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# hPEPT1 Affinity and Translocation of Selected Gln-Sar and Glu-Sar Dipeptide Derivatives

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**Abstract:** The intestinal di- and tripeptide transporter hPEPT1 is considered responsible for the absorption of di- and tripeptides arising from digestion, along with several drugs and prodrugs. In order to gather information on the binding site of the protein, several structure−affinity relationships have been suggested. However, these are not necessarily predictive of compounds that are actually translocated by hPEPT1. More information on affinity to and translocation via hPEPT1 of side-chain-modified dipeptides may be gained by conducting a study of selected dipeptide derivatives with variety in size, hydrophobicity, and bond type. The aim of the present study was to synthesize new esters and amides based on L-Glu-Sar and investigate the effects that bond type and size of modification of the N-terminal side chain of sarcosine-containing dipeptides have on the affinity to and translocation via hPEPT1. The esters L-Glu(O-*i*-Bu)-Sar and L-Glu($OCH_2$Ada)-Sar and the amides L-Gln(*N*,*N*-dimethyl)-Sar and L-Gln(*N*-piperidinyl)-Sar were synthesized, and affinity to and translocation via hPEPT1 were investigated in mature Caco-2 cell monolayers, grown on permeable supports. Affinity was estimated in a competition assay using $^{14}$C-labeled Gly-Sar. Translocation was measured as fluorescence ratios induced by the substrates using the fluorescent probe BCECF and an epifluorescence microscope setup. All compounds showed high affinity to hPEPT1, but only the amides L-Gln(*N*,*N*-dimethyl)-Sar and L-Gln(*N*-piperidinyl)-Sar were translocated by hPEPT1. hPEPT1 is very susceptible to modifications of the N-terminal amino acid side chain of dipeptidomimetic substrates, in terms of achieving compounds with high affinity for the transporter. However, as affinity is not predictive of translocation, derivatization in this position must be performed with great caution since some of the compounds investigated turn out not to be translocated by the transporter.

**Keywords:** Dipeptide derivatives; hPEPT1; Caco-2; intracellular pH; BCECF; epifluorescence microscopy

## Introduction

The intestinal di- and tripeptide transporter, hPEPT1, is considered responsible for the active uptake of di- and tripeptides arising from digestion. It is located in the apical membrane of the enterocytes of the small intestine. There are hundreds of naturally occurring dipeptides and thousands of tripeptides, and most of them are substrates of hPEPT1. Other types of compounds such as $\beta$-lactams and the valine ester prodrugs valacyclovir and valganciclovir are also substrates for hPEPT1. Consequently, this transporter is very interesting in a drug delivery setting.[1−3] The 3D structure of hPEPT1 is not yet known. In order to gather information on

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the binding site of the protein, attention has been directed toward structure−affinity relationships, and a number of models have been proposed using different approaches.[4,5] Structure−affinity relationships may be predictive of affinity, but they are not able to predict whether or not a compound will be translocated by the transporter, thus causing these models to be less relevant in a drug delivery perspective. Hence an investigation of structure−translocation relationships was the next logical step. Previous studies of sarcosine-containing dipeptide ester derivatives show that even relatively small modifications of the N-terminal amino acid side chain may constitute a problem for translocation via hPEPT1.[6] Furthermore, sarcosine-containing dipeptides with hydrophilic ester modifications proved not to be translocated by hPEPT1.[7,8] So far, only ester derivatives have been investigated. More information on affinity to and translocation via hPEPT1 of side-chain-modified dipeptides may be gained by conducting a study of selected dipeptide derivatives with variety in size, hydrophobicity, and bond type. The human colon adenocarcinoma cell line Caco-2 is commonly used in assays estimating mechanism and degree of intestinal absorption of compounds. In assessing the mechanism of di- and tripeptide absorption several assays have been established using Caco-2 cells. This includes assays investigating binding, carrier-mediated translocation across the apical membrane, and transcellular transport of compounds. In the present work, two different assays using Caco-2 cell monolayers were employed: an inhibition assay to ascertain the degree of binding of dipeptide derivatives to hPEPT1 and an epifluorescence assay monitoring the pH-dependent fluorescence of an intracellular fluorescent probe to detect hPEPT1-mediated proton transport. Previously a versatile way of synthesis of hydrophilic dipeptide ester derivatives was described.[8] The aim of the present study was to make use of this way of synthesis in order to produce new lipophilic esters and amides based on L-Glu-Sar and investigate the effects that bond type and size of modification of the N-terminal side chain of sarcosine-containing dipeptides have on the affinity to and translocation via hPEPT1. The esters L-Glu(O-*i*-Bu)-Sar and L-Glu($OCH_2$Ada)-Sar and the amides L-Gln(*N*,*N*-dimethyl)-Sar and L-Gln(*N*-piperidinyl)-Sar were synthesized, their affinity to hPEPT1 was investigated in Caco-2 cells by inhibition studies, and hPEPT1-mediated translocation of the compounds was estimated from studies of intracellular acidification of Caco-2 cells.

