Transepithelial Transport Properties of Peptidomimetic Thrombin Inhibitors in Monolayers of a Human Intestinal Cell Line (Caco-2) and Their Correlation to *in Vivo* Data

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Peptidomimetic thrombin inhibitors (TI), derived from L-Asp-D-Phe were examined in confluent monolayers of a human colon carcinoma cell line (Caco-2) to elucidate their transepithelial transport properties. Effect availabilities, based on activated partial thromboplastin time (aPTT) measurements in rats, after peroral administration of five TI correlated reasonably well with permeability coefficients obtained from *in vitro* transport studies in Caco-2 monolayers, whereas physicochemical properties, such as molecular mass, solubilities, pK_a and octanol-buffer partition coefficients failed to yield meaningful relationships. Substitution of the β-carboxylic group of L-Asp leads to analogues which are mainly transported by passive diffusion, while an unsubstituted carboxylic group favours carrier-mediated active transport. The effects of concentration, temperature, competitive inhibitors and direction dependence on *in vitro* transport were investigated. The results obtained are compatible with a saturable carrier-mediated transport, operating parallel to a passive paracellular route. The Michaelis-Menten parameters for the active transport component (K_m = 1.67 mM, V_{max} = 26.5 pmol min^{-1} mg $protein^{-1}$) indicate an involvement of the intestinal di/-tripeptide transport system for one of the TI. The Caco-2 transport model may be helpful for the design of perorally active peptidomimetics.

KEY WORDS: cell culture; Caco-2; thrombin inhibitors; passive diffusion; *in vitro/in vivo* correlation; carrier-mediated transport.

INTRODUCTION

For chronic therapy, the most desirable method of drug administration remains the oral route. Peptide drug delivery has been of considerable interest for the past decades (1). This is in part due to the rapid development in biotechnology and peptide synthesis, allowing the exploitation of the unique pharmacological activities of peptide drugs. Many investigators have attempted to develop new strategies for peptide delivery (2). One principal difficulty using peptides as therapeutic agents is the requirement for parenteral administration, since systemic bioavailability is usually very low after peroral administration (3). Thrombin is involved in many disorders of the hemostatic system and, therefore, control of thrombin activity may be clinically relevant in the treatment of inherited thrombotic disorders and antithrombic therapy (4). A number or protein inhibitors of thrombin are available, but peroral activity proved to be an elusive goal. Therefore, low molecular-weight peptidomimetic thrombin inhibitors have been developed as anticoagulants with peroral activity (4,5).

In addition to passive diffusion, specific carrier-mediated systems facilitate the absorption of di- and tripeptides in the gastrointestinal tract (6). Although hydrophilic and ionized at physiological pH, peptidomimetic drugs, such as β-lactam antibiotics and angiotensin-converting enzyme inhibitors, are absorbed via the di-/tripeptide transporter in the small intestine (7).

The human intestinal cell line Caco-2 provides a convenient model for the investigation of the differentiated functions of intestinal enterocytes (8). When Caco-2 cells are grown on microporous membranes, they form a confluent monolayer with several properties characteristic of differentiated epithelial cells in the small intestine (9,10). The monolayers are morphologically polar and develop brush borders at their apical surface. Furthermore, Caco-2 cells are known to express carrier mediated transport systems for vitamin B_{12}, D-glucose, amino acids and dipeptides (11–14). This cell culture model offers the opportunity of rapidly assessing the potential permeability and metabolism of a drug candidate. For perorally active peptide analogues an efficient tool for estimating and predicting bioavailability and metabolism is of considerable interest, especially in early development phases.

In the present study we want to estimate the oral bioavailabilities of five newly developed thrombin inhibitors and elucidate their cellular transport pathway in epithelial cells using the Caco-2 monolayers cell culture model.

MATERIALS AND METHODS

General Synthetic Methods

The thrombin inhibitors listed in figure 1 were synthesized by Behringwerke AG (Marburg, Germany) according to published results (5,15,16).

