Published in final edited form as:
Mol Pharm. 2013 February 4; 10(2): 477–487. doi:10.1021/mp300364k.

Expression profile and functional activity of peptide transporters in prostate cancer cells

Wanyi Tai, **Zhijin Chen**, and **Kun Cheng***
Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 2464 Charlotte Street, Kansas City, MO 64108, USA

Abstract

Peptide transporters are expressed predominantly in intestinal and renal epithelial cells. The functional expression of peptide transporters is also identified in other types of tissues, such as glia cells, macrophages, and the epithelia of the bile duct, the lungs and the mammary glands. However, their presence and role are poorly understood in carcinomas. We explored the expression profile and functional activity of peptide transporters in the prostate cancer cell lines LNCaP, PC-3 and DU145. Quantitative Real Time PCR (qRT-PCR) and Western blot were used to evaluate the expression profile of peptide transporter 1 (PEPT1), peptide transporter 2 (PEPT2), peptide histidine transporter 1 (PHT1) and peptide histidine transporter 2 (PHT2) in these cells. LNCaP expresses high levels of PEPT2 and PHT1, while PC-3 demonstrates strong expression of PEPT1 and PHT1. DU145 shows only weak expression of PEPT1 and PHT1. Functional activities were studied in these cell lines using radiolabeled Glycylsarcosine ([^{3}H]Gly-Sar) and *L*-Histidine ([^{3}H]*L*-Histidine). The uptake of [^{3}H]Gly-Sar and [^{3}H]*L*-Histidine was time- and pH-dependent. A kinetic study showed that the uptake of Gly-Sar and *L*-Histidine is saturable over the tested concentration range. The binding affinity (K_m) and the maximal velocity (V_{max}) exhibited in the three cell lines were consistent with the expression profiles we observed in qPCR and Western blot analysis. A competitive inhibition study revealed that peptide transporters in prostate cancer cells exhibited broad substrate specificity with a preference for hydrophobic dipeptides, such as Leu-Leu. Fluorescence microscopy revealed that the fluorescent dipeptide probe *D*-Ala-Lys-AMCA (a substrate of peptide transporters) specifically accumulated in the cytoplasm of LNCaP and PC-3, but not DU145 cells. Inhibiting the peptide transporter activity by Gly-Sar suppressed the growth of LNCaP and PC-3 cells. Our study indicated that PC-3 cells can be established as a new cell culture model for PEPT1 study, and LNCaP can be used as a model for PEPT2 study. Moreover, our results suggested that peptide transporters are over-expressed in prostate cancer cells and can be adopted as a promising target for tumor-specific drug delivery.

Keywords

peptide transporter; peptide histidine transporter; Gly-Sar; prostate cancer; PC-3; LNCaP; DU-145

*Corresponding author: Kun Cheng, Ph.D., Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 2464 Charlotte Street, Kansas City, MO 64108, Phone: (816) 235-2425, Fax: (816) 235-5779, chengkun@umkc.edu.

INTRODUCTION

Peptide transporters, also called proton-coupled oligopeptide transporters, are membrane proteins that mediate the cellular uptake of di/tri-peptides and peptidomimetic drugs by utilizing the inwardly directed proton-motive force.[1] In mammalian cells, the peptide transporter family consists of four members, including peptide transporter 1 (PEPT1), peptide transporter 2 (PEPT2), peptide histidine transporter 1 (PHT1) and peptide histidine transporter 2 (PHT2). PEPT1 and PEPT2 are the prototype transporters of this family and have been extensively studied since their discovery in the 1980s.[2–4] In contrast, PHT1 and PHT2 are newly identified peptide transporters, and their physiological roles and functions have not yet been fully defined. PEPT1 is expressed predominantly in the small intestine and mediates transportation of nutrients (di/tri-peptide) or peptidomimetic drugs from the lumen into enterocytes, whereas PEPT2 is strongly expressed in the kidneys and mediates the renal reabsorption of filtrated nutrients and peptidomimetic drugs.[5] Due to its strong expression in the small intestine, PEPT1 has been utilized to enhance the oral absorption of poorly permeable drugs, such as β-lactam antibiotics, angiotensin-converting enzyme inhibitors and peptide prodrugs.[6, 7] The capacity of peptide transporters to transport a broad spectrum of substrates has made them an attractive target for drug delivery.[7]

There has been growing interest in peptide transporters since the discovery of their high expression in cancer cells. Nakanishi et al. first reported that the human fibrosarcoma cell line HT1080 exhibits peptide transport activity.[8] Subsequently, Gonzalez et al. observed overexpression of PEPT1 in the pancreatic carcinoma cell lines AsPc-1 and Capan-2.[9] High expression of PEPT1 in gastric cancer cells has also been reported [10]. These findings suggest that peptide transporters might be expressed in various types of cancer cells. Considering their physiological function, it is reasonable to believe that the over-expression of peptide transporters helps cancer cells with the uptake of nutrients (di-/tri-peptide or *L*-Histidine) and supports the rapid growth of cancer cells.[11] It is also noteworthy that peptide transporters have been considered as an important target for drug delivery. By using peptide transporters in the small intestine, a variety of therapeutic agents have been efficiently transported.[12] The overexpressed peptide transporters in cancer cells therefore provide a specific pathway for therapeutic agents to enter cancer cells. However, although it is promising to utilize peptide transporters for drug delivery in cancer therapy, the expression profile and functional activity of peptide transporters are poorly elucidated in major cancer types, except pancreatic cancer and gastric cancer. Therefore, clarifying their expression patterns in new cancer types will expand the potential values of peptide transporters in cancer therapy.

Prostate cancer is the most common cancer and the second leading cause of death in American men.[13, 14] The expression profiles and functional activity of peptide transporters in prostate cancer cells have not been elucidated. In our previous study, we observed that the dipeptide prodrug Ser-Leu-TGX-D1 exhibited a 3-fold greater cellular uptake when compared to the parent drug TGX-D1 in LNCaP cells. The enhanced cellular uptake indicated that LNCaP cells might express functionally active peptide transporters.[15] In the present study, we examined the gene expression profiles and functional activity of peptide transporters in three of the most commonly used prostate cancer cell lines LNCaP, PC-3 and

DU145. For the first time, we confirmed the presence of peptide transporters *in vitro* in prostate cancer cell lines PC-3 and LNCaP. Our findings demonstrated that LNCaP and PC-3 cells express functionally active peptide transporters at high levels. Moreover, we proved that these peptide transporters facilitate the transportation of the fluorescent dipeptide probe *D*-Ala-Lys-AMCA, and the inhibition of their activity can suppress the growth of prostate cancer cells. These findings indicate the promising potential of peptide transporters for prostate cancer therapy.

EXPERIMENTAL SECTION

Materials

All reagents and solvents listed below were obtained from commercial sources and used without further purification. [^{3}H]Glycylsarcosine (0.2 Ci/mmol) and [^{3}H]*L*-Histidine (30 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA). Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, Cefadroxil, *L*-Histidine, glycylsarcosine (Gly-Sar) and 2-Amino-2-norbornanecarboxylic acid (BCH) were obtained from Sigma-Aldrich (St. Louis, MO). PCR primers were synthesized by Integrated DNA technologies Inc. (Coralville, IA). The fluorescent dipeptide probe *D*-Ala-Lys-AMCA was purchased from Biotrend Chemicals, LLC (Destin, FL). Primary Polyclonal Rabbit anti-PEPT1, Mouse anti-PEPT2 and Rabbit anti-PHT1 antibodies were provided by Abcam (Cambridge, MA). Primary polyclonal Rabbit anti-PHT2 was purchased from Abgent (San Diego, CA). Thiazolyl Blue was obtained from RPI Corp. (Prospect, IL). All of the organic solvents used for synthesis and HPLC were obtained from Fisher Scientific (Pittsburgh, PA).

Cell Culture

All of the cell lines used in this study were obtained from the ATCC. PC-3 cells were grown in RPMI-1640 media containing 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin. LNCaP cells were maintained in RPMI-1640 media supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate. Caco-2, HeLa, and DU145 cells were cultured in DMEM media supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every other day, and the cells were passaged when they reached 80–90% confluency. In the cellular uptake study, LNCaP cells were grown in Poly-*D*-Lysine coated wells to prevent detachment during washing.

Quantitative Real-Time RT-PCR (qRT-PCR, Copy number assay)

Quantification of the mRNA copy number for PEPT1, PEPT2, PHT1 and PHT2 in various cell lines was performed as reported.[16] Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Two hundred nanograms of RNA were converted to cDNA using random hexamer primers and the MultiScribe Reverse Transcriptase Reagent (Applied Biosystems, Inc., Branchburg, NJ). Eighty nanograms of the cDNA were amplified by real time PCR using SYBR Green-1 dye universal Master Mix on a Roche LightCycler 480 system (Roche Diagnostics, Indianapolis, IN). Human genomic DNA (Promega, Madison, WI) served as the universal external standard to quantify the

mRNA expressions of the four peptide transporter genes. Eighty nanograms of mRNA without reverse transcription were also directly amplified by PCR to monitor contamination of genomic DNA in the samples. Samples with no gDNA contamination (<0.3 copy/ng total RNA) were selected for qRT-PCR analysis. The primers for PEPT1, PEPT2, PHT1 and PHT2 were designed to recognize an extron and were synthesized by Integrated DNA technologies Inc. (Coralville, IA). The primers for PEPT1 were 5′-GCAGGATGGCTGCTGACCGT-3′ (forward) and 5′-GCTGAACTGGCCTGCCCCTG-3′ (reverse); for PEPT2, 5′-GCGGGGTCCAGCAGATAAG CAC-3′ (forward) and 5′-ACAGGGCCAGGAATTGGGGTCA-3′ (reverse); for PHT1, 5′-AATGGCGTGCCCACCAGCAG-3′ (forward) and 5′-GCCAGGAGACACGCTGCAGT-3′ (reverse); and for PHT2, 5′-ACATCAACAATTGCCGGATGGACC-3′ (forward) and 5′-ATAGCGTCCAGCGATCCAGACAAA-3′ (reverse). All groups were performed in triplicate, and the data were presented as the mean ± standard deviation (SD).

