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ORIGINAL ARTICLE

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Structural requirements for determining the substrate affinity of peptide transporters PEPT1 and PEPT2

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Abstract Peptide transporters PEPT1 and PEPT2 transport numerous compounds including small peptides, peptide-like drugs and nonpeptidic compounds such as valacyclovir. PEPT1 and PEPT2 show low and high affinity for most substrates, respectively, but β-lactam antibiotics without an α-amino group are the only known substrates that prefer PEPT1 to PEPT2. The aim of this study was to compare the recognition and affinity of various substrates between rat PEPT1 and rat PEPT2, and to determine the structural requirements influencing the substrate affinity. [^{14}C]Glycylsarcosine uptake by PEPT1- or PEPT2-expressing transfectant was inhibited by di- and tripeptides, but not by amino acids, tetrapeptides or most cyclic dipeptides. All dipeptides and tripeptides examined showed more potent inhibition of [^{14}C]glycylsarcosine uptake via PEPT2 than via PEPT1, irrespective of their charge and structure. Modification of the α-amino group of dipeptides reduced their substrate affinity to both transporters, as compared to unmodified dipeptides, but these dipeptides still showed potent inhibitory effects on PEPT2. Among the nonpeptidic substrates tested, only the eight-amino-octanoic acid displayed stronger inhibition of [^{14}C]glycylsarcosine uptake in PEPT1 than in PEPT2. These findings suggest that α- or β-amino carbonyl function is the key structure responsible for the higher affinity for PEPT2 than for PEPT1.

Key words α- or β-Carbonyl function · Cyclic dipeptides · Di- and tripeptides · Peptide transporters · Substrate affinity

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Introduction

Di- and tripeptides, as well as various peptide-like drugs such as β-lactam antibiotics, are taken up into intestinal and renal epithelial cells by H^+-coupled peptide cotransporters. Molecular cloning studies have identified two peptide transporters, PEPT1 and PEPT2, and many studies have been carried out to clarify the functional and molecular characteristics of both transporters [6, 13, 14]. For example, rat PEPT1 was found to be strongly expressed in the small intestine with weak expression in the kidney [18], whereas rat PEPT2 is predominantly expressed in the kidney [19]. Both transporters are localized in the brush-border membranes of these epithelial cells [16, 21, 22].

In addition to their tissue distribution, PEPT1 and PEPT2 show remarkable differences in substrate affinity. Rabbit [1, 2] and human [17] PEPT2 showed higher affinity for chemically diverse dipeptides as compared to rabbit and human PEPT1, respectively. Human [9] and rat [25] PEPT2 also exhibited higher affinity for amino β-lactam antibiotics as compared to human and rat PEPT1, respectively. These differences in substrate affinity are not necessarily limited to substrates with peptide bond(s). Nonpeptidic compounds such as valacyclovir, an oral L-valyl ester prodrug of the antiherpetic agent acyclovir, are preferentially recognized by rat PEPT2 rather than human [10] and rat [20] PEPT1. In addition, δ-aminolevulinic acid, which has a ketomethylene group instead of a peptide bond, was reported to have higher affinity for rabbit PEPT2 than for rabbit PEPT1 [7]. These findings suggest that PEPT1 and PEPT2 are low- and high-affinity peptide transporters, respectively. In contrast, however, anionic β-lactam antibiotics without an α-amino group, such as ceftibuten, appeared to have a higher affinity for rat PEPT1 than for rat PEPT2 [25]. This raised the question of whether the α-amino group of substrates is an important factor determining the substrate affinity of both transporters. To date, there have been few reports regarding the systematic comparison of substrate affinity between PEPT1 and

PEPT2 in the same species, and chemical structures affecting the substrate affinity remain unknown. In the present study, we compared the affinity of various substrates between rat PEPT1 and rat PEPT2, and explored the structural requirement for the higher affinity interaction with PEPT2.