## Materials and Methods

**Materials.** Cell culture media and Hanks balanced salt solution (HBSS) were obtained from Life Technologies (Høje Tåstrup, Denmark). [$^{14}$C]Glycylsarcosine ([$^{14}$C]Gly-Sar) with a specific activity of 49.94 mCi/mmol was purchased from New England Nuclear (Boston, MA). 2-[*N*-Morpholino]-ethanesulfonic acid (MES), 2[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid (Hepes), and glycylsarcosine (Gly-Sar) were purchased from Sigma-Aldrich (St. Louis, MO). 2′,7′-Bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF/AM), 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF acid), and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Cremophor EL was purchased from BASF AG (Ludwigshafen, Germany). Lys[Z($NO_2$)]-Pro was kindly donated by Professor Klaus Neubert, Department of Biochemistry, Marthin-Luther-Universität, Halle-Wittenberg, Germany. Transepithelial resistance (TEER) was measured in tissue resistance measurement chambers (Endohm) with a voltohmmeter from World Precision Instruments (Sarasota, FL). The Swip KS 10 Digi shaking plate used for cell culture experiments was from Edmund Bühler (Hechingen, Germany). Radioactivity was counted with a Tri-Carp 2110TR liquid scintillation analyzer from Packard (Perkin-Elmer Life and Analytical Sciences, Boston, MA). Intracellular pH was monitored using an upright Nikon Optiphot microscope with a Leitz (50 × W.I., NA. 1) objective. The microscope was equipped with a PTI Delta-RAM monochromator, coupled to the light port via fiber optics, and a PTI fluorescence detection system. $^1$H NMR spectra were recorded on a 300 MHz Varian instrument.

**Synthesis.** 1-Hydroxybenzotriazole monohydrate (HOBt, $H_2O$), adamantylmethanol ($AdaCH_2OH$), Raney nickel ca. 50 nm, surface area 80−100 $m^2$/g, and palladium 10% on carbon were purchased from Aldrich, and amino acid derivatives were purchased from BaChem AG and were used without further purification, except dimethylformamide (DMF) and dichloromethane (DCM), which were dried and stored over 3 Å molecular sieves. $^1$H NMR spectra were recorded at 300 MHz. Chemical shifts are given in parts per million ($\delta$-values) relative to tetramethylsilane (TMS) as an

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internal standard. All reactions involving air-sensitive reagents were performed under nitrogen. All glassware was flame-dried prior to use. *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDAC) was handled under a nitrogen atmosphere. The crude products were purified by flash chromatography (FC)[9] with Matrex silica, 60A/35−70 $\mu$m from Millipore.

**Boc-L-Glu(O-*i*-Bu)-OBn.** Boc-Glu-OBn 2.5 g (7.41 mmol) was dissolved in THF (22 mL) and DIPEA (6.5 mL). The solution was cooled under nitrogen to −60 °C, and 1.49 g (11.74 mmol) of oxalyl chloride was added. Stirring at −60 °C for 1 h, addition of *i*-BuOH (4.4 mL) and pyridine (4.4 mL), and stirring at −60 °C for 1 h and then at room temperature for 1 h followed by addition of EtOAc (80 mL) gave a solution, which was washed with saturated aqueous $NaHCO_3$ (3 × 50 mL), 10% aqueous citric acid (3 × 50 mL), and saturated aqueous NaCl (3 × 50 mL). Drying ($MgSO_4$), filtration, and removal of the EtOAc in vacuo afforded a yellow solid, which was purified by FC (DCM → EtOAc) to give 2.1 g (72%) of Boc-L-Glu(O-*i*-Bu)-OBn as an oil, $R_f$ 0.92 (95:5 DCM:MeOH). $\delta_H$ ($CDCl_3$): 7.35 (5H, s, Ph), 5.16 (2H, s, $CH_2$ in Bn), 5.12 (1H, d, $J$ = 4.9 Hz, NH), 4.37 (1H, m, CH in Glu), 3.85 (2H, d, $J$ = 6.7 Hz, $CH_2$ in *i*-Bu), 2.37 (2H, m, $CH_2$ in Glu), 2.18−1.95 (2H, m, $CH_2$ in Glu), 1.90 (1H, m, CH in *i*-Bu), 1.43 (9H, s, Boc), 0.92/0.90 (6H, s, $CH_3$ in *i*-Bu).

**Boc-L-Glu(O-*i*-Bu)-Sar-O-*t*-Bu (1).** Boc-L-Glu(O-*i*-Bu)-OBn was debenzylated by hydrogenation in a 10% solution of EtOAc containing 10% Pd on carbon to give 0.33 g (78%) of Boc-L-Glu(O-*i*-Bu)-OH as a clear oil, $R_f$ 0.11 (95:5 DCM:MeOH). This material (0.33 g, 1.09 mmol), HOBt, $H_2O$ (167 mg, 1.09 mmol), and EDAC (313 mg, 1.63 mmol) were dissolved in DMF (3.5 mL) and DIPEA (0.55 mL). After 10 min Sar-O-*t*-Bu, HCl (177 mg, 0.98 mmol) was added. The solution was stirred under nitrogen for 18 h and then evaporated to dryness in vacuo. The residue was dissolved in EtOAc (25 mL). Washing with saturated aqueous NaCl (1 × 20 mL), saturated aqueous $NaHCO_3$ (3 × 20 mL), 10% aqueous citric acid (3 × 20 mL), and saturated aqueous NaCl (20 mL), drying ($MgSO_4$), filtration, removal of the EtOAc in vacuo, and FC (1:3 EtOAc:heptane) yielded 0.67 g (71%) of Boc-L-Glu(O-*i*-Bu)-Sar-O-*t*-Bu **(1)** as an oil, $R_f$ 0.11 (1:3 EtOAc:heptane). $\delta_H$ ($CDCl_3$): 5.37 (1H, d, $J$ = 8.3 Hz, NH), 4.75/4.56* (1H, m, CH in Glu), 4.25 (1H, d, $J$ = 17.0 Hz, $H^A$ in $CH_2N$), 3.86 (2H, m, $CH_2$ in *i*-Bu) 3.75 (1H, d, $J$ = 17.1 Hz, $H^B$ in $CH_2N$), 3.18/2.96* (3H, s, $CH_3N$), 2.43 (2H, m, $CH_2$ in Glu), 2.13−1.70 (2H, m, $CH_2$ in Glu), 1.91 (1H, m, CH in *i*-Bu), 1.47/1.45* (9H, s, COO-*t*-Bu), 1.42/1.41* (9H, s, Boc), 0.93/0.91 (6H, s, $CH_3$ in *i*-Bu); (*) two conformers (4:1).

