

Tissue culture reagents were bought from Gibco (Eggenstein, Germany). Tissue culture articles were purchased from Nunc (Wiesbaden, Germany) and polycarbonate membrane cell culture inserts (Transwell™, Cat. No. 3412, Costar) were supplied by Integra Biosciences (Fernwald, Germany). All other chemicals and reagents were of analytical grade and obtained from E. Merck AG (Darmstadt, Germany).

General Synthetic Methods

Investigated compounds were provided by E. Merck AG and synthesized as described previously (5,6).

Apparent Octanol/Buffer Distribution Coefficients (log D)

The log D values of the compounds were calculated using the pKalc V.3.1. software (CompuDrug Chemistry Ltd., Budapest, Hungary) and the KowWin V.1.54c software (SRC Research Corp., North Syracuse, New York). Previous investigations in our laboratories have shown that data obtained with these computational methods were in good agreement with experimental data of octanol/buffer partition coefficients.

Cell Culture

Caco-2 cells at passages 40–47 were routinely maintained in DMEM, supplemented with 4.5 g glucose/L, 10% fetal calf serum, 1% nonessential amino acids, 2 mM glutamine and

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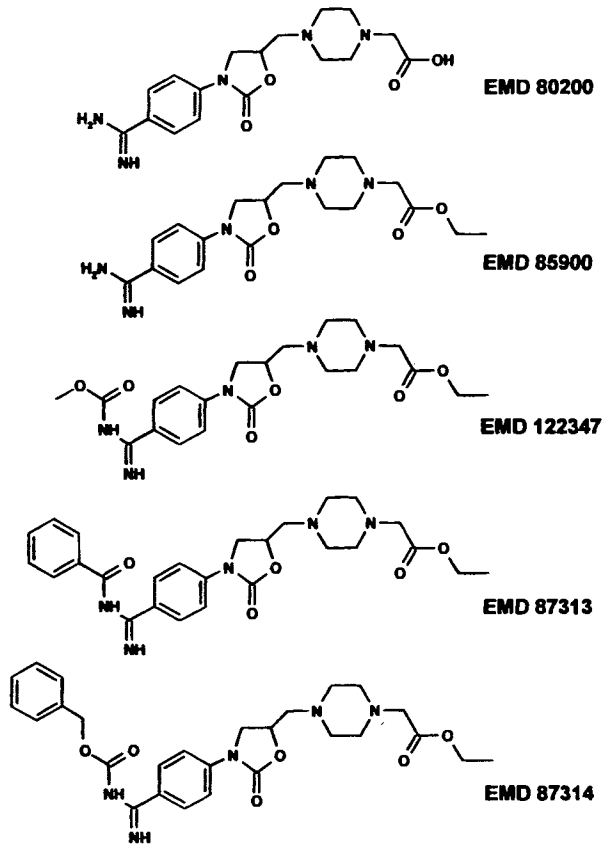

Fig. 1. Chemical structures of the $\alpha_{IIb}\beta_3$-antagonist and its prodrugs.

penicillin (100 IU/mL)/streptomycin (100 μg/ml) at 37°C in an atmosphere of 5% 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 (7).

Transport Studies

Transport studies were performed as described previously (7,8). Briefly, cells were seeded at a density of 6.5×10^4 cells/cm^2 onto collagen coated polycarbonate filters. Transport experiments were performed between 20th and 25th day post-seeding directly on the filter inserts in a 5%-CO_2 atmosphere. For studying apical to basolateral transport 1.5 mL of drug containing transport buffer (HBSS, 15 mM glucose, pH 6.7) was placed on the luminal and 2.6 mL transport buffer on the basolateral side. Basolateral to apical transport was performed vice versa. Drug concentration varied from 1000 μM for the hydrophilic compounds EMD 80200 and EMD 85900 to 300 μM for the more lipophilic compounds EMD 122347, EMD 87313 and EMD 87314 (for EMD 87313 and EMD 87314 addition of 0.5% (v/v) DMSO was necessary to enhance the aqueous solubility). After predetermined time intervals 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) (7–9).

