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Pyrimidine and nucleoside γ-esters of L-Glu-Sar: Synthesis, stability and interaction with hPEPT1

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Abstract

The aim of the present study was to improve the synthetic pathway of bioreversible dipeptide derivatives as well as evaluate the potential of using L-Glu-Sar as a pro-moiety for delivering three newly synthesised nucleoside and pyrimidine L-Glu-Sar derivatives. L-Glu(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-Sar (I), L-Glu(thymine-1-yl-methyl ester)-Sar (II) and L-Glu(acyclothymidine)-Sar (III) were synthesised and in vitro stability was studied in various aqueous and biological media. Affinity to and translocation via hPEPT1 was investigated in mature Caco-2 cell monolayers, grown on permeable supports. Affinity was estimated in a competition assay, using [^{14}C] labelled Gly-Sar (glycylsarcosine). Translocation was measured as pHi-changes induced by the substrates using the fluorescent probe BCECF and an epifluorescence microscope setup. All dipeptide derivatives released the model drugs quantitatively by specific base-catalysed hydrolysis at pH > 6.0. II was labile in aqueous buffer solution, whereas I and III showed appropriate stability for oral administration. In 10% porcine intestinal homogenate, the half-lives of the dipeptide derivatives indicated limited enzyme catalyzed degradation. All compounds showed good affinity to hPEPT1, but the Compounds I and III showed not to be translocated by hPEPT1. The translocation of the L-Glu-Sar derivative of acyclovir, L-Glu(acyclovir)-Sar was also investigated and showed not to take place. Consequently, L-Glu-Sar seems to be a poor pro-moiety for hPEPT1-mediated transport.

Keywords: Bioreversible derivatives; Dipeptide; Aqueous stability; hPEPT1; Caco-2; Intracellular pH

1. Introduction

Several chemotherapeutic agents share structural similarities to nucleosides or nucleobases and act by interrupting either the various steps of replication or various steps of the biosynthesis of nucleosides. Examples are the antiviral agents AZT, ganciclovir and acyclovir (Brigden et al., 1981; Matthews and Boehme, 1988; Mitsuya and Broder, 1987). Another example is 5-fluorouracil which is used in the treatment of cancer (Heidelberger et al., 1957; Longley et al., 2003). These drugs are often characterised by variable and low oral bioavailability (Morse et al., 1993), which may be ascribed to their relatively hydrophilic character, and hence limited passive permeability across intestinal mucosa. However, targeting the peptide transporter PEPT1 and intracellular esterases has proven to efficiently increase the oral bioavailability of aciclovir and ganciclovir when administered as the bioreversible valine ester pro-drugs (Beauchamp et al., 1992; Burnette and de Miranda, 1994; Crooks and Murray, 1994; Ganapathy et al., 1998; Han et al., 1998; Sugawara et al., 2000).

Another class of bioreversible model pro-drugs targeting hPEPT1 is based on metabolically stable dipeptidomimetic pro-moieties, such as L-Glu-Sar and D-Glu-Ala (Friedrichsen et al., 2001; Lepist et al., 2000; Nielsen et al., 2001c;

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Steffansen et al., 1999; Taub et al., 1997, 1998). These compounds showed promising drug delivery properties in vitro in terms of aqueous and enzymatic stability and affinity to hPEPT1. The parent drug release from γ-esters of L-Glu-Sar and D-Glu-Ala, is thus generally controlled by a specific base-catalysed hydrolysis in vitro, consequently suggesting that the pro-drugs will remain relatively stable during passage of the upper small intestine at pH 6.0, yet release parent drug within intracellular or blood environments at pH 7.4 (Thomsen et al., 2003). On the other hand, it has recently been shown that acyclovir when linked to L-Glu-Sar has limited bioavailability after oral administration to rats (Thomsen et al., 2004). The general application of the pro-moiety L-Glu-Sar in targeting of nucleoside analogues to PEPT1; however, still remains to be investigated.

The aim of the present study was to improve the synthetic pathway of bioreversible dipeptide derivatives as well as evaluate the potential of using L-Glu-Sar as a pro-moiety for delivering three newly synthesised nucleoside and pyrimidine L-Glu-Sar derivatives.

The following three L-Glu-Sar derivatives were synthesised: an ester linked cyclic nucleoside analogue L-Glu(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-Sar (I), an *N*-acyloxymethyl linked nucleobase L-Glu(thymine-1-yl-methyl ester)-Sar (II) and an ester linked acyclic nucleoside analogue L-Glu(acyclothymidine)-Sar (III). The potential of L-Glu-Sar as pro-moiety was evaluated in part by studying the rate and mechanism of the in vitro model drug release from the bioreversible derivatives in various aqueous and biological media. Furthermore, affinity to hPEPT1 was investigated in Caco-2 cells by inhibition studies. Translocation of compounds via hPEPT1 was estimated from studies of intracellular acidification.

2. Materials and methods

2.1. Materials

Chemicals used in the synthesis were purchased from Sigma–Aldrich (St. Louis, MO, USA) or BACHEM AG (Bubendorf, Switzerland) and used without further purification with the following exceptions: dimethyl formamide (DMF) and dichloromethane (DCM) were dried and stored over 3-Å molecular sieves.

HPLC solvents were of analytical grade and chemicals used in buffer preparations for stability studies were of laboratory grade. Flash chromatography was performed with Matrex silica, 60A/35-70 μm purchased from Millipore (Billerica, MA, USA).

Porcine intestine was kindly donated by The Danish Meat Trade College (Roskilde, Denmark). Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). 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, USA).

2-[*N*-Morpholino]ethanesulfonic acid (MES), 2[4-(2-hydroxyethyl)-1-piperazine]-ethanesulfonic acid (Hepes) and glycylsarcosine were purchased from Sigma–Aldrich (St. Louis, MO, USA). 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, USA). 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.

L-Glu(acyclovir)-Sar was synthesised as described by Steffansen and co-workers (Thomsen et al., 2003).

