molecular pharmaceutics

brief articles

Serine Side Chain-Linked Peptidomimetic Conjugates of Cyclic HPMPC and HPMPA: Synthesis and Interaction with hPEPT1†

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Received June 1, 2010; Revised Manuscript Received August 9, 2010; Accepted August 24, 2010

Abstract: Cidofovir (HPMPC), a broad spectrum antiviral agent, cannot be administered orally due to ionization of its phosphonic acid group at physiological pH. One prodrug approach involves conversion to the cyclic form (cHPMPC, **1**) and esterification by the side chain hydroxyl group of a peptidomimetic serine. Transport studies in a rat model have shown enhanced levels of total cidofovir species in the plasma after oral dosing with L-Val-L-Ser-OMe cHPMPC, **2a**. To explore the possibility that **2a** and its three L/D stereoisomers **2b**−**d** undergo active transport mediated by the peptide-specific intestinal transporter PEPT1, we performed radiotracer uptake and electrophysiology experiments applying the two-electrode voltage clamp technique in *Xenopus laevis* oocytes overexpressing human PEPT1 (hPEPT1, SLC15A1). **2a**−**d** did not induce inward currents, indicating that they are not transported, but the stereoisomers with an L-configuration at the N-terminal valine (**2a** and **2b**) potently inhibited transport of the hPEPT1 substrate glycylsarcosine (Gly-Sar). A "reversed" dipeptide conjugate, L-Ser-L-Ala-OiPr cHPMPC (**4**), also did not exhibit detectable transport, but completely abolished the Gly-Sar signal, suggesting that affinity of the transporter for these prodrugs is not impaired by a proximate linkage to the drug in the N-terminal amino acid of the dipeptide. Single amino acid conjugates of cHPMPC (**3a** and **3b**) or cHPMPA (**5**, **6a** and **6b**) were not transported and only weakly inhibited Gly-Sar transport. The known hPEPT1 prodrug substrate valacyclovir (**7**) and its L-Val-L-Val dipeptide analogue (**8**) were used to verify coupled transport by the oocyte model. The results indicate that the previously observed enhanced oral bioavailability of **2a** relative to the parent drug is unlikely to be due to active transport by hPEPT1. Syntheses of the novel compounds **2b**−**d** and **3**−**6** are described, including a convenient solid-phase method to prepare **5**, **6a** and **6b**.

Keywords: hPEPT1 transporter; SLC15A1; oral bioavailability; intestinal transport; *Xenopus laevis* oocyte; prodrugs

Introduction

Cidofovir (HPMPC) is a broad spectrum antiviral agent active against herpes-, adeno-, polyoma- and poxviruses.[1] It is currently used as a treatment for human cytomegalovirus (HCMV) retinitis in immune-suppressed individuals.[1] HPMPC, and other

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† Preliminary accounts of some of this work were presented: Peterson, L. W.; Kashemirov, B. A.; Blazewska, K. M.; Breitenbach, J. M.; Borysko, K.; Drach, J. C.; Kim, J. S.; Kijek, P.; Hilfinger, J. M.; McKenna, C. E. Synthesis and structure−activity aspects of some cyclic cidofovir peptidomimetic prodrugs; Oral-15, *20th International Conference on Antiviral Research*, Palm Springs, CA, April 29−May 3, 2007. Sala-Rabanal, M.; Peterson, L. W.; Serpi, M.; Krylov, I. S.; Kashemirov, B. A.; Kim, J. S.; Mitchell, S.; Hilfinger, J. M.; McKenna, C. E. Interactions between the human oligopeptide transporter, hPEPT1 and serine side chain-linked cidofovir prodrugs; Poster-119, *22nd International Conference on Antiviral Research*, Miami Beach, FL, May 3−7, 2009. Krylov, I. S.; Peterson, L. W.; Kashemirov, B. A.; Breitenbach, J.; Borysco, K.; Drach, J. C.; Kim, J. S.; Hilfinger, J. M.; McKenna, C. E. *In vitro* transport, activation and antiviral evaluation of new HPMPA prodrugs synthesized on a solid support; Poster-190, *22nd International Conference on Antiviral Research*, Miami Beach, FL, May 3−7, 2009.

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10.1021/mp100186b
Published on Web 10/07/2010

phosphate/phosphonate drugs, contain an ionizable $P(O)(OH)_2$ group, which exists as a dianion at physiological pH resulting in low membrane permeability and low oral bioavailability. To address this problem, a number of prodrug strategies have recently been developed.[2,3] In one approach,[2,4,5] the first negative charge is masked by intramolecular cyclization to form cyclic cidofovir (cHPMPC, **1**), while the second P−OH is esterified by the hydroxy side chain of an appropriate dipeptide, e.g. X-Ser where X is an amino acid with a hydrophobic side chain and the carboxyl group of the Ser is esterified to mask its ionizable OH and increase lipophilicity. The use of cHPMPC, which is converted to HPMPC by intracellular cyclic CMP phosphodiesterases, offers the advantage of decreased nephrotoxicity, while maintaining potency.[6] The amino acid and dipeptide promoieties offer the potential to "fine-tune" the properties of the prodrug and are expected to be toxically benign.

The application of prodrug strategies has led to numerous examples of polar drugs being made accessible orally.[2,7,8] Typically, these approaches involve creating a drug precursor having improved membrane permeability due to enhanced lipophilicity[3] or to targeting and exploiting the function of specific transporters located in the gastrointestinal tract. One example of the latter is valacyclovir (**7**), the 5′-valyl ester of acyclovir (ACV).[9−11] This drug does not contain a phosphonate group, but has a terminal hydroxyl that can esterify the carboxylic acid group of the valine to form the conjugated prodrug, which exhibits enhanced oral bioavailability. This has been attributed to uptake and transport by the human oligopeptide transporter, hPEPT1 (SLC15A1), based on direct evidence obtained using radiolabeled **7** in *Xenopus laevis* oocytes expressing hPEPT1.[9,12] hPEPT1 is a proton-coupled di- and tripeptide transporter located in the brush border membrane of enterocytes of the small intestine. Its broad substrate specificity allows it to also transport nonpeptidic compounds, such as β-lactam antibiotics, making it attractive as a drug delivery target.[13] While approaches to modeling SAR and STR properties of hPEPT1 have been recently reported,[14−17] predictive rules for affinity and transport have been elusive.[18]

We have reported the synthesis of several L-X-L-Ser-OMe cHPMPC conjugates where X = Ala, Val, and Leu and the dipeptide promoiety is attached to the drug via esterification of its phosphonic acid group by the serine side chain hydroxyl function.[4,5] When investigated for permeability in an *in situ* single pass perfusion rat model, these conjugates showed enhanced transport properties compared to the parent compound, **1**.[5] The L-X-L-Ser-OMe cHPMPC derivatives were also evaluated for transport in a murine model, and after oral gavage dosing, increased levels of **1** species were observed in the plasma compared to dosing with **1** itself.[4,5] To investigate the possibility that hPEPT1 was implicated