Degradation Studies

Analysis of the metabolic and hydrolytic stability of the compounds was performed using Caco-2 monolayers grown upon 6-well plates under conditions identical to those used for the Transwells. Confluent monolayers between day 21 and 25 postseeding were rinsed twice with HBSS (pH 6.7) containing 15 mM Glucose, allowed to equilibrate in buffer for 15 min and 3 mL of the drug solutions in the same concentrations as mentioned above were placed on the apical surface of the cells. After preset time intervals up to 8 h 150 μL samples were withdrawn and immediately frozen. Control experiments without cells were performed under identical conditions.

Analytical Methods

Aliquots of the samples were analyzed by reversed phase HPLC using a gradient pump (model L-6200-A), an automatic sampler (model AS-2000A), a column thermostat (model T-6300), an UV-detector (model L-4000) 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). All compounds were separated on a reversed-phase HPLC-column (Lichrosorb RP select B, 7 μm, 250 × 4 mm; Merck AG, Germany) using a binary gradient procedure: Eluent A contained 0.1% TFA in acetonitrile/water (10/90), eluent B consisted of acetonitrile gradient grade. With a flow of 1.2 mL/min retention times were as follows: EMD 80200, 3.0 min; EMD 85900, 5.9 min; EMD 122347, 6.8 min; EMD 87313, 8.4 min; EMD 87314, 9.1 min. Detection wavelength for UV-analysis was 270 nm (EMD 87313, 122347), for fluorimetric analysis 270 nm for excitation and 360 nm for emission wavelength. Limits of UV-detection were 0.3 μM, for fluorescent detection 0.05 μM (EMD 87314, 122347) and 0.01μM (EMD 80200, 85900).

Data Treatment

Apparent permeability coefficients (P_{app}) and secretory net flux were calculated as described previously (7,21). All results are expressed as the mean of at least 3 experiments ± standard deviation. Statistical analyses were performed using two sided independent t-test.

Animal Experiments

Oral bioavailability in cynomolgus monkey and the ex-vivo-inhibition of collagen induced platelet aggregation in guinea pigs were determined as described previously (5,6,10,11). Briefly, prodrugs and drugs were administered i.v. and/or peroral to laboratory animals (n = 3). Bioavailability data (monkey) were calculated from plasma levels of the active drug (EMD 80200) after oral prodrug application and i.v. application of the active parent drug. Oral effect bioavailability (guinea pig) after i.v. or p.o. application were determined measuring the ex-vivo-inhibition of collagen-induced platelet aggregation. After administration of the compound platelet rich plasma (PRP) was prepared and the platelet aggregation was induced by addition of collagen and adenosindiphosphate (ADP). The extent of aggregation was optically determined as change in light transmittance. The anti platelet aggregatory

Table 1. Transport of $\alpha_{IIb}\beta_3$-Antagonists and Their Prodrugs Through Caco-2 Monolayers: Permeability-Properties, Recovery Data, Transepithelial Electrical Resistance (TEER) Measurements and In Vivo Data [Partly from (5,6)]

Compound EMD	Molecular weight [g* Mol^{-1}]	log D	Transport direction	Permeability coefficient [* 10^{-7} cm/s][a]	Recovery after 120 min [%][b]	relative TEER after 120 min [%][c]	Oral bioavailability monkey [%]	ED_{50} Ex-vivo platelet aggregation (gp[d]) p.o. [mg/kg]
80200	361	−3.20	AP > BL	2.75 ± 0.7	96.1 ± 1.4	83 ± 6	3–10	3.3
			BL > AP	2.81 ± 0.4	95.9 ± 1.4	98 ± 9		
85900	389	−3.55	AP > BL	2.77 ± 0.6	96.2 ± 2.9	88 ± 15	3–5	3.0
			BL > AP	3.20 ± 1.1	97.7 ± 4.5	105 ± 21		
122347	447	−1.88	AP > BL	72.7 ± 1.3	97.7 ± 1.4	97 ± 3	40	0.20
			BL > AP	134 ± 4.8**	97.2 ± 1.2	90 ± 5		
87313	493	−0.03	AP > BL	291 ± 8.7*	108 ± 3.3	85 ± 11	15–20	0.16
			BL > AP	256 ± 12	97.5 ± 7.8	83 ± 14		
87314	523	−0.28	AP > BL	257 ± 1.7*	103 ± 0.8	92 ± 11	40	0.17
			BL > AP	240 ± 4.7	97.9 ± 8.6	89 ± 6		