2.2. Apparatus

^{1}H NMR and ^{13}C spectra were recorded on a 300 MHz Varian instrument. Chemical shifts are given in parts per million (*δ*-values) relative to tetramethylsilane (TMS) as an internal standard. Elemental analyses were performed by Microanalytical Laboratory, Department of Physical Chemistry, University of Vienna, Austria.

HPLC-UV used to quantify the compounds consisted of a Shimadzu Model LC-6A pump and a Rheodyne 7125 injection valve with a 20-μl loop connected to a Milton Roy Spectromonitor 3000 UV detector and a Merck D-2000 GPC integrator. Waters spherisorb S5OdS2 columns 4.6 mm × 250 mm connected to S5ODS2 precolumns were applied in all HPLC analyses.

Transepithelial resistance (TEER) was measured in tissue resistance measurement chambers (Endohm) with a voltohmmeter from World Precision Instruments. The Swip KS 10 Digi shaking plate used for cell culture experiments was from Edmund Bühler. Radioactivity was counted with a Tri-Carp 2110TR Liquid Scintillation Analyzer from Packard.

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 fibre-optics and a PTI fluorescence detection system.

2.3. Synthesis

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 as previously described (Friedrichsen et al., 2001). The crude products were purified by flash chromatography (Still et al., 1978). Intermediates (a–f) and final products (I–III) are shown in Fig. 1.

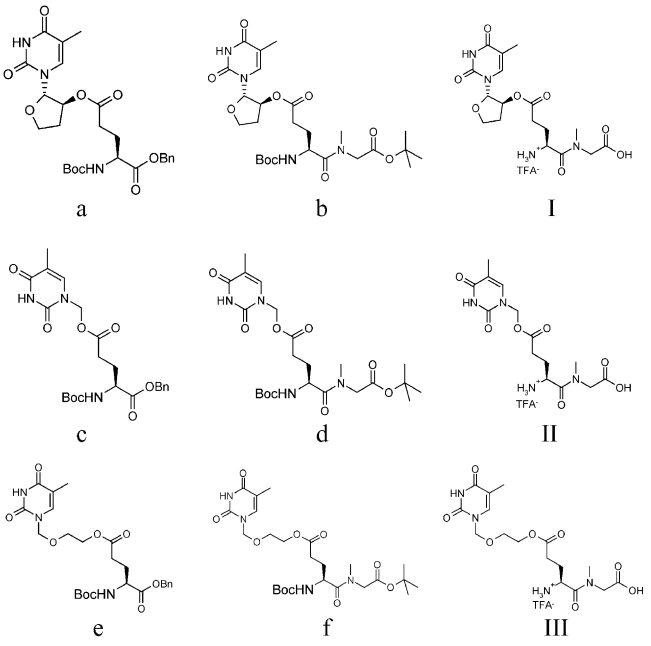


Fig. 1. The chemical structure of the three products L-Glu-(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-Sar (I), L-Glu-(thymine-1-yl-methyl ester)-Sar (II) and L-Glu-(acyclothymidine)-Sar (III) as their trifluoroacetates. The compounds (a–f) are intermediates in the synthesis as described in Section 2.

2.3.1. L-*Glu(trans-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-Sar-OH, trifluoroacetate (I)*

The ester linked cyclic nucleoside analogue was synthesised by a Vorbrüggen coupling between 2,4-bis(trimethylsilyl)thymine and acetic acid (2-methoxytetrahydrofuran-3-yl) ester catalyzed by $SnCl_4$ to give almost selectively the acetic acid (*trans*-2-thymin-1-yl-tetrahydrofuran-3-yl) ester (Lin et al., 1979). The key intermediate and acetic acid (2-methoxytetrahydrofuran-3-yl) ester were synthesised by treatment of 2-methoxytetrahydrofuran-3-ol with acetyl chloride/pyridine. 2,3-Dihydrofuran was treated with peracetic acid in methanol to give almost selectively the *trans*-2-methoxytetrahydrofuran-3-ol (Craig et al., 1999; Sweet and Brown, 1966). The acetyl group was removed using 1 M LiOH in THF.

By this procedure, 0.67 g of yellow–white, solid *trans* (*2-thymine-1-yl-tetrahydrofuran-3-ol*) was obtained, rf 0.25 (ethyl acetate). δ_H ($CDCl_3$) 10.30 (1H, s, thymine NH), 7.22 (1H, s, thymine H–C=C), 5.72 (1H, s, H-2′), 4.93 (1H, s, OH), 4.47 (1H, d, $J = 4.6$ Hz, H-3′), 4.32 (2H, m, H5′), 2.12 (1H, m, H-4′), 1.98 (1H, m, H-4′), 1.92 (3H, s, thymine CH_3). δ_C ($CDCl_3$) 164.6, 151.2, 134.6, 110.9, 94.3, 76.4, 70.5, 31.3 and 13.0. Anal. calc. for $C_9H_{12}N_2O_4$:C, 50.94; H, 5.70 and N, 13.20. Found: C, 50.74; H, 5.64 and N, 12.91. The 3-hydroxy group was then coupled to L-Glu-Sar using the following sequential procedure. *Boc-Glu*(*trans-2-thymine-1-yl-tetrahydrofuran-3-yl ester*)-*OBn* (*a*) was prepared by dissolving Boc-Glu-OBn (334 mg, 0.99 mmol), 1-hydroxybenzotriazole hydrate (152 mg, 0.99 mmol), EDAC (278 mg, 1.45 mmol) and DMAP (0.66 mmol, 81 mg) in DCM (2 ml). *Trans* (2-thymine-1-yl-tetrahydrofuran-3-ol) (0.6 g, 2.83 mmol) in DMF (2.00 ml) was added under nitrogen followed by stirring at rt for 16 h. Evaporation to dryness in vacuo gave oil, which was dissolved in boiling ethyl acetate (50 ml). Washing with saturated aqueous NaCl (2× 30 ml), saturated aqueous $NaHCO_3$ (2× 30 ml), 10% aqueous citric acid (2× 30 ml), saturated aqueous NaCl (30 ml), drying over $MgSO_4$, removal of the ethyl acetate and flash chromatography (ethyl acetate–heptane 1:1 → ethyl acetate) afforded 0.31 g (94%) of Boc-Glu(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-OBn (a), rf 0.12 (ethyl acetate-heptane 1:1). δ_H ($CDCl_3$) 7.37 (5H, s, phenyl CH), 7.06/7.03 (1H, s, C=C–H in thymine), 5.69 (1H, d, $J = 2.9$ Hz, H-2′), 5.37 (1H, m, H-3′) 5.21 (1H, m, NH in Glu), 5.17 (2H, s, CH_2 in benzyl), 4.40 (1H, m, CH in Glu), 4.32 (1H, m, H-5′), 4.09 (1H, m, H-5′), 2.44 (2H, m, CH_2 in Glu), 2.39 (1H, m, H-4′), 2.20 (1H, m, CH^A in CH_2 in Glu), 2.12 (1H, m, H-4′), 1.93 (1H, m, CH^B in CH_2 in Glu), 1.91 (3H, s, CH_3 in thymine), 1.42 (9H, s, Boc).

