Brief Articles

β- and γ-Di- and Tripeptides as Potential Substrates for the Oligopeptide Transporter hPepT1

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The hPepT1-mediated transport properties of a series of 11 synthesized β- and γ-peptides have been studied in Caco-2 cells. The results show that several of the compounds interact with the peptide transporter, but only two β-dipeptides act as substrates and are transported across the cell monolayers. These two are less-efficient substrates than α-peptides. Larger derivatives than β-dipeptides do not act as hPepT1 substrates, but instead, they appear to be substrates for P-glycoprotein efflux.

Introduction

Peptides composed of homologated amino acids, that is, β- and γ-peptides, are of increasing significance as novel peptidomimetic agents.[1] Although, intrinsically more flexible than α-peptides, β- and γ-peptides have been shown to form stable secondary structure elements, such as helices and turns,[2] more readily than α-peptides. In fact, only two β- or γ-amino acid residues are needed for the preparation of turn fragments that may act as somatostatin mimicking peptidomimetics with good binding affinities to the sst-receptors.[2c,3] Somewhat longer β-peptides, typically containing less than 10 residues, have also been shown to possess antibacterial,[4] antiproliferative, and hemolytic activities;[4a,b] examples of β-peptides capable of disrupting protein−protein interactions[5] are also known. Peptides composed of β- and γ-amino acids are completely resistant toward proteolytic and metabolic degradation,[6] which has fortified the interest of such molecules as peptidomimetic agents for medicinal and biological purposes.

Intrigued by the structural properties of β- and γ-peptides, we became interested in their potential interaction with the oligopeptide transport protein hPepT1 found mainly in the enterocytes in the human intestine. It has been shown that hPepT1 is involved in the transport of most di-and tripeptides as well as peptide-based drugs such as β-lactam antibiotics, ACE and renin inhibitors, and various prodrug derivatives.[7] The structure−transport relationships for hPepT1 have been studied,[8] as well as the use of dipeptidomimetics as drug transport vehicles.[9] β-Amino acids and β-dipeptides have also been investigated for their hPepT1 mediated transport, and it was shown that the introduction of β-amino acids in the *N*- or *C*-terminal positions of a dipeptide usually decreased the affinity, but was compatible with binding and transport.[8e,10]

In the present study, we report the hPepT1-mediated transport properties of a series of β- and γ-peptides. The results show that several of the compounds interact with the peptide transporter and that two compounds act as substrates and are actually transported across Caco-2 cell monolayers. However, larger derivatives than β-dipeptides, for example, γ-tripeptides, appear to be substrates for P-glycoprotein efflux.

Results and Discussion

Selection of β- and γ-Di- and Tripeptides. The structures of the β- and γ-di- and tripeptides **1**−**11** used in this study are shown in Figure 1. Based on the amino acids at hand, we aimed at making the selection of compounds as diverse as possible.

Among the tripeptides investigated were both mono- and disubstitited aliphatic γ-peptides, that is, **1** and **2**, β-peptides composed of all-β^3-amino acids (**3**−**5**), and of all-β^2-amino acids (**6**). Two β-dipeptides with a lysine side chain either in the 2-position (**7**) or in the 1-position (**8**) were tested, as well as the regioisomerically mixed β^2/β^3-peptides **9** and **10**. Also included was a β^3-dipeptide with a thioamide bond, that is, **11**. All β- and γ-peptides were synthesized in solution by standard EDC/HOBt[a]-mediated coupling of the protected homoamino acids (*N*-Boc- or *N*-Cbz-; *O*-Bn), as described in the Supporting Information.

hPepT1-Affinity Studies. A first assessment of the ability of the compounds to bind to the human peptide transporter hPepT1 was performed by testing whether the compounds were able to inhibit the uptake of the PepT1-substrate glycylsarcosine (GlySar). Due to the limited amount available of the test compounds, only one inhibitor concentration was used (4 mM). All the compounds except **1** and **3** showed significant inhibition of the [^{3}H]GlySar uptake but at a lower level than the positive control GlySar (Figure 2).

