**Regular Article**

**Evaluation of a Thiodipeptide, L-Phenylalanyl-Ψ[CS-N]-L-alanine, as a Novel Probe for Peptide Transporter 1**

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**Summary:** L-Phenylalanyl-Ψ[CS-N]-L-alanine (Phe-Ψ-Ala), a thiourea dipeptide, was evaluated as a probe for peptide transporter 1 (PEPT1). Uptake of Phe-Ψ-Ala in PEPT1-overexpressing HeLa cells was significantly higher than that in vector-transfected HeLa cells and the $K_m$ value was 275 ± 32 µM. The uptake was pH-dependent, being highest at pH 6.0, and was significantly decreased in the presence of PEPT1 inhibitors [glycylsarcosine (Gly-Sar), cephalexin, valaciclovir, glycylglycine, and glycylproline]. In metabolism assay using rat intestinal mucosa, rat hepatic microsomes, and human hepatocytes, the amount of Phe-Ψ-Ala was unchanged, whereas phenylalanylaminalanine was extensively decomposed. The clearance, distribution volume, and half-life of intravenously administered Phe-Ψ-Ala in rats were 0.151 ± 0.008 L/h/kg, 0.235 ± 0.012 L/kg, and 1.14 ± 0.07 h, respectively. The maximum plasma concentration of orally administered Phe-Ψ-Ala (2.31 ± 0.60 µg/mL) in the presence of Gly-Sar was significantly decreased compared with that in the absence of glycylsarcosine (3.74 ± 0.44 µg/mL), suggesting that the intestinal absorption of Phe-Ψ-Ala is mediated by intestinal PEPT1. In conclusion, our results indicate that Phe-Ψ-Ala is a high-affinity, metabolically stable, non-radioactive probe for PEPT1, and it should prove useful in studies of PEPT1, *e.g.*, for predicting drug-drug interactions mediated by PEPT1 in *vitro* and *in vivo*.

**Keywords:** peptide transporter 1 (PEPT1); Phe-Ψ-Ala; Gly-Sar

**Introduction**

Peptide transporter 1 (PEPT1 SLC15A1) is an H$^+$ conjugation-type peptide transporter expressed in brush-border membranes of small intestine and renal tubular epithelium cells.¹ It plays a nutritional role, transporting dietary di/tripeptides. Because of its broad substrate specificity, this transporter also contributes to intestinal absorption of various peptidomimetic compounds, including β-lactam antibiotics, antineoplastic agent bestatin, and angiotensin-converting enzyme inhibitors.²–⁴ PEPT1 can be utilized to increase intestinal transport of low-permeability compounds, if they can be converted to peptidomimetic prodrugs. For example, the l-valyl ester prodrugs valacyclovir and valgancyclovir increase the intestinal absorption of various peptidomimetic compounds, including l-valyl ester prodrugs valacyclovir and valgancyclovir increase the bioavailability of their parent drugs by three- to five-fold and 10-fold, respectively, in humans.⁵–⁰ Since PEPT1 can mediate drug absorption, as described above, drug-drug interactions may occur at this transporter. In this context, it was reported that oral absorption of valacyclovir was decreased upon coadministration of cephalexin.¹¹

Natural dipeptides are typical substrates of PEPT1 with high uptake activity, but they are metabolized by peptidases present in cell membranes. On the other hand, N-methyl dipeptide derivatives such as glycylsarcosine (Gly-Sar) are metabolically stable, and therefore, [$^3$H]Gly-Sar is often used as a PEPT1 probe.¹,¹² However, the measurement of a radiolabeled compound requires suitable facilities, and there is the problem of disposing of radioactive waste. Because Gly-Sar has high water solubility, it is also difficult to quantitate non-radiolabeled Gly-Sar by methods such as liquid chromatography-tandem mass spectrometry (LC/MS/MS). LC/MS/MS analysis of Gly-Sar by hydrophilic interaction chromatography (HILIC) column was reported.¹³ In our preliminary study, we measured Gly-Sar in rat plasma using the reported method with the detection limit of at most 100 nM. Thus, availability of a non-radiolabeled PEPT1 probe with metabolic stability and moderate lipophilicity would be advantageous.