**Boc-L-Glu($OCH_2$Ada)-OBn.** Boc-Glu-OBn (3.00 g, 8.89 mmol), HOBt, $H_2O$ (1.36 g, 8.89 mmol), DMAP (0.37 g, 3.03 mmol), and EDAC (2.50 g, 13.04 mmol) were dissolved in DMF (18 mL) and DCM (18 mL). After 10 min Ada$CH_2$-OH (0.99 g, 5.92 mmol) was added. Stirring under nitrogen for 16 h at room temperature followed by evaporation to dryness in vacuo gave a residue, which was dissolved in EtOAc (70 mL). Washing with saturated aqueous NaCl (40 mL), saturated aqueous $NaHCO_3$ (3 × 40 mL), 10% aqueous citric acid (3 × 40 mL), and saturated aqueous NaCl (40 mL), drying ($MgSO_4$), filtration, removal of the EtOAc in vacuo, and FC (1:4 EtOAc:heptane) yielded 2.84 g (99%) of Boc-L-Glu($OCH_2$Ada)-OBn as an oil, $R_f$ 0.24 (1:4 EtOAc:heptane). $\delta_H$ ($CDCl_3$): 7.34 (5H, s, Ph), 5.20 (1H, d, $J$ = 12.2 Hz, $CH_2^A$ in Bn), 5.16 (1H, d, $J$ = 12.2 Hz, $CH_2^B$ in Bn), 5.12 (1H, m, NH), 4.37 (1H, m, CH in Glu), 3.16 (2H, s, $OCH_2$Ada), 2.38 (2H, m, $CH_2$ in Glu), 2.18−1.97 (2H, m, $CH_2$ in Glu), 1.97 (3H, s, CH in Ada), 1.68 (6H, m, $CH_2$ in Ada), 1.51 (6H, s, $CH_2$ in Ada), 1.43 (9H, s, Boc).

**Boc-L-Glu($OCH_2$Ada)-Sar-O-*t*-Bu (2).** Boc-L-Glu($OCH_2$-Ada)-OBn was deprotected as described for **1** to give 1.80 g (88%) of Boc-L-Glu($OCH_2$Ada)-OH as an oil, $R_f$ 0.15 (1:1 EtOAc:heptane), which was coupled to Sar-O-*t*-Bu, HCl as described for **1**. FC (1:4 EtOAc:heptane → 1:1 EtOAc:heptane) yielded 1.40 g (72%) of Boc-L-Glu($OCH_2$Ada)-Sar-O-*t*-Bu **(2)** as a clear oil, $R_f$ 0.16 (1:4 EtOAc:heptane). $\delta_H$ ($CDCl_3$): 5.38 (1H, d, $J$ = 8.2 Hz, NH), 4.75/4.55* (1H, m, CH in Glu), 4.26 (1H, d, $J$ = 17.0 Hz, $H^A$ in $CH_2N$), 3.75/3.62* (2H, m, $OCH_2$Ada), 3.69 (1H, d, $J$ = 18.3 Hz, $H^B$ in $CH_2N$), 3.18/2.96* (3H, s, $CH_3N$), 2.45 (2H, m, $CH_2$ in Glu), 2.12−1.72 (2H, m, $CH_2$ in Glu), 1.97 (3H, s, CH in Ada), 1.70 (6H, m, $CH_2$ in Ada), 1.53 (6H, s, $CH_2$ in Ada), 1.47/1.45* (9H, s, COO-*t*-Bu), 1.42/1.41* (9H, s, Boc); (*) two conformers (4:1).

**Boc-L-Glu-Sar-O-*t*-Bu.** Boc-L-Glu(OBn)-Sar-O-*t*-Bu was debenzylated as described for **1** yielding 0.65 g (88%) of Boc-Glu-Sar-O-*t*-Bu as an oil, $R_f$ 0.09 (1:1 EtOAc:heptane). $\delta_H$ ($CDCl_3$): 5.58 (1H, d, $J$ = 8.4 Hz, NH), 4.78/4.61* (1H, m, CH in Glu), 4.26 (1H, d, $J$ = 17.1 Hz, $H^A$ in $CH_2N$), 4.03 (1H, d, $J$ = 18.0 Hz, $H^B$ in $CH_2N$), 3.75 (1H, d, $J$ = 17.1 Hz, $H^A$ in $CH_2N$), 3.16/2.96* (3H, s, $CH_3N$), 2.48 (2H, m, $CH_2$ in Glu), 2.12 (1H, m, H in $CH_2$ in Glu), 1.72 (1H, m, H in $CH_2$ in Glu), 1.45 (9H, s, Boc), 1.43/1.42* (9H, s, COO-*t*-Bu); (*) two conformers (4:1).