Boc-Glu(*trans-2-thymine-1-yl-tetrahydrofuran-3-yl ester*)-*Sar*-O*t-Bu* (*b*) was prepared by first dissolving Boc-Glu(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-OBn (a) (0.16 g, 0.301 mmol) in ethyl acetate (10 ml). Ten percent palladium on carbon (300 mg) was added and the solution was stirred under hydrogen for 16 h. Filtration, extraction with additional 100 ml of methanol and evaporation to dryness gave 0.10 g (75%) of Boc-Glu[1-(cis-3-hydroxy-2-tetrahydrofuranyl)-thymine]-OH as a white solid, rf 0.10 (ethyl acetate).

Sar was attached to Boc-Glu(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-OH by the following procedure: Boc-Glu(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-OH (100 mg, 0.23 mmol), 1-hydroxybenzotriazole, hydrate (33 mg, 0.22 mmol), diisopropylethylamine (0.2 ml) and EDAC (66 mg, 0.34 mmol) were dissolved in DMF (1.0 ml). H-Sar-O*t*-Bu·HCl (36 mg, 0.20 mmol) was added and the solution stirred under nitrogen for 20 h. The solution was evaporated to dryness in vacuo and the residue dissolved in boiling ethyl acetate (25 ml). Washing with saturated aqueous NaCl (2× 15 ml), saturated aqueous $NaHCO_3$ (2× 15 ml), 10% aqueous citric acid (2× 20 ml), saturated aqueous NaCl (1× 15 ml), drying over $MgSO_4$, filtration, removal of the ethyl acetate in vacuo and flash chromatography (ethyl acetate) yielded 80 mg (70%) of semi-solid Boc-Glu(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl ester)-Sar-O*t*-Bu (b), rf 0.70 (ethyl acetate). δ_H (CD_3OD) 7.45/7.38 (1H, s, thymine H–C=C), 5.73 (1H, m, H-2′), 5.43 (1H, m, H-3′), 4.70/4.51* (1H, m, CH in Glu), 4.35 (1H, m, H-5′), 4.20 (1H, d, $J = 17.2$ Hz, H^A in CH_2 in Sar), 4.08 (1H, m, H5′), 3.83 (1H, d, $J = 17.2$ Hz, H^B in CH_2 in Sar), 3.22/2.93* (3H, s, CH_3 in Sar), 2.50 (2H, m, CH_2 in Glu), 2.50 (beneath CH_2 signals from Glu) (1H, m, H-4′), 2.12 (1H, m, H^A in CH_2 in Glu), 2.12 (beneath CH_2 signals from Glu) (1H, m, H-4′), 1.88 (3H, s, CH_3 in thymine), 1.46 (9H, s, COO-*t*-Bu), 1.43 (9H, s, Boc). *Two conformers.

L-Glu(*trans-2-thymin-1-yl-tetrahydrofuran-3-yl ester*)-*Sar-OH, trifluoroacetate* (*I*) was achieved using the following deprotection procedure: Boc-Glu(*trans*-2-thymine-yl-tetrahydrofuran-3-yl ester)-Sar-O*t*-Bu (b) (80 mg, 0.14 mmol) was dissolved in dichloromethane (3 ml). After addition of TFA (1.5 ml), the solution was stirred for 3 h. The solvent was removed in vacuo. Co-evaporation with chloroform (3× 3 ml) yielded 74 mg (100%) of semi-solid L-Glu(*trans*-2-thymine-1-yl-tetrahydrofuran-3-yl-ester)-Sar-OH, trifluoroacetate (I). δ_H (CD_3OD) 7.38 (1H, s, thymine H–C=C), 5.79 (1H, m, H-2′), 5.42 (1H, m, H-3′), 4.57 (1H, m, CH in Glu), 4.38 (1H, m, H-5′), 4.30 (1H, d, $J = 17.4$ Hz, H^A CH_2 in Sar), 4.07 (1H, m, H5′), 4.03 (1H, d, $J = 17.2$ Hz, H^B in CH_2 in Sar), 3.19/3.00* (3H, s, CH_3 in Sar), 2.65 (2H, m, CH_2 in Glu), 2.40 (1H, m, H^A in CH_2 in Glu), 2.22 (1H, m, H^B in CH_2 in Glu), 2.17 (beneath CH_2 signals from Glu) (2H, m, H-4), 1.89 (3H, s, CH_3 in thymine). *Two conformers.