[a] Mean ± S.D. (n = 3).
[b] Summarized concentrations of the receiver compartment ($t_1 - t_n$) and donor compartment (t_{120}) related to the initial concentration of the donor compartment. Data represent only that compound initially added to the donor compartment. Mean ± S.D. (n = 3).
[c] In relation to the initial TEER-values. Mean ± S.D. (n = 3).
[d] Guinea pig.
* Significantly different from BL > AP transport at $p < 0.05$ (two sided independent t-test).
** Highly significant different from AP > BL transport at $p < 0.001$ (two sided independent t-test).

potency of the drugs was expressed as the administered dose which yield 50% platelet aggregation (ED_{50}). All treatments to animals were carried out by a licensed investigator in accordance with regulated procedures which adhere to the "Principles of Laboratory Animal Care" (NIH Publication # 85-23).

RESULTS

Permeability of the Compounds Across Caco-2 Cell Monolayers

As demonstrated in Table 1 striking differences in the transport rates of the five fibrinogen-receptor-antagonists were observed. The most lipophilic compounds EMD 87313 and 87314 showed permeability coefficients (AP > BL) of about 291 ± 8.7 × 10^{-7} cm/s and 257 ± 1.7 × 10^{-7} cm/s, respectively, which are nearly 100 × higher than those observed for the hydrophilic cationic parent drug EMD 80200 (2.75 ± 0.7 × 10^{-7} cm/s) and its hydrophilic ethylester EMD 85900 (2.77 ± 0.6 × 10^{-7} cm/s). The addition of DMSO (0.5% v/v), which was necessary to solubilize compounds EMD 87313, 87314, did not influence transport rates and transepithelial resistances (data not shown). Directional transport could not be observed for the hydrophilic compounds, whereas basolateral to apical transport of the lipophilic compounds EMD 87313 (256 ± 12 × 10^{-7} cm/s) and EMD 87314 (240 ± 4.7 × 10^{-7} cm/s) was somewhat lower ($p < 0.05$, two sided t-test) compared to the vice versa direction. Permeation of compound EMD 122347 (methoxycarbonyl-derivative of EMD 85900) through Caco-2 monolayers was found to be approximately 25 × (72.7 ± 1.3 × 10^{-7} cm/s, AP > BL) higher than permeability of the parent drug. We also observed an increased basolateral to apical drug transport (P_{app} = 134 ± 4.8 × 10^{-7} cm/s, BL > AP) at a concentration of 300 μM (1.86 fold, $p < 0.001$), pointing to possible efflux mechanism.

To further characterize this phenomenon several aspects were examined: Transport of EMD 122347 (300 μM) in the presence of verapamil (300 μM), a known inhibitor of the P-glycoprotein and concentration dependent transport of EMD 122347 (1800 μM, 900 μM, 300 μM, 60 μM, 30 μM, 12μM, 6 μM) were investigated.

As shown in Table 2 verapamil slightly increased EMD 122347 permeability from apical to basolateral (77.5 ± 2.8 × 10^{-7} cm/s), whereas the basolateral to apical P_{app} was reduced significantly ($p < 0.001$, two sided independent t-test) to 107 ± 2.7 × 10^{-7} cm/s. Lowering the donor concentration

Table 2. Concentration Dependent Directional Transport of EMD 122347, Influence of Verapamil (300 μM) on Transport Ratio

