Human PEPT1 Pharmacophore Distinguishes between Dipeptide Transport and Binding

Balvinder S. Vig,[†] Terry R. Stouch,[‡,§,||] Julita K. Timoszyk,[†] Yong Quan,[†] Doris A. Wall,[†] Ronald L. Smith,[†] and Teresa N. Faria*[,†]

Exploratory Biopharmaceutics and Stability, and Macromolecular Structure/CADD, Pharmaceutical Research Institute, Bristol-Myers Squibb Company, New Brunswick, New Jersey 08903-0191

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The human intestinal oligopeptide transporter (PEPT1) facilitates the absorption of dipeptides, tripeptides, and many peptidomimetic drugs. In this study, a large number of peptides were selected to investigate the structural features required for PEPT1 transport. Binding affinity was determined in a Gly-Sar uptake inhibition assay, whereas functional transport was ranked in a membrane depolarization assay. Although most of the peptides tested could bind to PEPT1, not all were substrates. As expected, single amino acids and tetrapeptides could not bind to or be transported by PEPT1. Dipeptide transport was influenced by charge, hydrophobicity, size, and side chain flexibility. The extent of transport was variable, and unexpectedly, some dipeptides were not substrates of PEPT1. These included dipeptides with two positive charges or extreme bulk in either position 1 or 2. Our results identify key features required for PEPT1 transport in contrast to most previously described pharmacophores, which are based on the inhibition of transport of a known substrate.

Introduction

The oligopeptide transporter, SLC15A1 (peptide transporter-1, PEPT1), mediates the transepithelial transport of dipeptides, tripeptides, and a number of peptide-like drugs, such as β-lactam and cephalosporin antibiotics, ACE inhibitors, renin inhibitors, and amino acid esters of nucleoside drugs, across the intestinal wall (see refs 1−3 for reviews).[1−3] PEPT1 is a low affinity, high capacity nutrient transporter, which actively transports substrates via a transmembrane electrochemical proton gradient.[4−7] Besides the intestinal tract, PEPT1 is also expressed in the pancreas,[8] bile ducts,[9] and kidneys,[10] where it may play a role in the re-uptake of the filtered peptides. Broad substrate specificity, expression in the small intestine, and the ability to enhance the permeability of drugs with poor biopharmaceutical properties make PEPT1 a very attractive target for enhancing oral drug delivery.[2] To design drugs that can be transported by PEPT1, the structural requirements of PEPT1 substrates must be understood. Because the 3D structure of PEPT1 is not available, structure−affinity relationships (SARs) and structure−transport relationships (STRs) can be used to shed light on the PEPT1 binding and transport requirements.

PEPT1 belongs to the major facilitator superfamily (MFS) of transporters, which includes the bacterial lactose permease LacY, whose crystal structure was recently determined.[11] LacY, like PEPT1, is an electrogenic transporter and co-transports substrates and protons. On the basis of the mechanism proposed for substrate/proton symport by LacY[11] and PEPT1,[12] one can envision the transport of substrates by PEPT1 to be comprised of the following steps: (1) protonation of the PEPT1 facing the extracellular site, (2) binding of the substrate to the PEPT1, (3) conformational change resulting in PEPT1 facing the cytoplasmic site, (4) release of the substrate, (5) deprotonation of the PEPT1, and (6) return of PEPT1 to its original state. The same steps may be followed for the transport of substrates by PEPT1 from the intracellular to the extracellular site, however, with different kinetics.[6] Compounds with appropriate structural features will bind to PEPT1,[13−16] but only those that are able to initiate correct conformational changes in PEPT1 will be transported. Thus, step 3 is key for a compound to be a PEPT1 substrate.

Numerous studies have been conducted during the past decade to investigate the structural requirements for PEPT1 binding.[13,17] The current understanding of the key features can be summarized as follows: the presence of a peptide bond is preferred for PEPT1 binding although not required.[18−21] The *N*-terminal amine and the *C*-terminal carboxyl group are not absolutely required;[19,22] however, compounds with these groups exhibit higher affinity for PEPT1, and substitutions at the *N*- and *C*-terminus result in reduced PEPT1 binding.[22−25] L-Amino acids are favored over the D-amino acids,[26,27] and only amide bonds in the trans configuration are recognized.[20] Dipeptides and tripeptides bind much better than amino acids and tetrapeptides.[28,29] Amino acids with more hydrophobic side chains are preferred.[22,24,30,31] It should be noted that the presence of these binding pharmacophoric features does not guarantee transport by PEPT1.

Binding and transport data obtained from different expression systems and under variable experimental conditions were used to propose early pharmacophoric models[26,32,33] and substrate templates for binding to PEPT1.[13] A 3D model of a PEPT1 pharmacophore has been assembled[16] using comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) of a large number of dipeptides and peptidomimetics. Most recently, CoMSIA was used to expand the model to tripeptides and β-lactam antibiotics.[14] A number of molecular descriptors (steric, electrostatic, hydrophobic/hydrophilic, hydrogen-bond donor, and hydrogen-bond acceptor properties) were found to be important for PEPT1 affinity. When hydrogen-bond acceptor and donor properties are considered electrostatic in nature, both models were dominated by electrostatic properties. To date, these models remain the most comprehensive PEPT1 SAR models and can be applied to predict affinity constants for new substrates.

* To whom correspondence should be addressed. Tel: (732) 227-6129. Fax: (732) 227-3764. E-mail: teresa.faria@bms.com.
[†] Exploratory Biopharmaceutics and Stability.
[‡] Macromolecular Structure/CADD Pharmaceutical Research Institute.
[§] Contact T.R.S. for modeling related questions.
[||] Present address: Lexicon Pharmaceuticals, 350 Carter Road, Princeton, NJ 09540.

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Recently, Vabeno et al.[15] investigated the conformational energy penalties involved in ligand binding to PEPT1 and found a significant contribution from the backbone conformational energy (ΔE_{bbone}) to the experimentally observed difference in affinity for PEPT1 ligands (log $1/K_i$). However, these models are based on the data from competition binding assays, which do not necessarily differentiate between substrates and nonsubstrates (compounds that bind but are not transported).

In the studies reported here, the structural features required for the transport of oligopeptides by PEPT1 were determined using a functional transport assay.[34] This assay distinguishes the substrates from binders and nonbinders among drug-like compounds[34] and was used to predict transport properties in a prodrug series.[34] The PEPT1-mediated transport of a large number of dipeptides displaying a wide range of physicochemical and structural properties was determined by measuring the membrane depolarization caused by the inward proton flux in hPEPT1-overexpressing Madin−Darby canine kidney (MDCK) cells. In addition, binding affinity was determined by measuring the compound concentration required to inhibit [^{14}C]Gly-Sar uptake in MDCK-PEPT1 cells. Using this model, the following questions were asked. (1) Are all dipeptides substrates of the PEPT1 transporter? (2) What are the structural requirements for the transport of dipeptides by the PEPT1 transporter?