2.3.2. L-*Glu(thymine-1-yl-methyl ester)-Sar, trifluoroacetate (II)*

Boc-Glu(*thymine-1-yl-methyl ester*)-*OBn* (*c*) was prepared by adding thymine to formaldehyde. A *N*-acyloxymethyl linker was introduced selectively in the 1-position by condensing 1,3 bis(hydroxy)thymine with Boc-Glu-OBn in the presence of dicyclohexylcarbodiimide (DCC) and catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP) (Ahmad et al., 1987). Boc-Glu(thymine-1-yl-methyl ester)-OBn (c) appeared as a solid, rf 0.70 (EtOAc). δ_H ($CDCl_3$) 8.14 (1H, s, NH in thymine), 7.36 (5H, s, benzyl CH), 7.28 (1H, s, H–C=C), 5.63 (2H, s, CH_2O), 5.17 (2H, s, benzyl CH_2), 4.40 (1H, m, CH in Glu), 2.47 (2H, m, CH_2 in Glu), 2.23-1.80 (2H, m, CH_2 in Glu), 1.92 (3H, s, thymine CH_3), 1.41 (9H, s, Boc).

Sar was attached by the following sequential procedure: Boc-Glu(thymine-1-yl-methyl ester)-OBn (c) (0.24 g, 0.50 mmol) was dissolved in ethyl acetate under nitrogen as described in Section 2.3.1. Ten percent palladium on carbon (200 mg) was added and the mixture stirred under hydrogen for 2 h. Subsequent filtration through Celite and evaporation to dryness gave 200 mg (100%) of Boc-L-Glu(thymin-1-yl-methyl ester)-OH as an oil. Boc-Glu(thymin-1-yl-methyl ester)-OH (0.20 g, 0.52 mmol) and 1-hydroxybenzotriazole hydrate (75 mg, 0.51 mmol) were dissolved in dry DMF under nitrogen. After 2–3 min, DIPEA (0.2 ml, 1.15 mmol), H-Sar-O*t*-Bu·HCl (82 mg, 0.45 mmol) and EDAC (0.15 g, 0.78 mmol) were added and the solution was stirred under nitrogen overnight. After the same evaporation and washing procedures as described in Section 2.3.1, 0.15 g (65%) of Boc-Glu(thymine-1-yl-methyl ester)-Sar-O*t*-Bu (d) appeared as an oil, rf 0.57 (ethyl acetate). δ_H ($CDCl_3$) 7.56 (1H, s, thymine H–C=C), 5.71 (2H, s, CH_2 in methyl linker), 5.40/5.30* (1H, s, NH in Glu), 4.70/4.54* (1H, m, CH in Glu), 4.20 (1H, d, $J = 17.1$ Hz, H^A in CH_2N), 3.79 (1H, d, $J = 17.2$ Hz, H^B in CH_2N), 3.20/2.94* (3H, s, NCH_3), 2.52 (2H, m, CH_2 in Glu), 2.06 (1H, m, H^A in CH_2 in Glu), 1.87 (3H, s, CH_3 in thymine), 1.73 (1H, m, H_B in CH_2 in Glu), 1.49/1.45* (9H, s, COO-*t*-Bu), 1.41 (9H, s, Boc). *Two conformers.

L-Glu(*thymin-1-yl-methyl ester*)-*Sar*, *trifluoroacetate* (II) was prepared from Boc-Glu(thymine-1-yl-methyl ester)-Sar-O*t*-Bu (d) (50 mg) using the same deprotection procedure as described in Section 2.3.1 to yield 46 mg (100%) of Boc-Glu(thymine-1-yl-methyl ester)-Sar, trifluoroacetate (II) as a semi-solid. δ_H ($CDCl_3$) 7.54 (1H, s, thymine H–C=C), 5.71 (2H, s, CH_2 in methyl-linker), 5.30 (1H, d, $J = 4.1$ Hz, NH in Glu), 4.58/4.40* (1H, m, CH in Glu), 4.29 (1H, d, $J = 17.5$ Hz, H^A in CH_2N), 4.01 (1H, d, $J = 17.4$ Hz, H^B in CH_2), 3.18/3.00* (3H, s, NCH_3), 2.66 (2H, m, CH_2 in Glu), 2.23 (1H, m, H^A in CH_2 in Glu), 2.15 (1H, m, H^B in CH_2 in Glu), 1.88 (3H, s, CH_3 in thymine). *Two conformers.

2.3.3. L-*Glu(acyclothymidine)-Sar, trifluoroacetate (III)*

The 1-(2-hydroethoxy)methyl linker was attached to *N*-1 of thymine by reacting 2,4-bis(trimethylsilyl)thymine with 1,3-dioxolane, potassium iodide and chlorotrimethylsilane in acetonitrile at room temperature (Ubasawa et al., 1995). A 3.4 g of (thymine-1-yl-methyl)-ethan-2-ol was obtained with rf 0.33 (DCM-MeOH 9:1), which was crystallised from 96% ethanol (18 ml) to give melting point of 175–177 °C. δ_H (CD_3OD) 7.51 (1H, s, thymine H–C=C), 5.18 (2H, s, NCH_2O), 3.64 (4H, m, CH_2O in linker), 1.89 (3H, s, CH_3 in thymine).

Boc-Glu(*acyclothymidine ester*)-*OBn* (*e*) was prepared from Boc-Glu-OBn (2.53 g, 7.49 mmol), 1-hydroxybenzotriazole hydrate (1.15 g, 7.48 mmol), DMAP (0.92 g, 7.53 mmol) and EDAC (2.10 g 10.95 mmol), which were dissolved in dry dichloromethane (15 ml). After stirring for 10 min, thymine-1-yl-methyl-ethan-2-ol (1.00, 5.00 mmol) dissolved in 15 ml hot dry DMF, was added. Upon stirring for 16 h and after evaporation and washing procedures as described in Section 2.3.1, 1.55 g (60%) of Boc-Glu(acyclothymidine)-OBn (e), rf 0.59 (ethyl acetate), in the form of a solidifying oil was obtained. δ_H ($CDCl_3$) 8.53 (1H, s, thymine NH), 7.36 (s, 5H, phenyl), 7.13 (1H, s, H–C=C in thymine), 5.23 (1H, d, $J = 8.4$ Hz, NH in Glu), 5.17 (2H, s, NCH_2O), 5.15 (2H, s, benzyl CH_2), 4.46 (1H, m, CH in Glu), 4.22 (2H, t, $J = 3.4$ Hz, CH_2 in linker), 3.77 (2H, t, $J = 4.6$ Hz, CH_2 in linker), 2.40 (2H, m, CH_2 in Glu), 2.18 (1H, m, H^A in CH_2 in Glu), 1.97 (1H, m, H^B in CH_2 in Glu), 1.93 (3H, s, CH_3 in thymine), 1.43 (9H, s, Boc).