Transport Parameter	P_{app} AP > BL [cm/s] × 10^{-7})*	P_{app} BL > AP [cm/s] × 10^{-7})*	Ratio BL > AP/AP > BL
6 μM	28.5 ± 2.8	94.0 ± 2.9**	3.30
12 μM	30.4 ± 1.3	94.3 ± 2.7**	3.10
30 μM	37.1 ± 1.3	105 ± 3.3**	2.83
60 μM	42.3 ± 2.0	156 ± 3.6**	3.69
300 μM	72.7 ± 1.3	134 ± 4.8**	1.84
900 μM	70.0 ± 1.5	115 ± 1.4**	1.64
1800 μM	64.7 ± 2.3	79.1 ± 5.7**	1.22
300 μM + 300 μM Verapamil	77.5 ± 2.8	107 ± 2.7***	1.37
Verapamil 300 μM	206 ± 8.4	235 ± 12.1	1.14

* Mean ± S.D. (n = 3).
** Highly significant different ($p < 0.001$) from AP > BL transport.
*** Highly significant different ($p < 0.001$) from BL > AP transport of EMD 122347 at 300 μM.

from 1800 μM down to 6 μM led to an increase in basolateral to apical transport ratio and to a decrease in absorptive P_{app}, respectively. The transport could be described by a nonlinear Michaelis Menten-type kinetics with K_m = 207 ± 59 μM and V_{max} = 0.275 ± 0.025 nmol cm^{-2} min^{-1} (Fig. 2). During all transport experiments no remarkable decrease in transepithelial resistance could be detected (max. 17%, Table 1) indicating the integrity of the monolayer up to a time of 120 min (12).

Metabolism

Transport Experiments

To investigate metabolic degradation during transport through the monolayers, we have calculated recovery values of each compound tested (Table 1). For this purpose we added up the amounts of initially added substance found in both compartments of the transport chambers and compared it with the initial donor-concentration, which was set to 100%. The recovery ratios were all around 90–100% indicating no or only small degradation caused by cytosolic or brush border membrane enzymes during transport experiment.

Apical Cell Surfaces

To distinguish between hydrolytic and metabolic degradation, antagonists were incubated over a time period of 480 min with and without cells. Neither compound EMD 80200, the unsubstituted parent drug, nor EMD 85900 (ethylester) showed any degradation during incubation in buffer or at the luminal surface of Caco-2 monolayers (data not shown). Cleavage of EMD 87313 could be observed in both, apical cell compartment and cell free buffer without significant differences (Fig. 3A). During an incubation time of 2 h ca. 90% and after 4 h approximately 83% of the prodrug remained intact in the apical compartment. The main degradation products seen here after 2 h were EMD 85900 (ca. 3%), EMD 80200 (0.2%) and a non identified compound (ca 5%). The spontaneous hydrolysis displayed a quite similar degradation pattern (not shown). EMD 87314 and EMD 122347 as alkoxy-carbonyl-amidino-derivatives showed significant higher stability than EMD 87313. Degradation experiments with and without cells displayed no or only slight differences. Recovery of the intact prodrugs after 120 min were ≥95% (Fig. 3B,C).

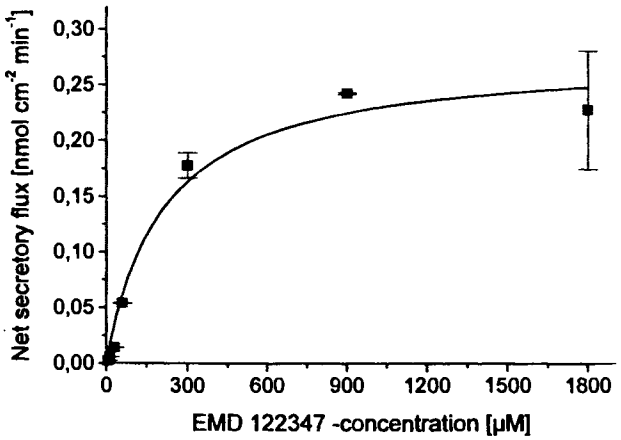

Fig. 2. Net drug secretion vs. drug concentration. Net drug fluxes (J_{BL-AP} − J_{AP-BL}) were determined at EMD 122347 concentrations up to 1800 μM. The solid line represents the χ^2-squares fit for Michaelis-Menten kinetics.