Western blot

Caco-2 cells were seeded in a 100 mm cell culture dish and cultured for 21 days. After differentiation, the Caco-2 cell monolayer was washed with PBS and lysed for 15 minutes on ice with RIPA buffer (50 mM Tris-cl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate) supplemented with protease inhibitors (Roche's complete protease inhibitor cocktail tablet). The lysate was centrifuged at 12,000 g for 10 minutes, and the supernatant was collected. Other cell lines (HeLa, LNCaP, PC-3 and Du145) were only cultured for two days before lysis. The cell lysate was prepared using the same method as described for Caco-2 cells. After measuring the total protein concentration, 20 μg of lysate was separated on a SDS-PAGE gel (10%) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 2 hours in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) plus 5% non-fat milk. The membrane was then incubated with a primary antibody (Polyclonal Rabbit anti-PEPT1, Mouse anti-PEPT2 and Rabbit anti-PHT1 antibodies, Abcam, Cambridge, MA) overnight at 4°C. After rinsing three times with TBST, the membrane was incubated for 2 hours with the secondary antibody linked with horseradish peroxidase (HRP) (anti-rabbit IgG and anti-mouse IgG, Invitrogen, Grand Island, NY). After washing with the TBST buffer, the membrane was incubated with substrate and the bands were detected using FluorChem HD2 Chemiluminescent imaging system (Alpha Innotech, Santa Clara, CA).

Cellular uptake of [^{3}H]Gly-Sar and [^{3}H]*L*-Histidine

A cellular uptake study was performed on the confluent cell monolayer as described previously.[9, 15] The uptake buffer was HBSS buffer (0.952 mM $CaCl_2$, 5.36 mM KCI, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 25 mM *D*-glucose, and 10 mM HEPES) adjusted to pH 7.4. The uptake experiment was initialized by washing monolayers with pre-warmed HBSS buffer and incubating with 0.5 mL of HBSS buffer containing [^{3}H]Gly-Sar (3 μCi/mL, 15 μM) or [^{3}H]*L*-Histidine (0.5 μCi/mL, 100 μM). After 15 minute incubation at 37°C, uptake was terminated by removing the buffer and washing 5 times with ice-cold HBSS buffer. Cells were lysed overnight in 1 mL of 0.3 M NaOH/0.1% Triton-100, and 0.7 mL of the lysate was mixed with 3 mL of a liquid scintillation cocktail.[17, 18] The radioactivity was measured using a Beckman Multipurpose

Scintillation Counter LS6500 (Beckman Coulter Inc., Brea, CA). Protein concentrations were determined using a Coomassie Protein Assay Reagent (Thermo Scientific, Rockford, IL).

Kinetic study of cellular uptake

To examine the uptake kinetics of Gly-Sar in prostate cancer cells, the uptake was measured in HBSS buffer (pH 7.4) over the concentration range of 62.5–2000 μM. The incubation time for the kinetic study of Gly-Sar is 30 minutes. The active uptake, which is mediated by peptide transporters, was calculated by subtracting the passive uptake from the total uptake. The passive uptake, which represents passive diffusion and nonspecific binding, was measured in the presence of 200 mM unlabeled Gly-Sar or *L*-Histidine. To obtain the kinetic parameters, the active uptake data were fitted to the Michaelis-Menten equation:

$$v=\frac{\mathrm{V_{max}}\,[\mathrm{S}]}{\mathrm{K_m}+[\mathrm{S}]}$$

Where *v* is the velocity of Gly-Sar uptake, V_{max} is the maximal velocity of Gly-Sar uptake, K_m is the Michaelis constant, and S is the Gly-Sar concentration. An Eadie-Hofstee plot was also performed to confirm a single transporter system of Gly-Sar uptake. The Eadie-Hofstee equation is:

$$v=\mathrm{V_{max}}-\mathrm{K_m}\frac{v}{[\mathrm{S}]}$$

Linear and nonlinear regressions were performed using Graphpad Prism 5 (Graphpad software, Inc., La Jolla, CA). The quality of fit was determined by evaluating the correlation coefficient r^2.

The kinetic study of *L*-Histidine uptake was performed in a similar way as that of Gly-Sar, but the concentration range was 125–4000 μM, and the incubation time was reduced to 15 minutes. The active uptake data of *L*-Histidine were also fitted to the Michaelis-Menten equation and the Eadie-Hofstee equation as described above.

Uptake of the fluorescent dipeptide probe *D*-Ala-Lys-AMCA

The uptake experiment was performed according to previous reports.[19–22] Cells were seeded in a 24-well plate one day before the experiment. After aspirating the culture media, cells were washed once with the HBSS buffer. *D*-Ala-Lys-AMCA (1 mM in HBSS) was then incubated with the cells at 37°C for 1 hour. For the inhibition studies, uptake was studied in the presence of 10 mM of the dipeptide Ser-Leu. The uptake study was terminated by rapidly washing the cells three times with a citrate buffer (150 mM NaCl, 15 mM Citric acid, and 3 mM EDTA, adjusted to pH 7.0). The cellular uptake of *D*-Ala-Lys-AMCA (excitation at 350 nm, emission at 460 nm) was examined by epifluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

Anti-proliferation assay

To profile the half maximal inhibitory concentration (IC_{50}) of Gly-Sar and BCH, LNCaP, PC-3 and DU145 cells were plated in 96-well plates (BD Falcon™, BD Biosciences) at a density of 5,000 cells/well. After 24 hours, serial dilutions of the drug (final concentration: 0.047–100 mM) were added and incubated with the cells for 72 hours. Cell survival was evaluated using a MTT assay according to the manufacturer's protocol. IC_{50} was calculated by fitting the concentration-absorbance curve using Graphpad Prism 5 (Graphpad software, Inc., La Jolla, CA).

Knockdown of PEPT1 and PEPT2 expressions by siRNA

Selencer® Select siRNAs targeting PEPT1 and PEPT2 genes were purchased from Life Technologies Corp. (Carlsbad, CA). The sequences of the PEPT1 siRNA are 5′-GCAUCGGAGUAAGGCAUUUtt-3′ (sense) and 5′-AAAUGCCUUACUCCGAUGCct-3′ (antisense). The sequences of the PEPT2 siRNA are 5′-GAUCGGCCUGAGUCUAAUAtt-3′ (sense) and 5′-UAUUAGACUCAGGCCGAUCaa-3′ (antisense). siRNA transfection was conducted as we previously reported.[23, 24] Briefly, cells were seeded in Poly-*D*-Lysine pretreated 24-well plates at a density of 7.0×10^4 cells/well 12 hr before transfection. The transfection mixture was prepared by mixing siRNAs and Lipofectamine-2000 in Opti-MEM®-I medium (Invitrogen) and incubating at room temperature for 25 min. The culture medium was replaced with 400 μL of Opti-MEM®-I medium, and 50 μL of the complex was then added into each well to product a final siRNA concentration of 50 nM siRNA per well. After 12 hr incubation, 500 μL complete cell culture medium containing 10% FBS was added to each well, and the cells were incubated for another 24 hr. The cells were then harvested for RNA isolation using Tri reagent (Molecular Research Center, Inc., Cincinnati, OH). The gene knockdown effect was measured by real-time RT-PCR. 18S ribosomal RNA was used as an internal control, and the primers are 5′-GTCTGTGATGCCCTTAGATG-3′ (forward) and 5′-AGCTTATGACCCGCACTTAC-3′ (reverse). The primers used for the PEPT1 gene are 5′-ATTGTGTTTGTCCTTGGCAGTGGG-3′ (forward) and 5′-TTTCTCTTTAGCCCAGTCCAGCCA-3′ (reverse). The primers for the PEPT2 gene are 5′-TGGCAGATGATGAGGTGAAGGTGA-3′ (forward) and 5′-TCTCCTGCACAGAATGCTCAGTGT-3′ (reverse).

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Differences between any two groups were determined by ANOVA. A p value of <0.05 was considered statistically significant.