Boc-Glu(*acyclothymidine*)-*Sar-Ot-Bu* (*f*) was prepared from Boc-Glu(acyclothymidine)-OBn (e) (0.77 g, 1.48 mmol) as described in Sections 2.3.1 and 2.3.2 to yield 0.60 g (87%) of *Boc-Glu*(*acyclothymidine*)-*Sar-Ot-Bu*(*f*) as an oil, rf 0.39 (ethyl acetate). δ_H ($CDCl_3$) 8.85 (1H, s, thymine NH), 7.16 (1H, s, thymine H–C=C), 5.46 (1H, d, $J = 8.4$ Hz, Glu NH), 4.74/4.56* (1H, m, CH in Glu), 4.23 (2H, m, CH_2O in linker), 4.23 (beneath CH_2O signal) (1H, H^A in CH_2N), 3.79 (2H, t, $J = 4.5$ Hz, CH_2O in linker), 3.79 (beneath CH_2O signal) (1H, H^B in CH_2N), 3.16/2.95* (3H, s, CH_3 in Sar), 2.44 (2H, m, CH_2 in Glu), 2.11 (1H, m, H^A in

CH_2 in Glu), 1.93 (3H, s, CH_3 in thymine), 1.75 (1H, m, H^B in CH_2 in Glu), 1.45 (9H, s, Boc), 1.40 (9H, s, COO-*t*-Bu). *Two conformers.

L-Glu(*acyclothymidine*)-*Sar, trifluoroacetate* (*III*) was obtained by deprotection of Boc-Glu(acyclothymidine)-Sar-O*t*-Bu (f) as described in Section 2.3.1 to yield 46 mg (100%) of L-Glu(acyclothymidine)-Sar, trifluoroacetate (III) as a brown oil. δ_H (CD_3OD) 7.90 (1H, s, thymine NH), 7.49 (1H, s, thymine H–C=C), 5.17 (2H, s, NCH_2O), 4.58/4.41* (1H, m, CH in Glu), 4.29 (beneath CH_2O signal) (1H, d, $J = 17.5$ Hz, H^A in CH_2N), 4.26 (2H, m, CH_2O in linker), 4.03 (1H, d, $J = 17.4$ Hz, H^B in CH_2N), 3.79 (2H, t, $J = 4.6$ Hz, CH_2O in linker), 3.19/3.00* (3H, s, NCH_3), 2.59 (2H, m, CH_2 in Glu), 2.22 (1H, m, H^A in CH_2 in Glu), 2.09 (1H, m, H^B in CH_2 in Glu), 1.88 (3H, s, CH_3 in thymine). *Two conformers are observed.

2.4. Stability in aqueous solution

The degradation of I–III as well as the appearance of the respective pyrimidine or nucleoside analogues was studied in various buffer solutions at 37 ± 0.5 °C. The degradation of III was investigated in the pH range from 2.0 to 10.0, whereas the degradation of I and II was investigated at pH 5.0–7.4. The applied buffers were phosphate (pH 2.0, 3.0, 6.0 and 7.4), acetate (pH 4.0 and 5.0) and borate (pH 8.0, 9.0 and 10.0). The total buffer concentrations were 0.02–0.06 M. The ionic strength of each solution was adjusted with potassium chloride to 0.15. The reaction was initiated by adding 100 μl stock solution to 10 ml pre-equilibrated buffer solution resulting in initial concentrations ranging from 10^{-5} to 10^{-4} M. At various times, samples were taken and either analysed immediately or kept for maximally 2 h at 5 °C until analysis. The mobile phase consisted of acetate buffer, methanol, acetonitrile and triethylamine (90:5:5:0.01 or 95:0:5:0.01). The flow rate was 1.0 ml/min and detection wavelengths were either 268 or 270 nm. The limit of quantification for the compounds in aqueous solution was generally approximately 5×10^{-6} M, and the retention times were 15 and 16 min for the two isomers of I, and 7 and 8 min for II and III. For Compound III, a peak appeared at 26 min. This peak was interpreted as the peak corresponding to the proposed cyclisation product arising from cyclisation of the dipeptide backbone.

The degradation of the dipeptide derivatives was also investigated at 37 °C ± 0.5 °C in 10% porcine intestinal mucosal homogenate prepared in saline 0.05 M phosphate buffer, pH 7.4 prepared as described previously (Krondahl et al., 1997). The reaction was initiated by adding a stock solution to 5 ml pre-equilibrated biological media resulting in initial concentrations ranging from 4×10^{-5} to 8×10^{-4} M. At various times, samples were taken and analysed as previously described; however, a protein precipitation step was included. This was done by adding a saturated solution of $(NH_4)_2SO_4$ to the sample at a ratio of 2:1 followed by mixing and centrifugation at 18,000 rpm for 5 min at 4 °C. The clear supernatant was stored for maximally 2 h at 5 °C until analysis.

2.5. Cell culture

Caco-2 cells were cultured as previously described (Nielsen et al., 2001a). 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 U/ml and 100 μg/ml, respectively), 1% L-Glutamine and 1% non-essential amino acids. For affinity, studies cells were seeded onto tissue culture treated Transwells® (1.13 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 32 to 45. TEER was measured at room temperature just before the experiments and ranged from 300 to 500 Ω cm^2. Cells for studying hPEPT1-mediated translocation were cultured under similar conditions except that they were seeded onto 4.52 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.

