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Note

Affinity and translocation relationships via hPEPT1 of H-X_{aa}-Ser-OH dipeptides: Evaluation of H-Phe-Ser-OH as a pro-moiety for ibuprofen and benzoic acid prodrugs

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ABSTRACT

The intestinal di/tri-peptide transporter 1 (hPEPT1) has been suggested as a drug delivery target for peptide-based prodrugs. The aim of the study was to synthesize a series of 11 serine-containing dipeptides (H-X_{aa}-Ser-OH) and to investigate the relationship between binding to and transport via hPEPT1. An additional aim was to design a dipeptide which could serve as a pro-moiety for prodrugs targeted to hPEPT1. X_{aa} was chosen from the 20 proteogenic amino acids. The dipeptides were synthesized using solid phase peptide synthesis. The K_i-values of H-X_{aa}-Ser-OH dipeptides for hPEPT1 in MDCK/hPEPT1 cells ranged from 0.14 mM ($\log IC_{50} = -0.85 \pm 0.06$) for H-Tyr-Ser-OH to 0.89 mM ($\log IC_{50} = -0.09 \pm 0.02$) for H-Gly-Ser-OH, as measured in a competition assay with [^{14}C]Gly-Sar. The dipeptides were translocated via hPEPT1 with K_m-values in the range of 0.20 ($\log IC_{50} = -0.69 \pm 0.04$) for H-Met-Ser-OH to 1.04 ($\log IC_{50} = 0.02 \pm 0.04$) mM for H-Gly-Ser-OH. The relationship between ligand and transportate properties indicated that the initial binding of the ligand to hPEPT1 is the major determinant for translocation of the investigated dipeptides. H-Phe-Ser-OH was selected as a pro-moiety, and two prodrugs were synthesized, i.e. H-Phe-Ser(Ibuprofyl)-OH and H-Phe-Ser(Bz)-OH. Both H-Phe-Ser(Ibuprofyl)-OH and H-Phe-Ser(Bz)-OH had high affinity for hPEPT1 with K_i-values of 0.07 mM ($\log IC_{50} = -0.92 \pm 0.12$) and 0.12 mM ($\log IC_{50} = -1.17 \pm 0.40$), respectively. However, none of the prodrugs were translocated via hPEPT1. This indicated that the coupling of the drug compounds to the peptide backbone did not decrease transporter binding, but abolished translocation, and that high affinity of prodrugs does not necessarily translate into favourable permeation properties.

1. Introduction

The proton-coupled di/tri-peptide transporter, PEPT1, remains one of the most studied absorptive transporters for biopharmaceutical exploitation, specifically within intestinal drug delivery and prodrug design [1]. Following the identification of valaciclovir as a substrate for PEPT1, the prodrug approach has been applied for targeting the transporter in order to increase bioavailability of poorly permeable drug candidates [2,3]. With valaciclovir, the oral bioavailability in rats increased from 19% of the parent drug to 63% of the valine prodrug [2].

Several different strategies have subsequently been pursued in order to increase intestinal permeation of low permeable drug candidates or model substances via hPEPT1. A number of prodrugs have been designed using pro-moieties such as (i) single amino acids with D- or L-configuration such as e.g. Ala, D-, L-Val, isoleucine or Phe, (ii) dipeptides such as enzymatically stabilized H-β-Ala-His-OH, D-Asp-Ala-OH, D-Glu-Ala-OH, dipeptides with keto-methylene amide bond bioisosters, thiodipeptides or natural dipeptides, (iii) tripeptides such as H-Phe-Ser-Ala-OH or tripeptides with keto-methylene amide bond bioisosters and (iv) other pro-moieties such as e.g. ethanol, which is used as pro-moiety for ester prodrugs such as enalapril and oseltamivir (tamiflu). It must be noted that enalapril has a low affinity for hPEPT1 and only seems to be transported to a limited degree [4]. Despite the many reports on prodrugs having affinities for hPEPT1, few systematic studies exist on L,L-configured dipeptides as pro-moieties. Even though peptides are degraded in the stomach and in the intestinal lumen, some dipeptides are absorbed intact or are degraded inside the small intestinal enterocytes rather than outside the cell [5]. We have therefore investigated whether dipeptides of L-configured

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proteogenic amino acids could serve as pro-moieties for prodrugs targeted to hPEPT1. We chose Ser as the amino acid for conjugation, since it has a hydroxyl group present in the side chain, which may be readily derivatized. We positioned Ser in the C-terminal end of the dipeptide. The 11 N-terminal amino acids, X_{aa}, were chosen from the proteinogenic amino acid in order to represent different physico-chemical properties of the amino acid with aliphatic (Gly, Ala, Val, Leu, Ile and Met), aromatic (Tyr, Phe and Trp), acidic (Asp) and basic (Lys) side chains.