RESULTS

Gene expressing profiles of peptide transporters in prostate cancer cell lines

A quantitative determination of mRNA levels in prostate cancer cell lines was performed using qRT-PCR. A differentiated Caco-2 cell line was used as the positive control and HeLa cells were used as the negative control.[25, 26] The expression levels of four subtypes of

peptide transporters were evaluated by measuring the mRNA copy numbers in one nanogram of total RNA (copy/ng). As illustrated in Table 1, Caco-2 cells showed the highest mRNA expression level of PEPT1, followed by PC-3 cells. The copy number of PEPT1 mRNA in Caco-2 cells (~37.4 copy/ng) is 3.6 times greater than what is found in PC-3 cells (~9.9 copy/ng) and more than 90 times greater than the negative cell lines HeLa and LNCaP. Expression of PEPT2 mRNA was observed in LNCaP, PC-3 and DU145 cells with the levels at 8.5, 6.1 and 2.4 copy/ng. LNCaP had the highest PEPT2 mRNA expression level of all three prostate cancer cell lines. It is slightly lower than what is found in Caco-2 cells (10.6 copy/ng), but much greater than HeLa (~2.9 copy/ng) and DU145 cells (~2.4 copy/ng). PHT1 and PHT2 are relatively new subtypes of peptide transporters and their expression profiles were also investigated using qRT-PCR. The expression levels of PHT1 mRNA in all five cell lines are similar (5–8 copy/ng). As for PHT2, PC-3 cells showed higher mRNA expression (~17 copy/ng) than other cell lines (4–8 copy/ng). The finding is partially different from a previous study, in which the authors stated that the mRNA expression levels of PEPT1 and PEPT2 were negligible *in vitro* in prostate PC-3 cell line but clearly detected *in vivo* in PC-3 xenograft.[27] The discrepancy between these *in vitro* results could be attributed to the different quantitative methods and external standards used in the mRNA copy number study. Instead of using probe-based real-time PCR (hybridization probe sets: Fluorescent probe and LCRed640 probe) in Mitsuoka's study, we used SYBR Green I as the dye to quantify the PCR amplification of target genes. In addition, we used genomic DNA as the universal external standard to calculate the absolute copy numbers of target genes. In contrast, Mitsuoka et al. did not mention which universal external standard was used in their study.[27]

To assess the protein expression profile, a Western blot was performed to detect PEPT1, PEPT2 and PHT1 proteins in total cell lysates. The blot confirmed that PEPT1 is expressed at a high level in Caco-2 and PC-3 cells (Figure 1). PEPT2 showed similar protein expression profile to that suggested by qRT-PCR. LNCaP cells had the highest expression level in the three prostate cancer cell lines, followed by PC-3 cells. DU145 expressed a negligible amount of PEPT2 protein. All the prostate cancer cell lines tested expressed PHT1 protein, especially the LNCaP and PC-3 cell lines. Although DU145 cells had the lowest expression of PHT1 protein of the three prostate cancer cell lines, the expression was much greater than what is found in Caco-2 and HeLa cells. PHT2 is a relatively new-identified peptide transporter, and there is only one anti-human PHT2 antibody available. However, we did not observe any PHT2 protein band in all samples in three independent experiments. Because this is the only anti-human PHT2 antibody in the market and we cannot validate the result with other anti-human PHT2 antibodies, we do not know whether this is because of the antibody itself or there is no PHT2 protein expression in these cell lines. Therefore, we did not include the PHT2 western blot result in this report.

Time course of [^{3}H]Gly-Sar and [^{3}H]*L*-Histidine uptake by prostate cancer cell lines

The time course of Gly-Sar and *L*-Histidine uptake in three prostate cell lines was studied first. As observed from the data in Figure 2A, the uptake of 15 μM of Gly-Sar was linear during the first 60 minutes in all three prostate cancer cell lines. Steady-state uptake was observed at 120 minutes in PC-3 cells. In contrast to PC-3, the uptake of LNCaP and DU145

did not reach plateau even at 240 minutes. Because the uptake of Gly-Sar was linear over the 60 minute incubation period in all three cell lines, the incubation time for all of the subsequent experiments was set at this linear range. The uptake of 100 μM of *L*-Histidine was also linear in the first 30 minutes of the experiment, but steady-state uptake was achieved at approximately 60 minutes in all three cell lines. On the basis of these results, the uptake of *L*-Histidine was determined at 15 minutes in subsequent experiments.

pH dependency of uptake

The effect of extracellular pH on the cellular uptake of Gly-Sar and *L*-Histidine was measured. As shown in Figure 2B, the uptake of Gly-Sar was pH dependent. The maximal uptake of Gly-Sar was observed at pH 6.0 in LNCaP cells and pH 6.5 in PC-3 cells. The pH dependency of Gly-Sar uptake on these two cell lines was similar to that observed for PEPT1 positive HT1080 cells and PEPT2 positive mouse astrocytes.[8, 28] The pH dependency of Gly-Sar uptake indicated that PEPT1 and PEPT2 acted as proton-coupled dipeptide transporters, and the inward proton gradient facilitated the uptake of Gly-Sar in LNCaP and PC-3 cells.[5] No significant pH dependency of Gly-Sar uptake was observed in DU145 cells. This is consistent with our observation that the expression levels of PEPT1 and PEPT2 were low in this cell line (Table 1 & Figure 1).

Uptake of *L*-Histidine also demonstrated pH dependency in the three prostate cancer cell lines (Figure 2B). Maximal uptake was observed at pH 6.5 in PC-3 cells and DU145 cells and at pH 6.0 in LNCaP cells. Compared to the pH dependency of Gly-Sar uptake, pH has a much weaker influence on the uptake of *L*-Histidine. The pH dependency observed in *L*-Histidine uptake suggested that PHT1 could operate in a similar manner and be driven by a similar proton-gradient force as PEPT1/PEPT2.[5]

Concentration dependency of uptake and kinetic analysis

To determine the kinetic parameters of Gly-Sar uptake in the three prostate cancer cell lines, the concentration-dependent uptake rate was investigated at pH 7.4 over the substrate concentration range of 62.5–2000 μM. The active uptake, which is mediated by peptide transporters, was calculated by subtracting the passive uptake from the total uptake. The passive uptake, which represents passive diffusion and nonspecific binding, was measured in the presence of 200 mM unlabeled Gly-Sar. The excess amount of unlabeled Gly-Sar can efficiently suppress active uptake, but minimally affect diffusion and nonspecific binding.[9, 29] As shown in Figure 3A, the passive uptake was linear over the entire concentration range in all three cell lines. In contrast, the active uptake was found to be hyperbolic over the concentration range (62.5–2000 μM), indicating the saturability of the peptide transporter system in LNCaP and PC-3 cell lines. The kinetic parameters were calculated by fitting the active uptake data into the Michaelis-Menten equation using non-linear regression. The LNCaP peptide transporter system exhibited a K_m of 871.2±201.5 μM and a V_{max} of 620.5±64.5 pmol/mg protein/30 min, whereas peptide transporters in PC-3 had a K_m of 603.0±136.7 μM and a V_{max} of 917.9±85.0 pmol/mg protein/30 min. A very weak active uptake was observed in the DU145 cell line (Figure 3A & Table 2). To further characterize the peptide transporter system, the active uptake data were converted into the Eadie-Hofstee plot (V vs V/S). The straight lines were obtained in both the LNCaP

(r^2=0.857) and PC-3 (r^2=0.913) groups, indicating a single transporter system for Gly-Sar uptake (Figure 4).

The kinetic parameters of *L*-Histidine uptake were determined using the same method as that of Gly-Sar. The active uptake of *L*-Histidine was also shown as a hyperbolic curve, and the passive uptake is linear over the entire concentration range (Figure 3B). The transporter systems exhibited K_m values of 4059.0±615.4 μM, 811.7±149.9 μM and 423.4±91.1 μM in LNCaP, PC-3 and DU145, respectively. The maximum uptake rate (V_{max}) obtained for *L*-Histidine uptake was 104.4±9.6 nmol/mg protein/15 min in LNCaP, 48.5±3.3 nmol/mg protein/15 min in PC-3 and 20.1±1.3 nmol/mg protein/15 min in DU145. As observed in Figure 4, Eadie-Hofstee plots calculated from the active uptake of *L*-Histidine were curvilinear, hence supporting a hypothesis of uptake by two transporter systems in all three cell lines.

Uptake of *L*-Histidine is mediated by two transporter systems

Although both PHT1 and PHT2 have the ability to transport *L*-Histidine, PHT1 was believed to play a major role in the cellular uptake of *L*-Histidine. PHT1 has been proven to be located in the plasma membrane of transiently transfected COS-7 cells.[30] Its active role in cellular uptake of *L*-Histidine has been reported in several studies.[30, 31] In contrast, expression of PHT2 protein was not found on cell membrane, but on lysosomal membrane, suggesting its role in lysosomes function rather than nutrient uptake.[32] In addition to the peptide histidine transporter system, an amino acid transporter system might also mediate the uptake of *L*-Histidine in prostate cancer cells. The Eadie-Hofstee plot has already suggested that multiple *L*-Histidine transporter systems exist in the three prostate cancer cell lines. For further verification, the competitive inhibition of *L*-Histidine uptake was conducted in the presence of various amino acids (competitive inhibitors of amino acid transporters). The uptake of *L*-Histidine can be significantly inhibited by a neutral amino acid *L*-Alanine (Ala), an acidic amino acid *L*-Aspartic acid (Asp), a basic amino acid *L*-Lysine (Lys) and a non-natural amino acid BCH. As shown in Figure 5A, *L*-Alanine showed the best inhibition in LNCaP cells, and BCH exhibited the best inhibitory effect in PC-3 and DU145 cells. Overall, *L*-Alanine demonstrated strong competitive inhibition in all three cell lines, with 42.8% in LNCaP, 45.6% in PC-3 and 49.2% in DU145 cells. This is likely because *L*-Alanine and *L*-Histidine possessed similar molecular geometry and charge, and both have been identified as the predominant substrates of many amino acid transporters, such as sodium-coupled neutral amino acid (system N/A) transporters (SNAT1, SNAT2 and SNAT3).[33] Additionally, synergistic inhibition of *L*-Histidine uptake was observed when the amino acid transporter and the peptide histidine transporters were simultaneously inhibited by *L*-Alanine and Leu-Leu (Ala + LL in Figure 5B). The effect of the synergistic inhibition was significantly higher than those of separate inhibitions and similar to that of *L*-Histidine (self-inhibition, which can also inhibit both transporter systems). In addition, different levels of synergistic effects were observed in the three prostate cancer cell lines. LNCaP and PC-3 cells exhibited a stronger synergistic effect than DU145 cells, possibly due to the high expression level of PHT1 in LNCaP and PC-3 cells (Figure 1).