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[a] Abbreviations: P_{app}, apparent permeability coefficient; P-gp, permeability-affecting glycoprotein; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; Bn, benzyl; Boc, *tert*-butoxycarbonyl; Cbz, benzyloxycarbonyl; 2-Cl-Cbz, 2-chlorobenzyloxycarbonyl; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride; ESI, electrospray ionization; HOBt, 1-hydroxy-1*H*-benzotriazole hydrate; TFA, trifluoroacetic acid. Three-letter amino-acid abbreviations are used for homologated amino acid derivatives: βhXaa (β^n, homoamino acid)[1] and γhhXaa ($\gamma^{m,n}$, doubly homologated amino acid), where *n* indicates the position of the proteinogenic side chain.

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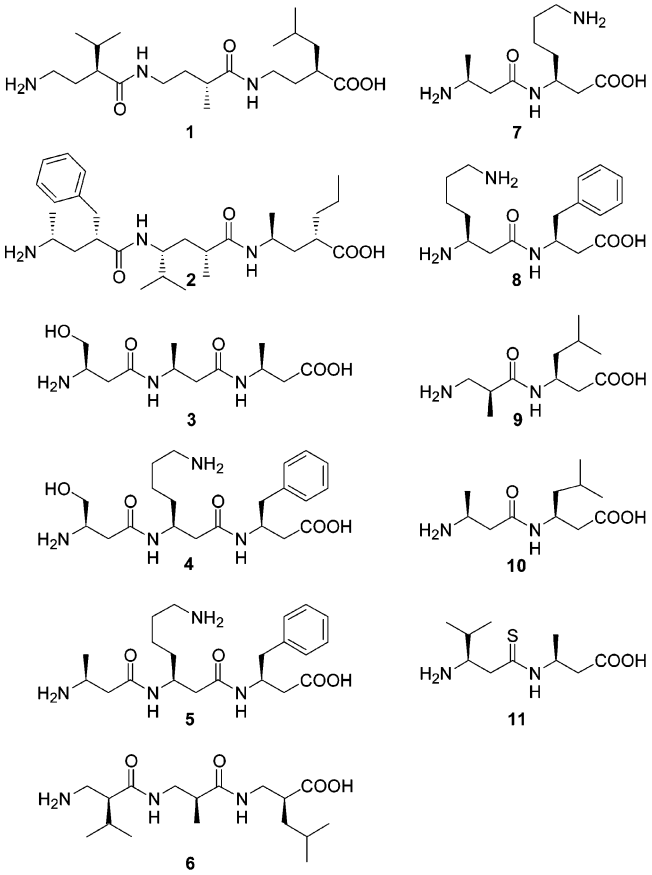

Figure 1. Structure of the synthesized and tested β- and γ-di- and tripeptides.

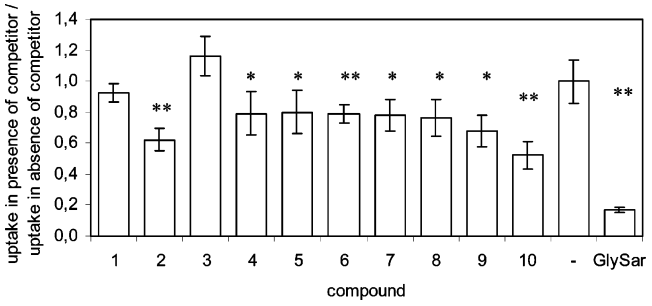

Figure 2. Relative [^{3}H]GlySar uptake into Caco-2 cell monolayers in the presence or absence of the 4 mM competitor (**1**−**10**, GlySar); error bars represent SD ($n = 3$); *significant difference against the control (−) at $p < 0.05$; **significant at $p < 0.005$.

Surprisingly, compounds **2** and **10** were found to be the most potent inhibitors, though the size of **2** would be expected to prevent an efficient interaction with the hPepT1 binding site. Compounds **4**−**9** were of equal activity. A comparison between the activities of **9** and **10** is of special interest as these compounds are positional isomers; in **9** the methyl group is positioned α to the *N*-terminal amino group but in **10** it is in the β-position. As shown in Figure 2, there is a significant difference in the inhibitory properties between the isomers, with compound **10** being most active. Thus, the results show that only small changes in substrate structures can have a large impact on binding to PepT1.