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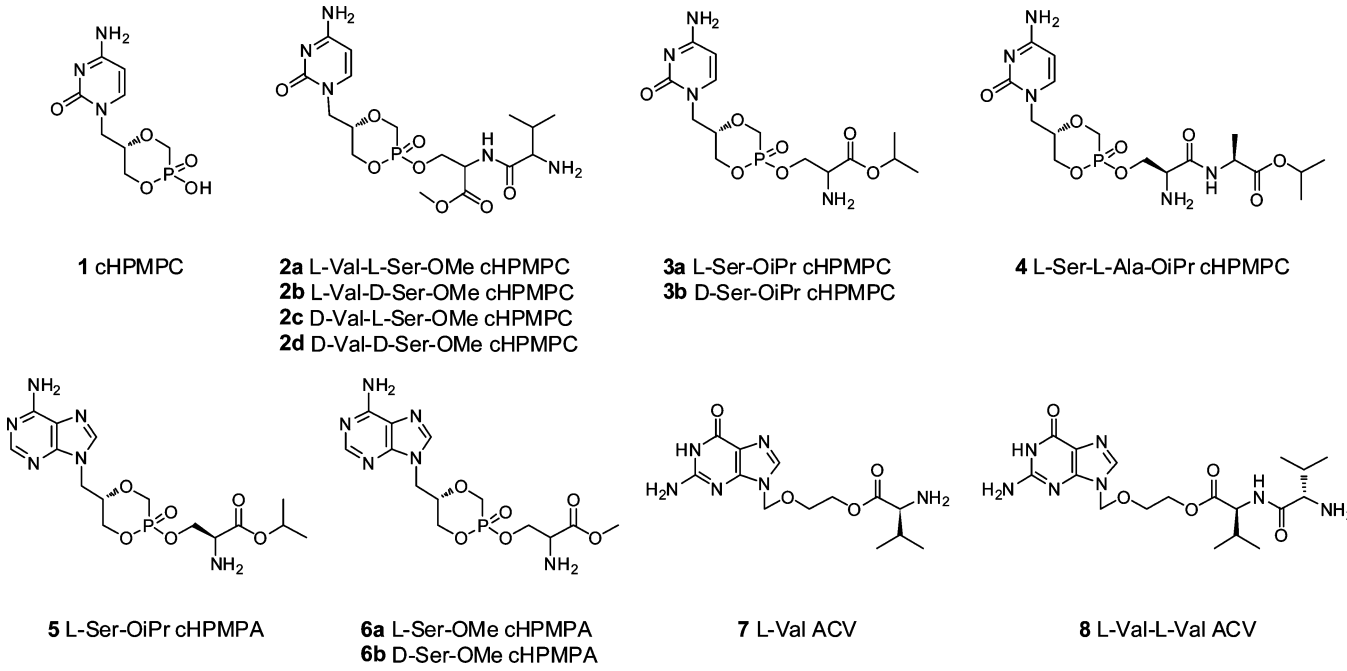

Figure 1. Compounds tested in the hPEPT1 oocyte expression system. Compounds **2**−**6** were isolated and evaluated as the diastereomeric mixtures obtained in synthesis due to generation of a new chiral center at phosphorus.

in the observed transport, these derivatives were codosed with glycylsarcosine (Gly-Sar), a well-known substrate for hPEPT1, in DC5 cells overexpressing hPEPT1.[5] All three compounds showed affinity for hPEPT1, with K_i values of 2.7 to 5.4 mM.[5]

In the present study, we report the synthesis of the three L/D stereoisomers of **2a** (**2b**−**2d**), the L/D stereoisomeric single amino acid serine side chain-linked conjugates of cHPMPC (**3a**,**b**) and its adenine analogue, cHPMPA (**5**, **6a** and **6b**) and a "reversed" dipeptide conjugate of cHPMPC (**4**) (Figure 1). To investigate the interactions between these prodrugs and hPEPT1, we employed a two-electrode voltage clamp (TEVC) technique and tracer uptakes in *Xenopus laevis* oocytes expressing the transporter.[13] In addition to Gly-Sar, valacyclovir (**7**, Figure 1) and its dipeptide analogue, L-Val-L-Val acyclovir (**8**, Figure 1), were included in the study as positive controls.

Experimental Section

General Experimental Methods. ^{1}H, ^{13}C, and ^{31}P NMR spectra were obtained on a 400 MHz Varian or 500 MHz Bruker spectrometer. Chemical shifts (δ values) are reported in ppm, and all coupling constants (J values) are quoted in Hz. The following NMR abbreviations are used: s (singlet), d (doublet), t (triplet), m (unresolved multiplet), dd (doublet of doublets), brs (broad signal). Chemical shifts (δ) are reported in parts per million (ppm) relative to internal $CHCl_3$ (δ 7.24, ^{1}H) or 85% H_3PO_4 (δ 0.00, ^{31}P). Data for ^{31}P NMR are ^{1}H decoupled. LC-MS analyses were done using a Thermo-Finnegan LCQ DECA XP_{max} system. HRMS spectra were recorded at the UCR High Resolution Mass Spectrometry Facility, Riverside, CA. All reagents were purchased from Sigma Aldrich or Alfa Aesar and used as obtained, unless specified otherwise. Cidofovir and also HPMPA were prepared as previously described,[4] and L-Val acyclovir was a kind gift from GlaxoSmithKline, Brentford, Middlesex, U.K. [Glycyl-2-^{3}H]-Gly-Sar (specific activity 60 Ci/mmol) was from American Radiolabeled Chemicals, St. Louis, MO.

General Method A. Synthesis of Amino Acid Isopropyl Esters. Isopropanol (1.5 mL/mmol amino acid) was added to a flask containing serine (500 mg, 4.8 mmol) or alanine (1.00 g, 11.2 mmol). TMSCl (2.7 equiv) was added to the reaction vessel, and the slurry was heated to reflux while stirring. Additional portions of TMSCl were added as necessary, while the reaction was monitored by ^{1}H NMR (after ~5 h, the solution became transparent, and the reaction was complete.) The sample was cooled to room temperature, and the volatiles were removed under reduced pressure yielding a solid. The excess isopropanol was coevaporated with acetonitrile, leaving a white solid.

Nomenclature. IUPAC-style names were generated with the assistance of ACD/Name (ACD Labs, Inc.). The Autonom nomenclature program, which gave somewhat different "IUPAC name" results for compounds such as **2a** as reported in our previous publication,[4] is no longer available.

Isopropyl L-Serinate Hydrochloride (9a). 9a was obtained, using general method A, as a white solid (759 mg, 87%). ^{1}H NMR (400 MHz, D_2O): δ = 1.17 (d, J = 6.3 Hz, 6H), 3.87 (dd, J = 12.5 and 3.5 Hz, 1H), 3.96 (dd, J = 12.5 and 4.4 Hz, 1H), 4.08 (dd, J = 3.5 and 4.4 Hz, 1H), 5.00 (m, 1H).

Isopropyl D-Serinate Hydrochloride (9b). 9b was obtained using general method A, as a white solid (558 mg, 64%). ^{1}H NMR (400 MHz, D_2O): δ = 1.18 (d, J = 6.3 Hz, 6H),

3.88 (dd, J = 12.5 and 3.5 Hz, 1H), 3.97 (dd, J = 12.5 and 4.4 Hz, 1H), 4.08 (dd, J = 3.5 and 4.4 Hz, 1H), 5.01 (m, 1H).

Isopropyl L-Alaninate Hydrochloride (10). 10 was obtained using general method A, as a white solid (972 mg, 52%). ^{1}H NMR (400 MHz, D_2O): δ = 1.17 (d, J = 6.3 Hz, 6H), 1.42 (d, J = 7.3 Hz, 3H), 4.01 (q, J = 7.3 Hz, 1H), 4.98 (m, 1H).

General Method B. Synthesis of Boc-Protected Dipeptides. Serine methyl ester hydrochloride (**9a**,**b**) or alanine isopropyl ester hydrochloride (**10**) (3.2 mmol, 1 equiv) and 1 equiv (3.2 mmol) of Boc-valine or Boc-L-serine, respectively, were dissolved in 30 mL of dry CH_2Cl_2. The reaction mixture was cooled to 0 °C before addition of HOBt hydrate (4.8 mmol, 1.5 equiv) and Et_3N (16 mmol, 5 equiv). The reaction mixture was kept at 0 °C for 15 min before EDC·HCl (4.0 mmol, 1.25 equiv) was added. The reaction mixture was stirred at rt overnight. An additional 100 mL of CH_2Cl_2 was added, and the organic layer was washed successively with a citric acid solution (1.6 M, 25 mL), saturated $NaHCO_3$ solution (25 mL), and saturated NaCl solution (20 mL). The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to yield the product.