The aim of the present study was thus to investigate the affinity and translocation properties of H-X_{aa}-Ser-OH dipeptides for hPEPT1 and to identify suitable dipeptide pro-moieties for generating dipeptide prodrugs utilising the side chain hydroxyl group of serine as a functional group for conjugating the drug moiety.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) was from Life Technologies (Høje Taastrup, DK). Hanks' Balanced salt solution (HBSS) was purchased from GIBCO, Invitrogen (Paislay, UK). 2-(N-morpholino)ethanesulfonic acid (MES) and bovine serum albumin (BSA) (St. Louis, MO, USA). [^{14}C]Gly-Sar (56 mCi/mmol) was from GE-Healthcare (Freiburg, Germany). Ultima Gold scintillation liquid was from Perkin Elmer (Boston, MA, USA). Corning Costar® 24 Well Clear Tissue-Culture Treated Microplates was from Corning Inc., Life Sciences (Lowell, MA, USA). FLIPR® membrane potential assay kit was purchased from Molecular Devices (Berkshire, UK). 96-well black with clear bottom tissue-culture-treated polystyrene plates were from BD Falcon (Franklin Lakes, NJ, USA).

2.2. Dipeptide synthesis – general procedure

The dipeptide synthesis is described in Supplementary material.

2.3. Synthesis of H-Phe-Ser-OH prodrugs

The prodrug synthesis is described in Supplementary material.

2.4. Cell culture

MDCK cells stably expressing hPEPT1 (MDCK/hPEPT1) or transfected with the empty pcDNA3.1 vector (MDCK/Mock) were cultured as previously described [6]. All experiments were conducted 3 days post-seeding. MDCK/hPEPT1 cells were used in passage 25–35, and the MDCK/Mock cells were in passage 20–31.

2.5. hPEPT1 affinity studies

Affinity for hPEPT1 was measured as concentration-dependent inhibition of the uptake of 1 μCi/ml [^{14}C]Gly-Sar in MDCK/hPEPT1 cell monolayers grown on the bottom of 24-well plates, as previously described [6]. The cells were transferred to scintillation vials, 2 ml scintillation fluid was added, and the radioactivity was counted in a liquid scintillation analyser (Tri-Carb 2100TR, Packard). Affinity studies were performed in triplicates.

2.6. hPEPT1 translocation studies

Translocation studies were performed as previously described [6]. The assay was carried out in a NOVOstar plate reader and was initiated by the addition of 50 μl test solution in MP probe, pH 6.0. The test compound induced change in fluorescence was measured at 37 °C, at wavelengths of 544 nm (excitation) and 590 nm (emission) for 72 s. Gly-Sar (20 mM) was used as a positive control and standard, HBSS served as a negative control, and MDCK/mock cells were used as a qualitative control to ensure that the signal observed was caused by substrate translocation via hPEPT1. Translocation studies were performed in triplicates.

2.7. Stability in buffer and on MDCK/hPEPT1 cells

The degradation of H-Phe-Ser(Bz)-OH was investigated in HBSS buffer pH 6.0 and 7.4 and on MDCK/hPEPT1 cells in HBSS buffer 6.0 and 7.4 at 37 °C. The reactions in buffer was initiated by the addition of 200 μl 20 mM H-Phe-Ser(Bz)-OH to 10 ml pre-equilibrated buffer kept in a water bath. The degradation reaction on the MDCK/hPEPT1 cells was initiated by addition of 20 μl 20 mM H-Phe-Ser(Bz)-OH to cells that were pre-equilibrated with 2 ml buffer solution. At various time points, samples were taken from the reactions and either analysed directly using HPLC-UV or kept on ice for later analysis. HPLC-UV analysis was performed with a Waters Spherisorb S5ODS2 reverse-phase column (5 μm, 250 × 4.6 mm) in a Merck/Hitachi system consisting of a 655A-11 pump, a 655 UV detector (operated at 210 nm) and a D-2520 GPC integrator. The mobile phase consisted of 60% 0.02 M phosphate buffer and 40% methanol, pH 6.0.