Materials and methods

Materials

[^{14}C]Glycylsarcosine (1.89 GBq/mmol) was obtained from Daiichi Pure Chemicals (Ibaraki, Japan). Valacyclovir was supplied by Glaxo Wellcome Research and Development (Hertfordshire, UK). Glycine and δ-aminolevulinic acid were obtained from Nacalai Tesque (Kyoto, Japan). Dipeptides, tripeptides, tetraglycine, 8-amino-octanoic acid and alanine-4-nitroanilide were purchased from Sigma (St. Louis, MO). All cyclic dipeptides were obtained from Bachem Feinchemikalien (Switzerland). All other chemicals used were of the highest purity available.

Cell culture

The parental LLC-PK$_1$ cells obtained from the American Type Culture Collection (ATCC CRL-1392) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (GIBCO, Life Technologies), supplemented with 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, Md., USA) without antibiotics in an atmosphere of 5% CO_2 and 95% air at 37°C. The LLC-PK$_1$ cells transfected with rat PEPT1 cDNA (LLC-rPEPT1) and with rat PEPT2 cDNA (LLC-rPEPT2) were used as described previously [24]. In the uptake experiments, the cells were cultured for 6 days in complete medium.

Uptake studies using cell monolayers

Uptake of [^{14}C]glycylsarcosine was measured in cells grown in 35- or 60-mm plastic dishes as described previously [25]. For determining the concentrations of the various substrates necessary to inhibit 50% of [^{14}C]glycylsarcosine uptake (IC_{50}), each transfectant was incubated with [^{14}C]glycylsarcosine (20 μM, pH 6.0) in the absence or presence of increasing concentrations of various substrates. Because [^{14}C]glycylsarcosine uptake by both transfectants was linear within 15 min [25], we made most measurements within this time, and determined the initial rate of uptake. [^{14}C]Glycylsarcosine uptake in the absence of the inhibitors was measured in four monolayers, and was taken as 100%. This value was highly reproducible (means ±SE of data from 20 monolayers from 5 separate cell cultures: 736±12 pmol mg^{-1} protein 15 min^{-1} for PEPT1 and 247±3 pmol mg^{-1} protein 15 min^{-1} for PEPT2), and was used to confirm the intra-experimental errors. [^{14}C]Glycylsarcosine uptake in the presence of the inhibitors was measured in two monolayers. The inhibitor concentrations of most substrates used were as follows: 10, 100, 300, 1000, 3000, 10000, and 20000 μM for PEPT1; 1, 10, 100, 300, 1000, 3000, and 10000 μM for PEPT2. The protein content of the cell monolayers solubilized in 1 N NaOH was determined by the Bradford method [4], using a Bio-Rad protein assay kit with bovine γ-globulin as the standard.

Data analysis

Data were analyzed statistically by one-way analysis of variance followed by Scheffé's test. IC_{50} values were determined by nonlinear regression analysis. The inhibition constant (K_i) values were calculated from IC_{50} values according to the method of Cheng and Prusoff [5].

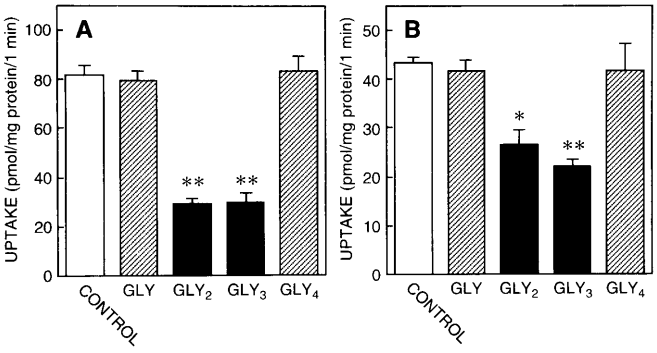


Fig. 1 Effects of $(glycine)_n$ (n=1–4) on [^{14}C]glycylsarcosine uptake in LLC-rPEPT1 (**A**) and LLC-rPEPT2 cells (**B**). Each transfectant was incubated for 1 min at 37°C with incubation medium containing [^{14}C]glycylsarcosine (20 μM, pH 6.0) in the absence or presence of each inhibitor (10 mM). *Columns* represent the means ±SE of three monolayers. $^{*}P$<0.05, $^{**}P$<0.01, significantly different from control