Methyl *N*-(*tert*-Butoxycarbonyl)-L-valyl-L-serinate (11a). 11a was obtained from 250 mg of L-serine methyl ester hydrochloride and 349 mg of Boc-L-valine using general method B, as an off-white film (386 mg, 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.89 and 0.92 (2d, J = 6.6 Hz, 6H), 1.36 (s, 9H), 1.97−2.05 (m, 1H), 3.70 (s, 3H), 3.78−3.96 (m, 3H), 4.61−4.63 (m, 1H), 5.43 (brs, 1H), 7.28 (brs, 1H). The ^{1}H NMR chemical shift values are similar to those previously reported for the same compound in CD_3OD.[4]

Methyl *N*-(*tert*-Butoxycarbonyl)-L-valyl-L-serinate (11b). 11b was obtained using general method B and starting from 500 mg of D-serine methyl ester hydrochloride and 698 mg of Boc-L-valine as an off-white film (907 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.97 (d, J = 7.0 Hz, 3H), 1.03 (d, J = 7.0 Hz, 3H) 1.48 (s, 9H), 2.25 (m, 1H), 3.82 (s, 3H), 3.98−4.05 (m, 3H), 4.68 (m, 1H), 5.08 (brs, 1H), 7.03 (brs, 1H).

Methyl *N*-(*tert*-Butoxycarbonyl)-D-valyl-L-serinate (11c). 11c was obtained using general method B and starting from 250 mg of L-serine methyl ester hydrochloride and 345 mg of Boc-D-valine, as an off-white film (352 mg, 69%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.87 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.8 Hz, 3H) 1.38 (s, 9H), 2.15 (m, 1H), 3.72 (s, 3H), 3.87−3.96 (m, 3H), 4.58 (m, 1H), 4.96 (brs, 1H), 6.86 (brs, 1H).

Methyl *N*-(*tert*-Butoxycarbonyl)-D-valyl-D-serinate (11d). 11d was obtained using general method B and starting from 1.250 g of D-serine methyl ester hydrochloride and 1.746 g of Boc-D-valine, as an off-white film (1.933 g, 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.92 (d, J = 6.5 Hz, 3H), 0.97 (d, J = 6.5 Hz, 3H) 1.41 (s, 9H), 2.13 (m, 1H), 3.68 (s, 3H), 3.73−4.18 (m, 3H), 4.56 (m, 1H), 5.93 (brs, 1H), 7.49 (brs, 1H).

Isopropyl *N*-(*tert*-Butoxycarbonyl)-L-seryl-L-alaninate (13). 13 was obtained using general method B and starting from 250 mg of L-alanine isopropyl ester hydrochloride (**10**) and 306 mg of Boc-L-serine, as an off-white film (353 mg, 74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.17 and 1.19 (2d, J = 6.5 and 6.8 Hz, 6H), 1.33 (d, J = 7.4 Hz, 3H), 1.37 (s, 9H), 3.62 (m, 2H), 3.90−3.93 (m, 1H), 4.19 (brs, 1H), 4.44 (m, 1H), 4.96 (m, 1H), 5.69 (brs, 1H), 7.30 (brs, 1H).

Boc-Protection of Serine Isopropyl Esters (12a,b). Serine isopropyl ester **9** (200 mg, 1.08 mmol, 1 equiv) was dissolved in 4 mL of CH_3CN along with 240 mg (1 equiv) of di-*tert*-butyl dicarbonate. To the resulting solution was added 0.25 μL of Et_3N at once. The resulting suspension was stirred at room temperature for 60 min and then diluted with 50 mL of CH_2Cl_2. The resulting solution was washed with 10 mL of H_2O, 10 mL of 10% citric acid solution, and 10 mL of saturated $NaHCO_3$ solution. An additional wash with saturated $NaHCO_3$ solution was done as necessary. The organic layer was dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to afford a clear oil.

Isopropyl *N*-(*tert*-Butoxycarbonyl)-L-serinate (12a). 12a was obtained as a transparent oil (269 mg, 99%). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.19 and 1.20 (2d, J = 6.3 Hz, 6H), 1.38 (s, 9H), 3.18 (brs, 1H), 3.80 (dd, J = 11.4 and 3.5 Hz, 1H), 3.85 (dd, J = 11.4 and 4.4 Hz, 1H), 4.23 (brs, 1H), 5.00 (m, 1H), 5.57 (brs, 1H).

Isopropyl *N*-(*tert*-Butoxycarbonyl)-D-serinate (12b). 12b was obtained as a transparent oil (232 mg, 86%). ^{1}H NMR (500 MHz, CD_3OD): δ = 1.19 and 1.20 (2d, J = 6.3 Hz, 6H), 1.38 (s, 9H), 3.17 (brs, 1H), 3.78−3.87 (m, 2H), 4.22 (brs, 1H), 5.00 (m, 1H), 5.57 (brs, 1H).

General Method C. Synthesis of the Peptidomimetic cHPMPC Conjugates. To HPMPC (0.30 mmol, 1 equiv), 5 mL of anhydrous DMF and 0.5 mL of dry DIPEA were added. The reaction flask was warmed by a heat gun to facilitate the dissolution of the HPMPC−DIPEA salt. The solvent was then removed under vacuum. To the residue, 4 mL of anhydrous DMF, DIPEA (5 equiv), the relevant Boc-protected amino acid or dipeptide (1.5 equiv) in dry DMF and PyBOP (2.1 equiv) were added, and the reaction mixture was stirred under N_2 at 35 °C for 2 h. The reaction was monitored by ^{31}P NMR, and additional portions of PyBOP were added as necessary. Solvent was removed under vacuum, and the brownish-red residue was washed with Et_2O. The product was purified by preparative TLC [2 mm silica gel on 20 × 20 glass support, Analtech]. The Boc-protected derivative was recovered after extraction in CH_3OH for 3 h. TFA (4 mL, 99%) was added to a solution of the Boc-protected derivative in 4 mL of dry CH_2Cl_2. After 5 h at room temperature, the solvent was removed under vacuum. Purification of the target compounds was conducted by preparative TLC (2 mm silica gel on 20 × 20 cm glass support). The desired product was found in the lower band, cut out, and extracted using 100 mL of methanol. The silica was removed by filtration, the solutions of the bis(trifluoroacetic acid) salts in methanol were concentrated, and the products were precipitated by the addition of Et_2O and

collected by filtration. The white crystals obtained were carefully dried in vacuo. The final products were analyzed by ^{1}H NMR and ^{31}P NMR, HRMS, and LC−MS.

Methyl L-Valyl-*O*-{(5*S*)-5-[(4-amino-2-oxopyrimidin-1(2*H*)-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-L-serinate (2a). 2a was obtained from 100 mg of HPMPC and 131 mg of **11a** using general method C, as a white powder (123 mg, 56%). Mobile phase for first TLC purification: CH_2Cl_2/CH_3OH (6:1). Mobile phase for second TLC purification: CH_2Cl_2/CH_3OH (10:3). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.09−1.16 (m, 6H), 2.22−2.34 (m, 1H), 3.78−4.60 (m, 14H), 5.91−5.94 (brs, 1H), 7.60 (d, J = 7.4 Hz, 0.7H), 7.65 (d, J = 7.4 Hz, 0.3H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.41 (s, 0.65P), 15.53 (s, 0.35P). The ^{1}H and ^{31}P NMR chemical shift values are similar to those previously reported for the same compound.[4] LC−MS: t_R = 9.83 and 11.17 min; $[M + H]^+$ 462.1.

Methyl L-Valyl-*O*-{(5*S*)-5-[(4-amino-2-oxopyrimidin-1(2*H*)-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-D-serinate (2b). 2b was obtained from 100 mg of HPMPC and 152 mg of **11b** using general method C, as a white powder (47 mg, 22%). Mobile phase for first TLC purification: CH_2Cl_2/CH_3OH (6:1). Mobile phase for second TLC purification: CH_2Cl_2/CH_3OH (4:1). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.06−1.14 (m, 6H), 2.26 (m, 1H), 3.79−4.58 (m, 14H), 5.88 (d, J = 7.3 Hz, 1H), 7.53 (d, J = 7.3 Hz, 0.7H), 7.57 (d, J = 7.5 Hz, 0.3H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.40 (s, 0.7P), 15.68 (s, 0.3P).