2.8. Data analysis

The uptake rate (J) of Gly-Sar (mass/time/area) at increasing concentration was fitted to a Michaelis–Menten-type equation with an additional non-saturable transport component:

$$J = \frac{J_{max} \cdot [S]}{K_m + [S]} + P_{app} \cdot [S] \quad (1)$$

The flux, J, is measured in nmol cm^{-2} min^{-1}, J_{max} is maximal flux, K_m is the Michaelis Menten constant (mM) and [S] is the Gly-Sar concentration (mM). P_{app} is the apparent permeability coefficient of the non-saturable uptake. The IC_{50}-value was determined using the equation:

$$U = U_{min} + \frac{U_{max} - U_{min}}{1 + 10^{\wedge}(\log[I] - \log IC_{50})} \quad (2)$$

U is the uptake of [^{14}C]Gly-Sar (0–100%), [I], is the concentration of inhibitor. U_{min} is the minimal uptake of [^{14}C]Gly-Sar and U_{max} is the control uptake (%) ([I] = 0). The IC_{50}-values of [^{14}C]Gly-Sar uptake inhibitors were converted to K_i-values according to competitive kinetics as described by Cheng and Prusoff [7]. The K_m-value for substrates determined from translocation studies were calculated as previously described [6]. All data were fitted using nonlinear regression in GraphPad Prism version 4.0.

2.9. Statistics

Affinity and translocation values are given as mean ± SEM unless otherwise stated (n = 3). The statistical significance of the results was determined by a two-tailed unpaired Students t-test. When means with different variances were compared, the Welch t-test was used. $P < 0.05$ was considered significant. The statistical analysis was made with GraphPad InStat v.3.06 from GraphPad software.

3. Results and discussion

A total of 11 dipeptides were synthesized and investigated for their affinity and transport via the intestinal di/tri-peptide transporter, hPEPT1 (Table 1, and Supplementary material). All investigated natural dipeptides bind to hPEPT1 and are also translocated

Table 1
Purity and kinetic parameters for H-X_{aa}-Ser-OH dipeptide binding (K_i) to and translocation (K_m) via hPEPT1 in MDCK/hPEPT1 cells (1 = 3).

	Purity (%)	K_i (mM)	log K_i	log SE	K_m (mM)	log K_m	log SE	Ratio (K_m/K_i)
H-Tyr-Ser-OH	0.68	0.14	−0.85	0.06	0.72	−0.14	0.04	5.14*
H-Phe-Ser-OH	0.86	0.15	−0.82	0.02	0.35	−0.46	0.03	2.33*
H-Leu-Ser-OH	0.61	0.18	−0.74	0.03	0.24	−0.62	0.03	1.33
H-Val-Ser-OH	0.89	0.20	−0.70	0.06	0.33	−0.49	0.05	1.65
H-Met-Ser-OH	0.93	0.21	−0.68	0.08	0.20	−0.69	0.04	0.95
H-Ala-Ser-OH	0.81	0.28	−0.55	0.05	0.25	−0.6	0.04	0.89
H-Ile-Ser-OH	0.57	0.35	−0.45	0.04	0.55	−0.26	0.03	1.57*
H-Trp-Ser-OH	0.79	0.47	−0.33	0.04	0.80	−0.1	0.03	1.70*
H-Lys-Ser-OH	0.82	0.77	−0.11	0.04	0.94	−0.03	0.05	1.22
H-Asp-Ser-OH	0.78	0.79	−0.1	0.04	0.87	−0.06	0.04	1.10
H-Gly-Ser-OH	0.89	0.8	−0.09	0.02	1.04	0.02	0.04	1.30

* K_m and K_i-values significantly different from each other, $P < 0.05$.

via the carrier. The benzoic acid and ibuprofen Phe-Ser ester prodrugs bind to hPEPT1 with high affinity, but to our surprise they were not translocated via hPEPT1. Our findings indicate that binding is generally predictive for translocation in the series of H-X_{aa}-Ser-OH dipeptides, but this relationship is lost upon the derivatization of the C-terminal amino acid.