Thiourea dipeptide derivatives are also resistant to peptidases, and L-phenylalanyl-Ψ[CS-N]-L-alanine (Phe-Ψ-Ala) (Fig. 1), a thiourea dipeptide, has been suggested to act as a PEPT1 substrate,¹⁴ based on results obtained with a single-pass method. However, there is no direct evidence that Phe-Ψ-Ala is a PEPT1 substrate. Therefore, in this study, we characterized and evaluated the usefulness of Phe-Ψ-Ala as a PEPT1 probe using PEPT1-
expressing cell models, metabolic stability assays, and rat in vivo studies.

Materials and Methods

Materials: Phe-Ψ-Ala, Gly-Sar, valaciclovir, cephalixin and phenylalanylalanylamine (Phe-Ala) were purchased from Operon Inc. (Tokyo, Japan), Tokyo Chemical Industry (Tokyo, Japan), LKT Laboratories (St. Paul, MN), Wako Pure Chemical Industries (Osaka, Japan), and Sigma Aldrich (St. Louis, MO), respectively. Glycylglycine (Gly-Gly), glycylproline (Gly-Pro) were from Peptide Institute (Osaka, Japan). PEPT1-overexpressing HeLa (HeLa/PEPT1) cells and HeLa cells transfected with vector alone (mock) were established as described previously. Cryopreserved human hepatocytes were purchased from Charles River (Lot No. Hu8110, Tokyo, Japan). William’s Medium E, Cryopreserved Hepatocytes Recovery Medium (CHRM) and Hepatocyte Plating Supplement Pack (CM3000) were purchased from Invitrogen (Durham, NC). G418 disulfate salt (Geneticin) was purchased from LKT Laboratories (St. Paul, MN), Wako Pure Chemical Industries (Osaka, Japan), and Sigma Aldrich (St. Louis, MO), respectively. LKT Laboratories (St. Paul, MN), Wako Pure Chemical Industries (Osaka, Japan), and Sigma Aldrich (St. Louis, MO), respectively.

Animals: Male Wistar rats (Japan SLC, Hamamatsu, Japan) were housed three per cage with free access to commercial chow and tap water, and were maintained on a 12 h dark/light cycle in a controlled environment (temperature, 24.0 ± 1°C; humidity, 55 ± 5%). All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals at the Takasaki University of Health and Welfare and approved by the Committee of Ethics of Animal Experimentation of the university.

Uptake study: HeLa/PEPT1 cells and mock cells were cultured in Dulbecco’s modified Eagle’s medium (Nakalai Tesque, Osaka, Japan) supplemented with 10% fetal bovine serum (Serum Source International, Charlotte, NC), penicillin (100 U/mL), streptomycin (100 µg/mL), 1% L-glutamine, 1% non-essential amino acids and G418 (0.5 mg/ml). The cells were grown in an atmosphere of 5% CO2 and 95% O2 at 37°C. HeLa/PEPT1 and mock cells were seeded at a density of 0.5 × 10^5 cells/cm² in 12-well plates, and experiments were conducted three days after seeding. Uptake studies using HeLa/PEPT1 and mock cells were performed at 37°C in Hank’s Balanced Salt Solution: [HBSS, 136.7 mM NaCl, 5.36 mM KCl, 0.952 mM CaCl2, 0.812 mM MgSO4, 0.441 mM KH2PO4, 0.385 mM Na2HPO4, 25 mM d-glucose, 10 mM 2-morpholinoethanesulfonic acid, monohydrate (MES)], Uptake was initiated by adding 400 µL of transport buffer containing Phe-Ψ-Ala (50 µM), a concentration much less than the estimated Michaelis-Menten constant (Km) value of Phe-Ψ-Ala (about 500 µM) in our preliminary study. For inhibition studies, Gly-Sar (10 mM), Gly-Gly (10 mM), Gly-Pro (10 mM), cephalixin (10 mM) and valaciclovir (10 mM) were used. Experiments were terminated by washing the cells three times with ice-cold HBSS. For quantitation of uptake, the cells were suspended in water/methanol (30:70; v/v), and collected, followed by centrifugation at 9,500 × g and 4°C for 5 min. The supernatant was evaporated by centrifugal evaporation at 30°C. The residue was dissolved in 0.1 mL of a mixture of 0.05% formic acid in water (pH 3.0) and 0.05% formic acid in methanol (50:50, v/v), and the solution was filtered through a 0.45-µm pore size membrane filter (Millipore Corporation, Billerica, MA). Aliquots of the filtrate were subjected to LC-MS/MS analysis. Protein concentration of HeLa/PEPT1 cells and mock cells were determined using the Lowry method.

