Transport of Peptidomimetic Renin Inhibitors Across Monolayers of a Human Intestinal Cell Line (Caco-2): Evidence for Self-Enhancement of Paracellular Transport Route

Elke Walter,[1] Thomas Kissel,[2,4] and Peter Raddatz[3]

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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. This is partly due to the rapid development in biotechnology and peptide synthesis allowing the exploitation of the unique pharmacological activities of peptide drugs. The principal difficulty in the use of these substances as therapeutic agents is the requirement for parenteral administration since they generally have very low systemic availability when administered orally (1).

The renin-angiotensin system is an important modulator of blood pressure and fluid balance. Because renin is highly specific for its substrate, angiotensinogen, renin inhibitors may emerge as clinically preferable alternative to ACE inhibitors, which affect multiple biological systems, including bradykinin and prostaglandin metabolism (2). Therefore, low molecular weight peptidomimetic renin inhibitors (RI) have been developed for the treatment of hypertension and congestive heart failure (2,3). Metabolic and transport barriers are limiting the oral absorption of peptides. Various approaches have been attempted to overcome these limitations, including the use of penetration enhancers, protease inhibitors, and various formulations. A more promising approach appears to be the design of peptide drugs that readily cross the intestinal epithelium. However, only very few information is available about the structural features of peptides that favor intestinal absorption.

The human intestinal cell line Caco-2 provides a convenient model for the study of the differentiated function of intestinal enterocytes. When Caco-2 cells are grown on microporous membranes, they form confluent monolayers with several properties characteristic of differentiated epithelial cells in the small intestine (4). This cell culture model affords the opportunity to rapidly assess the potential permeability and metabolism of a drug. The objective of this study was to determine whether a series of peptidomimetic RI would be transported across the intestinal epithelium and to elucidate their absorption behaviour.

MATERIALS AND METHODS

Materials

Lypressin was provided by Sandoz Pharma AG (Basel, Switzerland). D-Ala^2-Met-enkephalinamid was obtained from Bachem (Bubendorf, Switzerland) and Sulforhodamin from Sigma (Deisenhofen, Germany). All other chemicals were obtained from E. Merck (Darmstadt, Germany) in analytical quality. ^{14}C-Polyethylene glycol 4000 (15 mCi/g), ^{14}C-mannitol (310 μCi/g), ^{14}C-warfarin (117 μCi/g) were from Amersham (Braunschweig, Germany) and ^{14}C-acetylsalicylic acid (55.8 mCi/g) was obtained from New England Nuclear (Dreieich, Germany). Tissue culture reagents were 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). Polycarbonate (PC) membrane cell culture inserts were from Tecnomara (Fernwald, Germany)

General Synthetic Methods

The compounds 1–8 were synthesized as described previously and structures are shown in figure 1 (c.f. results) (3).

Partition Coefficient Determination

Lipophilicity of the respective compound was determined chromatographically between lipophilic solid phase and hydrophilic mobile phase according to (5). The characteristic value obtained is the chromatographic partition coefficient k′, which is proportional to the octanol/water partition coefficient.

Cell Culture

Caco-2 cells were routinely maintained in Dulbecco's Modified Eagle Medium, containing 10% fetal calf serum, 1% nonessential amino acids, 1% L-glutamine and penicillin (100 U/ml)/streptomycin (100 μg/ml) in an atmosphere of 10% CO_2 at 95% (r.h.) at 37°C. Cells grown in 100-mm-diameter plastic petri-dishes were passaged every 5 days at a split ratio 1:5 to 1:10. The mycoplasma-free cells (DKFZ, Heidelberg, Germany) were used between passage number 45–61.

For transport studies Caco-2 cells were seeded in cell culture inserts with polycarbonate membranes (Transwell^R, pores: 0.4 μm, area: 4.71 cm^2) at a cell density of 6.5×10^4 cells/cm^2 and incubated in six-well culture plates with medium change every second day. The confluent monolayers were used between days 21 and 25 after seeding.

Transport Studies

The transport studies were performed as described previously (6). Hank's balanced salts solution containing 15 mM glucose at pH 6.7 was used as transport buffer. After 15, 30,

[1] College of Pharmacy, The University of Michigan, Ann Arbor, Michigan 48109-1065.
[2] Department of Pharmaceutics and Biopharmacy, University of Marburg, Ketzerbach 63 D-35032 Marburg, Germany.
[3] E. Merck Darmstadt, Preclinical Pharmaceutical Research, D-64293 Darmstadt, Germany.
[4] To whom correspondence should be addressed.