3.1. Uptake and affinity studies of Gly-Sar and H-X_{aa}-Ser-OH dipeptides in MDCK/hPEPT1 cell monolayers

The uptake of the stable, radiolabelled dipeptide [^{14}C]Gly-Sar in the concentration range 0.002–10 mM was measured in MDCK/hPEPT1 cells. The uptake rates were fitted to an expression composed of a saturable as well as a non-saturable transport component (Fig. 1A) (Eq. (1)). The saturable uptake mechanism via hPEPT1 was characterised by a K_m-value of 1.15 ± 0.2 mM and a maximal uptake rate, V_{max}, of 261.5 ± 26.4 pmol cm^{-2} min^{-1}. The non-saturable uptake component was characterised by the following equation for passive uptake: $J_{passive}$ (pmol cm^{-2} min^{-1}) = 23.7 ± 2.3 [Gly-Sar] (mM). The passive uptake accounts for 9% of the total Gly-Sar uptake at 0.002 mM Gly-Sar and 57% at 10 mM Gly-Sar in the compartment. The uptake of 0.002 mM Gly-Sar in

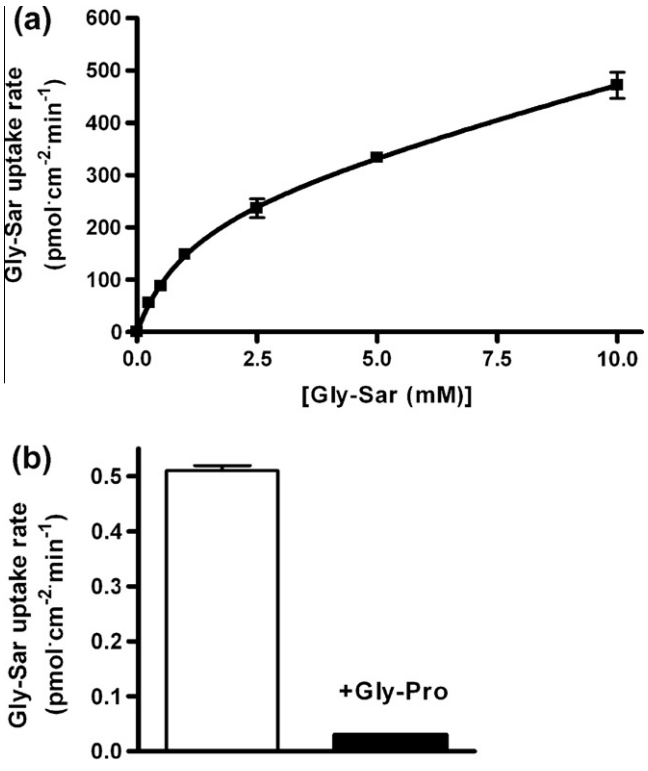

Fig. 1. (A) Uptake rate of [^{14}C]Gly-Sar (0.25 μCi/well) in MDCK/hPEPT1 cell monolayers as a function of increasing Gly-Sar concentrations. (B) Uptake rate of [^{14}C]Gly-Sar (0.25 μCi/well) in MDCK/hPEPT1 cell monolayers in the absence and presence of 20 mM Gly-Pro. Cells were cultured for 3 days, and uptake was measured for 5 min at pH of 6.0. The results are mean ± SD of three cell monolayers. The solid line represents the fit of experimental data to Eq. (1).