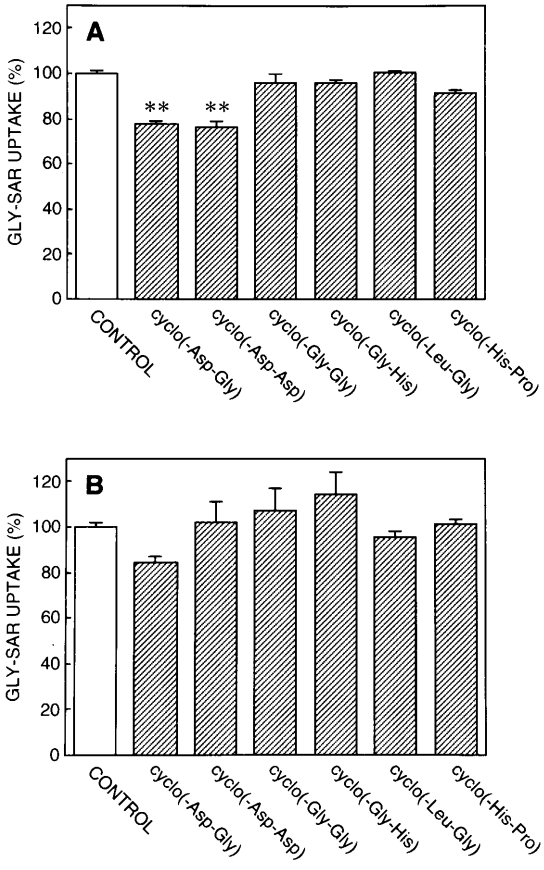


Fig. 2 Effects of cyclic dipeptides on [^{14}C]glycylsarcosine uptake in LLC-rPEPT1 (**A**) and LLC-rPEPT2 cells (**B**). Each transfectant was incubated for 15 min at 37°C with incubation medium containing [^{14}C]glycylsarcosine (20 μM, pH 6.0) in the absence or presence of each inhibitor (10 mM). Control uptake was taken as 100% (**A**, 793±32 pmol mg^{-1} protein 15 min^{-1}; **B**, 259±25 pmol mg^{-1} protein 15 min^{-1}). *Columns* represent the means ±SE of three monolayers. $^{**}P$<0.01, significantly different from control

Results

The substrate specificity of small peptides

Before comparing the substrate affinities of PEPT1 and PEPT2, we examined the effects of small peptides on [^{14}C]glycylsarcosine uptake by LLC-rPEPT1 and LLC-rPEPT2 cells. As shown in Fig. 1, [^{14}C]glycylsarcosine uptake was markedly inhibited by di- and triglycine, but not by glycine or tetraglycine.

Figure 2 shows the effects of cyclic dipeptides on [^{14}C]glycylsarcosine uptake by both transfectants. Cyclic dipeptides with neither free amino nor carboxyl groups have been reported to be recognized and transported by the peptide transporter [12, 15]. In contrast to these reports, most cyclic dipeptides had no inhibitory effect on either transfectant, although cyclo(–Asp–Asp) and cyclo(–Asp–Gly) had weak inhibitory effects on LLC-rPEPT1 cells. Recently, the free amino and/or carboxyl groups, rather than peptide bond(s), of substrates have been suggested to play an important role in the interaction with peptide transporters [7, 8, 10, 20]. Therefore, it might be reasonably hypothesized that cyclic dipeptides are not recognized by PEPT1 and PEPT2, although further studies are needed because we only examined the inhibitory effects of cyclic dipeptides.