Methyl D-Valyl-*O*-{(5*S*)-5-[(4-amino-2-oxopyrimidin-1(2*H*)-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-L-serinate (2c). 2c was obtained from 300 mg of HPMPC and 455 mg of **11c** using general method C, as a white solid (378 mg, 58%). Mobile phase for first TLC purification: CH_2Cl_2/CH_3OH (6:1). Mobile phase for second TLC purification: CH_2Cl_2/CH_3OH (10:3). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.07−1.13 (m, 6H), 2.25 (m, 1H), 3.75−4.58 (m, 14H), 5.92 (d, J = 7.3 Hz, 0.7H), 5.92 (d, J = 7.5 Hz, 0.3H), 7.59 (d, J = 7.3 Hz, 0.7H), 7.64 (d, J = 7.5 Hz, 0.3H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.21 (s, 0.8P), 15.26 (s, 0.2P).

Methyl D-Valyl-*O*-{(5*S*)-5-[(4-amino-2-oxopyrimidin-1(2*H*)-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-D-serinate (2d). 2d was obtained from 200 mg of HPMPC and 321 mg of **11d** using general method C, as a white powder (199 mg, 45%). Mobile phase for first TLC purification: CH_2Cl_2/CH_3OH (6:1). Mobile phase for second TLC purification: CH_2Cl_2/CH_3OH (10:3). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.09−1.16 (m, 6H), 2.29 (m, 1H), 3.78−4.57 (m, 14H), 5.89 (d, J = 7.5 Hz, 0.3H), 5.89 (d, J = 7.3 Hz, 0.7H), 7.53 (d, J = 7.3 Hz, 0.7H), 7.57 (d, J = 7.5 Hz, 0.3H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.77 (s, 0.7P), 15.73 (s, 0.3P). MS-ESI m/z $[M + H]^+$ calcd for $C_{17}H_{28}N_5O_8P$: 462.18, found 462.18. LC−MS: t_R = 13.34 and 14.88 min; $[M + H]^+$ 462.1.

Isopropyl *O*-{(5*S*)-5-[(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-L-serinate (3a). 3a was obtained from 150 mg of HPMPC and 176 mg of **12a** using general method C, as a white powder (110 mg, 37%). Mobile phase for first TLC purification: CH_2Cl_2/CH_3OH (6:1). Mobile phase for second TLC purification: CH_2Cl_2/CH_3OH (4:1). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.32−1.37 (m, 6H), 3.77−4.63 (m, 11H), 5.11−5.20 (m, 1H), 5.90 (d, J = 6.7 Hz, 1H), 7.55 (d, J = 6.7 Hz, 0.8H), 7.60 (d, J = 6.7 Hz, 0.2H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.63 (s, 0.78P), 15.51 (s, 0.22P). HRMS-FAB m/z $[M + H]^+$ calcd for $C_{14}H_{23}N_4O_7P$: 391.1377, found 391.1380. LC−MS: t_R = 9.22 min; $[M + H]^+$ 391.1.

Isopropyl *O*-{(5*S*)-5-[(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-D-serinate (3b). 3b was obtained from 150 mg of HPMPC and 176 mg of **12b** using general method C, as a white powder (62 mg, 21%). Mobile phase for first TLC purification: CH_2Cl_2/CH_3OH (9:1). Mobile phase for second TLC purification: CH_2Cl_2/CH_3OH (10:2.7). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.35−1.38 (m, 6H), 3.74−4.70 (m, 11H), 5.14−5.22 (m, 1H), 5.90 (d, J = 7.3 Hz, 1H), 7.52 (d, J = 7.3 Hz, 0.8H), 7.59 (d, J = 7.3 Hz, 0.2H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.51 (s, 0.80P), 15.73 (s, 0.20P). HRMS-FAB m/z $[M + H]^+$ calcd for $C_{14}H_{23}N_4O_7P$: 391.1377, found 391.1381.

Isopropyl *O*-{(5*S*)-5-[(4-Amino-2-oxopyrimidin-1(2*H*)-yl)-methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-L-seryl-L-alaninate (4). 4 was obtained from 150 mg of HPMPC and 257 mg of **13** using general method C, as a white powder (172 mg, 53%). Mobile phase for first TLC purification: CH_2Cl_2/CH_3OH (8:1). Mobile phase for second TLC purification: CH_2Cl_2/CH_3OH (4:1). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.27−1.31 (m, 6H), 1.44 and 1.47 (2d, J = 7.4 and 7.4 Hz, 3H), 3.75−4.64 (m, 11H), 5.01−5.07 (m, 1H), 6.00 (d, J = 7.5 Hz, 1H), 7.72 (d, J = 7.5 Hz, 0.6H), 7.77 (d, J = 7.5 Hz, 0.4H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.98 (s, 0.57P), 16.26 (s, 0.43P). HRMS-FAB m/z $[M + H]^+$ calcd for $C_{17}H_{28}N_5O_8P$: 462.1748, found 462.1752. LC−MS: t_R = 12.99 and 13.18 min; $[M + H]^+$ 462.2.

General Method D. TBDMS Protection of Serine Methyl and Isopropyl Esters. Serine methyl ester hydrochloride or serine isopropyl ester hydrochloride (**9a**) (2.3 mmol, 1 equiv) and *tert*-butylchlorodimethylsilane (4.8 mmol, 2.1 equiv) were dissolved in 20 mL of dry CH_2Cl_2. The reaction mixture was cooled to 0 °C before addition of imidazole (7.1 mmol, 3.1 equiv). The reaction mixture was stirred overnight at rt. An additional 100 mL of CH_2Cl_2 was added to the reaction mixture, and the organic layer was washed with a saturated citric acid solution (25 mL) and saturated $NaHCO_3$ solution (25 mL). Combined water phases were extracted twice with 30 mL of CH_2Cl_2 (to increase the yield, product as a salt is soluble in water). All organic phases were combined and dried over Na_2SO_4 and concentrated under reduced pressure yielding a white solid. The solid was washed on a filter with 20 mL of hexanes to remove the TBDMSOH, giving a clean product.

Isopropyl *O*-[*tert*-Butyl(dimethyl)silyl]-L-serinate (14). 14 was obtained using general method D (521 mg, 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.08 (s, 3H), 0.12 (s, 3H), 0.88 (s, 9H), 1.29 (d, J = 6.4 Hz, 6H), 4.13 (dd, J = 2.6

and 10.7 Hz, 1H), 4.20 (t, J = 2.6 Hz, 1H), 4.25 (dd, J = 2.6 and 10.7 Hz, 1H), 5.11 (sept, J = 6.7 Hz, 1H), 8.72 (brs, 3H).

Methyl *O*-[*tert*-Butyl(dimethyl)silyl]-L-serinate (15a). 15a was obtained using general method D (434 mg, 69%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.08 (s, 3H), 0.11 (s, 3H), 0.88 (s, 9H), 3.83 (s, 3H), 4.15 (dd, J = 3.0 and 10.7 Hz, 1H), 4.26 (dd, J = 3.0 and 10.7 Hz, 1H), 4.32 (m, 1H), 8.72 (brs, 3H).

Methyl *O*-[*tert*-Butyl(dimethyl)silyl]-D-serinate (15b). 15b was obtained using general method D (452 mg, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.07 (s, 3H), 0.10 (s, 3H), 0.87 (s, 9H), 3.82 (s, 3H), 4.14 (dd, J = 3.0 and 10.7 Hz, 1H), 4.25 (dd, J = 3.0 and 10.7 Hz, 1H), 4.32 (t, J = 3.0 Hz, 1H), 8.78 (brs, 3H).

General Method E. Solid-Phase Synthesis of the Peptidomimetic cHPMPA Conjugates. Addition of Amino Acid to the Trityl Chloride Polystyrene Resin (TCP). The TBDMS-protected serine alkyl ester (**14**, **15a** or **15b**, 4 equiv) was reacted with the trityl chloride polystyrene (TCP) resin (1 equiv) in CH_2Cl_2 in the presence of DIPEA (15 equiv) overnight. The resin was then filtered and washed using 150 mL of CH_2Cl_2. Removal of the TBDMS protecting group was accomplished with TBAF (4 equiv) in THF for 5 h at rt. The resin was then filtered and washed with 50 mL of THF followed by 100 mL of CH_2Cl_2. The resin (**16**) was dried under reduced pressure in a desiccator before being used for the coupling reaction.