Materials and Methods

Reagents and Equipment. Cell culture reagents and growth media were purchased from Mediatech, Inc. (Herndon, VA). The membrane potential assay kit was purchased from Molecular Devices Corporation (Sunnyvale, CA). [^{14}C]Glycyl-Sarcosine (Gly-Sar) and [^{3}H]Trp-Trp were purchased from Moravek Biochemicals (Brea, CA). A microplate reader coupled to a fluid transfer system, FLEXstation, was purchased from Molecular Devices Corporation (Sunnyvale, CA). Liquid scintillation supplies, including CulturPlates (96-well plates), Microscint-40 (liquid scintillation cocktail), and Topcount (scintillation counter) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Oligopeptides were purchased from Bachem Bioscience, Inc. (King of Prussia, PA) Chem-Impex International, Inc. (Chicago, IL), and Sigma Chemical Co. (St. Louis, MO); their purity ranged from 98 to 99% by TLC. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. A stably transfected MDCK-PEPT1 cell line expressing the hPEPT1 transporter was obtained as described previously.[35] The cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.2 mM L-Glutamine, 1% nonessential amino acids, and 0.3 mg/mL of Geneticin. The cells transfected with vector cDNA alone (mock-transfected cells, MDCK-mock) served as a control and were grown in the same conditions as the PEPT1 cells. Cell cultures were maintained in standard conditions (37 °C, 90% humidity, 5% CO_2) until 80−90% confluency. The growth medium was changed every other day. Prior to assays, MDCK-PEPT1 and MDCK-mock cells were grown for 2 days in 96-well plates in a complete DMEM medium without Geneticin.

Cellular Uptake Assays. [^{14}C]Gly-Sar and [^{3}H]Trp-Trp cell uptake was performed in triplicate, as described previously.[34] Briefly, the cells were washed twice and preincubated with bicarbonated Ringer's solution (BRS) at pH 6.0 for 30 min at 37 °C. The buffer was then replaced with BRS containing [^{14}C]Gly-Sar or [^{3}H]Trp-Trp (0.5−5 mM). After incubation for 10 min at 37 °C, the cells were washed twice with ice-cold phosphate buffered saline (PBS) and solubilized in situ with 200 μL of Microscint-40, and the radioactivity in each well was measured.

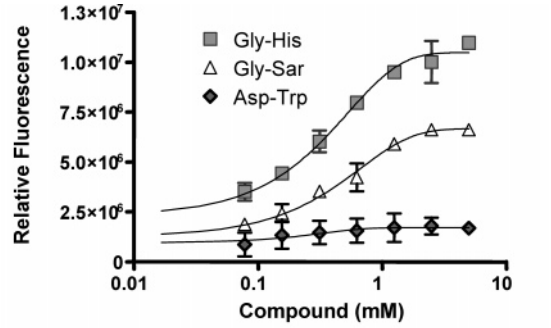
PEPT1 Binding Affinity Assay: Inhibition of [^{14}C]Gly-Sar Uptake. The binding affinity of the probes for PEPT1 was assessed by measuring the concentration at which they could inhibit [^{14}C]-Gly-Sar uptake. For inhibition studies, [^{14}C]Gly-Sar (10 μM) was added together with an excess of an unlabeled competitor (0.1−9 mM) in triplicate, and the uptake of [^{14}C]Gly-Sar was determined according to the procedure outlined above. The IC_{50} values were estimated by nonlinear regression analysis using Xlfit for Microsoft Excel (IDBS, Guilford, UK). Michaelis−Menten-like kinetic parameters (K_m, V_{max}) were determined by nonlinear curve fitting of specific uptake data to the following equation: $V_0 = V_{max}[S]/(K_m + [S])$, where V_0 is the initial uptake velocity, V_{max} is the maximal uptake velocity at saturating substrate concentrations, K_m is a constant analogous to the Michaelis−Menten constant, and S is the substrate concentration.

PEPT1 Activation Assay. The activation of PEPT1 transport by peptide probes was determined using a membrane potential assay as described previously.[34] Oligopeptides, including di, tri, and tetrapeptides were tested for their ability to induce changes of membrane potential in PEPT1 vs mock transfected MDCK cells. The compounds were dissolved in the assay buffer to a final concentration of 10 mM, or when insoluble, stock solutions were prepared in DMSO. Most of the compounds were tested at concentrations ranging from 0.08 to 5 mM. The final concentration of DMSO was less than 1%. The compounds were added in triplicate to cells preloaded with the membrane potential dye, and fluorescence was measured every 2 s for 2 min using 530 nm (excitation) and 565 nm (emission) wavelengths. Fluorescence responses were corrected for background changes in fluorescence caused by the addition of the dye solution containing no substrate into blank wells. Raw fluorescence data were analyzed with SOFTmaxPRO 4.0.1 (Molecular Devices, Sunnyvale, CA). The EC_{50} values (concentration of compound that results in half-maximum fluorescence response) were obtained by fitting the data to a four-parameter logistic, where the baseline response (A), the maximum response, Fl_{max}, the slope (B), and the EC_{50} values can be described by the following equation: $y = ((A - Fl_{max})/(1 + (X/EC_{50})\wedge B)) + Fl_{max}$.

Molecular Modeling. Molecular modeling and the conformational search were performed using the programs Insight, Discover, and the CFF2000 force field (Accelrys, Inc., San Diego, CA). Commonly available values for the physical properties of amino acids were used.[36,37] Data analysis was performed using statistical computing and graphics freeware, R[38] (www.R-project.org).