The substrate affinity of dipeptides and tripeptides

The linear di- and tripeptides were confirmed to have an inhibitory effect on [^{14}C]glycylsarcosine uptake by both transfectants (Fig. 1). Then, we performed concentration-dependent inhibition studies using various di- and tripeptides. Figure 3A and B shows the inhibition curves of Gly–Phe and Gly–Leu–Tyr in the transfectants, respectively. In both cases, [^{14}C]glycylsarcosine uptake was more potently inhibited in LLC-rPEPT2 than in LLC-rPEPT1 cells. The estimated K_i values of various dipeptides and tripeptides for PEPT1 and PEPT2 are

Fig. 3 Inhibition of [^{14}C]glycylsarcosine uptake by Gly–Phe (**A**), Gly–Leu–Tyr (**B**), valacyclovir (**C**) and 8-amino-octanoic acid (**D**) in LLC-rPEPT1 (○) and LLC-rPEPT2 cells (●). Each transfectant was incubated for 15 min at 37°C with incubation medium containing [^{14}C]glycylsarcosine (20 μM, pH 6.0) in the absence (□) or presence (○,●) of increasing concentrations of various substrates (PEPT1: **A**, 1–10,000; **B**, **C**, **D** 10–20,000; PEPT2: **A**, **B**, **C**, 1–10,000; **D**, 10–20,000 μM). [^{14}C]Glycylsarcosine uptake in the absence of inhibitor was taken as 100% (PEPT1: **A**, 764±17; **B**, 723±7; **C**, 748±10; **D**, 723±27; PEPT2: **A**, 241±9; **B**, 247±9; **C**, 257±5; **D**, 244±4 pmol mg^{-1} protein 15 min^{-1}). In **A**, **B**, and **C**, *points* in the absence of the inhibitors are the means of data from four monolayers, and *points* in the presence of the inhibitors are means of data from two monolayers. **D** *Points* in the absence of the inhibitors are the means of data from eight monolayers, and *points* in the presence of the inhibitors are means ±SE of data from four monolayers from two separate cell cultures. When the *error bars* are not shown, they are smaller than the *symbol*