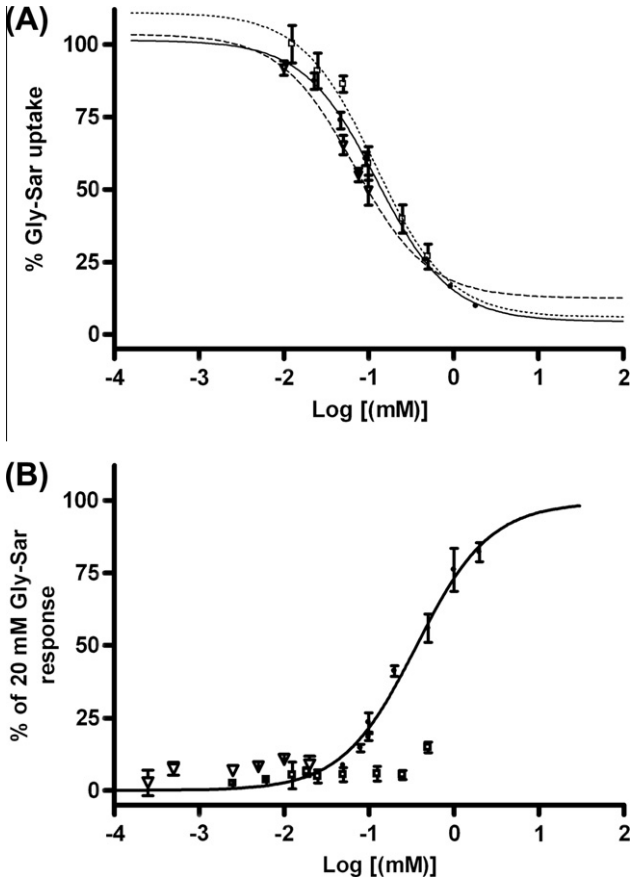

Fig. 2. (A) Uptake rate of [^{14}C]Gly-Sar (0.25 μCi/well) in MDCK/hPEPT1 cell monolayers cultured for 3 days as a function of increasing H-Phe-Ser-OH (closed circle, straight line), H-Phe-Ser(Ibu)-OH (open triangle, dashed line) or H-Phe-Ser(Bz)-OH (open squares, dotted line) concentrations. The uptake was measured for 5 min at pH 6.0. (B) Percentage change in fluorescence relative to the response elicited by 20 mM Gly-Sar measured in the MDCK/hPEPT1 FLIPR® membrane potential translocation assay after addition of increasing concentrations of H-Phe-Ser-OH (closed circle, straight line), H-Phe-Ser(Ibu)-OH (open triangle) or H-Phe-Ser(Bz)-OH (open squares). The fluorescence signal was measured for 72 s at pH 6.0. The results are mean ± SD of three cell monolayers. The solid lines represent the fit of experimental data to Eq. (2).

the presence of 20 mM H-Gly-Pro-OH was 6% of that without H-Gly-Pro-OH, confirming a negligible non-saturable uptake of Gly-Sar at these conditions (Fig. 1B). The affinity of a series of H-X_{aa}-Ser-OH dipeptides for hPEPT1 was determined in MDCK/hPEPT1 cell monolayers. In Fig. 2A, the uptake of [^{14}C]Gly-Sar in the presence of increasing H-Phe-Ser-OH concentrations is shown as a representative example. Although the C-terminal amino acids chosen for the H-X_{aa}-Ser-OH series differ in terms of charge, size and polarity, little variation was observed in the affinity values for hPEPT1. H-Tyr-Ser-OH and H-Phe-Ser-OH had the highest affinities for hPEPT1 of 0.14 and 0.15 mM, respectively, whereas H-Asp-Ser-OH and H-Gly-Ser-OH had the lowest affinities of 0.79 and 0.89, respectively (Table 1).

The affinities of H-X_{aa}-Ser-OH for hPEPT1 were surprisingly similar regardless of the physico-chemical properties of the N-terminal amino acid. For X_{aa}-Pro dipeptides, Brandsch et al. find similar close values with affinity values ranging from 0.15 mM for H-Ala-Pro-OH to 0.54 mM for H-Trp-Pro-OH [8]. Brandsch et al. suggested that the affinity of H-X_{aa}-Pro-OH dipeptides were generally similar when the affinity values were corrected for the amount of trans-conformation present, with the underlying idea that dipeptides only bind to hPEPT1 when the peptide bond is in the trans-conformation [8] and are translocated as such [9]. We find that the binding affinity for H-X_{aa}-Ser-OH dipeptides is generally indicative of actual translocation (Table 1 and Fig. 3). However, for H-X_{aa}-Ser-OH dipeptides with aromatic side chains in the N-terminal, i.e. H-Tyr-Ser-OH, H-Phe-Ser-OH, H-Trp-Ser-OH, translocation could be the rate limiting step. These dipeptides have significantly higher binding affinities than K_m-values. If dipeptides are only translocated in the trans-conformation, the lower translocation affinities could be due to higher cis-conformation fractions for H-Tyr-Ser-OH, H-Phe-Ser-OH and H-Trp-Ser-OH. Nevertheless, we have chosen H-Phe-Ser-OH as a lead dipeptide for generating prodrugs targeting hPEPT1. H-Phe-Ser-OH has a high affinity and K_m-value for hPEPT1, the phenyl-group makes the H-Phe-Ser-OH promoiety easily to detect analytically.