2.6. [^{14}C]Gly-Sar uptake studies

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 (Nielsen et al., 2001c). 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 applied apically and 7.4 applied basolaterally. The experiment was initiated 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.

2.7. pHi measurements

hPEPT1-mediated translocation of substrates across the apical membrane of Caco-2 cells was investigated by monitoring the intracellular pH of cells, which were perfused apically with test solutions (Thwaites et al., 1993). The solutions used were Krebs–Ringer's 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's solution, pH 6.0.

The experiments were carried out on filter grown Caco-2 cell monolayers. The cells were loaded in Krebs–Ringer's

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 upwards. The basolateral side was bathed in Krebs–Ringer's 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's solution, pH 7.4 for 5 min after loading and was then excited with light at 490 and 432 nm alternately (HBW 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 to approximate pHi by means of a calibration curve. The calibration curve was 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 one to three times the corresponding K_i value determined in inhibition studies. Based on Michaelis–Menten kinetics, a positive response would be expected to be between 50 and 75% of maximal response, and thus would be detectable. Experiments were performed at least in duplicate. Positive (Gly-Sar) and negative(Lys[Z(NO_2)]-Pro) controls were included for validation of the method (Knütter et al., 2001). Furthermore, the L-Glu-Sar derivative of acyclovir L-Glu(acyclovir)-Sar was included (Thomsen et al., 2003).

2.8. Data analysis

Kinetic analysis of model drug release in aqueous solutions was performed using observed rate constants (k_{obs}) obtained at various pH values and buffer concentrations extrapolated to zero buffer concentration (Larsen and Bundgaard, 1977). The degradation pathways of I and III at pH ≤ 7.4 were elucidated by following the release of the respective model drugs as well as following the disappearance of dipeptide derivative.

K_i for the dipeptide derivatives and K_m for Gly-Sar were calculated. In short the inhibition of [^{14}C]Gly-Sar uptake at various concentrations of test compound was determined. The degree of inhibition was fitted to a Michaelis–Menten-type equation (Equation (1)).

$$1 - \left(\frac{U}{U_0}\right) = \frac{1 - (U/U_0)_{max} \times [I]}{IC_{50} + [I]} \quad (1)$$

where U is the uptake of [^{14}C]Gly-Sar, U_0 the uptake of [^{14}C]Gly-Sar at zero inhibitor concentration, [I] the concentration of test compound and IC_{50} was the concentration of test compound causing 50% inhibition of [^{14}C]-Gly-Sar uptake. IC_{50}-values were transformed to a K_i-value using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

Data from pHi measurements were compiled by determining the slope of the pH versus time curve for each compound (app 150 data points) and testing it against the slope resulting from perfusion of a blank buffer (pH 6.0) using paired *t*-tests of the slopes from the respective blank and test solutions.

3. Results

3.1. Stability in aqueous solution

At fixed temperature, buffer concentration and ionic strength, the overall dipeptide derivative degradation as well as the model drug release followed first-order kinetics. The pH–rate profile of III was determined in the range between pH 2.0 and 10.0 (Table 1). The overall degradation was catalysed by specific acid and base and the overall stability of the dipeptide derivative was highest at pH ∼ 5. At pH < 5, the predominant route of degradation was through a proposed cyclisation of the dipeptide backbone similar to previous observations by Steffansen and co-workers (Thomsen et al., 2003).

The stability of the dipeptide derivatives as well as the model drug release from I and II was investigated in the pH range between 5.0 and 7.4 under the same conditions as for III. In this pH range, ester hydrolysis catalysed by specific base was predominant for both compounds. The overall half-lives of the three compounds at pH 6.0 and 7.4 in aqueous zero buffer solution are shown in Table 1. Standard error for half-lives in zero buffer concentration was obtained from the regression analysis of half-lives versus total buffer concentration.

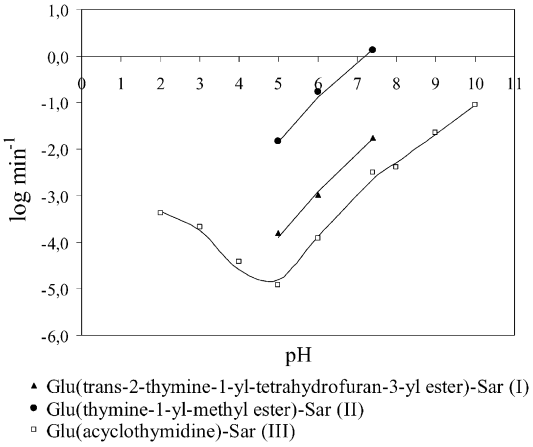
The stability of the dipeptide derivatives I and III was also followed in 10% porcine intestinal mucosal homogenate at pH 7.4 and at 37 °C. Compound II was not investigated in this biological medium due to the high lability in aqueous buffer solution. The degradation of I and III followed pseudo-first-order kinetics and their respective half-lives in 10% porcine intestinal mucosal homogenate is listed in Table 1. The degradation of III was faster in 10% intestinal mucosal homogenate than in aqueous solution at pH 7.4, whereas the degradation of I in this medium was in the same order of magnitude as in aqueous solution at pH 7.4.

3.2. [^{14}C]Gly-Sar uptake studies

The dipeptide derivatives' affinity to hPEPT1 was determined by displacement studies of [^{14}C]-Gly-Sar in Caco-2 cell monolayers as described in Section 2. The inhibition curves are shown along with the inhibition constants (K_i) in Fig. 2. Data are given as means ± S.E. All three compounds showed an affinity in terms of K_i less than 1 mM.