**Boc-L-Gln(*N*,*N*-dimethyl)-Sar-O-*t*-Bu (3)**. Boc-L-Glu-Sar-O-*t*-Bu (0.62 g, 1.66 mmol) was coupled to $Me_2NH$, HCl (123 mg, 1.51 mmol) with EDAC and HOBt as described for **1**. FC (EtOAc) yielded 0.51 g (85%) of Boc-L-Gln(*N*,*N*-dimethyl)-Sar-O-*t*-Bu **(3)** as a solidifying oil, $R_f$ 0.34 (EtOAc). $\delta_H$ ($CDCl_3$): 5.48 (1H, d, $J$ = 6.8 Hz, NH), 4.70/4.50* (1H, m, CH in Gln), 4.21 (1H, d, $J$ = 17 Hz, $CH_2^A$ in $CH_2N$), 3.77 (1H, d, $J$ = 17 Hz, $CH_2^B$ in $CH_2N$), 3.22 and 2.97 (6H, s, 2 × $CH_3N$), 2.95/2.94* (3H, s, $CH_3N$), 2.50−1.65 (4H, m, 2 × $CH_2$ in Glu), 1.44 (9H, s, 1.41, COO-*t*-Bu), (9H, s, Boc); (*) two conformers (3.5:1).

**Boc-L-Gln(*N*-piperidinyl)-OBn.** Boc-Glu-OBn (2.00 g, 5.93 mmol), HOBt, $H_2O$ (0.91 g, 5.93 mmol), and EDAC (1.7 g, 8.89 mmol) were dissolved in DMF (20 mL) and DIPEA (4 mL). After 10 min piperidine (455 mg, 5.34 mmol) was added. The solution was stirred for 16 h under nitrogen

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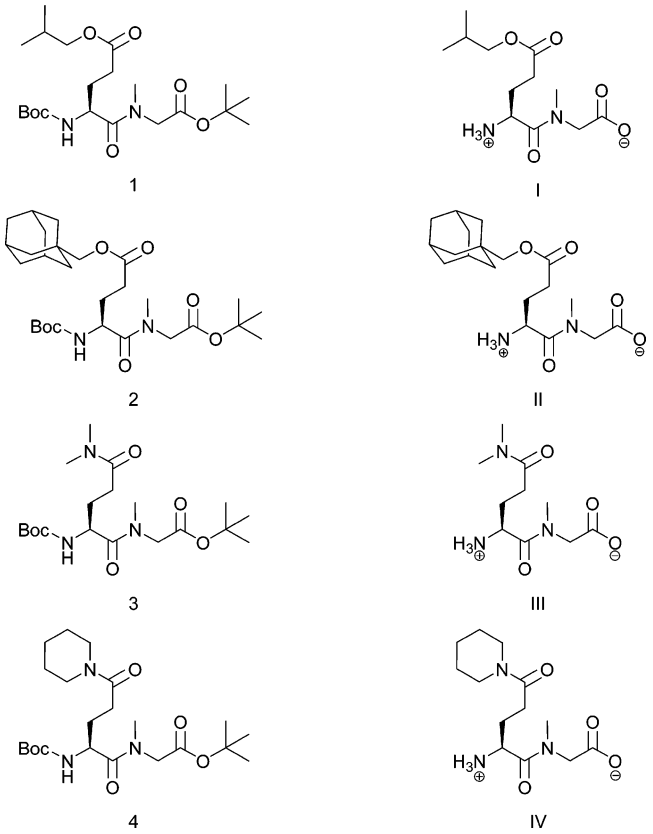
at room temperature. The solution was evaporated to dryness in vacuo, and the residue was dissolved in EtOAc (70 mL). Washing with saturated aqueous NaCl (40 mL), saturated aqueous $NaHCO_3$ (3 × 40 mL), 10% aqueous citric acid (3 × 40 mL), and saturated aqueous NaCl (40 mL), drying ($MgSO_4$), filtration, and removal of the EtOAc in vacuo followed by FC (DCM → 98:2 DCM:MeOH) yielded 1.70 g (79%) of Boc-L-Gln(*N*-piperidinyl)-OBn as a solidifying oil, $R_f$ 0.60 (95:5 DCM:MeOH). $\delta_H$ ($CDCl_3$): 7.35 (5H, s, Ph), 5.39 (1H, d, $J$ = 7.8 Hz, NH), 5.16 (2H, m, $CH_2$ in Bn), 4.33 (1H, m, CH in Gln), 3.51 (2H, m, $CH_2N$ in piperidine), 3.27 (2H, m, $CH_2N$ in piperidine), 2.32 (2H, m, $CH_2$ in Gln), 2.17−2.00 (2H, m, $CH_2$ in Gln), 1.61 (2H, m, $CH_2C$ in piperidine), 1.50 (4H, m, $CH_2C$ in piperidine), 1.42 (9H, s, Boc).