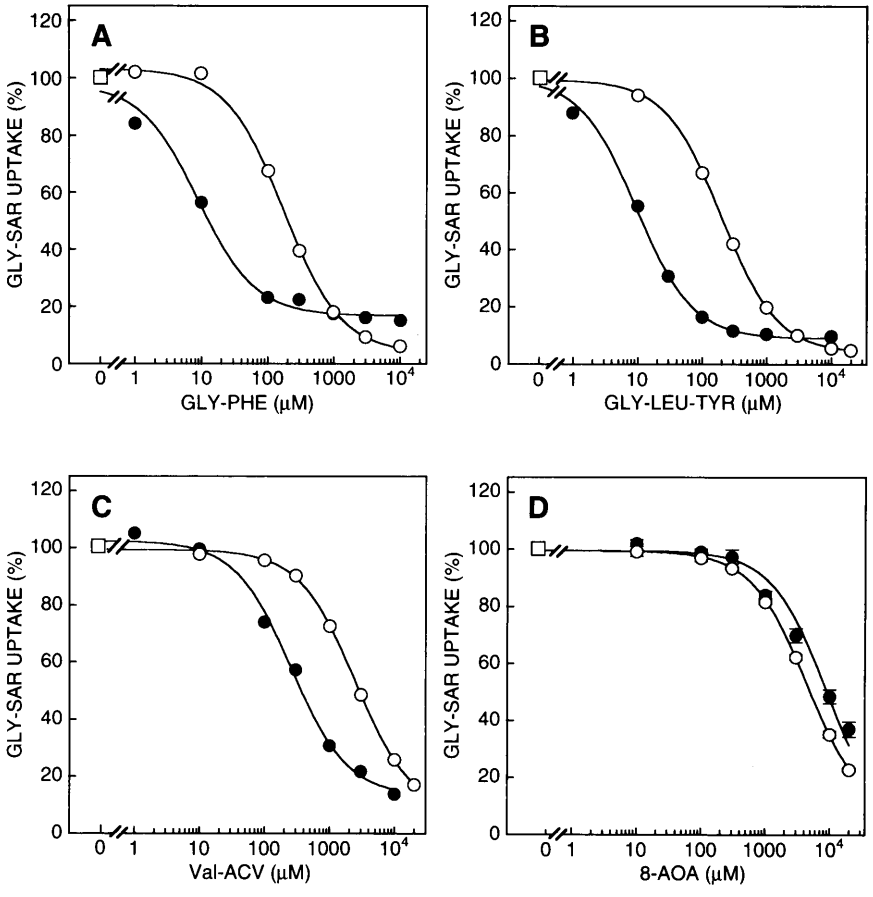


Table 1 Inhibition constant values for the various dipeptides and tripeptides for PEPT1 and PEPT2. Each value was calculated from the inhibition curves as described in Materials and methods

Substrates		K_i (μM)	
		PEPT1	PEPT2
Dipeptides	Zwitterion		
	Gly–Leu	110	6
	Gly–Phe	170	8
	Gly–Trp	1400	7
	Gly–Tyr	210	14
	Gly–Asn	270	22
	Phe–Gly	220	7
	Bestatin	1500	18
	Anion		
	Gly–Asp	300	60
	Asp–Gly	830	46
	β–Asp–Gly	>50,000	28,000
	Asp–Asp	990	150
	Cation		
	Gly–His	1400	39
	Gly–Lys	2200	210
	Lys–Gly	910	21
	Lys–Lys	8000	96
	Carnosine	8000	66
Tripeptides	Gly–Gly–Gly	1300	160
	Gly–Gly–Phe	610	29
	Gly–Leu–Tyr	190	8

summarized in Table 1. PEPT2, as compared to PEPT1, has a higher affinity for all dipeptides and tripeptides examined, even though they had different charges, sizes and chemical structures. Carnosine and the anticancer agent bestatin, which have a β-amino group instead of an α-amino group, also showed higher affinity for PEPT2. β–Asp–Gly had little inhibitory effect on [^{14}C]glycylsarcosine uptake by either transfectant.

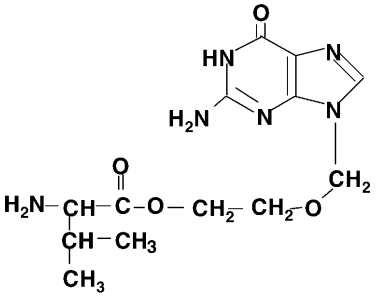
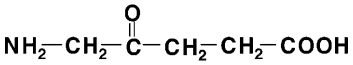
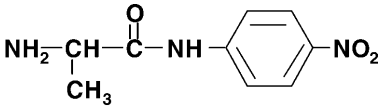
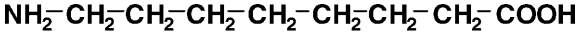
Table 2 Inhibition constant values for various dipeptides with a modified α-amino group for PEPT1 and PEPT2. Each value was calculated from the inhibition curves as described in Materials and methods

Substrates	K_i (μM)	
	PEPT1	PEPT2
Gly–Gly	860	42
Sar–Gly	13,000	2,200
Met–Ala	150	17
for–Met–Ala	24,000	10,000
Pro–Gly	13,000	2,000

The substrate affinity of dipeptides with a modified α-amino group

Next, we examined the substrate affinity of dipeptides with a modified α-amino group such as Sar–Gly (*N*-methyl-glycylglycine) and for–Met–Ala (*N*-formyl-methionylalanine). The K_i values of these substrates are shown in Table 2. The modification of an α-amino group of the dipeptides reduced the substrate affinity for both PEPT1 and PEPT2, i.e., Sar–Gly and for–Met–Ala had less affinity for both transporters than Gly–Gly and Met–Ala, respectively. Nevertheless, these dipeptides still had a higher affinity for PEPT2 than for PEPT1. Pro–Gly also had a higher affinity for PEPT2 than for PEPT1.