Results and Discussion

Interpretation of PEPT1 Activation Data. The structural requirements for transport by PEPT1 were assessed by determining transporter activation by a large number of di and tripeptides in a novel functional assay.[34] In this assay, the PEPT1-mediated transport of compounds was determined by measuring the membrane depolarization caused by the inward proton flux in MDCK-PEPT1 cells. In Figure 1, PEPT1 activation is represented by the dose-dependent increase in fluorescence caused by Gly-His, Gly-Sar, and Asp-Trp. The concentration required to achieve half of the maximum activation/membrane depolarization (EC_{50}) and maximum activation (V_{max}) were obtained by nonlinear regression analysis. Because the probes were tested on different days and the maximum fluorescence values exhibited some day-to-day variability, the V_{max} values were normalized to Gly-Sar (%GS_{max}, maximum activation relative to Gly-Sar), which was performed on each experiment as a control. To compare and rank order the different compounds, the ratio between %GS_{max} and EC_{50} (%GS_{max}/EC_{50}), which corresponds to V_{max}/K_m was obtained. On the basis of %GS_{max}/EC_{50}, Gly-His is a better PEPT1 substrate than Gly-Sar, which in turn is better than Asp-Trp, although Asp-Trp has a lower affinity than Gly-Sar (Figure 1). All compounds were also evaluated in mock transfected MDCK cells to account for nontransporter-mediated fluorescence fluctuations. For this



No.	Name	EC_{50} (mM) Mean ± SD	$\%GS_{max}$ Mean ± SD	$\%GS_{max}/EC_{50}$
29	Gly-His	0.40 ± 0.06	128 ± 31	320
36	Gly-Sar	0.54 ± 0.2	101 ± 7	190
14	Asp-Trp	0.47 ± 0.36	22 ± 5	46

Figure 1. PEPT1 activation represented by a dose-dependent increase in fluorescence for selected compounds: Gly-His, Gly-Sar, and Asp-Trp. The EC_{50} (mM), $\%GS_{max}$, and $\%GS_{max}/EC_{50}$ values for these compounds are shown in the table below the graph.

series of compounds, no increase in fluorescence upon compound addition was observed in the control cell line (data not shown).

Differential Activation of PEPT1 by Peptide Probes. Oligopeptide probes were tested for their ability to activate PEPT1. For all 81 probes tested, EC_{50} (mM), $\%GS_{max}$, and $\%GS_{max}/EC_{50}$ values, which correspond to K_m, V_{max}, and V_{max}/K_m values, respectively, were determined. Three independent experiments were performed for each probe (Table 1). In addition to PEPT1 activation, the compound binding affinity, expressed as the IC_{50} value (mM), was also determined in two independent experiments. The intraday variability was under 15%. The IC_{50} value was calculated from competition assays, where the uptake of [^{14}C]Gly-Sar was inhibited by increasing doses of a competitor. The IC_{50} values are generally used as a measure of binding affinity, with the assumption that PEPT1 inhibition is performed by competitive displacement of Gly-Sar from the transporter binding pocket. This dataset was rank ordered on the basis of $\%GS_{max}/EC_{50}$ values (Figure 2). An analysis of the data (Figure 2 and Table 1) shows a clear distinction in the ability of the compounds tested to activate PEPT1. Using Gly-Sar as a reference substrate ($\%GS_{max}/EC_{50} = 190$), dipeptides ($n = 73$) were arbitrarily divided into four categories: the best substrates (dipeptides that exhibit PEPT1 activation 5-fold greater than that of Gly-Sar; $\%GS_{max}/EC_{50} > 1000$; $n = 12$; e.g., Phe-Phe), good substrates (dipeptides that exhibit PEPT1 activation 2-fold greater than that of Gly-Sar; $\%GS_{max}/EC_{50} = 300-1000$; $n = 21$; e.g., Gly-Phe), intermediate substrates (dipeptides that exhibit activation similar to that of Gly-Sar, $\%GS_{max}/EC_{50} = 100-300$; $n = 15$; e.g., Lys-Glu), and poor substrates (dipeptides with low to negligible transporter activation, $\%GS_{max}/EC_{50} = 0-100$; $n = 25$; e.g., Asp-Trp). Of the 25 poor substrates, 21 induced no PEPT1 activation and can be classified as nonsubstrates. These results clearly show that not all dipeptides are substrates of the PEPT1 transporter. This finding contradicts the published assumption that PEPT1 can transport all di and tripeptides. Many dipeptides that failed to activate PEPT1 exhibited affinity toward this transporter in inhibition assays. Thus, affinity for PEPT1 does not always translate into transport. Currently, most of the PEPT1 pharmacophoric models are based on such competition studies, which cannot distinguish between inhibitors and substrates.[39] The present dataset based on a functional transport assay provides the basis for an improved pharmacophoric analysis of the structural features responsible for the transport by PEPT1.

Lack of Fluorescence Increase Is Due to the Lack of PEPT1 Activation. Figure 3 shows the activation of PEPT1 by Gly-Sar, Trp-Gly, Gly-Trp, and Trp-Trp. Even though all of these compounds have IC_{50} values lower than that of Gly-Sar (Table 1), it is clear that Trp-Trp and Gly-Trp are very poor activators of PEPT1. As will be discussed later, Trp at position 2 of the dipeptide seems to consistently affect the transportability but not the binding affinity of the dipeptide for PEPT1. To confirm that the failure of Trp-Trp to increase proton transport is due to the lack of transport, the uptake of [^{14}C]Gly-Sar and [^{3}H]Trp-Trp in MDCK-PEPT1 and MDCK-mock cells was evaluated (Figure 4). The dose-dependent uptake of [^{3}H]Trp-Trp in MDCK-PEPT1 and MDCK-mock cells had similar kinetic descriptors, with a V_{max} of 1.05 and 0.93 nmol/mg protein and a K_m of 0.11 and 0.18 mM, respectively (Figure 4a). This observation confirms that in these cells PEPT1 has no significant contribution toward [^{3}H]Trp-Trp transport. The total uptake of [^{3}H]Trp-Trp in MDCK-PEPT1 cells was much lower than that of [^{14}C]Gly-Sar (Figure 4b). [^{14}C]Gly-Sar showed a 44-fold higher V_{max} than that of [^{3}H]Trp-Trp. Finally, the uptake of [^{3}H]-Trp-Trp could not be blocked by Gly-Sar in either MDCK-PEPT1 or MDCK-mock cells (Figure 4c), again confirming the lack of significant contribution by PEPT1 to total [^{3}H]Trp-Trp uptake. This independent measurement of [^{3}H]Trp-Trp uptake confirms that the lack of activity of Trp-Trp in the fluorescence depolarization assay is due to the failure of this dipeptide to activate the PEPT1 transporter. A correlation between the response in the fluorescence assay and the lack of PEPT1-mediated transport has been reported previously for a series of amino acid prodrugs of floxuridine.[34]