Metabolic studies with small-intestinal homogenate and hepatic microsomes of rats: Rats weighing 280–300 g were starved overnight and, the next morning, were anesthetized with diethyl ether. The small intestine and liver were isolated from each rat and placed in ice-cold saline. All subsequent preparation procedures were carried out at 4°C. To obtain small intestinal homogenate, mucosa was collected by scraping the small intestines, suspended in 70 mM phosphate buffer and homogenized in a Potter-Elvehjem homogenizer. The obtained homogenates were used for the metabolic assays. Liver was homogenized in 1.15% KCl, and the homogenates were centrifuged at 9,000 × g for 10 min. The supernatants were centrifuged at 105,000 × g for 60 min, and the resulting pellets were suspended in 1.15% KCl and then centrifuged again at 105,000 × g for 60 min. Then, 1 g of each pellet was taken up in 3 mL of 100 mM phosphate buffer, pH 7.4. The intestinal homogenate and hepatic microsomes of rats were used for metabolic stability assays. Protein concentration of each tissue homogenate was determined by the Lowry method.

The metabolism of peptides in small-intestinal homogenate was investigated using 525 µL of homogenate (10 mg/mL) in 70 mM phosphate buffer at pH 7.4. A 25 µL aliquot of 50 µM Phe-Ψ-Ala or Phe-Ala was added and the mixture was incubated for 60 min, then the enzymatic reaction was terminated by adding 100 µL of acetic acid (50%). Metabolism of peptides by rat hepatic microsomes was investigated using microsomes (0.5 mg/mL) and NADPH (100 µM) in 190 µL of 100 mM phosphate buffer at pH 7.4. After pre-incubation of this mixture at 37°C for 5 min, a solution (10 µL) of Phe-Ψ-Ala (50 µM) or Phe-Ala (50 µM) was added. Incubation was continued for 60 min, and then 200 µL of acetonitrile was added to terminate the enzymatic reaction. For quantitation of analytes, each mixture was centrifuged at 9,500 × g and 4°C for 5 min and filtered through a 0.45-µm membrane filter. The supernatant was subjected to LC-MS/MS analysis.

Metabolic studies with human hepatocytes: Hepatocytes were stored in liquid nitrogen until required, and then immersed in water pre-warmed to 37°C, decanted into CHRM® and centrifuged at 100 × g for 10 min. The hepatocyte pellet was re-suspended in William’s Medium E containing HEPES (15 mM) and l-glutamine (2 mM), purged with 95% O2 and 5% CO2 (pH 7.4). The viability of the hepatocytes was assessed by trypan blue exclusion, and suspensions with viability of over 80% were used. Hepatocytes were pre-incubated at 37°C for 60 min in an atmosphere of 5% CO2 in air, after which reaction was initiated by adding 200 µL of the cell suspension (0.5 × 10⁶ cells/mL) to 300 µL of compound solution [Phe-Ψ-Ala (50 µM) or Phe-Ala (50 µM)]. Reactions were terminated by adding 500 µL acetonitrile at 60 min. For quantitation of analytes, the pellets were centrifuged at 9,500 × g and 4°C for 5 min. The supernatant was filtered through a 0.45-µm membrane filter and the filtrate was subjected to LC-MS/MS analysis.

In vivo pharmacokinetic studies: Rats were anesthetized with diethyl ether, and Phe-Ψ-Ala (0.125 mg/kg) was administered by femoral vein injection. Blood samples were collected at 2, 5, 15, 30, 60, 90, 120, 240, 360 and 480 min after dosing. For oral
administration of Phe-Ψ-Ala (1.25 mg/kg) was administered to conscious rats and samples were collected at 5, 15, 30, 45, 60, 120, 240 and 480 min after dosing. Blood samples were centrifuged at 1,700 × g for 15 min at 4°C, and the supernatants were used as plasma samples. Acetonitrile was added to the plasma samples, and each mixture was centrifuged at 21,500 × g for 10 min. The supernatant was subjected to centrifugal evaporation at 30°C. The residue was dissolved in 0.1 ml of a mixture of 0.05% formic acid in water (pH 3.0) and 0.05% formic acid in methanol (50:50, v/v), and filtered through a 0.45-µm membrane filter. The supernatants were subjected to LC-MS/MS analysis.