Substrate specificity of Gly-Sar and *L*-Histidine uptake

The uptake specificity of the three prostate cancer cell lines was investigated by determining the inhibition of Gly-Sar and *L*-Histidine uptake in the presence of transporter inhibitors. As listed in Table 3, the uptake of Gly-Sar was dramatically inhibited by various dipeptides in LNCaP and PC-3 cells. Ser-Leu and Leu-Leu are two extremely potent inhibitors in these two cell lines and both can reduce the uptake of Gly-Sar to ~30% and ~60% in LNCaP and PC-3, respectively. Similar to a previous report,[27] cellular uptake of [^{3}H]Gly-Sar (15 μM) in PC-3 cells was slightly inhibited (~15%) by excessive amount of unlabeled Gly-Sar. The pseudopeptide drug Cefadroxil, a 31 β-lactam antibiotic, also inhibited the uptake of Gly-Sar to ~38% in LNCaP cells, but no significant effect was observed on PC-3 cells. This is most likely because Cefadroxil could be recognized and transported by PEPT2 with a much higher affinity than by PEPT1 [34]. Peptide transporters were specific to di/tri-peptides in our study. The tetrapeptide Gly-Gly-Gly-Gly had a minimal inhibitory effect on the uptake of Gly-Sar. The specificity of *L*-Histidine uptake was also studied using the same inhibitors. In addition to *L*-Histidine itself, Ser-Leu and Leu-Leu were the two most potent inhibitors. Both reduced the uptake to 30–50% of control in LNCaP and PC-3 cells, and to 84–86% in DU145 cells. The variation of the inhibitory potency was mainly due to the difference of PHT1 expression levels in the three cell lines.

Visualization of peptide transport in prostate cancer cells by a fluorescent dipeptide probe

The fluorescent dipeptide *D*-Ala-Lys-AMCA is a widely used fluorescent probe to monitor the activity of peptide transporters.[35, 36] It has been proven to be a specific substrate of both PEPT1 and PEPT2.[22, 36] This fluorescent dipeptide probe could be advantageously used to follow the uptake of the compound either by fluorescence microscopy or by fluorescence plate readers. As shown in Figure 6, the visible accumulation of the fluorescent dipeptide probe was observed in both LNCaP and PC-3 cells, but not in DU145 cells. Differences in the intensity of *D*-Ala-Lys-AMCA staining were consistent with the gene expression profiles of peptide transporters in LNCaP, PC-3 and DU145. The uptake of the fluorescent probe can be effectively blocked by the dipeptides Ser-Leu and Leu-Leu.

Inhibition of peptide transporters suppresses prostate cancer cell growth

To examine the role of peptide transporters in the growth of prostate cancer cells, serial concentrations of the non-degradable dipeptide Gly-Sar (0.1–100 mM) were incubated with prostate cancer cells, and IC_{50} was determined after 72 hours using the MTT assay. The inhibition of peptide transporters by Gly-Sar dramatically suppresses the growth of LNCaP and PC-3 cells, but not DU145 cells. Gly-Sar displays IC_{50} values of 9.5 mM in LNCaP cells and 13.9 mM in PC-3 cells (Figure 7). No significant inhibition occurred in DU145 cells at low concentrations (< 33 mM), but a minor inhibition was observed at high concentration (100 mM). BCH, a non-metabolizable inhibitor of system *L* amino acid transporters, was used as a positive control. Blockage of amino acid uptake resulted in a depletion of ATP, which causes a cytotoxic effect in all cell lines.[37] BCH displayed similar IC_{50} values (~10 mM) and anti-proliferation potency (up to 20–30% of the control) as all three prostate cancer cell lines (Figure 7). When prostate cancer cells were treated with Gly-Sar and BCH simultaneously, no combinational anti-proliferation effect was observed when

compared to BCH alone (Data not shown). This may be because peptide transporters and amino acid transporters perform the same physiological function (nutrient uptake) in cell growth. Therefore, the anti-proliferation effect of Gly-Sar and BCH is not additive.

Knockdown of PEPT1 and PEPT2 genes reduces the transporter activity

We finally investigated whether knockdown of PEPT1 and PEPT2 can affect dipeptide uptake in PC-3 and LNCaP cells (Figure 8). *Selencer*® Select siRNAs targeting PEPT1 and PEPT2 genes were used to knockdown PEPT1 and PEPT2 in the cells. As shown in Figure 8, treatment of the cells with these siRNAs knockdown ~74% of PEPT1 in PC-3 cells and ~66% of PEPT2 in LNCaP cells respectively.

[^{3}H]Gly-Sar uptake (15 μM, 3 μCi/ml) was then measured at pH 7.4 in the PEPT1 and PEPT2 knockdown cells respectively. Active uptake was calculated by subtracting passive uptake from total uptake. After knockdown of the PEPT1 gene, the active uptake of [^{3}H]Gly-Sar in PC-3 cells was accordingly reduced by 47% compared with the control group (Figure 8B). The Gly-Sar transport activity in LNCaP cells was also reduced by 50% after the PEPT2 gene was silenced by the PEPT2 siRNA (Figure 8D). These results indicate that PEPT1 is the major transporter for Gly-Sar uptake in PC-3 cells and PEPT2 plays the dominant role in LNCaP cells. Our study also suggest that PC-3 can be established as a cell culture model for PEPT1 study and LNCaP as a model for PEPT2 study.

DISSCUSION

Peptide transporters can transport di- or tri-peptides, peptidomimetic drugs and peptide prodrugs into the cytoplasm of cells. They have been extensively characterized and studied since their discovery two decades ago.[2–4] Although peptide transporters are expressed predominantly in the intestinal and renal epithelial cells, functional expression of peptide transporters is also identified in other normal tissues, such as glia cells, macrophages, and the epithelia of the bile duct, the lungs, and the mammary glands.[5] However, little information is available regarding their presence and role in carcinomas. In this study, we have examined the expression profile and functional activity of peptide transporters in LNCaP, PC-3 and DU145 cells. Although these three cell lines exhibited different expression profiles of peptide transporters, transport activity was observed on the plasma membrane of all three cell lines. The uptakes of Gly-Sar and *L*-Histidine proceeded in a time- and pH-dependent manner (Figure 2). The uptake rates were concentration-dependent, and Michaelis-Menten fitting of the active uptake data gave hyperbolic curves which indicated the saturability of the peptide transporters. Further studies revealed that the uptake of Gly-Sar in LNCaP and PC-3 is mediated by a single transporter system, but multiple transporter systems were involved in *L*-Histidine uptake (Figure 4&5). In addition, the competitive inhibition study proven that the peptide transporters in the prostate cancer cells exhibited broad substrate specificity with a preference for hydrophobic dipeptides such as Leu-Leu. These results strongly indicate that prostate cancer cells express functionally active peptide transporters on the cytoplasmic membrane.

Because of the pharmaceutical interest and pharmacokinetic importance, peptide transporters have been commonly used for the *in vitro* evaluation of prodrugs.[38] In *in vitro*

cell culture model systems, Caco-2 cells and SKPT cells have been widely used for studying PEPT1 and PEPT2 mediated transport.[39] However, Caco-2 needs to grow for 21 days to differentiate into PEPT1 positive cells before they can be used in transport assays.[25] The long differentiation time (~21 Days) dramatically limits the use of Caco-2 cell culture model. SKPT cells are currently the standard cell culture model for PEPT2 studies.[34] As a rat renal cell line, SKPT expresses rat PEPT2, which only shares 83% amino acid identity with human PEPT2.[40] Our study indicates that PC-3 can be established as a new cell culture model for PEPT1 study and LNCaP as a model for PEPT2 study (Figure 8). As common carcinoma cell lines, PC-3 and LNCaP can be easily obtained and maintained. Moreover, the protein expression of PEPT1 and PEPT2 are continuous, and the cell culture models are ready to use after overnight culture. Lastly, LNCaP cells express natural human PEPT2, which makes it a better model than SKPT cells for PEPT2 study.

In addition to distinguishing PC-3 and LNCaP cells as *in vitro* models for peptide transporter study, the most important impact of our research is revealing the promising potential of peptide transporters in tumor-specific drug delivery. Because of the high expression in intestinal epithelial cells, peptide transporters have been widely utilized to enhance oral absorption of polar drugs by a prodrug approach. However, peptide transporters have not been considered as a specific target for tumor-specific drug delivery. The high expression of peptide transporters on prostate cancer cells provides a new approach for selectively targeting low-molecule anti-cancer drugs to cancer cells through the peptide transporters. Unlike ligand-mediated targeting, low molecular-weight anti-cancer dipeptide prodrugs can function as substrates of peptide transporters to enter into the cytoplasm of prostate cancer cells.[41] Peptide transporters provide a putative uptake pathway to selectively accumulate anti-cancer prodrugs in prostate cancer cells. However, the abundant expression of peptide transporters in the intestine, kidney and other normal tissues might limit their use as a target for drug delivery. Intravenous injection can circumvent some unwanted uptake by epithelial cells in the small intestine, but anti-cancer dipeptide prodrugs may still be taken up by renal and other non-cancer cells.[41] Therefore, it is essential to site-specifically deliver the dipeptide prodrug into the tumor where it could be taken up by cancer cells via peptide transporters. Dipeptide prodrugs would first be specifically delivered into prostate tumors by active targeting or passive targeting via the enhanced permeability and retention (EPR) effect. After accumulation in the interstitial space of the tumor, the dipeptide prodrug would be actively taken up by cancer cells via peptide transporters.[15] Further studies will be needed to evaluate the therapeutic effect of this system *in vivo*.