**Boc-L-Gln(*N*-piperidinyl)-Sar-O-*t*-Bu (4).** Boc-L-Gln(*N*-piperidinyl)-OBn was debenzylated as described for **1**, yielding 0.82 g (96%) of Boc-L-Gln(*N*-piperidinyl)-OH as an oil, $R_f$ 0.14 (95:5 DCM:MeOH), which was coupled to Sar-O-*t*-Bu, HCl as described for **1**. FC (1:1 EtOAc:heptane) yielded 0.72 g (78%) of Boc-L-Gln(*N*-piperidinyl)-Sar-O-*t*-Bu **(4)** as an oil, $R_f$ 0.12 (1:1 EtOAc:heptane). $\delta_H$ ($CDCl_3$): 5.46 (1H, d, $J$ = 8.0 Hz, NH), 4.75/4.54* (1H, m, CH in Gln), 4.23 (1H, d, $J$ = 17.1 Hz, $H^A$ in $CH_2N$), 3.76 (1H, d, $J$ = 17.1 Hz, $H^B$ in $CH_2N$), 3.54 (2H, br, $CH_2N$ in piperidine), 3.36 (2H, br, $CH_2N$ in piperidine), 3.22/2.95* (3H, s, $CH_3N$), 2.48−2.20 (4H, m, $CH_2$ in Gln), 1.61/1.54 (6H, m, $CH_2C$ in piperidine), 1.45 (9H, s, COO-*t*-Bu), 1.41 (9H, s, Boc); (*) two conformers (4:1).

**Boc and *tert*-butyl ester protecting groups** were removed by treatment with 33% TFA in DCM at room temperature for 1 h. Subsequent evaporation to dryness and coevaporation with chloroform gave the corresponding deprotected compounds as their trifluoroacetates (**I**−**IV**). The chemical structures of the Boc and *tert*-butyl ester protected model compounds (**1**−**4**) and their corresponding deprotected counterparts (**I**−**IV**) are shown in Chart 1.

**Cell Culture.** Caco-2 cells were cultured as described previously.[10] In short, cells were seeded in culture flasks and passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% fetal bovine serum, penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively), 1% L-glutamine, and 1% nonessential amino acids. For inhibition studies, cells were seeded onto tissue culture treated Transwells (1.1 $cm^2$, 0.4 μm pore size) at a density of $10^5$ cells/$cm^2$ and grown to confluent monolayers. Experiments were performed 25−27 days after seeding at passage numbers ranging from 27 to 34 and 42 to 45. TEER was measured at room temperature just before experiment and was in the range 150−300 and 500−600 Ω·$cm^2$, respectively. Cells for studying hPEPT1-mediated translocation were cultured under similar conditions, only they were seeded onto 4.5 $cm^2$, 0.4 μm pore size Transwells with clear PET filters. Translocation studies were performed 18−41 days after seeding at passage numbers ranging from 26 to 49.

***Chart 1.*** The Chemical Structures of the Boc-Protected and *tert*-Butyl Ester Protected Model Compounds (**1**−**4**) Based on L-Glu-Sar and Their Corresponding Deprotected Counterparts (**I**−**IV**)




**Affinity to hPEPT1 in Caco-2 Cells.** $K_i$ values for the dipeptide derivatives were determined by studying the inhibition of [$^{14}$C]Gly-Sar uptake into filter-grown Caco-2 cell monolayers as described previously.[11] In short, the applied buffers were HBSS supplemented with 0.05% BSA and 10 mM MES (pH 6.0) or 10 mM HEPES (pH 7.4). The cell monolayer was initially incubated for 15 min at 37 °C with buffers at pH 6.0 and 7.4 applied apically and basolaterally, respectively. The inhibition study was then performed by apically incubating the monolayer for 5 min at 37 °C with a mixture of 0.5 μCi [$^{14}$C]Gly-Sar and various concentrations of dipeptide derivative ranging from 0 to 3.0 mM. After incubation, the cells were washed with ice-cold HBSS, and filters were removed to count the cell-associated radioactivity by liquid scintillation analysis. Measurements were carried out in doublet on three different passages.

**hPEPT1-Mediated Translocation in Caco-2 Cells.** hPEPT1-mediated translocation of substrates across the apical

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membrane of Caco-2 cells was investigated by monitoring the intracellular pH of cells, which were perfused apically with test solutions.[12] The solutions used were Krebs−Ringer solution: NaCl (137.3 mM), KCl (5.1 mM), $CaCl_2$ (2.8 mM), $MgSO_4$ (1.0 mM), $KH_2PO_4$ (0.6 mM), glucose (10.0 mM), and MES (at pH = 6.0) or HEPES (at pH = 7.4) (20 mM). All test solutions were prepared in Krebs−Ringer solution, pH 6.0.

The experiments were carried out at room temperature on filter-grown Caco-2 cell monolayers. The cells were loaded in Krebs−Ringer solution pH 7.4 with a cocktail of BCECF/AM (2.6 mM), Pluronic F-127 (0.03% w/v), and Cremophor EL (0.03% w/v) for 60 min. The filters were mounted in an Ussing chamber on the stage of an upright microscope with the apical side facing upward. The basolateral side was bathed in Krebs−Ringer solution (pH 7.4) throughout the experiments. The apical side was exposed to a constant flow of solution (blank buffers as well as test solutions) by a peristaltic pump (6 mL/min). The objective was immersed in the apical solution.

The tissue was allowed to equilibrate with Krebs−Ringer solution, pH 7.4 for 5 min after loading and was then excited with light at 490 and 432 nm alternately (half-bandwidth 2−4 nm). The emitted light was collected at 530 ± 10 nm.