Coupling of HPMPA with Amino Acid Promoiety. PyBOP (1.2 equiv) was added to a solution of HPMPA (1 equiv) and DIPEA (15 equiv) in DMF (10 mL). The cyclization reaction proceeded at rt. After 1 h an additional portion of PyBOP (3−4 equiv) and the TCP resin containing serine alkyl ester (**16**) were added to the reaction mixture. The reaction mixture was shaken overnight at 38 °C. The resin was then filtered and washed with 60 mL of DMF followed by 200 mL of CH_2Cl_2. The product was cleaved from the resin with TFA (2 mL) in CH_2Cl_2 (10 mL) in the case of serine methyl ester cHPMPA conjugates (**17a** and **17b**) and with 1.4 M HCl in dioxane/CH_2Cl_2 (10 mL/5 mL) in the case of serine isopropyl ester conjugate **5**. Finally, TFA salts of the peptidomimetic cHPMPA conjugates were purified using silica gel TLC plates (2 mm silica gel on 20 × 20 cm glass support, Analtech) using CH_2Cl_2/CH_3OH (6:1) as the mobile phase. The products were precipitated as trifluoroacetic acid salts from methanol by addition of diethyl ether and collected by filtration. Conjugate **5** was purified by reprecipitation from a methanolic solution by the addition of diethyl ether.

Isopropyl *O*-{(5*S*)-5-[(6-Amino-9*H*-purin-9-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-L-serinate (5). 5 was obtained using general method E (96 mg, 30%). ^{1}H NMR (400 MHz, CD_3OD): δ = 1.30−1.39 (m, 6H), 4.04−4.20 (m, 1H), 4.32−4.76 (m, 9H), 5.13−5.20 (m, 1H), 8.36 (s, 0.4H), 8.41 (s, 0.6H), 8.47 (s, 1H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.18 (s, 0.44P), 15.13 (s, 0.56P). HRMS-FAB m/z $[M + H]^+$ calcd for $C_{15}H_{23}N_6O_6P$: 415.1489, found 415.1495.

Methyl *O*-{(5*S*)-5-[(6-Amino-9*H*-purin-9-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-L-serinate (17a). 17a was obtained using general method E, as a white powder (117 mg, 29%). ^{1}H NMR (400 MHz, CD_3OD): δ = 3.82 (s, 3H), 4.01−4.12 (m, 1H), 4.25−4.37 (m, 3H), 4.42−4.65 (m, 6H), 8.28 (s, 0.4H), 8.31 (s, 0.6H), 8.39 (s, 1H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.32 (s, 0.31P), 15.45 (s, 0.69P). HRMS-FAB m/z $[M + H]^+$ calcd for $C_{13}H_{19}N_6O_6P$: 387.1176, found 387.1181.

Methyl *O*-{(5*S*)-5-[(6-Amino-9*H*-purin-9-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-D-serinate (17b). 17b was obtained, using general method E as a white powder (106 mg, 26%). ^{1}H NMR (400 MHz, CD_3OD): δ = 3.90 and 3.92 (2s, 3H), 4.01−4.13 (m, 1H), 4.29−4.75 (m, 9H), 8.23 (s, 0.4H), 8.28 (s, 0.6H), 8.37 and 8.38 (2s, 1H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.15 (s, 0.33P), 15.54 (s, 0.67P). HRMS-FAB m/z $[M + H]^+$ calcd for $C_{13}H_{19}N_6O_6P$: 387.1176, found 387.1181.

General Method F. Conversion of TFA Salts to HCl Salts. The peptidomimetic cHPMPA conjugates as trifluoroacetic acid salts (**17a** or **17b**, 0.08 mmol) were dissolved in 3 mL of 3 N methanolic HCl and evaporated under reduced pressure. This process was repeated 4 times. Finally, the product was precipitated from a methanolic solution by the addition of ethyl ether. The solid hydrochloride salts were collected by filtration.

Methyl *O*-{(5*S*)-5-[(6-Amino-9*H*-purin-9-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-L-serinate Hydrochloride (6a). 6a was obtained using general method F, as a white powder (32 mg, 82%). ^{1}H NMR (400 MHz, CD_3OD): δ = 3.89 (s, 2H), 3.93 (s, 1H), 4.05−4.19 (m, 1H), 4.33−4.77 (m, 9H), 8.35 (s, 0.4H), 8.40 (s, 0.6H), 8.46 and 8.47 (s, 1H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.28 (s, 0.31P), 15.41 (s, 0.69P).

Methyl *O*-{(5*S*)-5-[(6-Amino-9*H*-purin-9-yl)methyl]-2-oxido-1,4,2-dioxaphosphinan-2-yl}-D-serinate Hydrochloride (6b). 6b was obtained using general method F, as an off-white solid (32 mg, 87%). ^{1}H NMR (400 MHz, CD_3OD): δ = 3.91 (s, 2H), 3.93 (s, 1H), 4.02−4.18 (m, 1H), 4.34−4.76 (m, 9H), 8.35 (s, 0.4H), 8.39 (s, 0.6H), 8.46 (s, 1H). ^{31}P NMR (202 MHz, CD_3OD): δ = 14.20 (s, 0.33P), 15.56 (s, 0.67P).

Synthesis of L-Val-L-Val Acyclovir (8). The product was obtained as a white solid as described in the literature[19] (140 mg, 39%).

2-[(2-Amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]-ethyl L-Valyl-L-valinate (8). ^{1}H NMR (400 MHz, D_2O): δ = 0.59 (d, J = 6.9, 6H), 0.72 (d, J = 7.0, 6H), 1.74−1.82 (m, 1H), 1.86−1.95 (m, 1H), 3.57 (d, J = 6.1, 1H), 3.67 (m, 2H), 3.89 (d, J = 5.7, 1H), 4.03−4.07 (m, 2H), 5.43 (s, 2H), 8.85 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ = 16.27, 17.01, 17.44, 17.85, 29.99, 30.23, 57.98, 58.13, 63.38, 67.03, 72.13, 115.77, 138.34, 151.95, 154.29, 157.82, 168.44,

170.94. The NMR chemical shift values are in agreement with the literature values:[19] LC−MS t_R = 19.15 min; $[M + H]^+$ 424.3.

Functional Expression of hPEPT1 in Oocytes. Mature female *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI). All animal protocols followed guidelines approved by the University of California Chancellor's Committee on Animal Research and the National Institutes of Health. Frogs were anesthetized with 0.1% tricaine (Sigma Chemical Company, St. Louis, MO) buffered with 0.1% $NaHCO_3$, a portion of the ovary was surgically removed, and the frogs were sacrificed by an overdose of nembutal (60 mg for 60 min). Stage V−VI oocytes were selected and maintained at 18 °C in modified Barth's solution, supplemented with antibiotics.[13] hPEPT1 plasmids were linearized with *Bam*HI (New England Biolabs, Ipswich, MA) and transcribed in vitro using the T7MEGAScript kit and RNA cap analogue (Ambion, Austin, TX), and the cRNAs were prepared as described.[20] Oocytes were injected with 50 ng of hPEPT1 cRNA one day after isolation, and incubated at 18 °C for 4−7 days postinjection. Experiments were performed at 20−22 °C. Noninjected oocytes served as controls.

Electrophysiology. A two-microelectrode voltage clamp system was used to determine the interactions between hPEPT1, glycylsarcosine (Gly-Sar) and the prodrugs in oocytes expressing the transporter.[13] Oocytes were superfused in a buffer containing (in mM) 100 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 Hepes/Tris (pH 7.5) or 10 2-(*N*-morpholino)ethanesulfonic acid (Mes)/Tris (pH 6.0), and the membrane potential was held at −50 mV. Continuous current data were acquired with a chart recorder, and the current responses to the addition of 0.5−10 mM of the hPEPT1 substrate glycylsarcosine (Gly-Sar) and/or 10 mM of the following compounds were recorded: L-valine (L-Val), L-serine (L-Ser), cHPMPC (**1**), L-Val-L-Ser-OMe cHPMPC (**2a**), L-Val-D-Ser-OMe cHPMPC (**2b**), D-Val-L-Ser-OMe cHPMPC (**2c**), D-Val-D-Ser-OMe cHPMPC (**2d**), L-Ser-OiPr cHPMPC (**3a**), D-Ser-OiPr cHPMPC (**3b**), L-Ser-L-Ala-OiPr cHPMPC (**4**), L-Ser-OiPr cHPMPA (**5**), L-Ser-OMe cHPMPA (**6a**), D-Ser-OMe cHPMPA (**6b**), L-Val ACV (**7**) and L-Val-L-Val ACV (**8**). At the concentrations used, none of the compounds induced currents in control oocytes (data not shown). Unless otherwise noted, experiments were repeated on at least three oocytes from two different donor frogs.