It is interesting that inhibition of peptide transporters by Gly-Sar suppresses prostate tumor cell growth to some extent (Figure 7). The anti-proliferation activity of Gly-Sar is possibly achieved by blocking the supply of nutrients mediated by the peptide transporters. However, it may be difficult to utilize peptide transporters as drug targets for cancer therapy. First, targeting peptide transporters may induce side effects in other tissues because of the wide distribution and important physiological roles of peptide transporters in the intestine and kidney. It has been reported that animals exhibited a remarkable phenotype of malnutrition when PEPT2 was knocked out. The long-term malnutrition in PEPT2-deficient animals also leads to retarded development and small brood size compared to wild-type animals.[42]

Second, the anti-tumor effect of Gly-Sar is weak (IC_{50} ~ 10 mM). Another concern is that tumor cells might rely on multiple routes, such as peptide transporter, amino acid transporters, albumin transporters and passive diffusion to absorb nutrients.[43, 44] Simply blocking the peptide transporters may not be efficient enough to suppress tumor cell growth.

In summary, we have demonstrated that peptide transporters exist in three common prostate cancer cells using qRT-PCR and Western blot. A cellular uptake study of Gly-Sar and *L*-Histidine demonstrated that peptide transporters are functional on the membrane surface of prostate cancer cells. The transport activity observed was also consistent with the expression profiles of peptide transporters. Furthermore, the transportation events were monitored in three prostate cancer cell lines using the fluorescent dipeptide probe *D*-Ala-Lys-AMCA. Inhibition of peptide transporter activity suppresses prostate cancer cell growth, which is consistent with observations from another PEPT1 overexpressing cancer cell line AsPC-1.[11] The selective expression of PEPT1 in PC-3 and PEPT2 in LNCaP indicate that these cell lines can be used as new *in vitro* cell culture models for PEPT1 and PEPT2 studies. The discovery of their over-expression in prostate cancer also validates the potential of these peptide transporters for tumor-targeted drug delivery.

Acknowledgments

This project has been supported by an award (NIH 1R21CA143683-01) from the National Cancer Institute (NCI).

References

1. Kottra G, Daniel H. Bidirectional electrogenic transport of peptides by the proton-coupled carrier PEPT1 in Xenopus laevis oocytes: its asymmetry and symmetry. J Physiol. 2001; 536(Pt 2):495–503. [PubMed: 11600684]
2. Silbernagl S, Ganapathy V, Leibach FH. H+ gradient-driven dipeptide reabsorption in proximal tubule of rat kidney. Studies in vivo and in vitro. Am J Physiol. 1987; 253(3 Pt 2):F448–57. [PubMed: 3631280]
3. Miyamoto Y, Ganapathy V, Leibach FH. Proton gradient-coupled uphill transport of glycylsarcosine in rabbit renal brush-border membrane vesicles. Biochem Biophys Res Commun. 1985; 132(3): 946–53. [PubMed: 4074356]
4. Barfuss DW, Ganapathy V, Leibach FH. Evidence for active dipeptide transport in isolated proximal straight tubules. Am J Physiol. 1988; 255(1 Pt 2):F177–81. [PubMed: 3293467]
5. Daniel H, Kottra G. The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. Pflugers Arch. 2004; 447(5):610–8. [PubMed: 12905028]
6. Takahashi K, Nakamura N, Terada T, Okano T, Futami T, Saito H, Inui KI. Interaction of beta-lactam antibiotics with H+/peptide cotransporters in rat renal brush-border membranes. J Pharmacol Exp Ther. 1998; 286(2):1037–42. [PubMed: 9694966]
7. Rubio-Aliaga I, Daniel H. Mammalian peptide transporters as targets for drug delivery. Trends Pharmacol Sci. 2002; 23(9):434–40. [PubMed: 12237156]
8. Nakanishi T, Tamai I, Sai Y, Sasaki T, Tsuji A. Carrier-mediated transport of oligopeptides in the human fibrosarcoma cell line HT1080. Cancer Res. 1997; 57(18):4118–22. [PubMed: 9307302]
9. Gonzalez DE, Covitz KM, Sadee W, Mrsny RJ. An oligopeptide transporter is expressed at high levels in the pancreatic carcinoma cell lines AsPc-1 and Capan-2. Cancer Res. 1998; 58(3):519–25. [PubMed: 9458100]
10. Inoue M, Terada T, Okuda M, Inui K. Regulation of human peptide transporter 1 (PEPT1) in gastric cancer cells by anticancer drugs. Cancer Lett. 2005; 230(1):72–80. [PubMed: 16253763]

11. Mitsuoka K, Kato Y, Miyoshi S, Murakami Y, Hiraiwa M, Kubo Y, Nishimura S, Tsuji A. Inhibition of oligopeptide transporter suppress growth of human pancreatic cancer cells. Eur J Pharm Sci. 2010; 40(3):202–8. [PubMed: 20307658]
12. Terada T, Inui K. Peptide transporters: structure, function, regulation and application for drug delivery. Curr Drug Metab. 2004; 5(1):85–94. [PubMed: 14965252]
13. Tai W, Mahato R, Cheng K. The role of HER2 in cancer therapy and targeted drug delivery. J Control Release. 2010; 146(3):264–75. [PubMed: 20385184]
14. Qin B, Tai W, Shukla RS, Cheng K. Identification of a LNCaP-specific binding peptide using phage display. Pharm Res. 2011; 28(10):2422–34. [PubMed: 21611873]
15. Tai W, Shukla RS, Qin B, Li B, Cheng K. Development of a peptide-drug conjugate for prostate cancer therapy. Mol Pharm. 2011; 8(3):901–12. [PubMed: 21510670]
16. Yun JJ, Heisler LE, Hwang II, Wilkins O, Lau SK, Hyrcza M, Jayabalasingham B, Jin J, McLaurin J, Tsao MS, Der SD. Genomic DNA functions as a universal external standard in quantitative real-time PCR. Nucleic Acids Res. 2006; 34(12):e85. [PubMed: 16840529]
17. Patel M, Vadlapatla RK, Shah S, Mitra AK. Molecular expression and functional activity of sodium dependent multivitamin transporter in human prostate cancer cells. Int J Pharm. 2012; 436(1–2):324–331. [PubMed: 22732670]
18. Jwala J, Vadlapatla RK, Vadlapudi AD, Boddu SH, Pal D, Mitra AK. Differential expression of folate receptor-alpha, sodium-dependent multivitamin transporter, and amino acid transporter (B (0, +)) in human retinoblastoma (Y-79) and retinal pigment epithelial (ARPE-19) cell lines. J Ocul Pharmacol Ther. 2012; 28(3):237–44. [PubMed: 22304562]
19. Zimmermann M, Stan AC. PepT2 transporter protein expression in human neoplastic glial cells and mediation of fluorescently tagged dipeptide derivative beta-Ala-Lys-Nepsilon-7-amino-4-methyl-coumarin-3-acetic acid accumulation. J Neurosurg. 2010; 112(5):1005–14. [PubMed: 19612975]
20. Otto C, Bauer K. Dipeptide uptake: a novel marker for testicular and ovarian macrophages. Anat Rec. 1996; 245(4):662–7. [PubMed: 8837724]
21. Otto C, tom Dieck S, Bauer K. Dipeptide uptake by adenohypophysial folliculostellate cells. Am J Physiol. 1996; 271(1 Pt 1):C210–7. [PubMed: 8760048]
22. Dieck ST, Heuer H, Ehrchen J, Otto C, Bauer K. The peptide transporter PepT2 is expressed in rat brain and mediates the accumulation of the fluorescent dipeptide derivative beta-Ala-Lys-Nepsilon-AMCA in astrocytes. Glia. 1999; 25(1):10–20. [PubMed: 9888294]
23. Tai W, Qin B, Cheng K. Inhibition of breast cancer cell growth and invasiveness by dual silencing of HER-2 and VEGF. Mol Pharm. 2010; 7(2):543–56. [PubMed: 20047302]
24. Qin B, Cheng K. Silencing of the IKKepsilon gene by siRNA inhibits invasiveness and growth of breast cancer cells. Breast Cancer Res. 2010; 12(5):R74. [PubMed: 20863366]
25. Buyse M, Berlioz F, Guilmeau S, Tsocas A, Voisin T, Peranzi G, Merlin D, Laburthe M, Lewin MJ, Roze C, Bado A. PepT1-mediated epithelial transport of dipeptides and cephalexin is enhanced by luminal leptin in the small intestine. J Clin Invest. 2001; 108(10):1483–94. [PubMed: 11714740]
26. Ganapathy ME, Brandsch M, Prasad PD, Ganapathy V, Leibach FH. Differential recognition of beta -lactam antibiotics by intestinal and renal peptide transporters, PEPT 1 and PEPT 2. J Biol Chem. 1995; 270(43):25672–7. [PubMed: 7592745]
27. Mitsuoka K, Miyoshi S, Kato Y, Murakami Y, Utsumi R, Kubo Y, Noda A, Nakamura Y, Nishimura S, Tsuji A. Cancer detection using a PET tracer, 11C-glycylsarcosine, targeted to H+/peptide transporter. J Nucl Med. 2008; 49(4):615–22. [PubMed: 18344442]
28. Wada M, Miyakawa S, Shimada A, Okada N, Yamamoto A, Fujita T. Functional linkage of H+/peptide transporter PEPT2 and Na+/H+ exchanger in primary cultures of astrocytes from mouse cerebral cortex. Brain Res. 2005; 1044(1):33–41. [PubMed: 15862787]
29. Knutter I, Rubio-Aliaga I, Boll M, Hause G, Daniel H, Neubert K, Brandsch M. H+-peptide cotransport in the human bile duct epithelium cell line SK-ChA-1. Am J Physiol Gastrointest Liver Physiol. 2002; 283(1):G222–9. [PubMed: 12065310]