3.2. Translocation studies of X_{aa}-Ser dipeptides in MDCK/hPEPT1 cell monolayers

We investigated the translocation of H-X_{aa}-Ser-OH dipeptides via hPEPT1 in a membrane potential sensitive fluorescence probe assay in MDCK/hPEPT1 cell monolayers [6] in order to establish whether a correlation existed between affinity and translocation properties. The translocation of Gly-Sar via hPEPT1 had a K_m-value of 1.25 ± 0.09 mM and a relative V_{max} of 104 ± 2% of the fluorescence change caused by 20 mM Gly-Sar. The K_m-value for hPEPT1-mediated translocation is similar to the K_m-values obtained from the uptake experiments using radiolabelled [^{14}C]Gly-Sar. As a representative example, the change in fluorescence normalised to the response caused by 20 mM Gly-Sar is displayed as a function of increasing H-Phe-Ser-OH concentrations (Fig. 2B). H-Met-Ser-OH and H-Leu-Ser-OH had the highest K_m-values for translocation via hPEPT1, i.e. 0.20 and 0.24 mM, respectively, whereas H-Lys-Ser-OH and H-Gly-Ser-OH had the lowest affinities of 0.94 and 1.04, respectively (Table 1). The K_m-values obtained for these dipeptides are not significantly different from the K_i-values obtained. The ratio between K_m and K_i-values for the H-X_{aa}-Ser-OH dipeptides were approximately 1, except for H-Tyr-Ser-OH, H-Phe-Ser-OH, H-Ile-Ser-OH and H-Trp-Ser-OH where the K_m-values were significantly higher than the K_i-values. The logarithm of the K_m-values is depicted as a function of the logarithm of the K_i-values in Fig. 3. The correlation between the two parameters are given by $\log(K_m) = 0.87\ (\pm 0.15)\ \log(K_i) + 0.068 \pm 0.077$ ($R^2 = 0.82$).

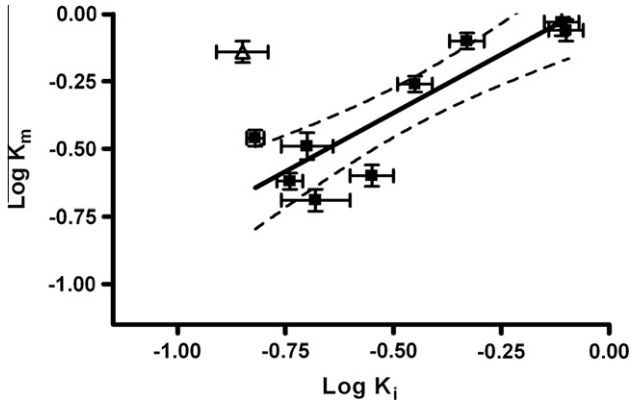

Fig. 3. The logarithm to the K_m-values obtained from translocation studies are shown as a function of the logarithm of the K_i-values obtained from competition studies in MDCK/hPEPT1 cells. The open triangle is the data for H-Tyr-Ser-OH. The solid line represents the linear regression of data (except H-Tyr-Ser-OH), and the stippled line is the 95% confidence interval.