Utility of an Inhibition Assay as a Screening Tool for PEPT1 Binding. Although binding to PEPT1 alone is not sufficient for transport, a binding afinity assay can be a very useful screening tool. Figure 5 shows the correlation between binding and transport ($\%GS_{max}/EC_{50}$ and IC_{50}). Thus, higher $\%GS_{max}/EC_{50}$ values generally correspond to higher affinities (lower IC_{50}) for PEPT1. This means that IC_{50} is a good first pass predictor of transportability. In our assays, all compounds with IC_{50} values >3 mM had a $\%GS_{max}/EC_{50} < 50$ (poor substrates). Thus, no low affinity compounds were significantly transported. This is not unexpected because binding is a prerequisite for transport. These results indicate that binding assays can be a good screening tool for the identification of nonsubstrates. A classification of PEPT1 substrates on the basis of IC_{50} values has been suggested,[40] where values >5 mM are classified as low affinity. In our dataset, dipeptides with an IC_{50} value >5 mM can be safely regarded as poor/nonsubstrates, for example, dibasic dipeptides and *C*-terminal modified dipeptides. However, this cutoff value (IC_{50} >5 mM) may be higher for compounds other than dipeptides such as β-lactam antibiotics, which exhibit IC_{50} >5 mM but are still PEPT1 substrates.[34]

Although the binding assay can distinguish between PEPT1 binders and nonbinders, it cannot distinguish between substrates and nonsubstrates. In the current dataset, most of the compounds/dipeptides have an IC_{50} value of <2 mM; however, many of them are poor/non substrates of PEPT1 as indicated by the membrane depolarization assay. Thus, although the binding assay is a good first pass screen, it is a poor surrogate measure of transporter activation.

PEPT1 Structure-Transport and Structure-Affinity Relationships. Fundamental Activation Determinant. The activity of Gly-Gly indicates that there exists a basic determinant

Table 1. PEPT1 Activity and Inhibition Data

no.	name	EC_{50} (mM) mean ± SD	$\%GS_{max}$ mean ± SD	$\%GS_{max}/EC_{50}$	IC_{50} (mM)	no.	name	EC_{50} (mM) mean ± SD	$\%GS_{max}$ mean ± SD	$\%GS_{max}/EC_{50}$	IC_{50} (mM)
1	Ac-Phe-di-iodo-Tyr	NC[a]	NA[b]		0.23	41	His-Gly	0.28 ± 0.03	128 ± 28	460	0.34
2	Ac-Phe-Tyr-NH_2	NC	NA		NA	42	His-His	0.37 ± 0.04	132 ± 33	350	0.40
3	Ala-Ala	0.08 ± 0.01	125 ± 19	1700	0.25	43	His-Trp	0.19	28	150	0.95
4	Ala-Asp	0.23 ± 0.04	124 ± 10	540	0.45	44	Leu-Leu	0.08 ± 0.03	108 ± 29	1400	0.17
5	Ala-Lys	0.22 ± 0.04	117 ± 16	540	0.28	45	Lys-Arg	NC	NA		7.20
6	Ala-Phe	0.08 ± 0.02	135 ± 34	1700	0.07	46	Lys-Glu	0.53 ± 0.05	121 ± 21	230	0.82
7	Ala-Trp	0.08[c]	64 ± 4	830	0.26	47	Lys-Gly	0.32 ± 0.06	130 ± 27	410	0.38
8	Ala-Tyr	0.06 ± 0.01	90 ± 23	1600	0.17	48	Lys-Lys	NC	NA		10.9
9	Arg-Arg	NC	NA		7.31	49	Lys-Pro	0.19 ± 0.03	138 ± 8	720	0.39
10	Arg-Gly	0.27 ± 0.06	136 ± 32	500	0.39	50	Lys-Trp	NC	NA		0.66
11	Arg-Lys	NC	NA		8.11	51	Lys-Val	0.14 ± 0.06	132 ± 6	960	0.25
12	Asp-Asp	0.99	99 ±11	100	0.63	52	Orn-Orn	NC	NA		NA
13	Asp-Gly	0.44	107 ± 15	240	0.81	53	Phe-Ala	0.11 ± 0.05	108 ± 20	1000	0.07
14	Asp-Trp	0.47 ± 0.36	22 ± 5	46	1.31	54	Phe-Ala-NH_2	0.85	39	50	2.99
15	Asp-Val	0.69 ± 0.28	78 ± 4	110	0.31	55	Phe-Gly	0.11 ± 0.00	120 ± 11	1100	0.17
16	Gln-Gln	0.10 ± 0.02	77 ±12	790	0.15	56	Phe-Phe	0.03 ± 0.02	105 ± 19	3100	0.08
17	Gln-Glu	0.42 ± 0.08	97 ± 4	230	0.51	57	Phe-Tyr	0.03 ± 0.01	78 ± 14	2900	0.02
18	Glu-Glu	1.00 ± 0.17	111 ± 3	110	0.62	58	Pro-Asp	>5	104 ± 30		9.16
19	Glu-Gly	0.51 ± 0.05	122 ± 10	240	0.39	59	Pro-Glu	>5	65 ± 14		12.3
20	Glu-Lys	0.31 ± 0.15	83 ± 12	270	0.72	60	Pro-Gly	NC	42 ± 27		>16
21	Gly	NC	NA	NC	NC	61	Pro-Leu	0.25 ± 0.06	76 ± 4	300	0.62
22	Gly-Arg	0.52 ± 0.05	55 ± 20	100	1.82	62	Pro-Lys	NC	NA		>16
23	Gly-Asp	0.55 ± 0.28	124 ± 16	230	0.38	63	Pro-Pro	0.70 ± 0.16	116 ± 31	170	0.80
24	Gly-Glu	1.10 ± 0.41	113 ± 12	100	0.65	64	Pro-Ser	1.6 ± 0.10	31 ± 25	20	>16
25	Gly-Gly	0.48 ± 0.18	112 ± 18	230	0.82	65	Ser-Ser	0.14 ± 0.02	108 ± 9	770	0.13
26	Gly-Gly-Gly	0.58 ± 0.06	109 ± 10	190	1.07	66	Trp-Ala	0.10 ± 0.02	98 ± 18	1000	0.26
27	Gly-Gly-Gly-Gly	NC	15		NA	67	Trp-Gly	0.26 ± 0.05	92 ± 10	350	0.73
28	Gly-Gly-Gly-NH_2				NA	68	Trp-Trp	NC	NA		0.25
29	Gly-His	0.40 ± 0.06	128 ± 31	320	0.81	69	Trp-Tyr	NC	NA		0.08
30	Gly-Leu	0.17 ± 0.11	103 ± 18	620	0.07	70	Trp-Val	0.05 ±0.02	58 ± 5	1100	0.09
31	Gly-Leu-Gly	0.21 ± 0.05	113 ± 29	530	0.24	71	Tyr-Ala	0.10 ±0.05	102 ± 21	1110	0.11
32	Gly-Leu-Phe	0.28 ± 0.07	83 ± 7	290	0.98	72	Tyr-Gly	0.24 ±0.06	99 ± 18	420	0.33
33	Gly-Lys	0.75 ± 0.37	78 ± 5	100	1.25	73	Tyr-Gly-NH_2	NC	NA		NA
34	Gly-Phe	0.13 ± 0.01	111 ± 17	830	0.17	74	Tyr-Tic-NH_2	NC	NA		NA
35	Gly-Phe-NH_2	NC			NA	75	Tyr-Trp	NC	46 ± 8		0.10
36	Gly-Pro	0.13 ± 0.03	111 ± 27	870	0.33	76	Tyr-Tyr	0.06 ± 0.01	69 ± 12	1200	0.06
37	Gly-Sar	0.54 ± 0.20	101 ± 7	190	1.16	77	Tyr-Tyr-NH_2	NC	NA		9.11
38	Gly-Trp	0.33 ± 0.08	23 ± 7	70	0.52	78	Val	NC	NA	NC	NC
39	Gly-Tyr	0.14 ± 0.01	125 ± 27	870	0.12	79	Val-Trp	0.04 ± 0.01	37 ± 21	950	0.10
40	Gly-Tyr-NH_2	NC	NA		NA	80	Val-Val	0.07 ± 0.02	74 ± 14	1000	0.21
						81	Val-Val-Val	0.21 ± 0.04	99 ± 28	480	0.23