30. Bhardwaj RK, Herrera-Ruiz D, Eltoukhy N, Saad M, Knipp GT. The functional evaluation of human peptide/histidine transporter 1 (hPHT1) in transiently transfected COS-7 cells. Eur J Pharm Sci. 2006; 27(5):533–42. [PubMed: 16289537]

31. Yamashita T, Shimada S, Guo W, Sato K, Kohmura E, Hayakawa T, Takagi T, Tohyama M. Cloning and functional expression of a brain peptide/histidine transporter. J Biol Chem. 1997; 272(15):10205–11. [PubMed: 9092568]

32. Sakata K, Yamashita T, Maeda M, Moriyama Y, Shimada S, Tohyama M. Cloning of a lymphatic peptide/histidine transporter. Biochem J. 2001; 356(Pt 1):53–60. [PubMed: 11336635]

33. Mackenzie B, Erickson JD. Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. Pflugers Arch. 2004; 447(5):784–95. [PubMed: 12845534]

34. Luckner P, Brandsch M. Interaction of 31 beta-lactam antibiotics with the H+/peptide symporter PEPT2: analysis of affinity constants and comparison with PEPT1. Eur J Pharm Biopharm. 2005; 59(1):17–24. [PubMed: 15567297]

35. Spence JR, Mayhew CN, Rankin SA, Kuhar MF, Vallance JE, Tolle K, Hoskins EE, Kalinichenko VV, Wells SI, Zorn AM, Shroyer NF, Wells JM. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature. 2011; 470(7332):105–9. [PubMed: 21151107]

36. Groneberg DA, Doring F, Eynott PR, Fischer A, Daniel H. Intestinal peptide transport: ex vivo uptake studies and localization of peptide carrier PEPT1. Am J Physiol Gastrointest Liver Physiol. 2001; 281(3):G697–704. [PubMed: 11518682]

37. Nawashiro H, Otani N, Shinomiya N, Fukui S, Ooigawa H, Shima K, Matsuo H, Kanai Y, Endou H. L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors. Int J Cancer. 2006; 119(3):484–92. [PubMed: 16496379]

38. Brandsch M, Knutter I, Bosse-Doenecke E. Pharmaceutical and pharmacological importance of peptide transporters. J Pharm Pharmacol. 2008; 60(5):543–85. [PubMed: 18416933]

39. Sugawara M, Huang W, Fei YJ, Leibach FH, Ganapathy V, Ganapathy ME. Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters PEPT1 and PEPT2. J Pharm Sci. 2000; 89(6):781–9. [PubMed: 10824137]

40. Saito H, Terada T, Okuda M, Sasaki S, Inui K. Molecular cloning and tissue distribution of rat peptide transporter PEPT2. Biochim Biophys Acta. 1996; 1280(2):173–7. [PubMed: 8639691]

41. Mrsny RJ. Oligopeptide transporters as putative therapeutic targets for cancer cells. Pharm Res. 1998; 15(6):816–8. [PubMed: 9647344]

42. Barbara Meissner, GC.; Boll, Michael; Daniel, Hannelore; Baumeister, Ralf. The knockout of the peptide transporter gene pep-2 results in delayed development and extended life-span in Caenorhabditis elegans. International C. elegans Meeting; 2001.;

43. Edinger AL. Controlling cell growth and survival through regulated nutrient transporter expression. Biochem J. 2007; 406(1):1–12. [PubMed: 17645414]

44. Francis GL. Albumin and mammalian cell culture: implications for biotechnology applications. Cytotechnology. 2010; 62(1):1–16. [PubMed: 20373019]

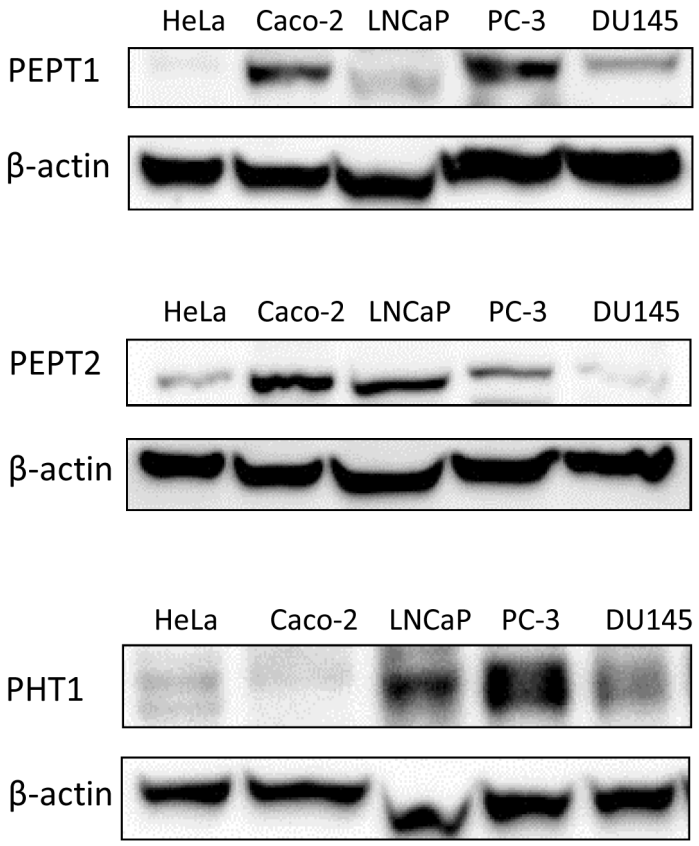

Figure 1.

Expression profiles of PEPT1, PEPT2 and PHT1 proteins in five cell lines. Cell lysates of HeLa, Caco-2 (after 21-day differentiation), LNCaP, PC-3 and DU145 cells (orresponding to Lane 1–5) were analyzed by Western blot. Beta-actin was used as an internal control for equal loading. HeLa cells were used as a negative control, and no signal could be spotted at corresponding sites. Caco-2 was used as a positive control, and both PEPT1 and PEPT2 could be detected.

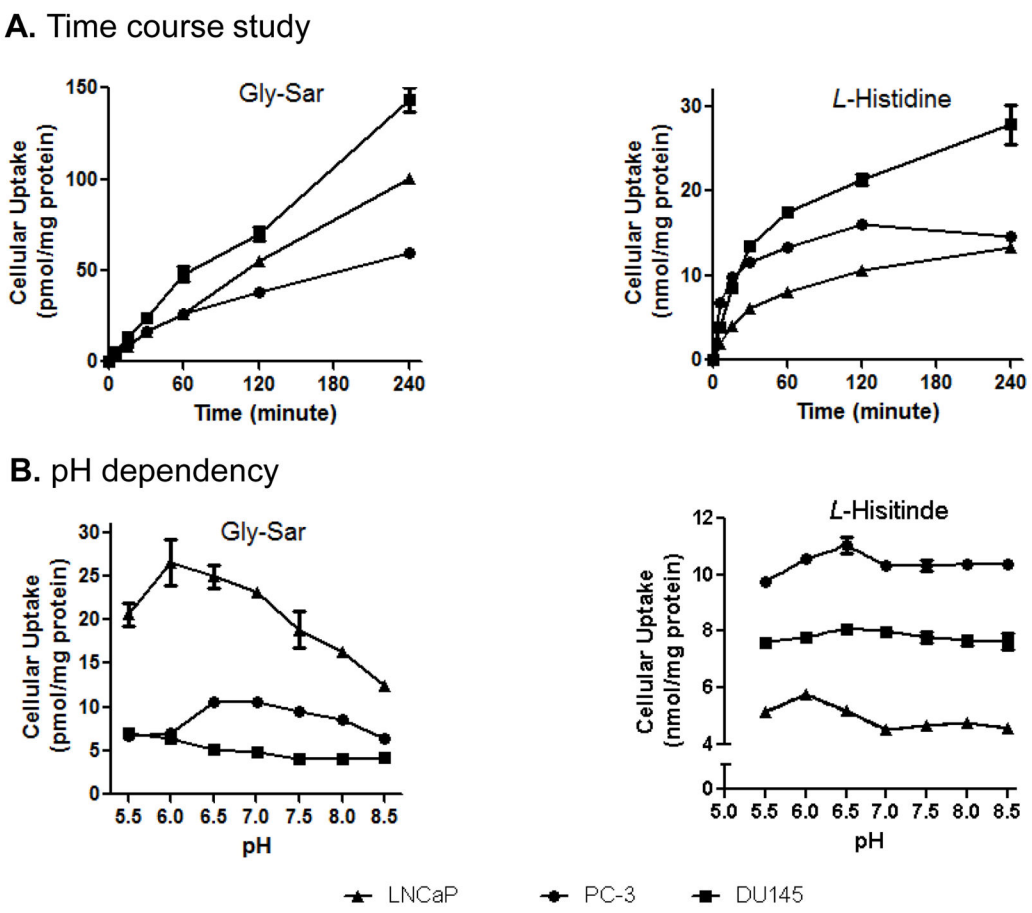

Figure 2.