The ratio between light emitted when the probe was excited at 490 and 432 nm, respectively, was used as an estimate of intracellular pH since a declining ratio was an indication of a corresponding decline in intracellular pH. The rate of decline was used as a measure of flux of protons into the cell. In order to approximate $pH_i$, a calibration curve was also obtained by solutions of BCECF in Krebs−Ringer-like media (NaCl (5.1 mM), KCl (137.3 mM), $CaCl_2$ (2.8 mM), $MgSO_4$ (1.0 mM), $KH_2PO_4$ (0.6 mM), glucose (10.0 mM), and MES (at pH = 6.0 and 6.5) or HEPES (at pH = 7.0, 7.5, and 8.0) (20 mM)). The viscosity of the solutions for calibration was increased by adding PVP (25 g/L). Compounds were tested in concentrations ranging from 1 to 3 times the corresponding $K_i$ value determined in inhibition studies. On the basis of Michaelis−Menten kinetics an expected positive response would range between 50% and 75% of maximal response and would thus be detectable. Experiments were performed at least in duplicate. Positive and negative controls (Gly-Sar and Lys[Z($NO_2$)]-Pro, respectively) were included for validation of the method.[13]

**Data Analysis.** $K_i$ for the dipeptide derivatives and $K_m$ for Gly-Sar were calculated as previously described.[11] In short, the concentration of dipeptide derivative causing 50% inhibition ($IC_{50}$) of total [$^{14}$C]Gly-Sar uptake in Caco-2 cells was transformed to a $K_i$ value using the Cheng−Prusoff equation.[14]

The translocation data were compiled by determining the slope of the ratio vs time curve for each compound (approximately 150 data points) and testing it against the slope resulting from perfusion of a blank buffer (pH = 6.0).

## Results

**Affinity to hPEPT1 in Caco-2 Cells.** The affinity to hPEPT1 of the dipeptide derivatives was determined by displacement studies of [$^{14}$C]Gly-Sar in Caco-2 cell monolayers as described in Materials and Methods. The inhibition data are shown along with the inhibition constants ($K_i$) in Figure 1. All four compounds showed an affinity in terms of $K_i$ less than 0.5 mM. The curves do not show 100% inhibition of [$^{14}$C]Gly-Sar uptake. This might be an artifact due to residual amount of extracellular isotope. Indeed measurements of extracellular volume in a separate experiment demonstrated an extracellular volume of 0.22 $\mu$L after washing (data not shown), which, using the specific activity of [$^{14}$C]Gly-Sar, nicely could account for the apparent lack of 100% inhibition.

The Glu-Sar derivatives had higher affinities than the Gln-Sar derivatives. Glu($OCH_2$Ada)-Sar showed the highest affinity of the tested compounds with an inhibition constant of approximately 10 $\mu$M, comparable to that of Lys[Z($NO_2$)]-Pro (6.7 ± 0.4 $\mu$M), which in turn corresponds well with the previously published value (19 $\mu$M) determined in a similar system.[13]

**hPEPT1-Mediated Translocation in Caco-2 Cells.** The hPEPT1-mediated translocation of the dipeptide derivatives and the compounds Gly-Sar and Lys[Z($NO_2$)]-Pro was investigated by monitoring the emission ratio of BCECF in Caco-2 cells (Figure 2). The ratio interval 9.70−10.50 corresponds to the $pH_i$ interval 7.34−7.51. When the pH of the perfusion buffer was changed from pH 7.4 to pH 6.0, an initial drop in intracellular pH was seen and the pH leveled at a new, lower level. Continuing perfusion with blank pH 6.0 buffer resulted in a slight decrease in intracellular pH. When a known substrate of hPEPT1 (Gly-Sar) was added to the perfusion buffer, a significant additional drop in intracellular pH was seen, whereas perfusion with a known inhibitor of hPEPT1 as a negative control (Lys[Z($NO_2$)]-Pro) did not lead to further intracellular acidification.

Slopes of the ratio vs time curve are depicted in Figure 3. Gly-Sar, L-Gln(*N*,*N*-dimethyl)-Sar, and L-Gln(*N*-piperidinyl)-Sar proved to induce a drop in intracellular pH significantly different ($p < 0.001$) from what is induced by a blank buffer (pH = 6.0). The compounds Lys[Z($NO_2$)]-Pro, L-Glu(O-*i*-Bu)-Sar, and L-Glu($OCH_2$Ada)-Sar did not induce an intracellular acidification indicating that these compounds are not translocated via a proton cotransporter such as hPEPT1.