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45, 60, 75, 90, 105 and 120 min, 750 µl samples were withdrawn from the receiver chamber and replaced by fresh transport buffer. The integrity of the monolayer was checked at the beginning and at the end of each experiment by determination of the transmembrane resistance (Endohm^R, WPI, Germany).

Sample Analysis

The radioactive samples were analyzed in a liquid scintillation counter (Hewlett Packard Tricarb 4660).

HPLC. The instrumentation consists of a pump (Model L-6200A), an automatic sampler (Model AS-2000A), a column thermostat (Model T-6300), and a fluorescence detector (Model L-1050) all from Hitachi, Merck (Darmstadt, Germany). Data acquisition and integration was carried out by Millennium 2010 software (Millipore Waters, Eschborn, Germany). A derivatisation for compounds 1–8 was performed using fluorescamine at pH 9.0. The renin inhibitors were stable under these conditions as tested by HPLC. All fluorescamine-derivates were separated on a reversed-phase column (Lichrospher 100, RP 18, 5 µm, 125 × 4 mm, Merck) with mobile phase consisting of acetonitril (A) and 0.01 M ammoniumphosphate buffer pH 7.0 (B). With a flow of 1.0 ml/min at 30°C retention times were as follows: 1 (35% A), 4.4 min; 2 (40% A), 4.0 min; 3 (41% A), 4.1 min; 4 (41% A), 3.1 min; 5 (40% A), 3.7 min; 6 (40% A), 3.5 min; 7 (38% A), 7.0 min and 8 (39% A), 3.4 min. Detection wavelengths were 390 nm for excitation and 475 nm for emission. Quantitation of the compounds was performed by measuring peak areas in relation to those of standards chromatographed under identical conditions. The sensitivity of the assay was ca. 50 ng/ml.

All other substances were separated without derivatisation and conditions were varied as follows: Fluorescein, 14% acetone and 86% 0.01 M phosphate buffer pH 7.0 (488 nm, 514 nm); sulforhodamine, 30% acetonitril, 70% 0.01 M phosphate buffer pH 7.0 (586 nm, 610 nm); lypressin, 15% acetonitril, 0.01 M phosphate buffer pH 2.0 (215 nm, UV detector, Model L-4000, Hitachi, Merck). The separation of D-Ala^2-Leu-enkephalinamid was performed as described previously (7).

Data Treatment

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

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

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 transported amount versus time (which was linear up to 120 min after a short lagtime of 1–5 min) (6).

RESULTS AND DISCUSSION

When transported across Caco-2 monolayers, striking differences in the permeability of the eight peptidomimetic RI were found as outlined in Table I. Compound 7 with P_{eff} = 21.7 ± 0.50 × 10^{-6} cm/s displayed the highest transport rate and compound 8 with P_{eff} = 0.0581 ± 0.0093 × 10^{-6} cm/s displayed the lowest transport rate. This corresponds to a factor of 370 between the two extremes. The molecular structures of the compounds do not offer a straightforward explanation for these deviations in transepithelial transport properties. The compounds 2–8 have similar structures which differ only slightly in their substitution pattern at the R1 residue (Fig. 1). The molecular masses of the renin inhibitors are comparable in the range of 793 to 656 g/mol (Table I). Moreover, the lipophilicities of all eight RI are a very similar. To evaluate metabolic degradation of RI on the Caco-2 brush border membrane, we calculated mass balance values shown in Table I. The balances are approaching 100% and, therefore, no prominent metabolic degradation due to brush border enzymes could be detected.