The time course and pH dependency of [^{3}H]Gly-Sar and [^{3}H]*L*-Histidine uptake in three prostate cancer cell lines. (**A**). Uptakes of [^{3}H]Gly-Sar and [^{3}H]*L*-Histidine at pH 7.4 were measured over a 240 minute time course. (B). The uptakes by prostate cancer cell lines were pH-dependent. Uptake of Gly-Sar was measured after 15 minutes of incubation at various pH values (Range 5.5–8.5). Similar uptake studies were performed for [^{3}H]*L*-Histidine. Data points: mean of three samples; bar: *SD*.

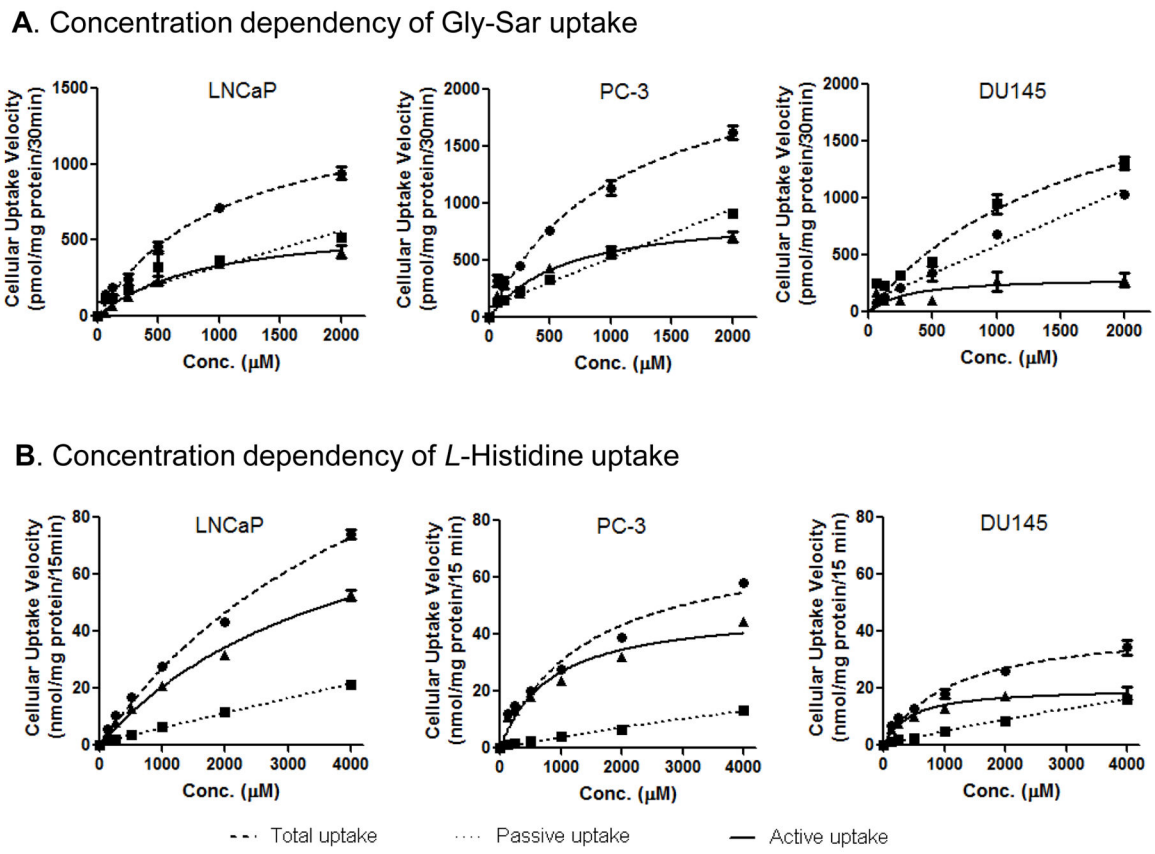

Figure 3.

Concentration dependency of [^{3}H]Gly-Sar and [^{3}H]*L*-Histidine uptake. Uptake was measured at pH 7.4 with an incubation time of 30 minutes for [^{3}H]Gly-Sar uptake and 15 minutes for [^{3}H]*L*-Histidine uptake. Passive uptake was determined by measuring the uptake in the presence of 200 mM unlabeled Gly-Sar or *L*-Histidine. Active uptake was obtained by subtracting passive uptake from total uptake. Data points represented mean ± SD for three samples.

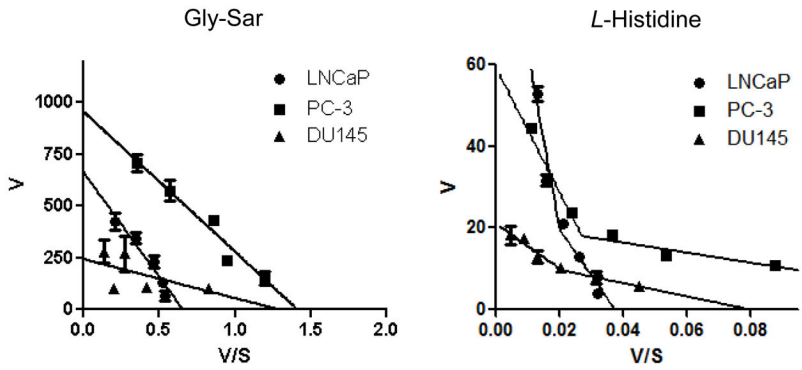

Figure 4.

Eadie-Hofstee plots calculated from the active uptake data of [^{3}H]Gly-Sar and [^{3}H]*L*-Histidine in three prostate cancer cell lines. Eadie-Hofstee plot for [^{3}H]Gly-Sar uptake showed straight lines, which indicated a single transporter system. Curvilinear lines were observed in the Eadie-Hofstee plots of [^{3}H]*L*-Histidine uptake, therefore multiple transporter systems mediated the uptake.

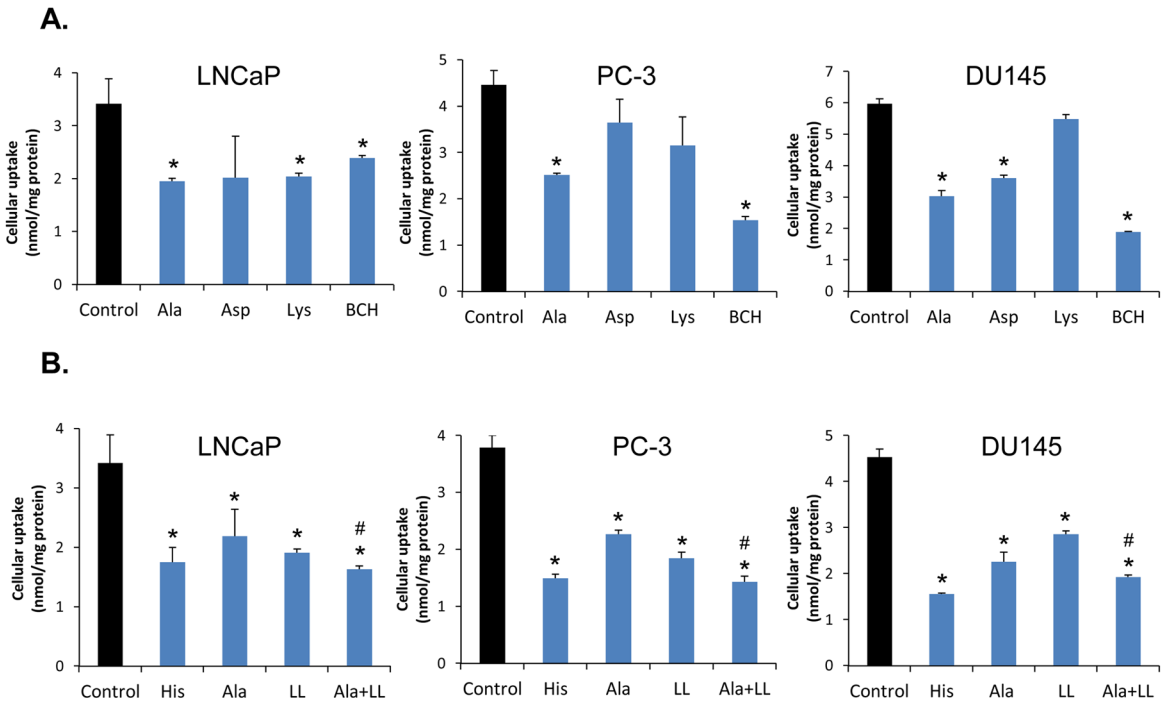

Figure 5.

Amino acid transporters are involved in the uptake of [^{3}H]*L*-Histidine in three prostate cancer cell lines. (**A**). Uptake of [^{3}H]*L*-Histidine can be inhibited by various amino acids; (**B**). Uptake of [^{3}H]*L*-Histidine can be synergistically inhibited by Ala and LL, which separately represents an amino acid transporter inhibitor and a peptide histidine transporter inhibitor, respectively. (* $P < 0.05$ compared to control; # $P < 0.05$ compared to Ala and LL groups).