Quantitation of peptides by LC-MS/MS: Phe-Ψ-Ala and Phe-Ala were measured by LC-MS/MS. A 10 µL aliquot of each sample was injected into the LC-MS/MS system (API 3000, Applied Biosystems, Carlsbad, CA) for analysis. The HPLC system consisted of a pump, ultraviolet spectrophotometric detector, fluorescence spectrophotometric detector, and data processing system. C18 ODS MGII (3 µm 2.0 mm I.D × 50 mm) and C18 MGG S-3 (2.0 mm I.D. × 10 mm) were used as analytical columns at 50°C. The mobile phase was composed of a mixture of 0.05% formic acid in water (pH 3.0) and 0.05% formic acid in acetonitrile (60:40, v/v) for Phe-Ψ-Ala and a mixture of 0.05% formic acid in water (pH 3.0) and 0.05% formic acid in methanol (50:50, v/v) for Phe-Ala at the flow rate of 0.1 mL/min. In the LC-MS/MS system, the Turbo Ion Spray interface was operated in the positive ion mode at 5,500 V and 500°C. The MS/MS transitions were as follows: Phe-Ψ-Ala (253.0→86.0) and Phe-Ala (237.1→120.2). Analyst software version 1.4.2 was used for data manipulation. The detection limit was 10 nM.

Data analysis: Uptake results are shown as cell-to-medium ratio (microliters per milligram protein), which was obtained by dividing the cellular uptake amount by the initial concentration of test compound in the uptake medium. The PEPT1-mediated uptake rate was obtained by subtracting the uptake in mock cells from that in HeLa/PEPT1 cells. Kinetic parameters were calculated by means of nonlinear least-squares analysis using MULTI. The \( K_m \) of Phe-Ψ-Ala uptake mediated by PEPT1 and its maximal velocity \( V_{max} \) were obtained by fitting the observed data to the following Eq. (1):

\[
v = \frac{V_{max} \times C}{K_m + C}
\]

where \( v \) and \( C \) are the initial uptake rate and concentration of substrate, respectively. All data were expressed as means ± S.E.M., and statistical analysis was performed by the use of Student’s t-test with \( p < 0.05 \) as the criterion of significance.

Results

Uptake of Phe-Ψ-Ala in HeLa/PEPT1 cells: As shown in Figure 2, the uptake of 50 µM Phe-Ψ-Ala in HeLa/PEPT1 cells was significantly higher than that in mock cells at all time points examined. Since the uptake of Phe-Ψ-Ala in HeLa/PEPT1 cells increased linearly up to 2 min (93.3 ± 3.7 µL/mg protein/2 min in HeLa/PEPT1 cells; 80.87 ± 0.82 µL/mg protein/2 min in mock cells), the initial rate was evaluated at 2 min in the following studies. From the concentration dependency, the \( V_{max} \) and \( K_m \) values were obtained as 1.78 ± 0.29 nmol/mg protein/2 min and 275 ± 32 µM, respectively (Fig. 3). The uptake of Phe-Ψ-Ala was highest at pH 6.0 (Fig. 4). As shown in Figure 5, the uptake of Phe-Ψ-Ala was decreased to 3.67 ± 0.31%, 22.7 ± 5.1%, 7.93 ± 3.13%, 0.960 ± 2.463%, and 2.98 ± 0.29% in the presence of Gly-Sar, cephalaxin, valaciclovir, Gly-Gly, and Gly-Pro, respectively.

Stability of Phe-Ψ-Ala in the presence of intestinal homogenerate and hepatic microsomes of rats, and human hepatocytes: To clarify metabolic stability of Phe-Ψ-Ala in rats and humans, the metabolism stability of Phe-Ψ-Ala (50 µM) and Phe-Ala (50 µM) using rat and human samples were investigated. The remaining
amounts of Phe-Ψ-Ala were 95.8 ± 1.3%, 100 ± 18%, and 99.0 ± 14.2% in intestinal homogenates and hepatic microsomes of rats, and human hepatocytes after 60 min, respectively. On the other hand, the corresponding values of Phe-Ala were 15.0 ± 5.1%, and 46.2 ± 7.2%, respectively (data not shown).

**In vivo pharmacokinetic studies:** Figure 6 shows the plasma concentration-time curves of intravenously and orally administered Phe-Ψ-Ala. The pharmacokinetic parameters are listed in Table 1.