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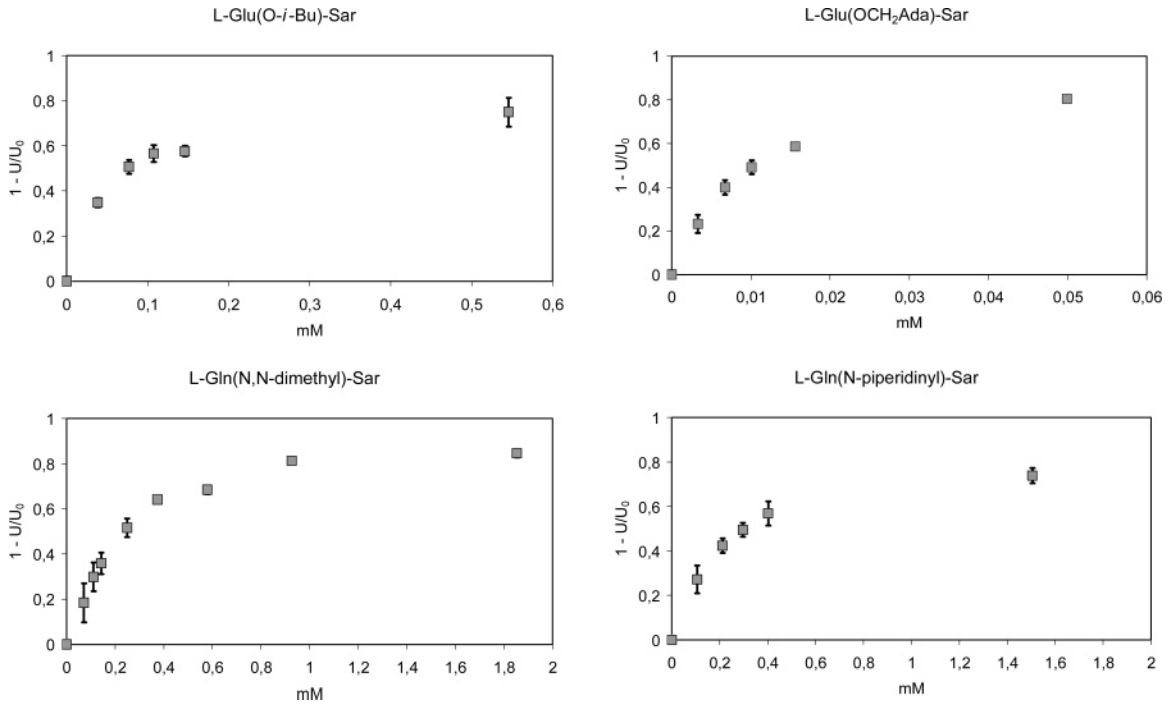


***Figure 1.*** Inhibition data for compounds **I**−**IV**. Inhibition of [$^{14}$C]Gly-Sar uptake ($1 - U/U_0$) as a function of concentration of test compound. Values of $K_i \pm$ SE (mM) for the four compounds were determined as described in Data Analysis to be as follows: L-Glu(O-*i*-Bu)-Sar (**I**), 0.049 ± 0.003; L-Glu($OCH_2$Ada)-Sar (**II**), 0.0097 ± 0.0011; L-Gln(*N*,*N*-dimethyl)-Sar (**III**), 0.22 ± 0.04; and L-Gln(*N*-piperidinyl)-Sar (**IV**), 0.22 ± 0.04. The values are means from at least three different passages and were calculated on the basis of a $K_m$ for Gly-Sar of 1.37 ± 0.10 mM (mean ± SE, $n = 3$).

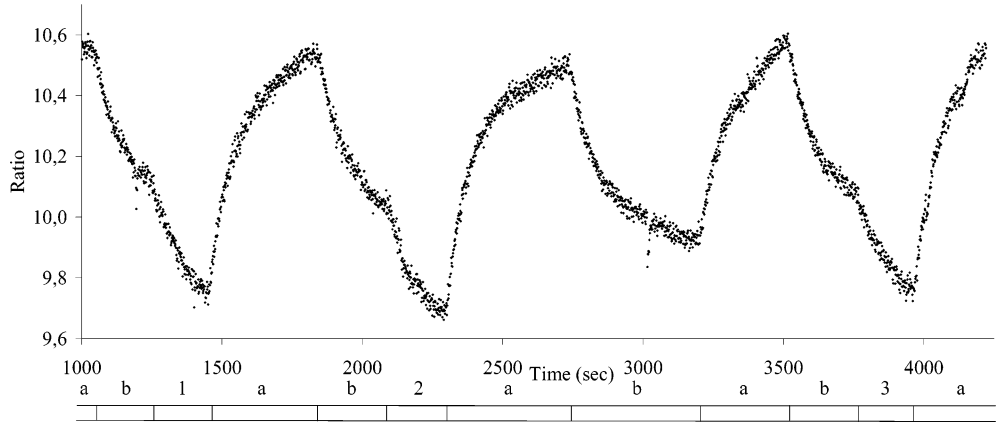


***Figure 2.*** Translocation studies on Caco-2 cell monolayers. Filters mounted in Ussing-like chambers with Krebs−Ringer solution pH 7.4 basolaterally were perfused apically with (a) buffer pH = 7.4, (b) buffer pH 6.0, (1) 2 mM Gly-Sar in buffer pH 6.0, (2) L-Gln(*N*,*N*-dimethyl)-Sar (0.32 mM) and (3) L-Gln(*N*-piperidinyl)-Sar (0.61 mM).

## Discussion

The aim of the present study was, first, to explore a new way of synthesis[8] in order to produce new lipophilic esters and amides based on L-Glu-Sar, and second, to investigate the effects that bond type and size of modification of the N-terminal side chain of sarcosine-containing dipeptides have on the affinity to and translocation via hPEPT1. L-Glu(O-*i*-Bu)-Sar, L-Glu($OCH_2$Ada)-Sar, L-Gln(*N*,*N*-dimethyl)-Sar, and L-Gln(*N*-piperidinyl)-Sar were synthesized successfully, proving the synthetic pathway to be robust toward alternative bond types and variability in hydrophilicity of the derivatives. All four compounds showed high affinity to hPEPT1. The Glu-Sar ester derivatives showed particularly high affinity, with L-Glu($OCH_2$Ada)-Sar as the compound with the highest affinity. The Glu-Sar derivatives are more lipophilic than the Gln-Sar amide derivatives, indicating that lipophilic properties in the N-terminal side chain may increase affinity. Furthermore, the adamantylmethyl derivative contains a bulky group, and the high affinity corresponds well to previous QSAR analyses predicting that bulky groups in this position will increase affinity.[5] The two Gln-Sar derivatives (a dimethyl and a piperidinyl derivate) resulted in the same derivative affinity, indicating that smaller structural changes