Table 1
Half-lives of the dipeptide derivatives at pH 6.0 and 7.4 at zero buffer concentration as well as in the biological medium 10% intestinal porcine mucosal homogenate, mean ± S.E. ($n = 3$)

Compound	$t_{1/2}$ ± S.E.		
	Zero buffer concentration		Biological medium
	pH 6.0	pH 7.4	10% intestinal porcine mucosal homogenate
I	11.3 ± 1.2 h	40 ± 1 min	59 ± 4 min
II	8.7 ± 0.7 min	27 ± 1 s	nd
III	3.74 ± 0.38 days	3.6 ± 0.5 h	39 ± 3 min



nd: not determined. *Inset:* pH–rate profile with log k_{obs} (min^{-1}) vs. pH for the dipeptide derivatives at 37 °C and zero buffer concentration. The curves are the results of the fits to the theoretical expression for degradation.

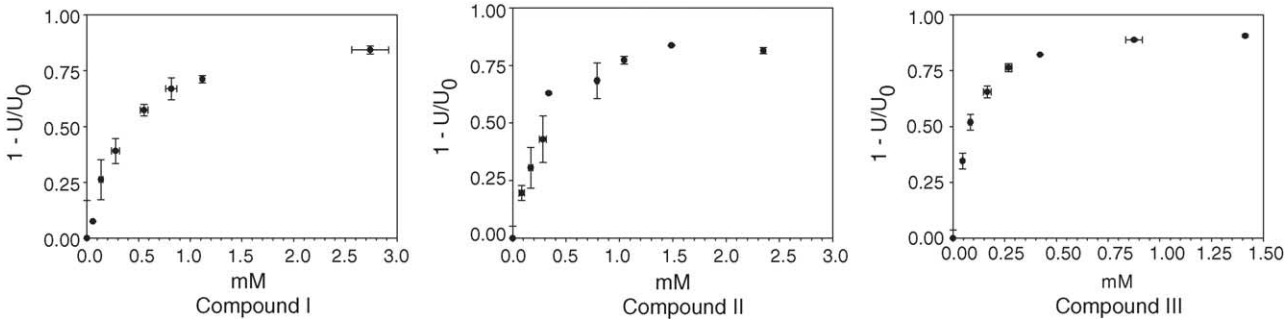


Fig. 2. Inhibition data for Compounds I–III. Inhibition of [^{14}C]Gly-Sar uptake ($1 - U/U_0$) as a function of concentration of test compound. K_i ± S.E. (mM) for the three compounds were determined as described in Section 2 to be: Compound I: 0.38 ± 0.05, Compound II: 0.37 ± 0.05 and Compound III: 0.075 ± 0.004. 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 ± S.E., $n = 3$).

3.3. pHi measurements

The hPEPT1-mediated translocation of Compounds I and III, Gly-Sar, Lys[Z(NO_2)]-Pro and L-Glu(acyclovir)-Sar were investigated by monitoring the intracellular pH of Caco-2 cell monolayers (Fig. 3). Slopes of the ratio versus time curve are depicted in Fig. 4. Only Gly-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 III and L-Glu(acyclovir)-Sar proved not to induce an intracellular acidification indicating that they are not translocated via a proton cotransporter such as hPEPT1. The magnitude of the responses for the compounds I and Lys[Z(NO_2)]-Pro indicate that these compounds are not translocated either.

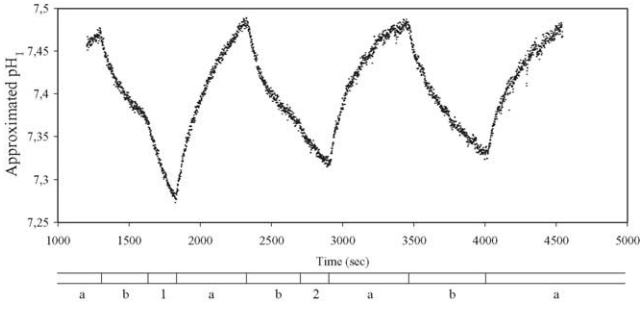


Fig. 3. Translocation studies on Caco-2 cell monolayers. Filters mounted in Ussing-like chambers with Krebs–Ringer solution pH 7.4 basolaterally are perfused apically with (a) buffer pH 7.4, (b) buffer pH 6.0, (1) 2 mM Gly-Sar in buffer pH 6.0 and (2) 0.23 mM III (L-Glu(acyclothymidine)-Sar).

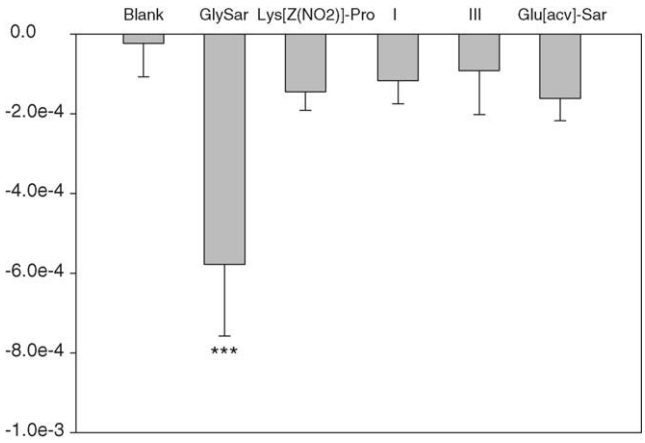


Fig. 4. pH (per s) ± S.D. for blank buffer (pH 6.0, $n = 15$), Gly-Sar (2 mM, $n = 5$), Lys[Z(NO_2)]-Pro (20 μM, $n = 2$), I (0.37 mM, $n = 2$), III (0.16 mM, $n = 3$) and L-Glu(acyclovir)-Sar (0.85 mM, $n = 3$); *** indicates significant ($p < 0.001$) difference from blank buffer.

4. Discussion

The aim of the present study was to improve the synthetic pathway of bioreversible dipeptide derivatives as well as evaluate the potential of using L-Glu-Sar as a pro-moiety for delivering three newly synthesised nucleoside and pyrimidine L-Glu-Sar derivatives.