[a] NA = No activity. [b] NC = Not calculable. [c] At places, the standard deviation is not provided. This may be due to either compounds causing insufficient activation of PEPT1 for parameter calculation or not enough repeats ($n = 2$) for few of the compounds.

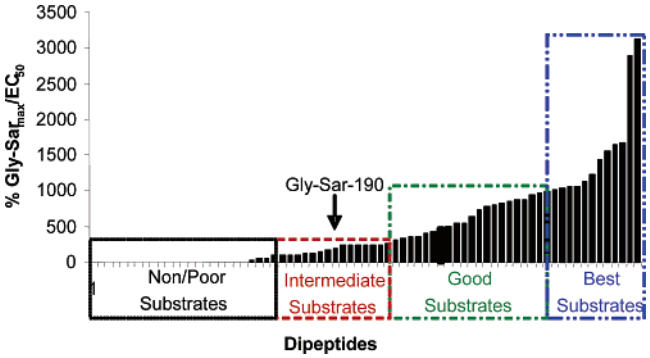
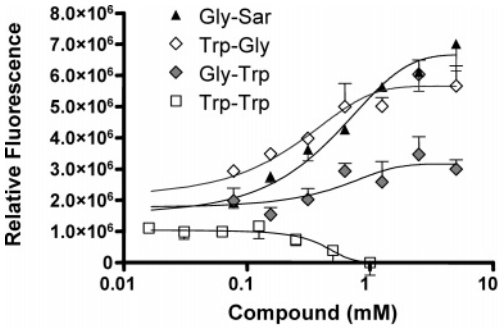


Figure 2. Classification of the dipeptides on the basis of the maximum depolarization achieved in a functional assay relative to the Gly-Sar response ($\%GS_{max}/EC_{50}$). Best substrates >1000, good substrates = 300−1000, intermediate substrates = 100−300, and poor substrates = 0−100.

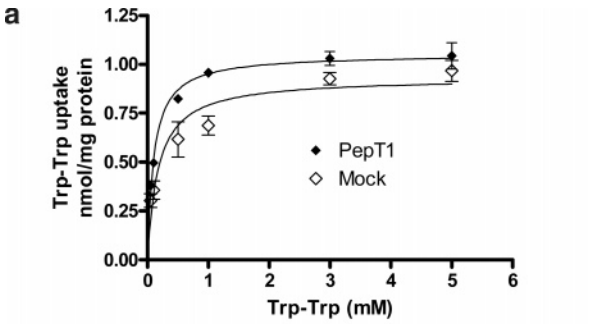


No.	Name	EC_{50} (mM) Mean ± SD	$\%GS_{max}$ Mean ± SD	$\%GS_{max}/EC_{50}$
37	Gly-Sar	0.54 ± 0.20	101 ± 7	190
38	Gly-Trp	0.33 ± 0.08	23 ± 7	70
68	Trp-Trp	NC	NA	---
67	Trp-Gly	0.26 ±0.05	92 ± 10	350

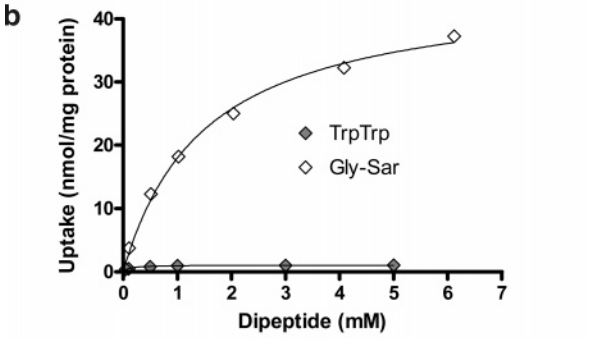
NA; No activity, NC; Not calculatable

Figure 3. Concentration vs fluorescence plots for Gly-Sar, Trp-Gly, Gly-Trp, and Trp-Trp. The EC_{50} (mM), $\%GS_{max}$, and $\%GS_{max}/EC_{50}$ values are shown in the table below the graph.

for PEPT1 activity, not dependent on the side chains. This minimal determinant appears to be the *N*-terminal amino group, the *C*-terminal carboxyl group, and possibly the carbonyl group of the amide linkage. The slightly enhanced activity of Gly-Sar relative to Gly-Gly shows that the amide NH group is not important for the activity. These observations are supported by the earlier binding-based pharmacophore models[13] and the ability of PEPT1 to transport simple molecules, such as ω-amino fatty acids,[19] δ-aminolevulinic acid,[41] and 4-aminophenylacetic acid.[42] However, Gly-Gly's modest activity also makes it clear that the nature of the side chains plays a significant role in further enhancing or abolishing the activity due to the fundamental motif.