Chemicals and Materials

TRH (pyro-L-Glu-L-His-L-Pro-NH_2), were obtained from Bachem (Bubendorf, Switzerland). All other chemicals were purchased from E. Merck (Darmstadt, Germany) in analytical quality ^{14}C-Polyethylene glycol 4000 (15 mCi/g) and ^{14}C-mannitol (310 μCi/mg) were from Amersham (Braunschweig, Germany) and ^{3}H-TRH ([L-proline-3,4-^{3}H(N), L-histidine-3-^{3}H(N)] (83.0 Ci/mmol) was from New England Nuclear (Dreieich, Germany).

Tissue culture reagents were bought from Gibco (Eggenstein, Germany) except for fetal calf serum (FCS) which was from Biozol (Eching, Germany). Tissue culture articles were purchased from Nunc (Wiesbaden, Germany).

Determination of pK_a Values

Ionization constants (pK_a) of substances were deter-

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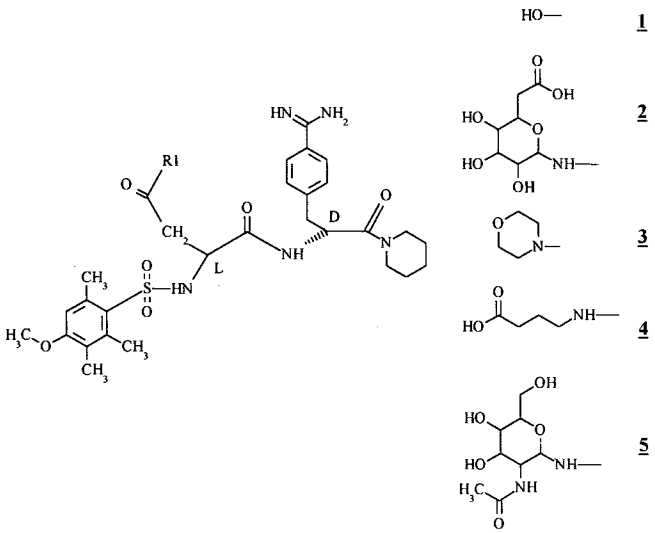

Fig. 1. Chemical structures of the thrombin inhibitors 1–5.

mined potentiometrically using the PCA 101 equipment (Sirius Analytical Instruments) (17). This instrument allows automatic titration and calculation of the pK_a. CO_2 contamination was avoided by working under argon. Each substance was dissolved in 0.1 M KNO_3 yielding concentrations of about 1 mM. The titration span ranged from pH 1.8 to 12.2 at 25°C. The calculation procedure involved a weighted nonlinear regression analysis.

Partition Coefficients

The determination of the partition coefficient (log P) was carried out using an additional potentiometric assay in the presence of octanol in the equipment mentioned above. Exploiting the difference between the pK_a value in the pure aqueous system and the apparent pK_a of the organic solvent, log P and the effective partition at any pH (log D) of the substance could be calculated (17). All measurements were carried out at 25°C under argon.

Cell Culture

Caco-2 cells were cultivated as described recently (18). The mycoplasma-free cells were used between passage numbers 45 and 55.

For transport studies Caco-2 cells were seeded on polycarbonate cell culture inserts (Transwell^R, 0.4 μm pores, 4.71 cm^2, Tecnomara) at a cell density of 6.5×10^4 cells/cm^2. Confluent monolayers were used between days 21 and 25 after seeding for transport experiments.

Transport Studies

Transport studies were performed according to the method described in (18). The integrity of the monolayers was checked at the beginning and end of each experiment by measurement of the transmembrane resistance (Endohm, WPI, Germany) with values in the range of 280-310 ohms × cm^2. The permeabilities of the monolayers were routinely controlled using fluorescein as hydrophilic marker substance (19). Protein content of the Caco-2 monolayers was determined according to Lowry (20).

Sample Analysis by HPLC

The instrumentation consisted of a pump (Model 126), an automatic sampler (Model 507) and diodearray detector (Model 168) all from Beckman (München, Germany). Data acquisition and integration was carried out using the Beckman Gold V711 software. Compounds 1–5 were separated on a reversed-phase column (Nucleosil 100, C18, 3 μm, 60 × 20 mm) protected with a precolumn (Guardcartridge K1, Nucleosil 100, C18, 5 μm). Detection wavelength was 205 nm. Quantification of the compounds was carried out by measuring peak areas in relation to those of standards chromatographed under the same conditions. The sensitivity of the assay was ca. 50 ng/ml. Analysis for TRH was performed as described previously (18).