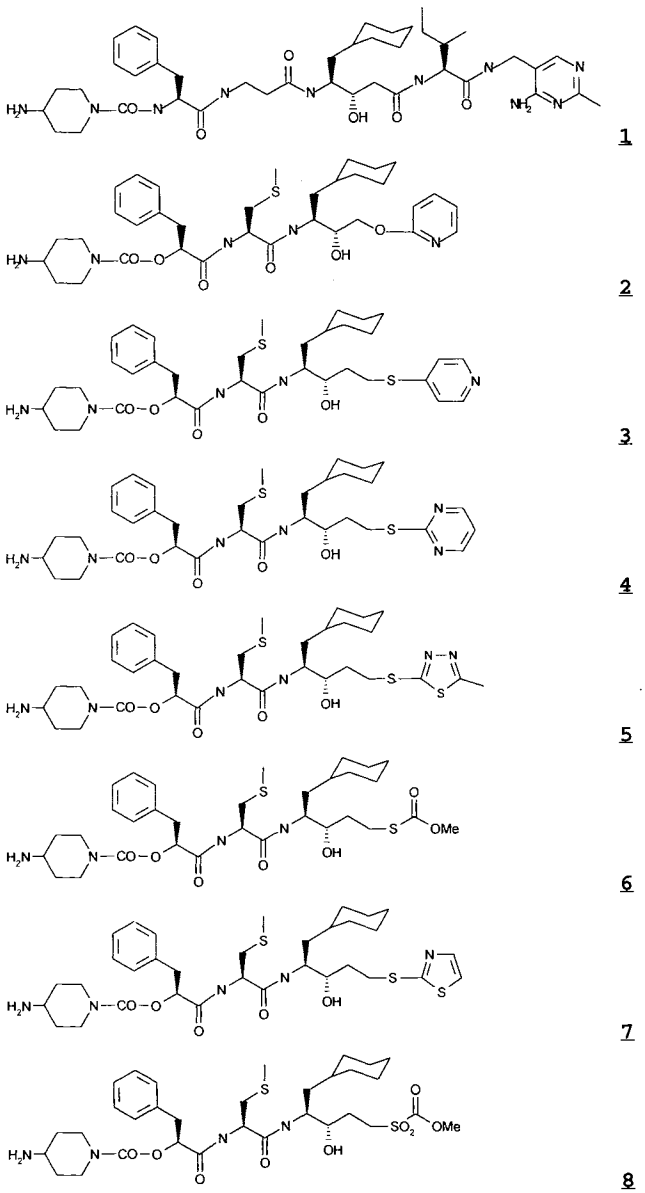

Fig. 1. Structures of the renin inhibitors 1–8.

Table I. Physico-chemical and Transport Properties of Eight Renin Inhibitors Through Caco-2 monolayers

Compound	Molecular weight	log (k')[a]	Permeability coefficient ($\times 10^{-6}$ cm/s)[b]	Relative permeability (%)[c]	Balance after 120 min (%)[d]	Relative TEER after 120 min (%)[e]	Relative TEER after 17 h (%)[e]
1	793	4.6 ± 0.21	0.123 ± 0.027	0.57	107.8 ± 0.9	119 ± 16	—
2	656	4.9 ± 0.15	1.59 ± 0.53	7.3	92.7 ± 0.9	12 ± 4	111 ± 6
3	686	5.2 ± 0.17	6.63 ± 0.95	30	104.8 ± 1.5	3 ± 1	147 ± 36[f]
4	687	4.7 ± 0.16	0.960 ± 0.297	4.4	96.7 ± 2.6	19 ± 3	120 ± 6
5	707	5.1 ± 0.17	0.904 ± 0.063	4.2	102.8 ± 1.5	48 ± 5	—
6	667	5.2 ± 0.16	0.0720 ± 0.0248	0.33	94.8 ± 1.3	198 ± 12	121 ± 4
7	692	5.3 ± 0.19	21.68 ± 0.50	100	95.7 ± 1.3	0	—
8	699	4.3 ± 0.17	0.0581 ± 0.0093	0.27	89.0 ± 0.9	169 ± 15	—

[a] Mean ± S.D.
[b] Mean ± S.D. (n = 3).
[c] Calculated fromm P_{eff} of the compound 7 set as 100%.
[d] Calculated from the amount found in the apical chamber plus amount found in the basolateral chamber related to the initial concentration. Mean ± S.D. (n = 3).
[e] Calculated with TEER values set as 100% at t = 0. Mean ± S.D. (n = 6).
[f] After 27 h.

When incubating Caco-2 monolayers with 1 mg/ml of the respective RI for 120 min, we observed a remarkable decrease in transepithelial electrical resistance (TEER) for compounds 2, 3, 4, 5 and 7.

After transport experiments, the monolayers were rinsed with transport buffer and put back into their maintaining culture medium. As outlined in table I, a recovery of the TEER, to initial values occurred after 17 h.

Permeability studies with lipophilic highly permeable drugs as warfarin and acetylsalicylic acid as well as hydrophilic substances, transported paracellulary show a similar wide range in transport rates without causing remarkable changes in TEER during 120 min incubation on Caco-2 monolayers (data not shown). Nevertheless, differences in transport rate of RI could not be explained by their lipophilicities as shown by k' values. Furthermore, different hydrophilic marker substances with variations in molecular weight, structural features and charge yielded less dramatic differences in transport rates compared to RI. The permeability coefficients of the poorly transported peptides lypressin and D-Ala^2-Leu-enkephalinamid are in accordance with some of the RI, showing lower Peff. No metabolic degradation could be detected for both peptides during transport experiments. The relatively low transport rates of some RI, similarity in structure, molecular weight and hydrophilicity indicate that RI are mainly transported by the paracellular pathway.