	PEPT1	Mock
V_{max} (nmol/mg)	1.05 ± 0.02	0.93 ± 0.05
K_m (mM)	0.11 ± 0.01	0.18 ± 0.05



	Trp-Trp	Gly-Sar
V_{max} (nmol/mg)	1.05 ± 0.02	45.3 ± 1.7
K_m (mM)	0.11 ± 0.01	1.11 ± 0.15

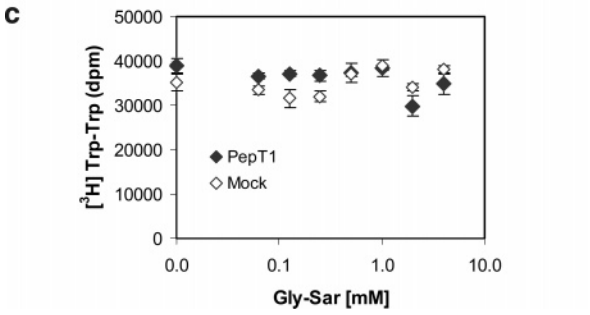


Figure 4. PEPT1 does not transport [^{3}H]Trp-Trp. (a) Dose-dependent uptake of [^{3}H]Trp-Trp in MDCK-PEPT1 and MDCK-mock (control) cells. (b) Total uptake of [^{3}H]Trp-Trp and [^{14}C]Gly-Sar by MDCK-PEPT1 cells. (c) Inhibition of [^{3}H]Trp-Trp uptake by Gly-Sar in MDCK-PEPT1 and MDCK-mock (control) cells.

Peptide Size. PEPT1 activation induced by one tetrapeptide, and several tripeptides, dipeptides, and single amino acids was compared (Table 1). To be transported, compounds must be larger than single amino acids and smaller than tetrapeptides because Gly-Gly-Gly-Gly and Gly and Val are not substrates of PEPT1. This data is in agreement with previous studies demonstrating that amino acids and peptides larger than tripeptides do not bind to PEPT1.[29] Tripeptides and dipeptides were substrates of the PEPT1 transporter. Dipeptides (Gly-Gly, Val-Val, and Gly-Leu) were better substrates than tripeptides (Gly-Gly-Gly, Val-Val-Val, and Gly-Leu-Phe/Gly). These data indicate that the PEPT1 binding pocket is large enough to accommodate tripeptides but not tetrapeptides, and the individual amino acids do not exhibit the structural features required to bind. The higher affinity and transport of dipeptides suggest that they contain the optimum properties for binding and transport.

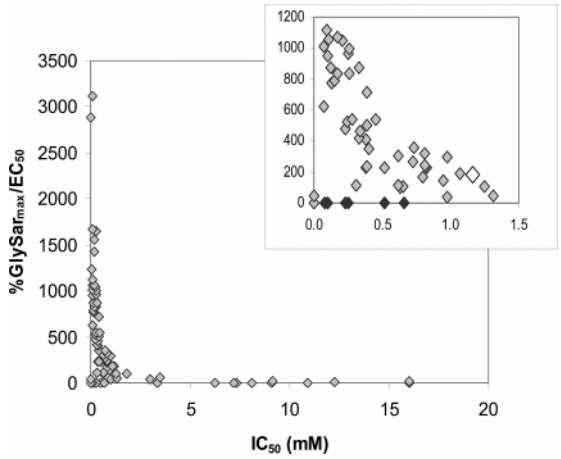


Figure 5. Correlation between PEPT1 activation (%Gly-Sar$_{max}$/EC$_{50}$) and binding (IC$_{50}$) for the dipeptides tested. Inset: magnification of area in large graph containing 75% of the dipeptides tested. (♦): dipeptides showing good binding affinity for PEPT1 (less than 1 mM), which fail to activate the transporter. (◊): Gly-Sar.

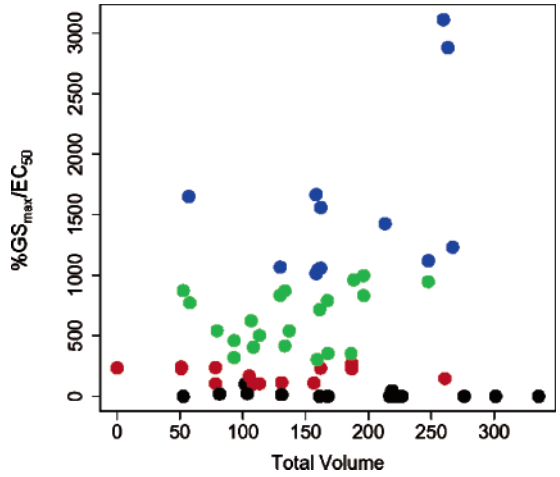


Figure 6. %GS$_{max}$/EC$_{50}$ vs total volume of the dipeptides side chains. Biological activities (%GS$_{max}$/EC$_{50}$) are highlighted by color. Blue, best substrates >1000; green, good substrates = 300−1000; red, intermediate substrates = 100−300; black, poor substrates = 0−100.

Substitutions at the *N*- and *C*-Termini. Substitutions at the *N*-terminal amine and *C*-terminal carboxylic group of dipeptides resulted in the loss of PEPT1 activation. Substitutions also resulted in the loss of binding affinity, except for Phe-Ala-NH_2 and Ac-Phe-diiodo-Tyr. These results are in agreement with independent observations that modifications at the *N*- and *C*-termini of the dipeptide are generally not tolerated by the PEPT1 transporter.[16,25]

Effect of Size/Hydrophobicity/Aromaticity. Several groups have shown that hydrophobicity increases binding affinity for the PEPT1 transporter.[16,30,31,43] For example, Gebauer and co-workers showed that binding affinity of dipeptides correlated positively with *N*-terminus hydrophobicity in the X-Ala series. However, it has not been established if this higher binding affinity also results in enhanced transport by PEPT1. The current transport data address this issue.

It is difficult to deconvolute all of the properties of the individual amino acids, because volume (size), aromaticity, and hydrophobicity are all correlated, and as noted below, the effects of the side chains appear to be synergistic. It is clear that at the *N*-terminus, larger, more hydrophobic amino acids resulted in increased PEPT1 activation (Figure 6): Phe-Gly, Tyr-Gly, and Trp-Gly were better substrates than Gly-Gly. Ala, also in

position 1, showed greater transport than Gly: Ala-Phe, Ala-Tyr, Ala-Leu, Ala-Lys, and Ala-Asp exhibited higher PEPT1 activation than did the corresponding Gly-X dipeptides. Aromaticity further accentuates activity: Phe-Tyr and Phe-Phe exhibited greater activity than Leu-Leu despite very similar hydrophobicities. The more hydrophobic Phe-Phe was more active than Tyr-Tyr (which has a bigger size). However, the increased activity with increase in size (and/or aromaticity) correlation is limited because Trp at the *N*-terminus results in lower activity. Thus, for X-Gly, X-Ala, and X-Tyr, the larger Trp was less preferred. This outcome could be distinguished from the effect of hydrophobicity because Leu was associated with enhanced activity despite similar hydrophobicity.