Table 3 Inhibition constant values of nonpeptidic compounds for PEPT1 and PEPT2. Each value was calculated from the inhibition curves as described in Materials and methods

Substrates	Chemical structures	K_i (μM)	
		PEPT1	PEPT2
Valacyclovir		2500	220
δ-ALA		2200	230
Alanine-4-nitroanilide		61	29
8-AOA		4400	7900

The substrate affinity of nonpeptidic substrates

Finally, the substrate affinities of nonpeptidic compounds were compared. Valacyclovir [11], 8-amino-octanoic acid (8-AOA) [8], δ-aminolevulinic acid (δ-ALA) [7] and alanine-4-nitroanilide [3], which are all reported to be substrates of peptide transporters, were used for the inhibition studies. Figure 3C and D shows the results for valacyclovir and 8-AOA, respectively, and the K_i values for nonpeptidic compounds are summarized in Table 3. Valacyclovir, δ-ALA and alanine-4-nitroanilide had potent inhibitory effects on PEPT2 rather than on PEPT1, whereas 8-AOA did not. These results suggest that, of the compounds examined, only 8-AOA has a higher affinity for PEPT1 than for PEPT2.

Discussion

Previous studies have shown that rabbit and human PEPT2 have a higher affinity for chemically diverse dipeptides than rabbit and human PEPT1 [1, 2, 17]. These studies mainly focused on the differences in charge among the dipeptides tested, and demonstrated that PEPT2 has a higher affinity than PEPT1, not only for zwitterionic dipeptides but also for anionic and cationic dipeptides. However, little information is available regarding whether structural factors other than charge are able to affect the substrate affinities of PEPT1 and PEPT2. Furthermore, there are no systematic comparisons of the affinities of tripeptides and nonpeptidic substrates between both transporters.

Here, we demonstrated that PEPT2 has a higher affinity for various dipeptides than PEPT1, irrespective of their charge, side-chain structure and modification of their α-amino group. Tripeptides also had a higher affinity for PEPT2. Dipeptides with a β-amino group such as bestatin also exhibited higher affinity for PEPT2 than for PEPT1. This result indicates that even if dipeptides have a β-amino group instead of an α-amino group, the order of substrate affinity between PEPT1 and PEPT2 is maintained.

β–Asp–Gly also has the β-amino group, but it was found to have little affinity for either transporter. Döring et al. [8] compared the substrate affinities of 8-AOA and 2-amino-octanoic acid (2-AOA) for rabbit PEPT1. When the amino group was moved from position 8 to position 2, 2-AOA failed to inhibit dipeptide influx, whereas 8-AOA had a strong inhibitory effect. Based on these observations, Döring et al. [8] suggested that charged amino and carboxyl groups in close proximity prevent the interaction of substrates with the transporter binding site. Similarly, β–Asp–Gly has charged amino and carboxyl groups attached to the same carbon atom, and therefore this dipeptide might fail to show the interaction for both transporters.

The substrate affinity for PEPT1 and PEPT2 was reduced by modifying an α-amino group of the dipeptides. That is, Sar–Gly and for–Met–Ala, which have the secondary α-amino group, were found to have lower affinity for both transporters than Gly–Gly and Met–Ala, respectively. As well as these modified dipeptides, Pro–Gly also has the secondary α-amino group, and has lower affinity for both transporters as compared to the other zwitterionic dipeptides indicated in Table 1. The α-amino group of substrates was suggested to bind to the imidazole ring of the histidine residue in PEPT1 and PEPT2 [23]. The substrate affinity of dipeptides with the secondary α-amino group may be reduced by preventing this interaction.