Animal Studies

Bioavailability studies in female Wistar rats (200 g) were carried out administering aqueous solutions via gavage at dosage ranges between 10 and 90 mg/kg. Plasma levels were monitored by measuring the activated partial thromboplastin time (aPTT) at time points 5, 15 and 45 min. The areas under the effect time curves after oral and intravenous administration were compared to calculate relative "effect"-bioavailabilities.

Data Treatment

Effective permeability coefficients (P_{eff}) were calculated using the following equation:

$$P_{eff} = \frac{dc \cdot V}{dt \cdot A \cdot c_0} \qquad [cm/s] \qquad (1)$$

where dc/dt is the flux across the monolayer (mM/s), V the volume of the receiver chamber (ml), A the surface area of the monolayer (cm^2), and c_0 the initial concentration (mM) in the donor compartment. The flux across the monolayer was calculated from the slope of the regression line describing the amount transported versus time (which was linear up to 120 min) (21). The non-passive transport component is characterized by a Michaelis-Menten kinetic described by the following equation:

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]} + k_d \cdot [S] \qquad (2)$$

with the Michaelis-Menten constant K_m, the maximum transport rate V_{max}, the initial concentration of substrate [S] and the first order rate constant for passive transport k_d.

Statistical analysis was performed using two-sided t-test. All data are expressed as means ± standard deviations.

RESULTS

Permeability of the Compounds Across Caco-2 Cell Monolayers and Correlation with Data Obtained in Rats and Octanol-Water Partition Coefficients

When transported across Caco-2 monolayers, we found

Table I. Summary of in Vitro Data of the Five Thrombin Inhibitors

Compound	Molecular weight (g/mol)	Permeability coefficients ($\times 10^{-6}$ cm/s)[a]	Relative permeability (%)[b]	Extrapolated oral bioavailability (%)[c]	log D[d]	pK_a
1	638	0.126 ± 0.008	100	10	0.1	10.9
2	819	0.0311 ± 0.0022	25	0.4	1.6	10.2
3	699	0.0406 ± 0.0012	32	1.3	−2.2	9.9
4	715	0.0517 ± 0.0012	41	2.5	−0.4	9.9
5	832	0.0600 ± 0.0041	48	3.3	−1.5	9.8

[a] In Caco-2 monolayers, mean ± S.D., n = 3.
[b] Based on permeability of compound 1.
[c] Calculated from P_{eff} values of the compounds in the plot of figure 2 [22, 23].
[d] log D octanol/water.

striking differences in the permeability of the five dipeptide-derived TI as outlined in table 1. No metabolic degradation could be detected on either side of the monolayers using the HPLC assay. All five compounds are analogues of the dipeptide L-Asp-D-Phe and possess comparable molecular weights as described in table I. Comparing effective permeability coefficients of the TI determined in Caco-2 monolayers under *in vitro* conditions with frequently used marker molecules, such as mannitol, TRH and PEG 4000, we observe a rank order in P_{eff} of PEG 4000 < 2 < 3 < 4 < 5 ≈ TRH < 1 < mannitol. Using the published peroral bioavailabilities of PEG 4000 (22), TRH (23) and mannitol (22) in humans we obtain the relationship as shown in Figure 2. From this relationship we estimate the peroral bioavailability to be comparatively low lying in the range of 0.4 − 10%. A similar rank order is obtained when the relative peroral effect bioavailability in rats (Table II) is compared to the effective permeability of the thrombin inhibitors in Caco-2 monolayers. In figure 3, where the flux across Caco-2 monolayers was plotted against oral effect bioavailability in rats, the correlation coefficient for the linear regression line was found to be r = 0.977.