Gly-Sar Uptake Assays. Oocytes were incubated for 30 min in the presence of 5 μM Gly-Sar (0.1 μM [^{3}H]-Gly-Sar) in buffer containing (in mM) 100 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes)/Tris (pH 7.5) or in Na^+ buffer at pH 6, and in the presence or in the absence of 10 mM Gly-Sar, cHPMPC (**1**), L-Val-L-Ser-OMe cHPMPC (**2a**), D-Val-D-Ser-OMe cHPMPC (**2d**), L-Ser-OiPr cHPMPC (**3a**), D-Ser-OiPr cHPMPC (**3b**), L-Ser-OMe cHPMPA (**6a**), and D-Ser-OMe cHPMPA (**6b**), L-Val ACV (**7**), or L-Val-L-Val ACV (**8**). After incubation, oocytes were rinsed thoroughly with ice cold Na^+ 7.5 buffer, individually lysed with 5% sodium dodecyl sulfate, and assayed for radioactivity in commercial scintillation cocktail (Ultima Gold, PerkinElmer, Waltham, MA). Unless otherwise noted, data are shown as mean ± SEM of at least 3 experiments from different frogs, with 5−8 oocytes each. At the concentrations used, none of the compounds had an effect on Gly-Sar uptake in noninjected oocytes (data not shown).

Results and Discussion

Chemistry. The synthesis of **2**−**4** required gram amounts of HPMPC, which was prepared as previously described.[4,5] The Boc-protected dipeptide promoieties **11a**−**d** and **13** were prepared using EDC coupling, whereas the amino acid promoieties **12a** and **12b** were synthesized by acid-catalyzed esterification and standard Boc-protection of the amino acid. Conjugates **2**−**4** were synthesized as described in Scheme 1. The Boc-protected intermediates were prepared using PyBOP to convert HPMPC to **1** and attach the promoiety in one pot.[4,5] The reaction was easily monitored by ^{31}P NMR, and when neither HPMPC nor **1** remained, the reaction was stopped. The PyBOP byproduct and excess promoiety were removed with a diethyl ether wash before purification by preparative TLC. Following Boc-deprotection with TFA, the target compounds **2**−**4** were purified by preparative TLC. The bis(trifluoroacetic acid) salts of **2a**−**d** were obtained by precipitation with diethyl ether from a saturated methanolic solution (overall yields 21−58%). The structures of all the cHPMPC conjugates were confirmed by ^{1}H and ^{31}P NMR and by HRMS. Purity was verified by LC−MS. A pair of diastereomers is formed (owing to the chiral phosphorus atom) when the promoiety is conjugated to **1**, detectable by ^{31}P NMR. It should be noted that each cHPMPC conjugate was isolated and evaluated as the diastereomeric mixture. Thus, pairs of ^{31}P NMR signals are observed, but a single parent ion is seen in the mass spectrum. The stereoisomer with the more upfield chemical shift is formed predominantly, in a ratio of from 7:3 to 4:1 relative to the more downfield stereoisomer. The latter is relatively less polar, based on its longer retention on a C-18 LC column.[4]

The cHPMPA prodrugs were prepared using a solid-phase method (Scheme 2), wherein the Boc protecting group is essentially replaced with a resin.[21] This new method has the advantage of easier product purification, avoiding exposure to silica gel. Briefly, the side chain hydroxyl group of the

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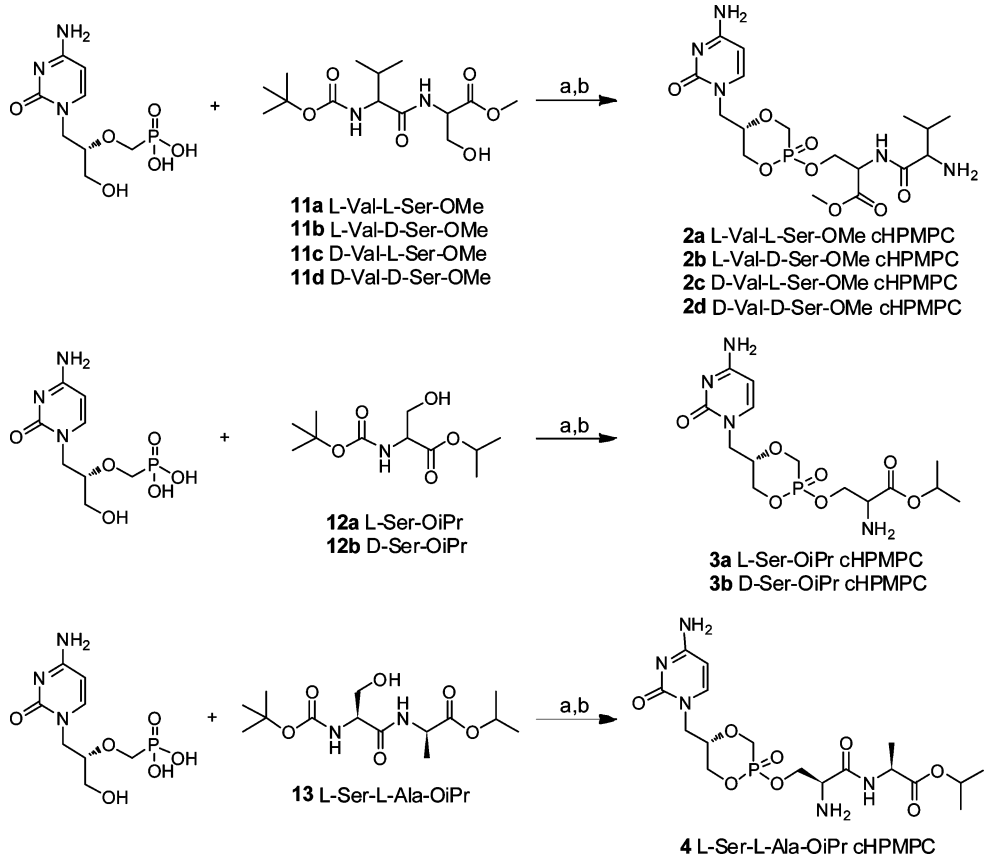
Scheme 1. Synthesis of the Peptidomimetic cHPMPC Conjugates **2**−**4**[a]



[a] Reagents and conditions: (a) PyBOP, DIPEA, DMF, 35 °C, 4 h; (b) TFA, CH_2Cl_2, 35 °C, 5 h.