Transepithelial Transport Studies. As most of the compounds were found to interact with PepT1, transepithelial transport studies were carried out to determine whether they also acted as substrates. These studies were done both in the apical-to-basolateral (absorptive) and in the basolateral-to-apical direction to determine if there is a vectorial transport. The transport in the absorptive direction was also studied in the presence of the competitive inhibitor GlySar (33-fold excess). The results are shown in Table 1. The apparent permeability coefficient (P_{app}) values measured at the different conditions show that the permeability for most of the β- and γ-peptides is considerably lower than for α-peptides (for example, Val−Val[11], $P_{app} = 1.9 \times 10^{-7}$ cm s^{-1} or the metabolically stable GlySar, $P_{app} = 1.1 \pm 0.1 \times 10^{-5}$ cm s^{-1}, own data). The P_{app} values indicate active, PepT1-mediated transport only for the smaller β-dipeptides **10** and **11**. These compounds show significantly higher transport in the apical-to-basolateral direction compared to the reversed direction and the apical-to-basolateral transport is significantly reduced when GlySar is added at the pH value when PepT1 is activated (pH 6). Again, a comparison between the isomeric **9** and **10** shows that **10** interacts more favorably with PepT1, indicating that a substituent in the β^2-position seems to induce a more favorable conformation for proper PepT1 interaction than in the β^3-position.

Table 1. Apparent Permeability Coefficients (P_{app}) for the Transepithelial Transport of **1**−**11** Across Caco-2 Monolayers (24 ± 1 days after Seeding)[a]

	$P_{app} \times 10^{-8}$ cm s^{-1} ± S.D.		
cmpd	apical-to-basolateral	apical-to-basolateral in the presence of 5 mM GlySar	basolateral-to-apical
1	3.4 ± 0.4	2.6 ± 0.1	9.4 ± 1.1 ($n = 2$)
2	3.3 ± 0.2	2.8 ± 0.3	9.7 ± 1.0
3	4.4 ± 0.3	4.3 ± 0.6	n.d.
4	3.7 ± 0.7	3.1 ± 0.2	7.1 ± 1.4
5	4.9 ± 0.1	3.9 ± 0.7	6.1 ± 0.9
6	2.4 ± 0.1	2.3 ± 0.3	7.1 ± 1.0
7	3.7 ± 0.5[b]	n.d.[c]	7.8 ± 0.5
8	7.2 ± 0.1	4.5 ± 0.2	7.5 ± 1.1
9	9.5 ± 0.6	5.5± 0.6	8.4 ± 0.9
10	61.3 ± 3.3	29.1 ± 1.8	12.9 ± 1.5[d]
11	75.4 ± 6.6	39.3 ± 3.0	12.4 ± 0.4

[a] Measurements done in buffered HBSS, apical pH 6.0, basolateral pH 7.4 (n = 3−4). See Supporting Information for experimental conditions. [b] Because some time-dependent samples (45, 90, and 120 min) were below the detection limit, the apical-to-basolateral value is based on measurements after one single time point (90 min). [c] n.d. = not determined. [d] Evaluation 45−90 min.

However, the size of the whole compound is also of importance, it cannot be too large, as, for example, **2**, **5**, or **7**.

It has previously been shown that an *N*-terminal valine residue enhances the affinity and transport by PepT1 of both peptidomimetics[9c] and of drugs such as valacyclovir[12] and valganciclovir.[13] However, for the γ- and β^2-derivatives **1** and **6**, it is likely that the size prevented an efficient binding to PepT1, whereas the smaller β^3-dithiopeptide **11** fits considerably better and can even be translocated across the Caco-2 cell monolayer by PepT1. Thioamides have previously been shown to be good substrates for PepT1, studies on AlaΨ[CS−N]Pro showed that the *trans*-amide conformer of the thioamide is preferentially transported by PepT1.[14]

To investigate if the difference in PepT1 transport properties of the tested dipeptide derivatives **9**−**11** depended on their conformational properties, the compounds were subjected to conformational analysis using molecular mechanics calculations (Amber force field as implemented in the MacroModel program, version 7.1). The results showed that **10** and **11** had similar conformational preferences, whereas the low-energy conforma-

tion of **9** was slightly different, especially in the N-terminal region (data not shown). These differences might prevent the proper interaction of **9** with hPepT1. It was, however, observed that the low-energy conformations of all three compounds were quite similar, and the analysis could not clearly illustrate which small modifications of a structure are needed for a compound to act as substrate for a transporter.