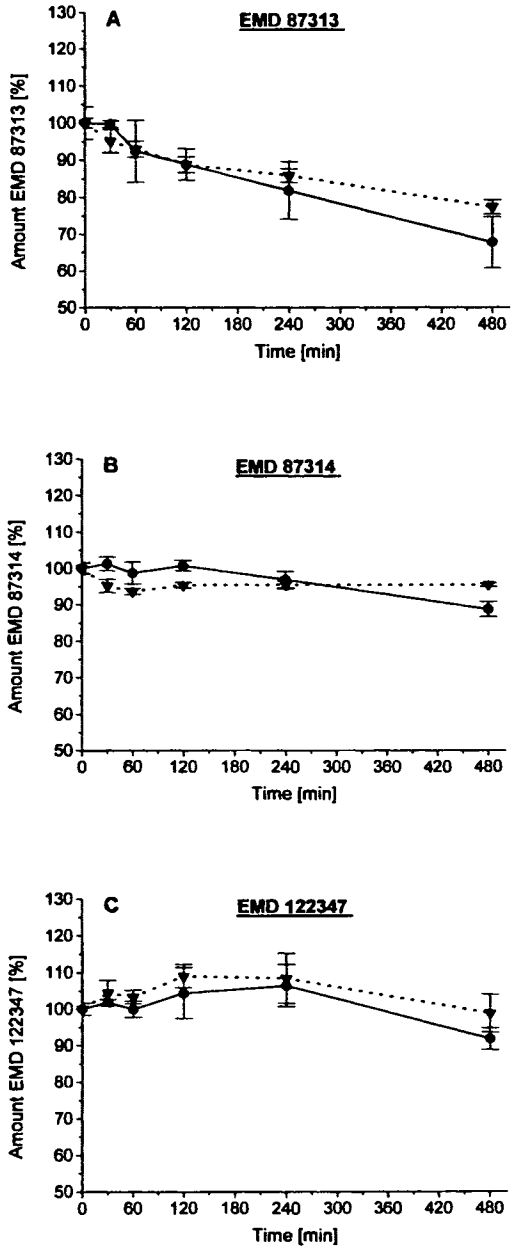

Fig. 3. Time dependent apical (circles) and spontaneous (down-triangles) degradation of the prodrugs EMD 87313 (A), 87314 (B) and 122347 (C). Each point represents the mean of at least three determinations ± SD.

Animal Data

Oral Bioavailability

Oral bioavailability was related to the appearance of the active drug (EMD 80200) in blood circulation. In cynomolgus monkey oral bioavailability was in the range of 3 to 10% for the hydrophilic drugs (parent and ethylester) and could be increased 5 to 10 fold, namely 40% for both, EMD 87314 and

EMD 122347. With EMD 87313 (benzoyl-amidino-derivative) an oral bioavailability between 15–20% was obtained (Table 1).

Inhibition of the Ex-Vivo Platelet Aggregation (Oral Effect Bioavailability)

Following oral administration to guinea pigs ED_{50} doses of the compounds inhibiting the collagen induced ex-vivo platelet aggregation were determined. ED_{50} p.o. doses of EMD 122347 (0.2 mg/kg) and the more lipophilic prodrugs EMD 87313 (0.16 mg/kg) and 87314 (0.17 mg/kg) were about 16–20 times lower than the ED_{50} doses of the parent drug EMD 80200 (3.3 mg/kg) and its ethylester EMD 85900 (3.0 mg/kg) (Table 1). The calculated ratio ED_{50} i.v./ED_{50} p.o. demonstrating the oral anti-aggregatory potency of a drug was about 30% for EMD 87313 and 18% for EMD 87314, whereas hydrophilic drugs EMD 80200 and 85900 showed quotients <1%.