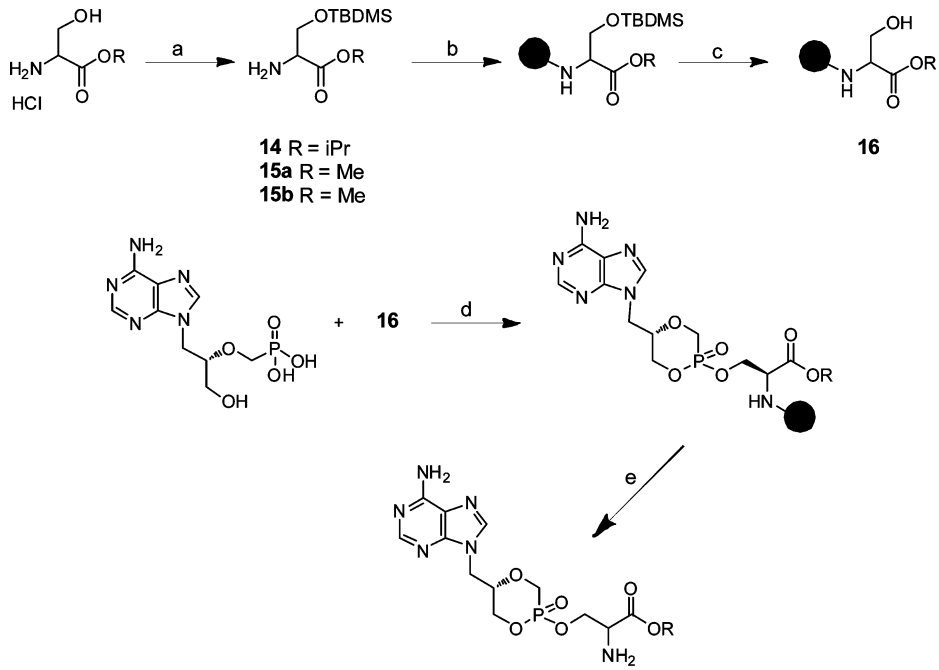
amino acid was first protected by a *tert*-butyldimethylsilyl (TBDMS) group, and the resulting TBDMS-protected serine alkyl ester (**13**, **14a** and **14b**) was then immobilized by reaction with a tritylchloride polystyrene (TCP) resin. After removal of the TBDMS protecting group with TBAF, the coupling reaction to the drug was accomplished using PyBOP to cyclize HPMPA and conjugate the amino acid, which can be visualized by ^{31}P gel NMR (Figure 2). The product conjugates were cleaved from the resin using either TFA in dichloromethane (**17a** and **17b**) or HCl in dioxane (**5**). Purification of **17a** and **17b** by preparative TLC, followed by treatment with 3N HCl in MeOH afforded final products **6a** and **6b** as hydrochloride salts (overall yields 26−30%). The structures of all conjugates were confirmed by ^{1}H and ^{31}P NMR and by HRMS. Purity was verified by LC−MS. As demonstrated by their ^{31}P NMR spectra, like the cHPMPC conjugates they are obtained as diastereomeric mixtures, however the major stereoisomer (which is present only in small excess) is that one having the more downfield ^{31}P resonance. The cHPMPA conjugates were prepared as hydrochloride salts because the reduced basicity of the adenine ring appears to make the TFA salts less stable.

L-Val-L-Val ACV (**8**) was synthesized according to the literature procedure,[19] and obtained as a trifluoroacetic acid salt, which was purified by preparative TLC. The structure was verified by ^{1}H and ^{13}C NMR, which were in agreement with the reported values,[19] and the purity was confirmed by LC−MS.

Interactions between hPEPT1 and the Peptidomimetic cHPMPC and cHPMPA Prodrugs 2−6. Cidofovir prodrugs were tested for recognition by hPEPT1 using the two-electrode voltage clamp (TEVC) technique and radiotracer uptake assays. First, the compounds were examined for hPEPT1 translocation, based on their ability to generate inward currents in *Xenopus* oocytes. The interactions of the prodrugs with hPEPT1 were further investigated by measuring their effect on the inward currents induced by 0.5 mM of the known hPEPT1 substrate Gly-Sar (Figure 3B), and on the uptake of 5 μM Gly-Sar (Figure 4). All prodrugs were tested at 10 mM; test solutions were prepared immediately before each assay, and the pH was checked before analysis. Solutions of 10 mM L-valine (L-Val), L-serine (L-Ser), and cHPMPC (**1**) were used as controls.

Figure 3A shows a record from a representative TEVC experiment, in which an hPEPT1-expressing oocyte voltage-clamped at −50 mV was superfused with 0.5 mM Gly-Sar or 10 mM **3a** at pH 6. Addition of Gly-Sar resulted in the generation of an inward H^+ current, 100 nA. **3a** did not induce a detectable current, indicating that it is not transported by hPEPT1 (data not shown). When the oocyte was

Scheme 2. Solid-Phase Synthesis of Peptidomimetic cHPMPA Conjugates[a]



[a] Reagents and conditions: (a) TBDMSCl, imidazole, CH_2Cl_2, 0 °C to rt, 24 h; (b) TCP-resin, DIPEA, CH_2Cl_2, 0 °C to rt, 18 h; (c) TBAF, THF, rt, 5 h; (d) PyBOP, DIPEA, DMF, 38 °C, 18 h; (e) TFA, CH_2Cl_2 and 3N HCl in MeOH or HCl, dioxane, rt, 18 h. Isolated products are HCl salts.

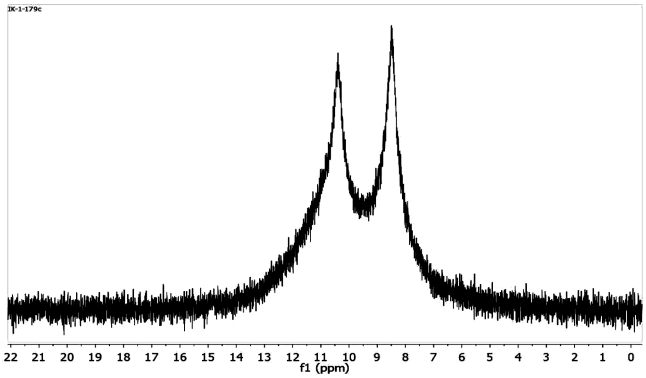


Figure 2. Gel ^{31}P NMR of immobilized **5** prior to cleavage from solid-phase support.

exposed to 0.5 mM Gly-Sar, the addition of 10 mM **3a** produced a 55% reduction in the current.

Neither L-Val nor L-Ser evoked detectable currents, and both had little effect on Gly-Sar transport (Figure 3B), as expected since amino acids are not hPEPT1 substrates.[22] Cyclic cidofovir (**1**) was not transported and inhibited Gly-Sar transport weakly (Figures 3B and 4), suggesting poor recognition by hPEPT1. cHPMPC conjugates **2a**−**d** did not evoke an inward current, indicating they are not transported by hPEPT1 (data not shown). Compounds **2a** and **2b**, both N-terminal L-valine dipeptide conjugates, abolished 100% and 93% of the Gly-Sar current, respectively, while the D-valine analogues **2c** and **2d** decreased the Gly-Sar current by only about 40% (Figure 3B).

These results demonstrate the importance of an L-configuration at the N-terminal amino acid for hPEPT1 affinity, whereas the configuration of the C-terminal, drug-linked serine has little influence on binding to the transporter. Despite the occurrence of interaction between hPEPT1 and **2a** and **2b**, it appears that the steric profile of these prodrugs, which approach tetrapeptides in size, and/or their polarity, prevents transport of the bound molecule.[23]

In order to better define structural effects on the interactions of serine side chain-linked peptidomimetic prodrugs with hPEPT1, we prepared the analogues **3**−**6**. First, the effect of removing the nonlinking, second amino acid from the dipeptide moiety, as in the cHPMPC conjugates **3a** and **3b**, was ascertained. These compounds did not evoke an inward current, indicating they are not substrates for hPEPT1, and inhibited the Gly-Sar current only by about 50% (Figure 3B). In addition, the difference between the L- and D-isomers was small, most likely due to fewer points of bonding interaction with the transporter.

(22) Liang, R.; Fei, Y. J.; Prasad, P. D.; Ramamoorthy, S.; Han, H.; Yangfeng, T. L.; Hediger, M. A.; Ganapathy, V.; Leibach, F. H. Human intestinal H+/peptide cotransporter - cloning, functional expression, and chromosomal localization. *J. Biol. Chem.* **1995**, *270* (12), 6456–6463.

(23) Rubio-Aliaga, I.; Daniel, H. Peptide transporters and their roles in physiological processes and drug disposition. *Xenobiotica* **2008**, *38* (7−8), 1022–1042.