Furthermore, we determined the octanol-water partition coefficients of the five thrombin inhibitors in order to evaluate their lipophilicities (table I). No obvious correlation was found when we compared the octanol-water coefficients with peroral effect bioavailibities in rats and Caco-2 transport data. The correlation coefficients for a linear regression are in both cases r < 0.1 (data not shown). The pK_a values of the amidino-groups in para-position of D-Phe are for all compounds in a similar range of 10.9 to 9.8, as listed in table I. Compounds 1 and 4 show additional pK_a values of 3.8 and 4.5 due to their free carboxyl-groups in R1. Compound 5 possess a further pK_a of 11.8 due to the acetamide-functionality. Concerning the charge of the compounds at physiological pH, the molecules are likely to carry a positive charge, except in compounds 1 and 4, where zwitterionic structures are to be expected due to the deprotonated carboxyl-groups.

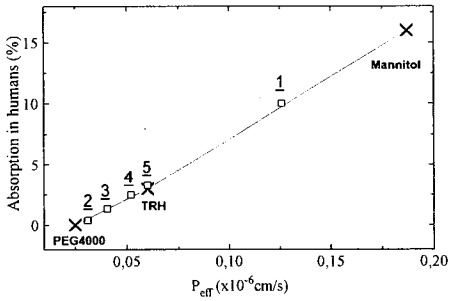

Fig. 2. Estimation of relative peroral bioavailabilities for five thrombin inhibitors. Crosses indicate the determined peroral bioavailabilities of PEG 4000, TRH and mannitol related to their permeabilities through Caco-2 monolayers. Open squares display the predicted oral absorption rates of the thrombin inhibitors calculated from their Caco-2 permeabilities. Each point represents the mean of three to four determinations.

Characterization of the Transport Pathway of Compound 1

It is evident, that compound 1 yields a significantly higher permeability coefficient across Caco-2 cell monolayers and higher effect bioavailabilities in rats. Although all compounds have comparable chemical structures, different transport mechanisms seem to apply. To characterize the transport system for compound 1 several factors were examined as outline in figure 4. The effect of the dipeptide Gly-L-Pro (27 mM) and cephradine (27 mM) on the transport of 0.8 mM of compound 1 were tested. Coadministration of an excess of Gly-L-Pro and cephradine, both well known substrates for the di-peptide carrier, inhibit the transport of

Table II. Oral Bioavailability and Variability of Synthetic Thrombin Inhibitors in Rats

Compound	Dose (mg/kg)	n	Relative peroral effect bioavailability (%)[a] mean	S.D.
1	20, 60	30	3.8	5.3
2	30	5	0.0	0.0
3	20	5	0.4	0.6
4	60, 90	10	0.0	0.0
5	10	5	0.7	1.0

[a] Bioavailability was calculated based on aPTT values at time points 5, 15, 45 min after oral application. Mean aPTT values obtained after intravenous application of various dosages were set as 100% bioavailability.

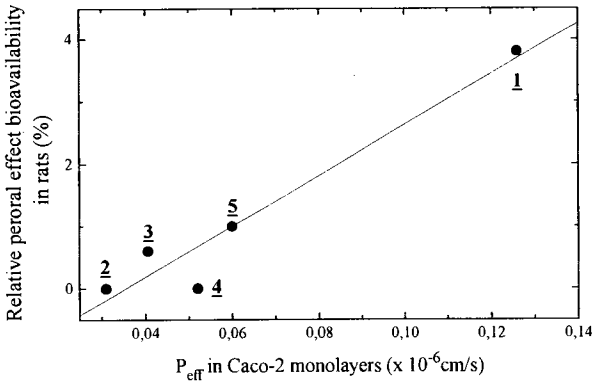

Fig. 3. Relationship of the permeability coefficients for the compounds 1–5 across Caco-2 cell monolayers and peroral effect availability in rats. The correlation coefficient for the linear regression line shown is r = 0.977.

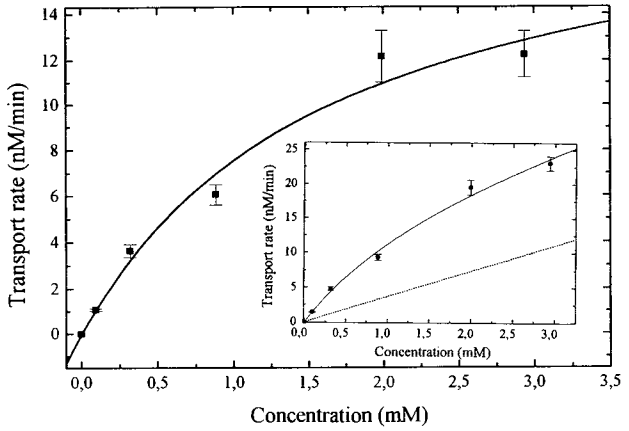

Fig. 5. Concentration dependence of compound 1 transport across Caco-2 monolayers after correction for the nonsaturable component. Inset shows the nonsaturable transport (dashed line) and the total transport rates measured at 37°C (solid line). Each point represents the mean of three determinations ± S.D.