Similar trends were seen at the *C*-terminus. Although most substitutions at this position conferred PEPT1 activation, bulky/hydrophobic amino acids were generally preferred. For Gly-X dipeptides, improved PEPT1 activity correlated with increases in bulk and hydrophobicity at position 2. For example, Gly-Tyr and Gly-Phe were superior substrates to Gly-Gly, Gly-Asp, and Gly-Glu. This correlation was also observed in other series such as Ala-X, Phe-X, Trp-X, and Tyr-X. As observed for the *N*-terminus, Trp at the *C*-terminus resulted in either a reduction or a complete loss of activation while retaining affinity for PEPT1 (Figure 6). Accordingly, Trp-Trp and Gly-Trp could not activate the transporter (Figure 4), despite high binding affinity. This indicates that PEPT1 has a size limitation for amino acids at both *N*-and *C*-termini.

Effect of Charge on PEPT1 Activation. Neutral dipeptides exhibited higher PEPT1 activation than did those containing a single charged residue: Ala-Ala exhibited higher activation than Ala-Asp and Ala-Lys, Gly-Leu and Gly-Gly were better than Gly-Glu, Gly-Arg, and Gly-Lys, and Gln-Gln exhibited higher activation than Gln-Glu. These data are in agreement with reports that charged dipeptides tend to lower the affinity for the PEPT1 transporter.[24,29,40] The introduction of a second charged residue in the dipeptide further reduced PEPT1 activation. Thus, Asp-Asp was not as good as Asp-Gly, and Asp-Val and Glu-Glu exhibited lower activation than Glu-Gly. Dipeptides with acidic residues at both positions were substrates, albeit poor, of PEPT1. However, dipeptides with basic amino acids at both positions were neither transported by nor bound to PEPT1. Thus Arg-Arg, Arg-Lys, Lys-Lys, Lys-Arg, and Orn-Orn are not substrates of PEPT1. These results indicate that the presence of basic amino acids at both positions is not allowed by PEPT1, whereas a basic amino acid at either position 1 or 2 of the dipeptide is tolerated. The effect of substitution of charged amino acids in dipeptides on PEPT1 activation can be summarized as follows: neutral−neutral > charged−neutral ∼ neutral−charged > acidic−acidic > basic−basic.

Role of Proline. Dipeptides with a proline at the *C*-terminus (Gly-Pro and Lys-Pro) exhibited both high affinity and activation of the PEPT1 transporter. However, dipeptides with proline at the *N*-terminus exhibited more complex activities. Generally, Pro-X dipeptides, where X is a small/hydrophilic amino acid, were both poor binders and poor substrates; Pro-Gly, Pro-Ser, Pro-Asp, Pro-Glu, and Pro-Lys exhibited very low binding affinity and were poor/nonsubstrates. However, Pro-X dipeptides, where X is a branched/hydrophobic amino acid (e.g., Pro-Leu and Pro-Pro), were substrates of the PEPT1 transporter. Previously, the role of proline was systematically studied using binding assays,[24] and it was found that dipeptides with proline at the *C*-terminus exhibit high affinity for the PEPT1 transporter, whereas Pro-X dipeptides exhibited much lower affinity. Brandsch et al. suggested that the binding of Pro-X dipeptides is influenced by the hydrophobicity and rigidity of the *C*-terminal amino acid.[24]

PEPT1 Transport Pharmacophore. Synergistic Effects of the Residues in Dipeptides: The Nature of the Binding Pocket. As detailed above, the properties of the individual residues at both the *N*- and the *C*-termini are important, and each site appears to have its own preferred characteristics. Volume has a substantial effect on activity, and the most active compounds contain the larger amino acids Phe, Tyr, Trp, Leu, Val. Aromaticity seems to accentuate activity. Although charge tends to diminish activity, one charged side chain is still allowed, and one basic residue (Arg, Lys) seems to accentuate activity.

The data also shows that the activity of the dipeptides is not the sum of the contributions from the individual residues. For example, Table 1 and Figure 6 show that, although Trp is individually tolerated at both positions, Trp-Trp is not transported. Furthermore, the dipeptides incorporating an additional large amino acid, such as Tyr or Phe in addition to Trp, are not activators despite the fact that Tyr-Tyr and Phe-Phe are the best activators. Similarly, although the presence of Arg can individually enhance activity at any position, Arg-Arg is also not transported.

These points, in addition to the analysis presented below, suggest that PEPT1 has one contiguous binding pocket that is influenced by both side chains rather than separate binding pockets for the individual side chains. The data also suggest that key residues in the binding site are aromatic. Aromatic binding site residues could form favorable pi stacking interactions with the dipeptide aromatic residues (including arginine) and can also participate in cation pi stacking with both arginine and lysine. The high activity of both Tyr-Tyr and Phe-Phe suggests that desolvation and hydrogen-bonding proclivity is probably not an overriding issue for binding. If correct, then desolvation can be discounted as an explanation for Trp-Trp inactivity. These trends indicate that Trp-Trp has exceeded the size limitations for the pocket and that a larger total volume also will not be supported.

Conformational Analysis of the Dipeptides. An independent conformational analysis of several dipeptides (Pro-Pro, Ala-Ala, Phe-Phe, Trp-Trp, and Glu-Lys) provides a consistent picture as shown in the overlay of low energy conformations of these peptides (Figure 7). Pro-Pro, the most rigid of the dipeptides, shows the fewest low energy conformations, and a key low energy conformation places both Cβ positions on approximately the same side of the dipeptide. A similar conformation is independently achieved for Ala-Ala. Additionally, one of the lowest energy conformations of Phe-Phe forms a similar conformation, supported by intrapeptide pi stacking between rings. This conformation, which shows the interaction between the side chains, is in agreement with the activation data, which shows synergy between the dipeptide side chains. The distance between the *N*- and *C*-termini for dipeptides is 5.6 Å (data not shown), which is similar to that reported for omega fatty acids[19] and dipeptides.[44]

Charge plays an important role in both activity and conformation. In terms of charge states and activity, Glu-Arg (or Glu-Lys) would appear to be an anomaly, having two charges yet providing reasonable activation. However, its side chains readily form an intramolecular salt bridge, allowing it to assume a conformation in good agreement with the other dipeptides and effectively neutralizing its charge. Single basic amino acids are allowed, and the potential pi stacking with PEPT1 residues has already been addressed. However, the dibasic dipeptides, Lys-Lys and Arg-Arg, show no activation. This could be explained