Previously, Steffansen and co-workers have prepared Boc-Glu-Sar-O*t*-Bu and Boc-D-Glu-Ala-O*t*-Bu and coupled the dipeptide directly to other compounds such as acyclovir and 1,2 hydroxyethylthymine (Thomsen et al., 2003). This strategy seems to have some disadvantages due to the very ineffective synthesis involving the Fm protection of the γ-carboxylic acid. According to our experiments, the deprotection of the Fm-group with triethylamine followed by ion-exchange chromatography was very time consuming. Instead, L-Glu was introduced to the pyrimidine/nucleoside analogues. The benzyl- and Boc-protecting groups in Boc-L-Glu(model drug)-OBn are orthogonal to deprotection and various amino acids can subsequently be introduced in either the C- or N-terminal. This synthetic pathway proved more flexible giving more opportunities for future new combinations of pro-drugs.

In evaluating the potential of L-Glu-Sar as pro-moiety, the strategy applied was to investigate the in vitro stability to gather qualitative as well as quantitative information on mechanisms and rates of degradation. Furthermore, targeting to hPEPT1 was investigated in Caco-2 cells by means of inhibition studies and hPEPT1-mediated intracellular acidification.

The pH in the upper small intestine is estimated to range from 5 to 6 and the transit time to be between 2 and 3 h (Daugherty and Mrsny, 1999). Since hPEPT1-mediated absorption is considered to take place in the upper small intestine, a drug or pro-drug targeted to hPEPT1 must remain stable under these conditions.

A number of dipeptide coupled ester model pro-drugs have been characterised recently (Nielsen et al., 2001b; Steffansen et al., 1999; Thomsen et al., 2003). This class of model pro-drugs releases typically the model drug by specific base-catalysed ester hydrolysis in the pH range between 5 and 10. Consequently, the model pro-drugs are considerably more stable at the luminal pH than at intracellular and plasma pH with half-lives typically in the range of days or hours at pH 6.0 and hours or minutes at pH 7.4. The mechanism of specific base-catalysed ester hydrolysis may be similar to the mechanism proposed by Johansen and Bundgaard, suggesting that the protonated amine is involved in an intramolecular intermediate facilitating the ester bond cleavage (Johansen and Bundgaard, 1981). However, the much shorter half-life of Compound II might indicate a different, kinetically equivalent, mechanism of release for this particular compound. The very short half-life of I (9 min) at pH 6.0 indicates that the derivative would be degraded rapidly in the small intestine before interaction with hPEPT1. Therefore, the drug delivery properties of this model drug (thymine) seem to not have improved by this approach.

The dipeptide derivatives I and III proved sufficiently stable at pH 6.0 in order for the compounds to be available for hPEPT1-mediated absorption with half-lives of close to 4 days and 9 h, respectively, at pH 6.0. Furthermore, the predominant mechanism of degradation of the dipeptide derivatives at pH > 5 was specific base-catalysed hydrolysis, which indicates that the model drugs will be released quantitatively once absorbed.

It has previously been proposed that dipeptide pro-drugs with an L-Glu-Sar dipeptide backbone form a cyclic product in parallel to drug release (Thomsen et al., 2003). A similar reaction has been reported on dipeptidic structures in ACE inhibitors (Gu and Strickley, 1987, 1988). The stability studies in aqueous solution suggest that the same is the case for Compound III. However, since this proposed mechanism of degradation is predominant at pH < 5 it could be avoided in a potential drug formulation by a gastro-resistant formulation.

The in vitro metabolism studies of Compounds I and III further indicate that model drug release is controlled by pH and to a lesser extent enzymatic activity in 10% intestinal mucosal homogenate.

All three Compounds I–III showed high affinity to hPEPT1, comparable to other known substrates such as valaciclovir ($K_i = 0.51$ mM) and cephalexin ($K_i = 11$ mM) and to affinities of other similar nucleoside and pyrimidine dipeptide pro-drugs such as L-Glu(acyclovir)-Sar ($K_i = 0.28$ mM) and L-Glu[1-(2-hydroxyethyl)-thymine]-Sar ($K_i = 0.21$ mM) (Nielsen et al., 2001c; Thomsen et al., 2003). On the other hand, it has recently been demonstrated that L-Glu(acyclovir)-Sar has limited in vivo bioavailability when administered orally to rats (Thomsen et al., 2004).

The present γ-derivatives I, III and L-Glu(acyclovir)-Sar were not translocated by hPEPT1 and L-Glu-Sar, therefore seems to be a poor pro-moiety for hPEPT1-mediated transport. A similar observation has been made for ε-derivatives

of Lys-Pro (Knütter et al., 2001). Based on a series of dipeptide derivatives with variations of the *N*-terminal amino acid, Brandsch and co-workers proposed that hPEPT1-mediated transport of these derivatives rely on the size of the side chain modification along with the distance from the α-carbon of the amino acid. Furthermore, the hydrophobicity of the introduced group was suggested to be of importance (Knütter et al., 2004).

The compounds presented in this study are less lipophilic than previously reported inhibitors with side-chain modifications, thus indicating that size alone may be a restrictive parameter. A contributing factor to the lack of translocation of the investigated compounds may also be the *N*-methylation of the dipeptide backbone. The potential of hPEPT1-mediated pro-drug delivery, is however still evident. So far, only a limited number of dipeptide backbones have been investigated and there are still possibilities in terms of other side chain positions available for derivatising. Furthermore, the dipeptide backbone is available for other forms of modification. Promising ketomethylene dipeptidomimetic backbone pro-moieties have recently been proposed by Luthman and co-workers (Våbenø et al., 2004a,b). Viable future strategies for targeting pro-drugs to hPEPT1 may be to explore combinations of alternative dipeptidomimetic backbone pro-moieties and drug attachment in various positions.

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