compound 1 to 31% for Gly-L-Pro and to 51% for cephradine. Transport was direction dependent and significantly higher from the mucosal to serosal side. Temperature reduction to 4°C decreased the transport rate to 52%. The concentration dependence was studied in the range of 0.016–3.0 mM at pH 6 and 37°C. Under this condition compound 1 was insoluble at concentrations above 3.2 mM. The rate constant of passive diffusion was obtained from transport at 4°C with $k_d = 4.87 \pm 0.41 \times 10^{-3}$ nmol min^{-1} mg $protein^{-1}$ mM^{-1} (fig. 5, inset). After correction of the nonsaturable component, we estimated the Michaelis-Menten parameters for the active transport component. The curve in figure 5 shows the result of nonlinear regression analysis with the Michaelis-Menten constant for $K_m = 1.67 \pm 0.62$ mM and the maximum transport rate $V_{max} = 26.5 \pm 4.8$ pmol min^{-1} mg $protein^{-1}$.

DISCUSSION

Permeability of the Compounds Across Caco-2 Cell Monolayers and Correlation with Data Obtained in Rats and Octanol-Water Partition Coefficients

In our study we investigated the permeability of five structurally related TI across intestinal cell membranes using the Caco-2 cell culture model. The Caco-2 cells have been shown by several laboratories to represent a sensitive and reproducible *in vitro* model of the intestinal epithelium for the study of transport mechanisms (9,10,13). A good correlation between extent of drug absorption in rat intestinal *in situ* model and in the Caco-2 model was obtained for a series of β-blocking agents (24).

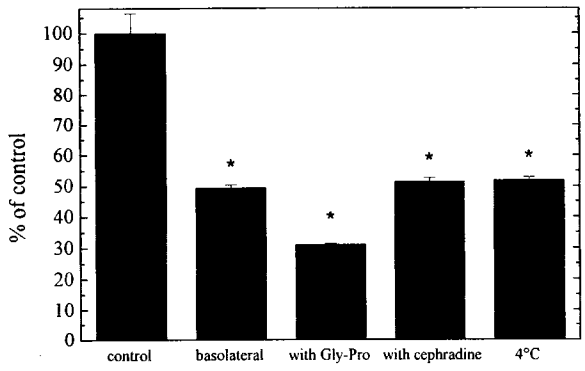

Fig. 4. Relative permeability of compound 1 across Caco-2 cell monolayers under different transport conditions. Data are represented as means ± S.D. (n = 3) and two-sided t-test results expressed as differences from control value (*p < 0.01).

All TI are derivates of the L-Asp-D-Phe which differ only in their substitution of the L-asparagyl-β-carboxyl group. Despite structural similarities, we found striking differences in transport rates across Caco-2 monolayers ranging from $P_{eff} = 0.126 \times 10^{-6}$ cm/s for compound 1 to $P_{eff} = 0.031 \times 10^{-6}$ cm/s for compound 2.

For a molecule to traverse passively across an organized epithelium, both paracellular and transcellular mechanisms have to be considered (25). For passive transcellular transport the partitioning behaviour of a solute between octanol and water is a good predictor for its membrane permeability (26). Partition coefficients of 10–1000 are generally regarded as optimal (27–29). For polar solutes, with log octanol-water partition coefficients ≤0, transport across cell membranes is slow due to poor membrane partitioning properties. These solutes will cross the cell monolayers by the paracellular route. To further elucidate the transport properties of the TI, we determined log octanol-water partition coefficients (log D) to obtain information on the partition behaviour of the compounds. Interestingly, we could not find a relationship between membrane permeability in Caco-2 monolayers and log D. Compound 2 with a relatively high log D value of 1.6 showed the lowest membrane permeability. Recently, for peptide-related substances the octanol-buffer partition coefficients (PC) were demonstrated not to be a good predictor for absorption processes (25). In a series of homologous peptides no significant correlation between P_{eff} across Caco-2 cells and log PC was obtained. The total hydrogen binding capacity of the molecules was proposed as an alternative explanation for the absorption behaviour of peptides via the passive transcellular pathway (25). Regarding the peroral effect availability in rats, we confirm the lack of correlation between log D and oral absorption of TI both *in vitro* and *in*