In the present study, valacyclovir and δ-ALA had higher affinity interactions with PEPT2 than with

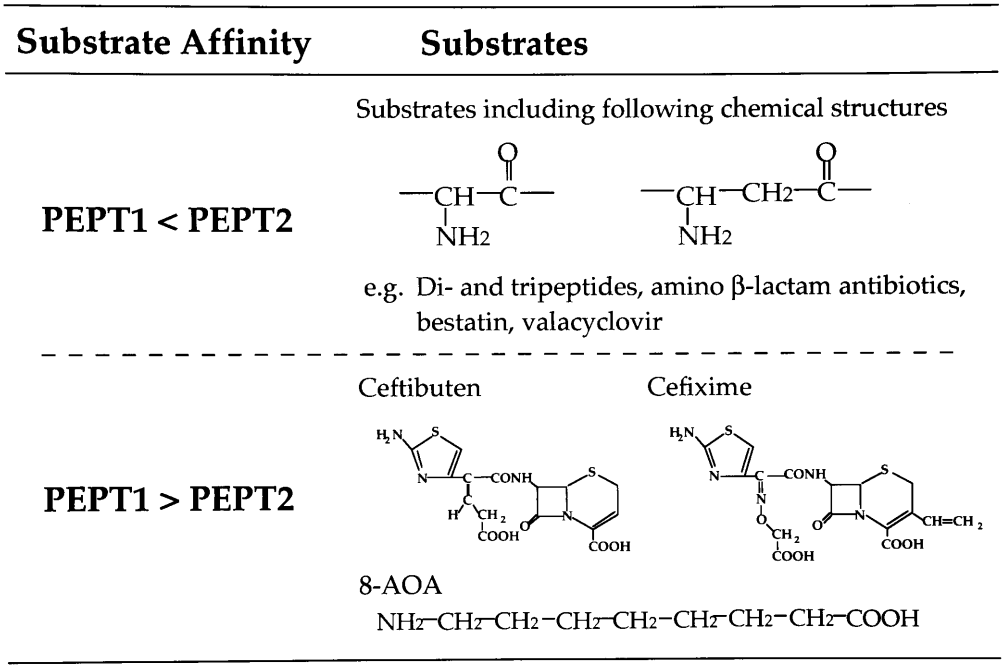


Fig. 4 Structural factors influencing the substrate affinities of PEPT1 and PEPT2. The order of substrate affinity between PEPT1 and PEPT2 is retained even if the α-amino group of dipeptides is modified

PEPT1, and this finding is consistent with the previous reports [7, 10, 20]. With regard to the L-amino acid ester compounds, we recently reported that L-valine methyl ester also has a higher affinity for PEPT2 than for PEPT1 [20]. Furthermore, the present study shows that alanine-4-nitroanilide, which has an amide bond and a free amino group, inhibits PEPT2 more strongly than PEPT1. In contrast, only 8-AOA was found to have a greater affinity for PEPT1 than PEPT2. Previously, we have demonstrated that anionic β-lactam antibiotics without an α-amino group, such as ceftibuten, prefer PEPT1 to PEPT2 as well as 8-AOA [25]. Taking all these information into consideration, we compared the chemical structures of various substrates such as small peptides, oral β-lactam antibiotics, bestatin and non-peptidic substrates. As shown in Fig. 4, the α- or β-amino carbonyl function appears to be the common structure that exhibits a higher affinity for PEPT2 than for PEPT1. Even if the dipeptide α-amino group is modified, the order of substrate affinity (PEPT1<PEPT2) is retained. Further studies are needed to clarify how the α- and β-amino carbonyl functions are involved in determining the substrate affinity of both transporters. Additional substrates showing higher affinity for PEPT1 could provide useful information about the precise structural requirements.

In conclusion, we have clearly demonstrated that PEPT2 has a higher affinity than PEPT1 for various substrates including di- and tripeptides, valacyclovir, δ-ALA and alanine-4-nitroanilide. On the other hand, 8-AOA prefers PEPT1 to PEPT2. Cyclic dipeptides, as well as amino acids and tetrapeptides, could not be recognized by the peptide transporters. The α- or β-amino carbonyl function was suggested to be the key structure that enables the differences in affinity between PEPT2 and PEPT1.

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