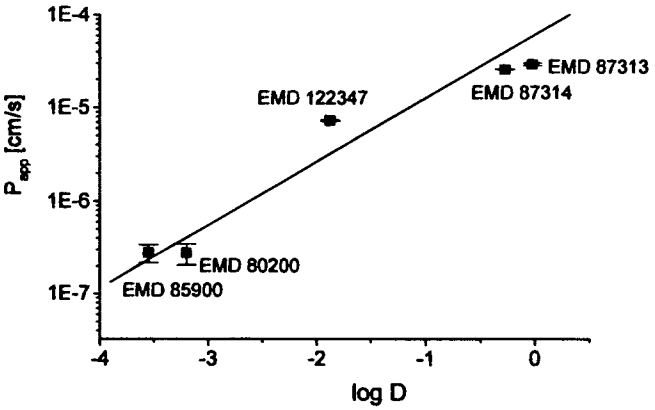

Fig. 4. Apparent permeability coefficients of the compounds as a function of lipophilicity. P_{app} data are the mean of 3 determinations ± SD. The correlation coefficient for the linear regression line shown is 0.96.

DISCUSSION

Masking of highly charged residues to enhance the lipophilicity of peptidomimetic drugs might be an useful approach to overcome the intestinal membrane barrier. However, fundamental requirements for any successful prodrug strategy are both rapid permeation through intestinal barrier with negligible degradation and reliable conversion to the parent drugs after reaching the central compartment (13).

Since bioavailability data from monkey and guinea pig indicated that described prodrugs were well absorbed orally and converted rapidly to the active drug (5,6), the aim of our study was the evaluation of these lipophilic amidine-prodrugs in a human intestinal cell line Caco-2, whose suitability for predicting absorption in humans is well documented (14,15).

Transport Experiments

For the hydrophilic compounds EMD 80200 (parent) and 85900 (ethylester) with positively charged amidino-groups we have obtained permeability coefficients of about 2.8×10^{-7} cm/s without directional differences between apical to basolateral flux and vice versa, suggesting a lacking of active transport processes (8). The low lipophilicities (log D < −3) and the low permeability coefficients point to paracellular transport pathway in agreement with data published for hydrophilic compounds and peptides, e.g., TRH ($P_{app} = 1.8 \times 10^{-7}$ cm/s) (8,16). Esterification of EMD 80200 to 85900 did not increase the permeability coefficients which can be explained by similar lipophilicities and the effects of the positively charged amidino group. These results are in agreement with Kim *et al.* (17) who showed that esterification had no influence on membrane permeation of the peptidomimetic thrombin inhibitor Argatroban in Caco-2 monolayers. Lipophilisation of the amidino-group yielded prodrugs with alkoxy-carbonyl-amidine-(EMD 122347, EMD 87314) and benzoylamidine-structures (EMD 87313). These prodrugs showed 25 to 100 × higher transport rates in monolayers than the charged drugs (Table 1). P_{app} data correlate in a satisfactory way (R = 0.96) with distribution coefficients (log D) of the investigated compounds, suggesting that the lipophilicity seems to have a predictive value for drug transport in this case (Fig. 4). These results were in agreement with data from Artursson *et al.* (14), Yamashita *et al.* (15) and Aungst *et al.* (18). Comparing directional fluxes the most lipophilic derivatives EMD 87313 and 87314 with large residues displayed small but statistically significant ($p < 0.05$) higher transport rates from apical to basolateral, suggesting an active transport component. In contrast prodrug EMD 122347 with a small methoxycarbonyl group showed a markedly enhanced basolateral to apical flux.

Characterization of the Enhanced BL to AP Transport of Compound EMD 122347

To characterize the transport mechanism for the prodrug EMD 122347 we have examined concentration dependent transport properties and the effect of coadministration of verapamil a known inhibitor of the p-glycoprotein (P-gp). P-gp is responsible for polarized basolateral to apical drug efflux and was shown to be expressed in human epithelial Caco-2 cell monolayers (19–21). Permeability coefficients as well as transport-ratio BL > AP/AP > BL were markedly dependent on drug concentration (Table 2). Secretory net flux showed nonlinear kinetics (Fig. 2), which could be described by the Michael-Menten model suggesting the presence of a carrier mediated polarized efflux process. Our hypothesis of a possible involvement of P-gp in that process was supported by the significant decrease of EMD 122347 transport-ratio BL > AP/AP > BL in the presence of verapamil. In the past, several drugs were reported to be substrates for p-glycoprotein: Some β-receptor antagonists (22), anti-cancer drugs as vinblastine (21) and actinomycin D (20) and peptides, e.g., tripeptides, pentapeptides and undecapeptides, i.e., cyclosporin A (23–25,19). Little is known about structural requirements for P-gp binding. Literature data suggest that substrates of P-gp are generally positively charged at neutral pH and/or rather hydrophobic (26). Aungst *et al.* (18) reported recently that a cyclic-fibrinogen-antagonist with low lipophilicity (DMP 728, log D = −1.88) was potential substrate for P-glycoprotein mediated secretion in intestine. In our experiments neither hydrophilic compounds with unsubstituted benzamidino-group (EMD 80200, 85900) nor derivatives with large lipophilic residues (EMD 87313, 87314) showed directional BL to AP transport, whereas the small methoxy-carbonyl-amidino residue in EMD 122347 obviously mediated net secretory transport. Therefore, the chemical environment of the amidino-group