vivo suggesting that the passive transcellular route of transport is of minor importance.

We found, however, a good correlation of permeability coefficients in Caco-2 monolayers with peroral effect bioavailabilities in rats. It should be noted, however, that estimates of peroral bioavailabilities based upon pharmacological effect data are of preliminary nature only, for a variety of reasons: aPTT is a convenient, but not very sensitive biological signal for thrombin inhibition. The relationship between the plasma-levels and effects is not necessarily linear. The influence of elimination and metabolism of the thrombin inhibitors under investigation can not be estimated from the effect data with certainty, therefore, only a very global bioavailability pattern can be discussed.

The permeabilities of the TI across Caco-2 monolayers are grouped between the flux of the hydrophilic marker substances PEG 4000 and mannitol with molecular masses of 4000 g/mol and 182 g/mol. Estimates of the extent of peroral absorption for the five TI from Caco-2 experiments predict bioavailabilities to fall into the range of 0.4 to 10%. These estimates are in good agreement with our data for oral effect availabilities obtained in rats. From the literature it is evident, that oral bioavailabilities in the range of 0.4 to 3.3% are not unusual for peptides and peptide-related drugs (3). Typical representatives are dDAVP (1-deamino-8-D-arginine-vasopressin, MW = 1071 Da) with 1% peroral bioavailability in humans or cefoxitin showing a bioavailability of 2% after oral administration in rats (22,30). From our investigations we conclude that the transport of the TI 2–5 is restricted to the less effective paracellular transport pathway, presumably due to their more polar molecular structure. Compound 1, for which we estimate a significantly higher oral bioavailability from our transport data with Caco-2 cells and which showed a relative peroral effect bioavailability of 3.8% in rat, is absorbed by a different transport pathway, e.g. active transport.

Characterization of the Active Transport Component

To characterize the transport system for compound 1 different factors were examined. Temperature reduction to 4°C inhibits the transport of compound 1. Transport was direction dependent and significantly higher from the mucosal to serosal side. Compound 1 showed nonlinear kinetics, which could be described by the Michaelis-Menten-type carrier-mediated transport model with parallel passive transport, probably via the peptide carrier. This suggestion is supported by the inhibition by Gly-L-Pro and cephradine, both well known substrates for the peptide carrier.

The peptide carrier required for dipeptide and tripeptide absorption plays a substantial role in peptide-type drug absorption (7). It is widely distributed throughout the small intestine and shows a low substrate specifity combined with high transport rates. Minimal requirements for binding to the di-peptide carrier were shown to consist of a free carboxylic acid group and an amide bond. A peptide-like structure appears to be necessary, but an α-amino group is not essential (6,7). TRH was still found to be orally absorbed by the peptide transporter without having free N-terminal α-amino and C-terminal carboxyl-groups (23,31). However, the structural requirements for peptide transport are yet to be fully clarified.

Compound 1 fulfills the minimal structural requirements with a free carboxyl- and one amide-group. D-amino-acids in TI does not seem to inhibit the affinity for the dipeptide transporter (6). Nevertheless, the uptake of small peptides is stereoselective. Dipeptides of L-L form have the highest uptake rate, followed by mixed isomers (L-D and D-L) and then D-D-isomers. Therefore, from structural considerations, compound 1 may be a potential substrate for the di-/tripeptide transporter.

Regarding the Michaelis-Menten parameters for the active transport component, the K_m value of 1.67 mM is in a typical range for the peptide carrier (7,13,14). For Gly-L-Pro and cephradine, which inhibit the transport of compound 1 to some extend, K_m's were 0.9 mM in rabbit small intestine brush border vesicles and 1.5 mM in rat perfusion studies in the small intestine, respectively (13,32). These findings suggest that TI is transported by the dipeptide carrier.