Compounds **2** and **7** showed a considerably higher transport rate in the basolateral-to-apical direction than in the apical-to-basolateral direction in the pH gradient system (which is needed for PepT1-activity) and were therefore studied further at pH 7.4 to try to find the cause of this apparent efflux. For **7**, the P_{app} values measured at pH 7.4 did not show any significant difference between apical-to-basolateral and basolateral-to-apical transport measured over a time interval of 180 min and neither did the P-gp inhibitor GF120918[15] influence the transport [the values were $P_{app} = 4.4 \pm 1.7 \times 10^{-8}$ cm s^{-1} (apical-to-basolateral), $5.1 \pm 0.4 \times 10^{-8}$ cm s^{-1} (basolateral-to-apical), and, in the presence of 2 μM GF120918, $P_{app} = 2.9 \pm 1.3 \times 10^{-8}$ cm s^{-1} (apical-to-basolateral) and $4.9 \pm 0.7 \times 10^{-8}$ cm s^{-1} (basolateral-to-apical)]. From these results, we conclude that **7** is not a substrate for an efflux transporter. Furthermore, P_{app} values measured in the absorptive direction, either with or without a pH gradient, are comparable, confirming that PepT1 should not be involved in the transepithelial transport of **7**.

On the other hand, the P_{app} values measured in the secretive direction for the γ-tripeptide **2** differ in absence ($11.5 \pm 1 \times 10^{-8}$ cm s^{-1}) and presence ($5.3 \pm 0.9 \times 10^{-8}$ cm s^{-1}) of the P-gp inhibitor, indicating that **2** is a potential P-gp substrate; the absorptive transport of **2** was not significantly changed at pH 7.4 ($P_{app} = 2.1 \pm 1 \times 10^{-8}$ cm s^{-1}) compared to the pH gradient, but seems slightly higher in presence of GF120918 ($P_{app} = 5.3 \pm 2.4 \times 10^{-8}$ cm s^{-1}), an observation that would strengthen the hypothesis of **2** being a substrate for P-gp. In line with these observations, Gao et al. have reported that three Ala-Phe hydroxyethylamine bioisostere-containing peptidomimetics are substrates for P-gp.[16]

Conclusions

The β- and γ-peptides studied here are less-efficient substrates for hPepT1 than their α-homologues. Larger compounds than β-dipeptides do not act as substrates for hPepT1, instead they appear to be substrates for P-glycoprotein efflux. However, many of the tested derivatives act as inhibitors of Gly-Sar transport, which show that they do interact with the binding site of hPepT1. Interestingly, also for this type of derivative the results show that small modifications in structure result in loss of transport properties.

Experimental Section

Peptide Synthesis and Purification. HPLC Analysis and Purification. All HPLC related to peptide purification and analysis after preparation was done on a Merck LaChrom HPLC system (L-7150 pump, L-7400 UV detector (variable wavelength monitor)). RP-HPLC analysis of the crude product was performed on a Merck Lichrospher 100 column (RP-18, 100 × 4.6 mm) using a linear gradient (5−100% A in 30 min) of A (acetonitrile with 0.1% TFA) and B (H_2O with 0.1% of TFA) at a flow rate of 1.2 mL/min, with UV detection at 220 nm. Crude products were separated and purified by preparative RP-HPLC on a Macherey-Nagel C_{18} column/Nucleosil 100-7 C_{18} (250 × 21 mm) using a gradient of A and B (Method A: 5 → 30% A over 25 min, 30% isocratic for 3 min, 30 → 50% A over 2 min; Method B: 10 → 40% A over 30 min, 40% isocratic for 2 min, 40 → 50% A over 2 min) at a flow rate of 20 mL/min, with UV detection at 220 nm, and then lyophilized. The purified peptides were then analyzed by analytical RP-HPLC on a Merck Lichrospher 100 column (RP-18, 100 × 4.6 mm) with the gradient given for each peptide and by analytical HPLC on a Thermo HyPurity Aquastar column (250 × 2.1 mm) using another gradient system (see Supporting Information).

Mass Spectrometry. Mass spectrometrical analyses of the synthesized peptides were performed on a Finnigan MAT TSQ 700 (ESI) and reported as *m*/*z* (% of basis peak).