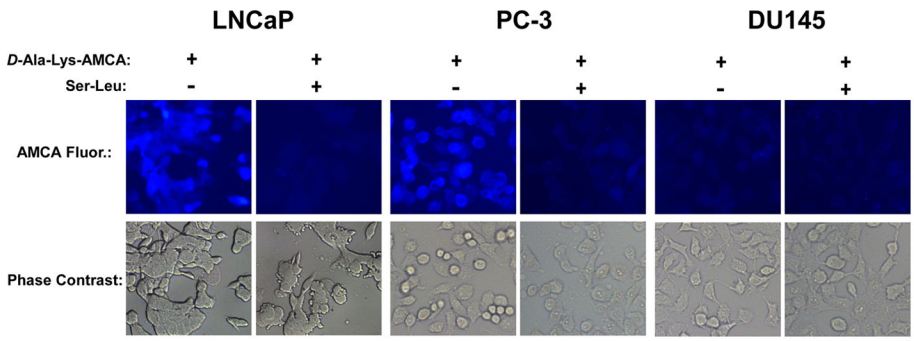

Figure 6.

Visualization of peptide transport in prostate cancer cells by the fluorescent dipeptide probe *D*-Ala-Lys-AMCA. The fluorescent dipeptide probe was taken up via peptide transporters by LNCaP/PC-3 cells, but not DU145 cells. With the competitive inhibitor Ser-Leu, fluorescence was reduced. Data represents typical results of two independent experiments with triplicate samples.

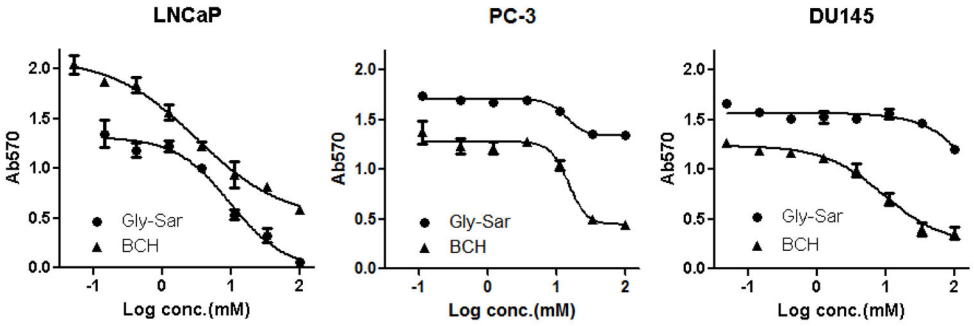

Figure 7.

Comparison of growth inhibition of prostate cancer cells by Gly-Sar and BCH. By inhibiting peptide transporters, Gly-Sar suppressed the growth of LNCaP and PC-3 cells.

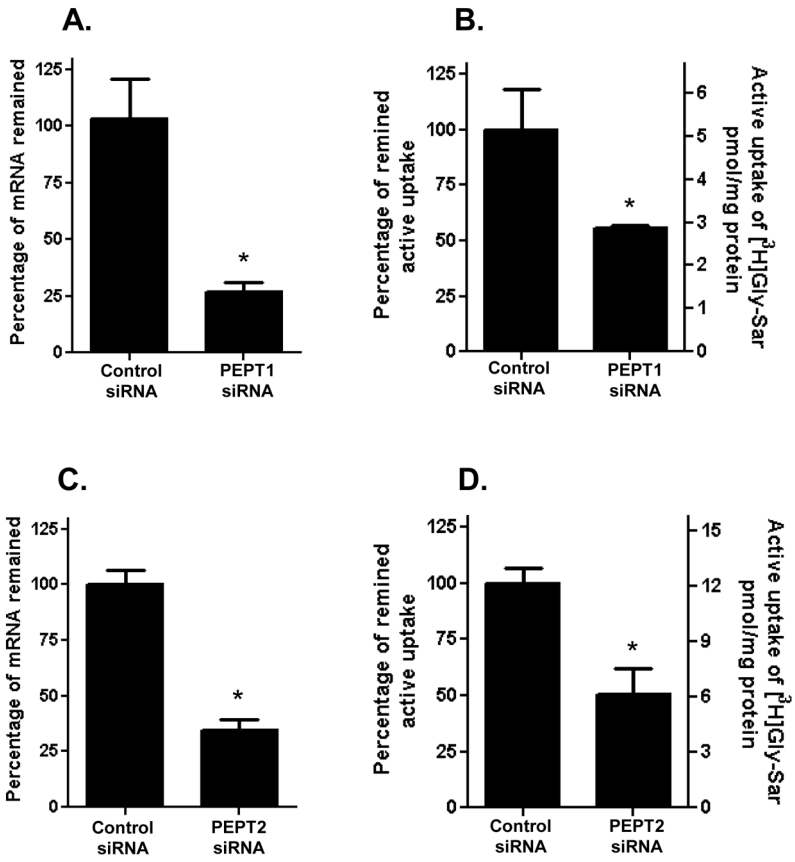

Figure 8.

Knockdown of PEPT1 and PEPT2 genes reduces the active uptake of [^{3}H]Gly-Sar in PC-3 and LNCaP cells. (A). The PEPT1 siRNA down-regulates PEPT1 mRNA level in PC-3 cells. (B). PEPT1 knockdown reduces the active uptake of [^{3}H]Gly-Sar in PC-3 cells. (C). The PEPT2 siRNA down-regulates PEPT2 mRNA expression in LNCaP cells. (D). PEPT2 knockdown reduces the active uptake of [^{3}H]Gly-Sar in LNCaP cells. [^{3}H]Gly-Sar uptake (15 μM, 3 μCi/ml) was measured at pH 7.4 in PEPT1 or PEPT2 knockdown cells. Active uptake was obtained by subtracting passive uptake (in the presence of 100 mM unlabeled Gly-Sar) from total uptake. All experiments were conducted in triplicate, and the results are presented as the mean ± SD. (* $P < 0.05$)

Table 1

mRNA expression profiles of peptide transporters in various cell lines.

Cell line	PEPT1	PEPT2	PHT1	PHT2
HeLa	0.32±0.55	2.98±0.06	5.62±0.99	4.54±1.84
Caco-2	37.42±1.77	10.63±0.20	5.14±0.70	4.20±0.28
LNCaP	0.32±0.04	8.51±0.58	5.42±1.00	5.15±1.42
PC-3	9.91±2.14	6.09±2.16	6.19±1.18	17.28±2.84
DU145	1.17±0.34	2.41±0.38	8.40±1.05	8.16±1.14

Expression levels were reported as copy/ng total RNA. Values given are mean ± SD for three samples.

Table 2

Kinetic parameters of Gly-Sar and *L*-Histidine uptake measured on three prostate cancer cell lines.

Cell lines	Gly-Sar		*L*-Histidine	
	K_m [a]	V_{max} [b]	K_m [a]	V_{max} [b]
LNCaP	871.2±201.5	620.5±64.5	4059.0±615.4	104.4±9.6
PC-3	603.0±136.7	917.9±85.0	811.7±149.9	48.5±3.3
DU145	300.5±218.9	301.5±72.5	423.4±91.1	20.1±1.3

[a] Unit for K_m is μM.

[b] Unit for V_{max} of Gly-Sar is pmol/mg protein/30 min, while Unit for V_{max} of *L*-Histidine is nmol/mg protein/15 min.

Table 3

Substrate specificity of peptide transporters in LNCaP, PC-3 and DU145 cells.

Inhibitors	Conc. (mM)	% Gly-Sar uptake[a]			Conc. (mM)	% *L*-Histidine uptake[a]		
		LNCaP	PC-3	DU145		LNCaP	PC-3	DU145
Gly-Sar	1	46.3±3.3	84.9±7.6	89.3±6.7	10	92.4±6.2	100.1±1.3	99.2±6.9
L-Histidine	1	79.7±1.0	90.1±7.1	103.2±5.8	1	55.0±4.7	39.5±1.7	26.2±0.6
Cefadroxil	1	37.6±8.3	95.1±13.7	85.1±5.0	1	89.8±4.8	92.6±1.2	96.6±2.2
Ala-Ala	1	25.3±2.5	74.6±12.4	92.1±9.5	1	51.8±2.2	61.2±6.1	98.1±2.6
Val-Val	1	36.0±6.3	62.9±5.4	77.8±3.4	1	75.1±0.5	60.0±2.6	82.0±3.2
Ser-Leu	1	31.0±1.9	57.1±5.1	86.1±12.2	1	34.8±3.3	45.4±3.1	84.0±4.0
Leu-Leu	1	28.2±3.9	60.8±10.3	72.8±13.4	1	51.3±1.7	46.5±7.3	85.8±4.1
Gly-Gly	1	42.1±1.9	70.4±11.7	96.0±11.7	1	89.6±3.6	86.9±3.1	88.1±2.7
Gly-Gly-Gly	1	68.6±6.7	91.4±3.8	82.7±6.6	1	87.3±1.7	90.7±2.7	89.4±0.4
Gly-Gly-Gly-Gly	1	91.9±4.9	110.0±18.8	94.8±9.2	1	98.4±3.7	96.6±0.5	94.7±4.8

[a]The uptakes of [^{3}H]Gly-Sar (15 μM, 3 [Ci/ml) and [^{3}H]*L*-Histidine (100 μM, 0.5 μCi/ml) were measured separately at 37°C by incubating for 15 min in HBSS buffer (pH 7.4) at presence of each inhibitor. Each value represents % of control uptake (total uptake) and shows as mean ± SD of three experiments.