The carrier-mediated transport accounts for 50% of total transport of compound 1. The carboxyl-group, which was reported to be essential for recognition by the carrier, is esterified in TI 2–5 with bulky side chains. This may be a possible explanation for the differences in transport mechanism of TI 2–5. Further investigations are clearly necessary to define the structural requirements for substrates of the dipeptide carrier.

CONCLUSION

In summary, we demonstrated in this study, that the Caco-2 cell culture model offers a suitable tool in investigating gastrointestinal absorption mechanisms, as well as, estimating oral bioavailability. A satisfactory correlation between permeability coefficients in Caco-2 monolayers and peroral effect availabilities in rats was obtained for a series of peptidomimetic TI. The log D as predictor for peptide-absorption failed to give an approximate indication of drug absorption and should be interpreted carefully, especially when active transport components are involved. The transport of compound 1 in Caco-2 monolayers has been shown to consist of a passive and active component involving the peptide carrier-mediated transport system. This model may be helpful for the design of orally active peptides.

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REFERENCES

1. P. L. Smith, D. A. Wall, C. H. Gochoco, and G. Wilson. Routes of delivery: Case studies. (5) Oral absorption of peptides and proteins. *Adv. Drug Delivery Rev.* 8:253–290 (1992).
2. J. G. Hardy, S. S. Davis, and C. G. Wilson. *Drug delivery to the gastrointestinal tract*, Ellis Horwood Limited, New York, 1989.
3. M. J. Humphrey and P. S. Ringrose. Peptides and related drugs: A review of their absorption, metabolism, and excretion. *Drug Met. Rev.* 17:283–310 (1986).
4. L. J. Berliner. *Thrombin: structure and function*, Plenum Press, New York, 1992.
5. W. Stueber, R. Koschinsky, D. Dickneite, and C. Kolar. Patent: EP 0513543, (1993).
6. J. B. F. Bai and G. L. Amidon. Structural specifity of mucosal-cell transport and metabolism of peptide drugs: Implication for oral peptide drug delivery. *Pharm. Res.* 8:969–978 (1992).