in our series seems to affect the affinity to a polarized efflux system, presumably P-gp. With regard to the Michaelis Menten parameters for the polarized transport of EMD 122347, the calculated K_m-value of 207 μM ± 59 μM was markedly higher than data reported for known P-gp substrates, i.e., vinblastine (18.99 ± 5.55 μM, suggesting only a small affinity to the efflux transporter (19). Moreover this was supported by the still existing high absorptive permeability of EMD 122347 ($72.7 \pm 1.3 \times 10^{-7}$ cm/s, 300 μM) and the rather high oral bioavailability as well as oral effect availability (40%) obtained in monkey and guinea pig respectively (Table 1). However the latter observations might be also due to different expression of efflux mediating P-gp in colorectal carcinoma and in the small intestine (27).

Metabolism

With respect to the structure of the prodrugs, a cleavage of the peptide like bond between amidino group and the carbonyl residue as well as the ethylester could occur in the presence of intracellular and brush border- esterases such as exo- and endopeptidases (28,29). This reaction would generate the hydrophilic drugs, whose mucosal absorption and oral bioavailability were observed to be rather low. Weller *et al.* reported that only dual prodrugs with modified amidino- and carboxy-group markedly enhanced oral bioavailability in mice (30).

No significant cleavage of each component was observed during transport through tight monolayers as indicated by recovery values between 90 and 100%. Since prodrugs were expected to be transported mainly transcellularly due to their high permeability coefficients, one might speculate that they fail to be suitable substrates for cytosolic enzymes. In an additional experiment compounds were exposed for 8 h to both cell free buffer and apical cell compartment to evaluate the hydrolytic stability. The decrease in prodrug concentration in cell free buffer was not significantly different from that in the apical cell compartment, indicating that cleavage occurs predominantly by non-enzymatic hydrolysis. Apical decomposition half time of the benzoyl-amidino derivative EMD 87313 ($t_{1/2}$ = 14h) was significant lower than those observed for the benzyl-oxycarbonyl EMD 87314 ($t_{1/2}$ = 42h) and the methoxy-carbonyl-derivatives EMD 122347 ($t_{1/2}$ = 70h). This could be due to the more peptidic structure of EMD 87313 whereas the latter two are formally derivatives of carbamic acid esters, whose hydrolytic stability at pH below 7 recently was reported (31). In summary, the degradation profiles did not affect the transport studies over a time period of 120 min, as demonstrated by permeability coefficients.

Comparison of In Vivo Data with Apparent Permeability Coefficients (P_{app})

Compared to the parent drug EMD 80200, the structurally related ethylester EMD 85900 did not affect both transport characteristics in Caco-2 experiments and peroral bioavailabilities as well as effect bioavailabilities, indicating that the highly charged amidino group is responsible for low intestinal permeation. These observations are in agreement with data from Kim *et al.* (17) and Weller *et al.* (30) who demonstrated that esterification of a zwitterionic $\alpha_{IIb}\beta_3$-antagonist did not alter oral activity compared to the parent drug.