An effect on the tight junctions increasing the paracellular permeability, will not necessarily be detected in transport experiments. After administration of the compound, the absorption curve may be still linear, but steeper. Using the hydrophilic marker substance fluorescein, we found that the resistance of a cell monolayer is inversely proportional to the paracellular flux (figure 2). In this regard, the correlation of changes in TEER caused by the RI are in good accordance with their permeability data as demonstrated in figure 3 (r = 0.996).

Our results give evidence that some of the renin inhibitors seem to influence the permeability in the Caco-2 monolayers which is reflected on one hand in higher transport rates, as well as, in decreased transepithelial electrical resistances. The origin of these permeability changes is unclear and requires further investigations. However, the involvement of active efflux transport systems as has been demonstrated recently for peptides (8,9). Polarized efflux systems usually would actively return the peptide back to the apical media and therefore contributes to a reduction of transport rates.

The observed effects of some of the RI on Caco-2 TEER are similar to earlier work reported by Yen and Lee (10), where the respective compounds are shown to enhance their own paracellular permeability in Caco-2 monolayers. Possibly the same phenomenon is occurring in the present case. Recovery of the TEER after removal of the respective compound is consistent with reversible effects on tight junction

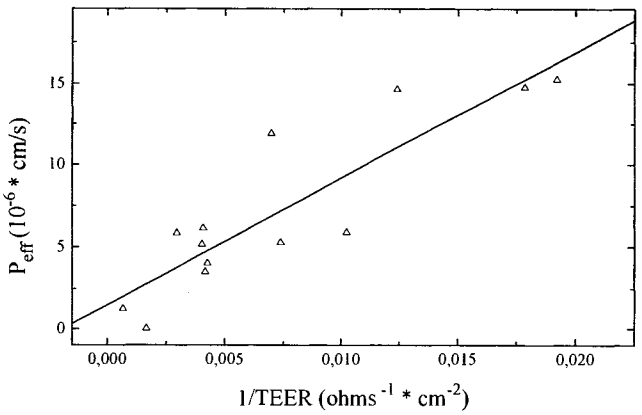

Fig. 2. Correlation of permeability coefficients for the hydrophilic marker fluorescein and TEER values in Caco-2 cell monolayers. Linear regression was performed for P_{eff} and 1/TEER with r = 0.881. Each point represents the mean of three determinations.

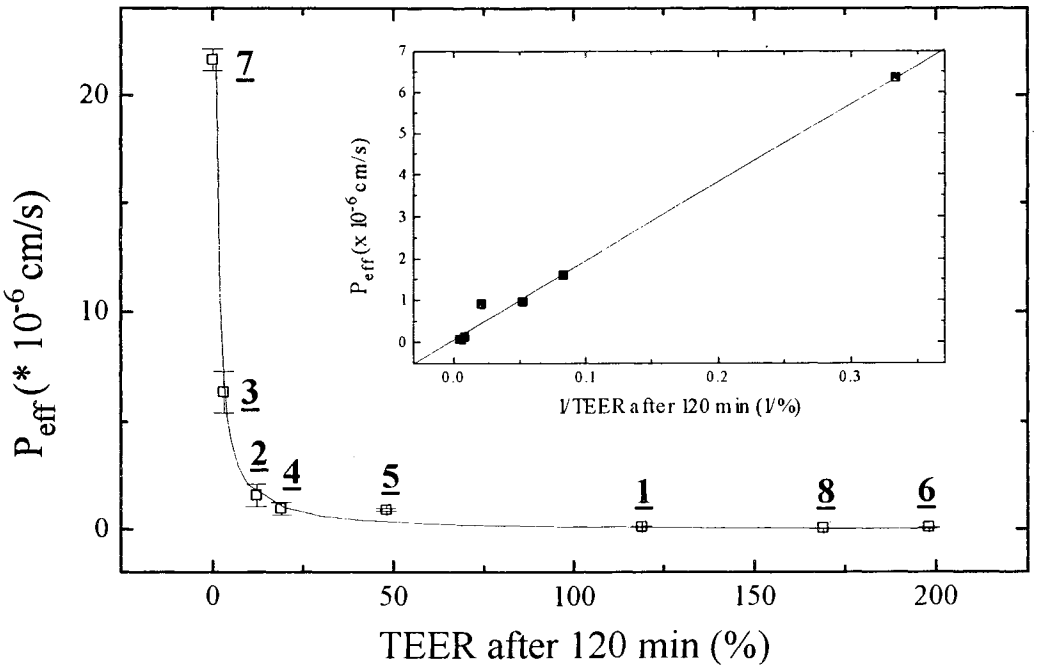

Fig. 3. Correlation of permeability coefficients of RI and relative TEER measurements in Caco-2 cell monolayers after 2 h incubation with transport solution containing 1 mg/ml of the respective compound. Nonlinear curve fit was performed for P_{eff} and %TEER with $y = k \cdot x^{-1} + m$ (inset: linear regression for P_{eff} and 1/%TEER, RI 7 excluded) yielding in both cases $r = 0.996$. Each point represents the mean of three determinations ± S.D.