7. D. I. Friedman and G. L. Amidon. Characterization of the intestinal transport parameters for small peptide drugs. *J. Contr. Rel.* 13:141–146 (1990).
8. P. Artursson. Cell culture as models for drug absorption across the intestinal mucosa. *Crit. Rev. Ther. Drug Carrier Syst.* 8:305–330 (1991).
9. I. J. Hidalgo, T. J. Raub, and R. T. Borchardt. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 96:736–749 (1989).
10. G. Wilson, I. F. Hassan, C. J. Dix, I. Williamson, and M. Mackay. Transport and permeability properties of human Caco-2 cells. An in vitro model of the intestinal epithelial cell barrier. *J. Control. Rel.* 11:25–40 (1990).
11. C. J. Dix, H. Y. Orbay, I. F. Hassan, and G. Wilson. Vitamin B_{12} transport through polarized monolayers of a colon carcinoma cell line. *Biochem. Soc. Trans.* 15:443–440 (1987).
12. A. Blais, P. Bissonnette, and A. Berteloot. Common characteristics for Na-dependent sugar transport in Caco-2 cells and human fetal colon. *J. Membr. Biol.* 99:113–125 (1987).
13. A. H. Dantzig and L. Bergin. Uptake of the cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal cell line, Caco-2. *Biochim. Biophys. Acta* 1027:211–217 (1990).
14. K. I. Inui, M. Yamamoto, and H. Saito. Transepithelial transport of oral cephalosporins by monolayers of intestinal epithelial cell line Caco-2: Specific transport systems in apical and basolateral membranes. *J. Pharm. Exp. Ther.* 261:195–201 (1992).
15. W. Stüber, G. Dickneite, R. Koschinsky, M. Reers, D. Hoffmann, and E. P. Paques. Development of highly potent thrombin inhibitors based on amidinophenylalanine derivates. *Keystone Symp., Prospects and Progress in Drug Based on Peptides and Proteins.* 1993.
16. W. Stueber, R. Koschinsky, C. Kolar, M. Reers, G. Dickneite, D. Hoffmann, J. Czech, K. H. Diehl, and E. P. Paques. Inhibition of thrombin by derivates of the dipeptide aspartic acid-amidinophenylalanine. *Peptides: Chemistry and Biology, Proceed. 13th American Peptide Symp.*, in press.
17. A Avdeef. Fast simultaneous determination of log P and pK_a by potentiometry: para-alkoxyphenol series (methoxy to pentoxy). In C. Silipo, V. Vittoria (eds.), *QSAR: rational approaches to the design of bioactive compounds,* Elsevier Science Publishers B.V., Amsterdam, 1991, p. 119–122.
18. E. Walter and T. Kissel. Transepithelial transport and metabolism of thyrotropin-releasing hormone (TRH) in monolayers of a human intestinal cell line (Caco-2): Evidence for an active transport component? *Pharm. Res.* 11:1576–1581 (1994).
19. A. B. J. Noach, Y. Kurosaki, M. C. M. Blom-Rosemalen, A. G. de Boer, and D. D. Breimer. Cell-polarity dependent effect of chelation on the paracellular permeability of confluent Caco-2 cell monolayers. *Int. J. Pharm.* 90:229–237 (1993).
20. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol red. *J. Biol. Chem.* 193:265–275 (1951).
21. A. R. Hilgers, R. A. Conradi, and P. S. Burton. Caco-2 cell monolayer as a model for drug transport across the intestinal mucosa. *Pharm. Res.* 9:902–910 (1990).
22. P. Artursson and J. Karlsson. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem. Biophys. Res. Commun.* 175:880–885 (1991).
23. S. Yokohama, K. Yamashita, H. Toguchi, J. Takeuchi, and N. Kitamori. Absorption of thyrotropin-releasing hormone after oral administration of TRH tartrate monohydrate in the rat, dog and human. *J. Pharmacobio-Dyn.* 7:101–111 (1984).
24. P. Artursson. Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. *J. Pharm. Sci.* 79:476–482 (1990).
25. P. S. Burton, R. A. Conradi, and A. R. Hilgers. (B) Mechanisms of peptide and protein absorption. (2) Transcellular mechanism of peptide and protein absorption: Passive aspects. *Advanced Drug Delivery Rev.* 7:365–386 (1991).
26. A. Leo, C. Hansch, and D. Elkins. Partition coefficients and their uses. *Chem. Rev.* 71:525–616 (1977).
27. S. H. Yalkowsky, W. Morozowich, and W. I. Higuchi. A physical chemical basis of the design of orally active prodrugs. *Drug Design* 9:122–185 (1980).
28. N. F. H. Ho, J. Y. Park, W. Morozowich, and W. I. Higuchi. Physical model approach to the design of drugs with improved intestinal absorption. In E. B. Roche (ed.), *Design of biopharmaceutical properties through prodrugs and analogs,* American Pharmaceutical Association, Academy of Pharmaceutical Sciences, 1977, p. 136–227.
29. I. Osiescka, P. A. Porter, R. T. Borchardt, J. A. Fix, and R. C. Gardner. In vitro drug absorption models: brush border membrane vesicles, isolated mucosal cells and everted intestinal rings: characterization and salicylate accumulation. *Pharm. Res.* 2:284–293 (1983).
30. P. A. Porter, I. Osiecka, R. T. Borchardt, J. A. Fix, I. Frost, and C. Gardner. In vitro drug absorption models. II. Salicylate, cefoxitin, α-methyldopa and theophylline uptake in cells and rings: correlation with in vivo bioavailability. *Pharm. Res.* 2:293–297 (1985).
31. S. Yokohama, T. Yoshioka, K. Yamashita, and N. Kitamori. Intestinal absorption mechanism of thyrotropin-releasing hormone. *J. Pharmacobio-Dyn.* 7:445–451 (1984).
32. H. Yuasa, G. L. Amidon, and D. Fleisher. Peptide carrier-mediated transport in intestinal brush border membrane vesicles of rats and rabbits: Cephradine uptake and inhibition. *Pharm. Res.* 10:400–404 (1993).