### Table 1. Pharmacokinetic parameters of Phe-Ψ-Ala

<table>
<thead>
<tr>
<th>Parameter</th>
<th>i.v. (0.125 mg/kg)</th>
<th>p.o. (1.25 mg/kg)</th>
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<tbody>
<tr>
<td>CL (L/h/kg)</td>
<td>0.151 ± 0.008</td>
<td>—</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>0.235 ± 0.012</td>
<td>—</td>
</tr>
<tr>
<td>t½ (h)</td>
<td>1.14 ± 0.07</td>
<td>—</td>
</tr>
<tr>
<td>AUC (µg/mL·h)</td>
<td>0.811 ± 0.045</td>
<td>4.89 ± 0.56</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>3.74 ± 0.44</td>
<td>2.31 ± 0.60*</td>
</tr>
<tr>
<td>BA (%)</td>
<td>60.8 ± 6.9</td>
<td>53.3 ± 10.1</td>
</tr>
</tbody>
</table>

Each result represents the mean ± S.D. (n = 3 or 4). *Indicates a significant difference from the control (p < 0.05) by Student’s t-test.

**Discussion**

The uptake of Phe-Ψ-Ala by HeLa/PEPT1 cells was over one hundred times higher than that in mock cells (Fig. 2), and the uptake was optimal at pH 6.0 (Fig. 4). A bell-shaped pH profile with maximal activity at pH 5.5–6.0 is consistent with those of uptake of known PEPT1 substrates, such as natural dipeptides (glycylleucine, glycyglutamate, and glycyllysine) and [14C]Gly-Sar,1,18,19. The transport mechanism was reported as binding of H+ to the substrate-binding site, and causing the inhibition of PEPT1 transport.20 Moreover, saturable uptake of Phe-Ψ-Ala was observed with a Km value of 275 µM (Fig. 3). These results confirmed that Phe-Ψ-Ala is a PEPT1 substrate.

Phe-Ψ-Ala was not readily metabolized by peptidases, as expected since it has a thiourea structure unlike natural peptides, whereas Phe-Ala was extensively metabolized. Moreover, since Phe-Ψ-Ala was stable in the intestinal homogenates and hepatic microsomes of rats, and human hepatocytes, it is not expected to undergo general metabolism by metabolic enzymes such as cytochrome P450. Lys-FITC-OCH₃, a fluorescent thiourea compound, has been reported as a non-radiolabeled PEPT1 substrate,21) but the ester structure of this compound is hydrolyzed in Caco-2 cells, with release of Lys-FITC-OH. Therefore, Phe-Ψ-Ala could be more useful as a PEPT1 probe than Lys-FITC-OCH₃.

In *in vivo* pharmacokinetic studies, the Cmax of orally administered Phe-Ψ-Ala in the presence of Gly-Sar was significantly decreased compared with that in the absence of Gly-Sar (Fig. 6), suggesting that Phe-Ψ-Ala was absorbed via PEPT1. In addition, AUC in the presence of Gly-Sar was decreased compared with that in the absence of Gly-Sar, though not significantly. Since Gly-Sar (10 mM) inhibited Phe-Ψ-Ala uptake in HeLa/PEPT1 (Fig. 5), the initial concentration of Gly-Sar (40 mM) was sufficient to inhibit PEPT1 in the rat small intestine. The observed insignificant change might have been caused by rapid absorption and dilution of Gly-Sar in the small intestine. Since the reported fluid volume in the rat...
small intestine was 4.6 mL,\(^2\) administrated Gly-Sar was estimated to be diluted by between 5 and 6 times. Moreover, PEPT1 is abundantly expressed in the upper region of the rat small intestine.\(^2\) These factors might decrease the inhibition effects of Gly-Sar. The \(V_0\) of Phe-\(\text{-}\alpha\)-Ala was 0.235 L/kg, suggesting that this compound was mainly distributed into plasma. These results suggest that the passive permeability of Phe-\(\text{-}\alpha\)-Ala is low, and Phe-\(\text{-}\alpha\)-Ala may be mainly taken up into PEPT1-expressing tissues, such as normal intestine and various cancers.\(^2,3\) On the other hand, the reported \(V_0\) of Gly-Sar in mice was 0.43 L/kg (8.6 mL/body), suggesting that Gly-Sar could be distributed into intracellular space.\(^2\) Therefore, uptake of Gly-Sar may not be specific to PEPT1. In addition, the reported uptake rate of \([^{14}\text{C}]\text{Gly-Sar}\) was about 8 \(\mu\text{L/mg protein}\) (8 times higher than that in mock cells) in the same expression

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