widening as has been observed for substances like EDTA and sodium caprate (11,12), indicating a reversible interaction of RI with regulatory factors responsible for tight junction permeability. The regulation of tight junctions and conditions which disrupt junctional complexes are still incompletely understood. Nevertheless interactions of peptide hormones with tight junctions are known, e.g. IGF I or II and TNF α (13,14), although exposure from the basolateral side seems to be a prerequisite. Other effects interfering with the perijunctional actin-myosin ring, such as oxidants (15), protein kinase C activators or Ca^{2+} depletion could also be responsible for decrease of the TEER and increased permeability of the RI.

From our results, we conclude, that some of the RI facilate their own penetration by enhancing the tight junction permeability through an as yet unknown mechanism. These peptides warrant further investigation, because they may provide the crucial information to guide their preferential transport via the paracellular pathway *in vitro* in monolayers of the intestinal cell line Caco-2 and maybe *in vivo* in the intestinal epithelium. The above findings have raised the question about the structural features of the peptides that are important to their enhanced paracellular penetration. Furthermore, the mechanism underlying this effect is of interest and may possibly lead to structure relationships for compounds with higher oral absorption rates.

REFERENCES

1. 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).
2. W. J. Greenlee. Renin inhibitors. *Med. Res. Rev.* 10:173–236 (1990).
3. P. Raddatz, A. Jonczyk, K.-O. Minck, C. J. Schmitges and J. Sombroek. Substrate analogue renin inhibitors containing replacements of histidine in P_2 or isosteres of the amid bond between P_3 and P_2 sites. *J. Med. Chem.* 34:3267–3280 (1991).
4. 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).
5. W. Butte, C. Fooken, R. Klußmann and D. Schuller. Die Messung der Lipophilität mit Hilfe der reversed-phase HPLC und HPTLC. *Kontakte (Merck)* 3:25–31 (1982).
6. 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.* 11:1575–1580 (1994).
7. E. Walter and T. Kissel. Metabolic differences in cubclones of a human intestinal cell line (Caco-2): presystemic cleavage of leu- and met-enkephalin. *Pharm. Pharmacol. Lett.* 3:221–224 (1994).
8. P. F. Augustijns, T. P. Bradshaw, L. S. Gan, R. W. Hendren and D. R. Thakker. Evidence for a polarized efflux system in Caco-2 cells capable of modulating cyclosporin A transport. *Biochem. Biophys. Res. Commun.* 197:360–365 (1993).
9. P. S. Burton, R. A. Conradi, A. R. Hilgers and N. F. H. 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).
10. W. C. Yen and V. H. L. Lee. Paracellular transport of a proteolytically labile pentapeptide across the colonic and other intestinal segments of the albino rabbit: implications for peptide drug design. *J. Control. Rel.* 28:97–109 (1994).
11. A. B. J. Noach, M. C. M. Roosemalen, Y. Kurosaki, A. G. de Boer and D. D. Breimer. Effect of apical and/or basolateral ap-

plication of EDTA on the permeability of hydrophilic compounds in a human intestinal epithelial cell-line (Caco-2). *J. Control. Rel.* **21**:206–207 (1992).
12. E. K. Anderberg, T. Lindmark and P. Artursson. Sodium caprate elicits dilatations in human intestinal tight junctions and enhances drug absorption by the paracellular route. *Pharm. Res.* **10**:857–864 (1993).
13. J. A. Roberts and N. E. Riley. Regulation of T84 cell monolayer permeability by insulin-like growth factors. *Am. J. Physiol.* **262**:C207–C213 (1992).
14. J. M. Mullin and K. V. Schock. Effect of tumor necrosis factor on epithelial tight junctions and transepithelial permeability. *J. Cancer Res.* **50**:2172–2176 (1990).
15. D. M. Shasby, M. Winter and S. Shasby. Oxidant and conductance of cultured epithelial cell monolayers: inositol phospholipid hydrolysis. *Am. J. Physiol.* **255**:C781–C788 (1988).