3.3. Affinity, translocation and stability of Phe-Ser prodrugs

H-Phe-Ser-OH was chosen as lead pro-moiety for further investigations. Ibuprofen is a non-steroid anti-inflammatory drug substance (NSAID) almost completely absorbed from the intestine after oral administration. Therefore, ibuprofen is not an optimal drug substance for a prodrug targeting hPEPT1. However, Ibuprofen was chosen due to the relatively easy procedures required for synthesizing H-Phe-Ser(ibuprofyl)-OH. Benzoic acid is often used as a preservative in oral solutions, but is also a drug substance used together with opium as a cough suppressing agent. The prodrugs, H-Phe-Ser(Bz)-OH and H-Phe-Ser(ibuprofyl)-OH, were then investigated for their affinity for hPEPT1 in MDCK/hPEPT1 cells. The prodrugs have high affinity for hPEPT1 (Fig. 2A). The affinity for hPEPT1 was estimated to be 0.12 ($\log IC_{50} = -0.92 \pm 0.12$) and 0.07 ($\log IC_{50} = -1.17 \pm 0.40$) mM for H-Phe-Ser(Bz)-OH and H-Phe-Ser(Ibuprofyl)-OH, respectively. We have quite recently shown that ibuprofen itself is a non-competitive inhibitor of hPEPT1-mediated transport [10]. However, the affinity of ibuprofen is almost six times lower than the affinity of the ibuprofen prodrug. To investigate if the prodrugs were substrates for hPEPT1, translocation experiments were performed. It is evident that the prodrugs are, however, not translocated via hPEPT1 (Fig. 2B). Since prodrugs must be converted into the pro-moiety and parent compound *in vivo* by either chemical or enzymatic degradation, we investigated the stability of H-Phe-Ser(Bz)-OH in buffer and with cells. H-Phe-Ser(Bz)-OH was relatively stable in aqueous buffer at pH 6.0 and 7.4 with half-life of 220 and 357 h, respectively (Table 2). In the presence of MDCK cells, the stability of the prodrug was much lower with half-life of 2.0–2.8 h (Table 2). Thus, the conversion rate of the prodrug into parent drug and pro-moiety in the

Table 2
Stability of H-Phe-Ser(Bz)-OH in HBSS pH 6.0, HBSS pH 7.4, HBSS pH 6.0 on MDCK/hPEPT1 cells and HBSS pH 7.4 on MDCK/hPEPT1 cells.

	k_{obs} (h^{-1})	$T_{½}$ (h)
HBSS pH 6.0	$3.2 \pm 0.4 \times 10^{-3}$	220 ± 25
HBSS pH 7.4	$2.1 \pm 0.4 \times 10^{-3}$	357 ± 62
MDCK/hPEPT1, HBSS pH 6.0	0.25 ± 0.01	2.8 ± 0.1
MDCK/hPEPT1, HBSS pH 7.4	0.35 ± 0.03	2.0 ± 0.1

Values are mean ± SEM ($n = 3$).

presence of MDCK cells is fast enough to expect a good bio-reversibility and at the same time sufficiently slow to avoid degradation during the experiments conducted here. The stability of H-Phe-Ser(ibuprofen)-OH was not investigated due to a limited solubility of the prodrug. In terms of hPEPT1 targeting, H-Phe-Ser(ibuprofen)-OH and H-Phe-Ser(Bz)-OH have high affinity for hPEPT1 of approximately 0.1 mM. The prodrugs thus have improved affinities compared to the H-Phe-Ser-OH pro-moiety. To our surprise, H-Phe-Ser(ibuprofen)-OH and H-Phe-Ser(Bz)-OH are not translocated via hPEPT1. This indicates that the prodrugs are high-affinity ligands which are not translocated, i.e. inhibitors of hPEPT1-mediated substrate transport. For H-Phe-Ser-OH prodrugs, the translocation process must be affected by the drug substances conjugated to H-Phe-Ser-OH via the serine side chain hydroxyl group. This could be due to an interaction between the two phenyl groups causing an increased fraction of cis-conformers around the peptide bond. However, the two prodrugs have high affinity, but this could be due to hydrophobic interactions increasing the affinity but abolishing translocation, due to an interaction which somehow interferes with the conformational shift in the hPEPT1 protein during translocation. Further experiments are required to investigate these speculations. Irrespectively of the mechanism causing the prodrugs to have high affinity while not being translocated, it may be concluded that H-Phe-Ser-OH is not a suitable pro-moiety for phenyl contained drug substances. It might be a possibility that aliphatic drug substances could be conjugated to the serine residue and retain affinity; however, this awaits further experiments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2010.12.009.

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