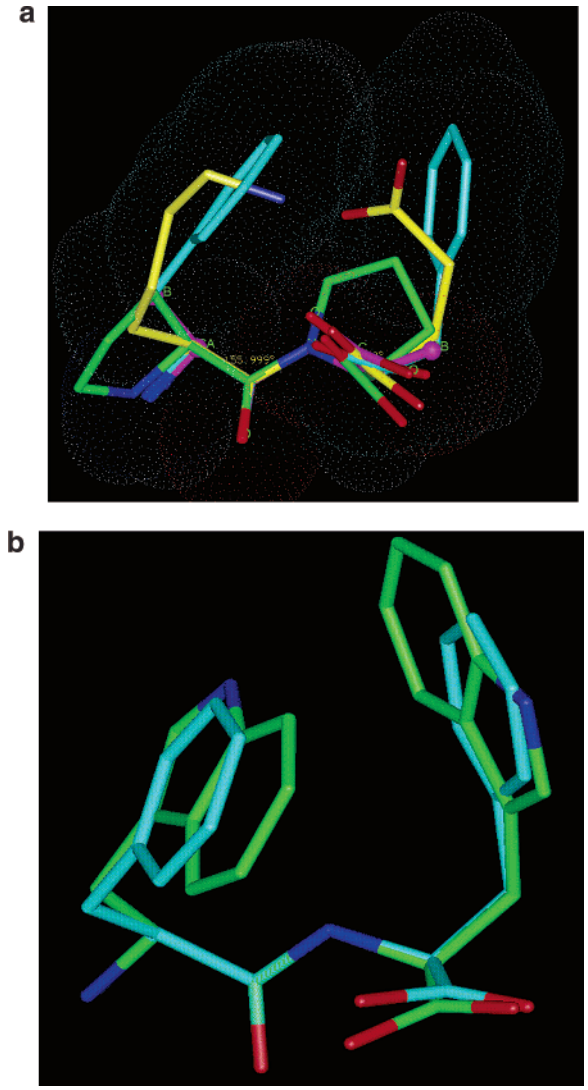


Figure 7. Overlay of low-energy conformations of key dipeptides. Oxygen, red; nitrogen, blue; carbon is colored per dipeptides: Pro-Pro, green; Ala-Ala, purple; Phe-Phe, cyan; and Glu-Lys, yellow. The carboxylic acid moieties of all dipeptides point out of the plane of the figure on the lower right of the structures. (a) Dot representation of the solvent accessible surface area of the dipeptides. (b) Overlay of Phe-Phe and Trp-Trp in the preferred low energy conformation of the proposed pharmacophore, graphically illustrating the enhanced size of the Trp residues.

by the fact that the conformation we have seen as consensus between the most active dipeptides could not be assumed by these peptides. At physiological pH, both Lys and Arg would be protonated, causing their like charges to repel each other. This would lead to an extended conformation, placing the side chains at opposite faces of the peptide, and preventing the consensus conformation from occurring. Glu-Glu shows modest activity. The less extreme pK_a of the side chain and the predilection of Glu to form hydrogen bonds with water might allow it to form an intramolecular bridge, perhaps water mediated, between its side chains. In this way, as with Glu-Arg and Glu-Lys, it could assume the consensus conformation.

The high activity of Ala-Ala is somewhat at odds with the other data. It has small side chains of low hydrophobicity yet elicits very high activation. A likely explanation is that the limited bulk of its side chains allow its backbone to readily assume an ideal conformation. The substantial flexibility of Ala-Ala allows its small hydrophobic moieties to interact at exactly the right position with the binding pocket without any of the entropic penalty required by the other amino acids which contain larger, more flexible side chains.

The low activity of the *N*-terminal proline dipeptides is likely due to two reasons: a nonideal position of the side chain and a nonideal position of the *N*-terminal amino group. When the common backbone of the dipeptides is overlaid, it can be seen that the cyclized ring of proline prevents the placement of the amino group at the same position, as is seen for the other dipeptides. Thus, for a key part of the binding and transport determinant, the COO^- to NH_4^+ distance, Pro-X peptides do not completely match dipeptides with more flexible amino acids at the *N*-terminus.

Trp-Trp can assume the proposed consensus active conformation as can other inactive dipeptides such as Tyr-Trp. We must assume that their reduced activity lies in their large total volume, which is greater than the capacity of the binding site.

Conclusions

These data clearly show that not all dipeptides are PEPT1 substrates, in contradiction to the current implicit assumption that PEPT1 transports all dipeptides and tripeptides. Whereas binding is a prerequisite for transport, it does not ensure transport because many peptides that were found to bind do not show any evidence of transport. Confirmation of this was established by a direct measure of the uptake of radio-labeled dipeptides.

The key structural features required for PEPT1 transport were identified. The binding pocket is large enough to accommodate tripeptides but not tetrapeptides, and individual amino acids are not transported. Free terminal carboxyl and amino functions appear to be an important feature. The nature of the side chains plays a key role in either enhancing or abolishing the activity due to the minimal determinants provided by the dipeptide backbone.

The observations linking PEPT1 activation with amino acid properties coupled with the conformational analysis of several key dipeptides provide a picture of the binding site and a pharmacophore consistent with the data. In addition to the properties mentioned above (charge (net and individual amino acids), hydrophobicity, volume (net and individual)), hydrogen-bonding ability and side chain entropic freedom (number of rotatable bonds) help to rationalize these data.

It appears that PEPT1 has one contiguous binding pocket, which is substantially aromatic. We hypothesize that aromatic residues within the binding site form favorable pi stacking interactions with substrate peptide aromatic side chains (including the pi system of arginine) and also cation pi stacking with arginine and lysine. A proposed PEPT1 computer model[45,46] indicates that the putative transporter channel is lined by a number of charged and aromatic amino acids that could be involved in peptide transport.

We suggest that the optimum conformation of substrate dipeptides places both side chains in close position with an optimum total volume between that of Phe-Phe and Trp-Trp. This conformation is stabilized by intramolecular pi-stacking, hydrophobic collapse, or salt-bridges, when available. We propose that the binding site might not be completely desolvated. The fundamental binding determinant exhibited by Gly-Gly provides a carboxyl moiety, likely charged, within 5.6 Å of a basic amine. It is likely that an additional hydrogen-bond acceptor (the amide carbonyl in these molecules) is also required. Work is underway to further define the structure−transport relationships of PEPT1 with an eye toward application to transported drugs.

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