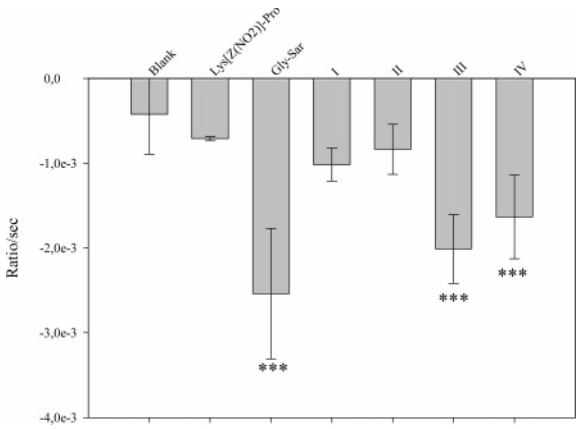


***Figure 3.*** Average slopes of ratio−time curves from translocation studies. Ratio/s ± SD for blank buffer (pH 6, $n = 15$), Lys[Z($NO_2$)]-Pro (20 $\mu$M, $n = 2$), Gly-Sar (2 mM, $n = 5$), L-Glu(O-*i*-Bu)-Sar (**I**, 0.17 mM, $n = 3$), L-Glu($OCH_2$Ada)-Sar (**II**, 20 $\mu$M, $n = 5$), L-Gln(*N*,*N*-dimethyl)-Sar (**III**, 0.41 mM, $n = 5$), and L-Gln(*N*-piperidinyl)-Sar (**IV**, 0.69 mM, $n = 6$); (***) significant ($p < 0.001$) difference from blank buffer.

of the Gln side chain are possible without influencing the affinity.

The Gln-Sar amide derivatives induced an intracellular acidification similar to the signal induced by the positive control Gly-Sar. We interpreted this as being caused by a proton cotransport mediated by hPEPT1. Other sources of an intracellular acidification might be other proton cotransporters such as amino acid transporters. However, we find this less likely due to the structural restrictions of substrates of such transport systems. The Glu-Sar ester derivatives did not induce an intracellular acidification significantly different from the response to a blank pH 6 buffer, nor did the negative control Lys[Z($NO_2$)]-Pro. Previous studies on sarcosine-containing dipeptide derivatives all involved ester derivatives, and no hPEPT1-mediated translocation was seen for these compounds. It has been proposed that the size of the modification could be determinant for the hPEPT1-mediated translocation.[7,8] The adamantylmethyl derivative is lipophilic as well as relatively bulky. Hence, the lack of transporter-mediated translocation might be caused by an interplay of these properties. The isobutyl derivative, however, is not bulky but is still not translocated. Size probably not being the determining factor in this case, lipophilicity may be the primary reason for the lack of translocation, especially in light of the observed translocation of the less lipophilic compounds L-Gln(*N*,*N*-dimethyl)-Sar and L-Gln(*N*-piperidinyl)-Sar.

An alternative explanation could be suggested considering the differenct bond types of the compounds. L-Glu(O-*i*-Bu)-Sar and L-Gln(*N*-piperidinyl)-Sar are of equal size. The difference in translocation might then be explained by the difference between the amide bond and the ester bond present in the two compounds. This might indicate that the properties of the amide bond are more beneficial for translocation than the properties of the ester bond. The ester and the amide bonds differ in electronegativity as the nitrogen atom of the amide bond is less electronegative than the oxygen atom of the ester bond. More studies will be required to shed light on the factors influencing the hPEPT1-mediated translocation of side-chain-modified sarcosine-containing dipeptides.

It might be relevant to consider alternative dipeptidic backbones. Other studies have shown that the composition of the dipeptidic backbone is of importance for retaining the translocation of derivatives.[15,16] A recent investigation of alanine-containing dipeptide derivatives showed translocation of compounds like Tyr(Bzl)-Ala and Lys(Bu)-Ala, which are of size similar to or larger than the compounds investigated in this study.[15] Derivatives based on alternative dipeptidic backbones such as ketomethylene dipeptidomimetic isosters have been shown to be translocated by hPEPT1.[17] Furthermore, a di- or tripeptidic backbone offers different points of derivatization and it is thus relevant to investigate these approaches. Previous studies have shown that derivatization at the C-terminal side chain in dipeptides offers more opportunities for modifications while retaining translocation.[15,16]

To summarize the findings, hPEPT1 is very tolerant of modifications of the N-terminal amino acid side chain in terms of achieving compounds with high affinity for the transporter. However, as it has been shown in this study as well as in previous studies, affinity is not predictive of translocation, which must obviously remain the goal when investigating compounds in a drug delivery perspective. Hence, derivatization in this position should be carried out with great caution since some of the compounds investigated turn out not to be translocated by the transporter. The present results indicate that dipeptide side-chain modification using ester linkages might have drawbacks in terms of unfavorable translocation properties. This possibility must be investigated using translocation as well as affinity studies of a rationally designed compound library covering a wide range of derivatives with variations in side-chain size and bond type.

In a wider perspective, hPEPT1 is already known as a drug and prodrug carrier facilitating oral drug delivery. Studies of transporter-mediated translocation of compounds with affinity for hPEPT1 and development of predictive models for hPEPT1-translocated substrates are thus highly relevant

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for the development of new compounds aimed at absorption from the intestine by hPEPT1.

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