Amidine prodrug formation leads to a markedly increase in transepithelial in vitro transport (25–100 fold) as well as oral bioavailabilities (5–10 fold, monkey) and oral effect bioavailabilities (16–20 fold, guinea pig). In vivo–in vitro correlations of these data are depicted in Fig. 5A,B. Even if a larger number of compounds would be desirable, reasonably linear relationships could be observed between P_{app} and oral bioavailability (r = 0.76, Fig. 5A) as well as P_{app} and oral effect bioavailability (r = 0.97, Fig. 5B). The scattering in Fig. 5A is due to the significant lower oral bioavailability of EMD 87313 in monkey (15–20%) compared to the alkoxy-carbonyl prodrugs (40%). Since this was not observed in guinea pigs, this might point to species specific elimination processes of this prodrug after absorption in blood and liver (e.g., first pass effect). In summary, animal pharmacokinetic and pharmacodynamic data are showing the same rank order as the permeability coefficients in the Caco-2 monolayers, indicating that the intestinal permeability of the compounds is the limiting process for peroral application.

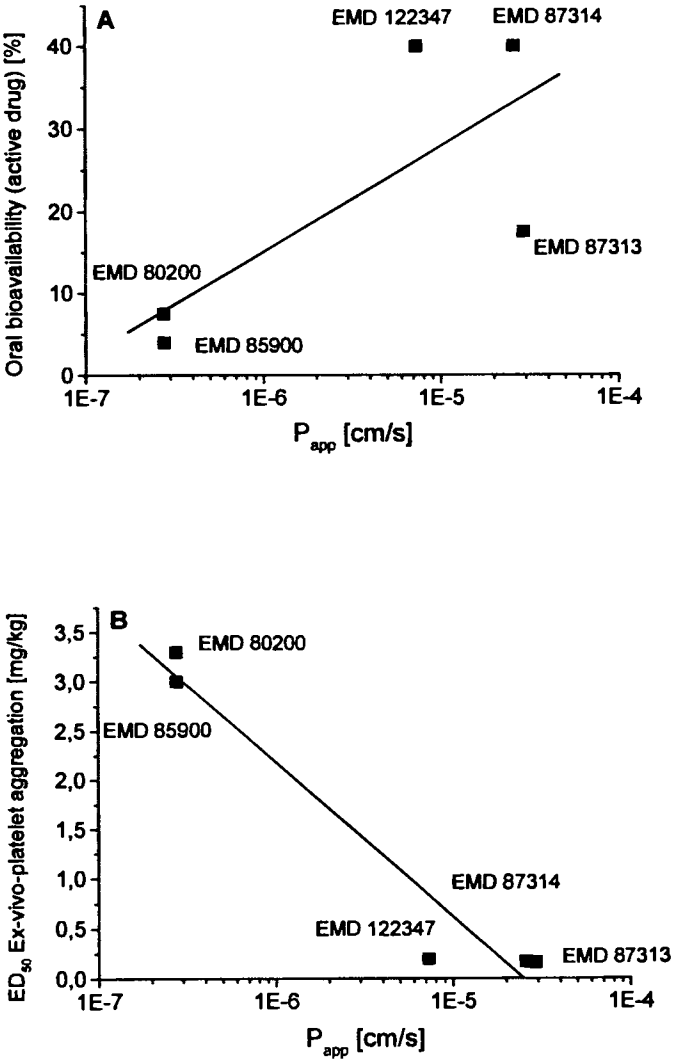

Fig. 5. A, Relationship of oral bioavailability (cynomolgus monkey) of the active drug and apparent permeability coefficient in Caco-2 monolayers. Each point represents the mean of three determinations ± SD, B, Oral effect bioavailability (Inhibition of ex-vivo platelet aggregation) as a function of apparent permeability coefficients (Caco-2). The data shown are the mean of three determinations ± SD.

CONCLUSIONS

Alkyloxy-carbonyl-and benzoyl-amidine prodrugs of the highly potent RGD mimetic drug EMD 80200 displayed high intestinal absorption rates with negligible enzymatic degradation using Caco-2 cell monolayers. In vitro permeation in this human model could be confirmed by high oral bioavailability- and effect bioavailability data (monkey, guinea pig) suggesting that these prodrugs were promising candidates for an oral antithrombotic therapy.

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