Synthetic Procedures. Peptide Synthesis. In short, all peptides were synthesized in solution by standard EDC/HOBt-mediated coupling of the protected homoamino acids (*N*-Boc- or *N*-Cbz-; *O*-Bn). Detailed descriptions for the preparation of protected β- and γ-peptides can be found in references 2a, 17, and 18 and in the Supporting Information.

Catalytic Hydrogenation of Peptides (GP 1). The Cbz-, 2-Cl-Cbz-, or -OBn-protected peptide was dissolved in MeOH (0.1 M), and Pd/C (10%; 0.1 equiv) was added after careful degassing and saturation of the solvent with argon. The reaction flask was evacuated and flushed with H_2 several times, and the reaction mixture was stirred under an atmosphere of H_2 (1 bar) for 16 h. Subsequent filtration through Celite and concentration under reduced pressure afforded the desired product.

Boc-Deprotection of Amino Acids or Peptides (GP 2). The Boc-protected peptide was dissolved in CH_2Cl_2 (1 M) at rt under argon. After cooling to 0 °C, the same amount of TFA was added, and the resulting homogeneous reaction mixture was stirred for 2 h at rt. The solvent was removed under reduced pressure, and the resulting oily residue was stripped three times with diethyl ether. Finally, the residue was dried at high vacuum (0.01−0.1 Torr) overnight.

H_2N-(*S*)-β^3hLys-(*S*)-β^3hPhe-OH (8). Boc-β^3hLys(2-Cl-Cbz)-β^3-hPhe-OBn (100 mg; 0.14 mmol)[6] was hydrogenated as described in GP 1. The Bn-deprotected peptide was directly treated with TFA (GP 2) to yield crude **8**. Purification by preparative HPLC (Method A), followed by lyophilization, gave β-dipeptide H_2N-β^3hLys-β^3-hPhe-OH as a di-TFA salt (15 mg; 19% yield). Analytical HPLC (5% A for 15 min; 5 → 50% A over 15 min; C_{18}): t_R 23.7 min, purity >98%. ESI-MS (positive mode): 322.2 (100, $[M + H]^+$).

Caco-2 Cell Studies. In short, the studies were performed on Caco-2 cell monolayers (ATCC, passage 94−100) grown on cell culture inserts maintained at 10% CO_2 in an incubator with a humidified atmosphere at 37 °C. Cells were seeded on polycarbonate cell culture inserts (4.4×10^5 cells per cm^2, Transwell system, diameter 12 mm, pore size 0.4 μm, Corning Costar, The Netherlands) and were allowed to differentiate for 24 ± 1 days in DMEM supplemented with 10% fetal calf serum, 1% nonessential amino acids, and PEST.

The transepithelial resistance values of the monolayers were 285 ± 45 Ω cm^2 ($n = 49$ filters) before the experiment and had decreased in average 30 Ω cm^2 after the transport studies.

Briefly, experiments were performed in Hank's balanced salt solution (HBSS) and a pH-gradient was applied, which is required for PepT1 activity (apical side pH 6.0 by addition of 10 mM MES, basolateral side pH 7.4 by addition of 25 mM HEPES); exceptions were the experiments performed to evaluate P-gp interaction, which were done at pH 7.4. For solubility reasons, a final concentration of 0.5% DMSO was included during the experiments. During transport experiments, samples were withdrawn at regular time intervals and replaced by the same volume of pretempered HBSS + 0.5% DMSO. The P_{app} were calculated according to $P_{app} = dQ/dt \times 1/(A \times c_0)$, where dQ/dt is the steady-state flux (μmol/s), A is the surface area of the filter (cm^2), and c_0 is the initial concentration in the donor chamber at each time interval (μM).

For affinity studies, cells monolayers grown on the filter supports were incubated for 5 min, including 1 μCi [^{3}H]GlySar/mL (250 nM) with or without addition of 4 mM of the respective test compound in the apical donor chamber. Uptake was stopped by removing the uptake solution, followed by three washes of the filters in ice-cold HBSS, and the radioactivity of the monolayers was determined.

Peptide Stability During the Experiments. The stability of the compounds is judged by the recovery after the Caco-2 transport experiments; the recovery was determined as 101 ± 4% (average of all experiments). No compound showed significant deviation.

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Supporting Information Available: Detailed experimental procedures for the Caco-2 cell studies and the synthesis of compounds **1**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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