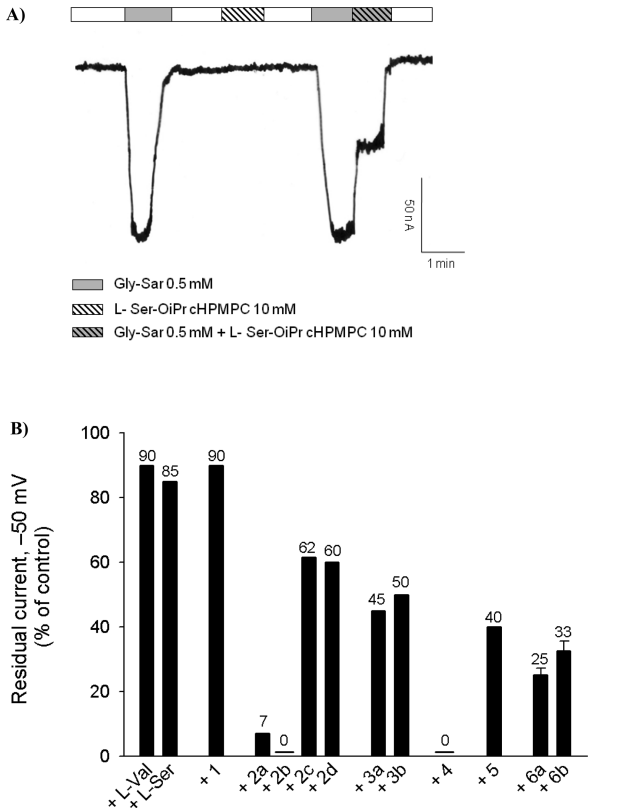


Figure 3. Interactions between prodrugs and hPEPT1. Four to seven days postinjection with hPEPT1 cRNA, *X. laevis* oocytes were mounted in a two-microelectrode voltage clamp setup, superfused with Na^+ buffer at pH 6, and held at −50 mV. Currents in response to the addition of 0.5 mM of the hPEPT1 substrate glycylsarcosine (Gly-Sar) and/or 10 mM of the following compounds were recorded: L-Val, L-Ser, cHPMPC (**1**), L-Val-L-Ser-OMe cHPMPC (**2a**), L-Val-D-Ser-OMe cHPMPC (**2b**), D-Val-L-Ser-OMe cHPMPC (**2c**), D-Val-D-Ser-OMe cHPMPC (**2d**), L-Ser-OiPr cHPMPC (**3a**), D-Ser-OiPr cHPMPC (**3b**), L-Ser-L-Ala-OiPr cHPMPC (**4**), L-Ser-OiPr cHPMPA (**5**), L-Ser-OMe cHPMPA (**6a**), and D-Ser-OMe cHPMPA (**6b**). (A) Individual trace for Gly-Sar and **3a**. (B) Effect of the compounds on inward currents evoked by Gly-Sar. Data were normalized to the current due to 0.5 mM Gly-Sar (control), 112 ± 4 nA (mean ± SEM of at least 5 measurements in 3 oocytes from different frogs), and represent the percentage of Gly-Sar current that remained upon addition of each compound. Results are from individual oocytes (L-Val, L-Ser, **1**, and prodrugs **2** to **5**); all observations were confirmed in at least one additional oocyte from a different batch. For **6a** and **6b**, data are shown as mean ± SEM of three oocytes from two donor frogs.

It is important to mention that weak inward currents were produced in the model when **3a** was purified by HPLC using 0.1% TFA followed by lyophilization (data not shown). Nevertheless, these signals were not reproducible and the same compound, purified by preparative TLC, gave no signal.

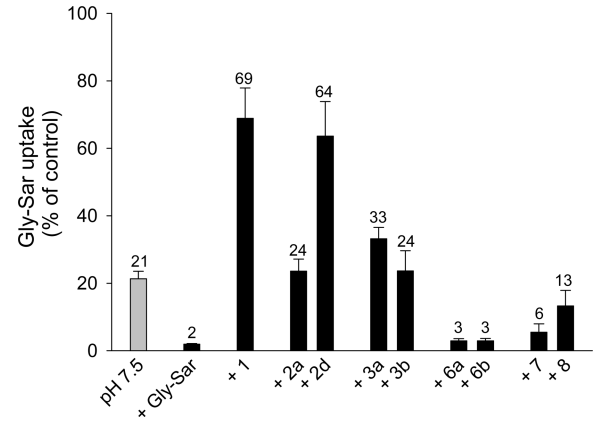


Figure 4. Effect of prodrugs on Gly-Sar uptake. Uptake of 5 μM Gly-Sar (0.1 μM [^{3}H]-Gly-Sar) was measured in Na^+ buffer at pH 7.5 (gray bar) or pH 6 (black bars), in the absence or in the presence of 10 mM Gly-Sar, cHPMPC (**1**), L-Val-L-Ser-OMe cHPMPC (**2a**), D-Val-D-Ser-OMe cHPMPC (**2d**), L-Ser-OiPr cHPMPC (**3a**), D-Ser-OiPr cHPMPC (**3b**), L-Ser-OMe cHPMPA (**6a**), and D-Ser-OMe cHPMPA (**6b**), L-Val ACV (**7**), or L-Val-L-Val ACV (**8**). Results are shown as means ± SEM of 5−8 oocytes and were normalized to the Gly-Sar uptake in the absence of external inhibitors (control), 15 ± 1 pmol h^{-1} $oocyte^{-1}$ (mean ± SEM of 4 experiments with different donor frogs). Uptake in noninjected oocytes was 0.6% ± 0.1% of control, regardless of pH and was not affected by any of the test compounds. Compounds **1** to **8**: data are from individual experiments, but were confirmed in at least one additional trial with oocytes from a different frog.

A possible explanation of this effect could be that HPLC purified samples contain an excess of TFA compared to those purified by TLC. This conclusion is based on ^{19}F NMR and combustion analysis for CHN, which support the presence of excess TFA in the HPLC-purified preparations. Thus, to avoid this artifact, we recommend that compounds tested in the oocyte system be carefully purified to remove excess TFA, or alternatively, that HCl salts be used.

We also prepared and evaluated in the model three single serine side chain-linked conjugates of the adenine analogue of cHPMPC, cHPMPA: **5** (L-Ser-OiPr), **6a** (L-Ser-OMe), and **6b** (D-Ser-OMe). These compounds also did not evoke an inward current and thus were not transported. However, they inhibited Gly-Sar currents (Figure 3B) to a higher extent than **3a** and **3b**, suggesting that the larger size of the drug heterocycle does not greatly affect interaction with the transporter. In the L/D stereoisomer pair **6a** and **6b**, inhibition of the Gly-Sar current by the conjugate with an L-configuration at the serine was modestly more pronounced than with the corresponding cHPMPC conjugates, **3a**/**3b** (Figure 3B).

We next prepared the "reversed" dipeptide cHPMPC conjugate **4** to investigate the effect of the linking serine position in the dipeptide sequence. Compound **4** again evoked no inward current, indicating absence of translocation by hPEPT1, but it abolished the current induced by Gly-

Sar, suggesting good recognition by the transporter. These observations show that the position of the linked L-serine does not affect hPEPT1 binding or improve the ability of the prodrug to undergo transport.

Next, we evaluated the effect of **1**, **2a**, **2d**, **3a/3b** and **6a/6b** on the uptake of radiolabeled Gly-Sar; results are summarized in Figure 4. In hPEPT1 oocytes, influx of 5 μM Gly-Sar (0.1 μM [^{3}H]-Gly-Sar) at pH 6 was 15 $\pm$ 1 pmol h^{-1} oocyte^{-1} (control), 150-fold higher than in noninjected oocytes, and dropped 98% in the presence of 10 mM unlabeled Gly-Sar. At pH 7.5, Gly-Sar uptake was 20% of control at 3 $\pm$ 0.2 pmol h^{-1} oocyte^{-1} (Figure 4, gray bar), indicating that hPEPT1 has a limited, but significant. ability to transport Gly-Sar in the absence of an inwardly directed H^+ gradient. hPEPT1-mediated Gly-Sar uptake decreased 75% in the presence of **2a** but only ~30% due to **1** or **2d** (Figure 4), in accordance with poor recognition of the parent drug by hPEPT1, and further shows stereospecificity in the interactions between the carrier and our dipeptide prodrugs. **2a** and **2b** inhibited nearly 100% of the Gly-Sar induced H^+-currents (Figure 3B), but uptake in the presence of **2a** was similar to that at pH 7.5. This may indicate that, while in the presence of **2a** or **2b** H^+-coupled Gly-Sar transport is fully blocked (Figure 3B), uncoupled transport of the dipeptide is still possible. On the other hand, 10 mM **6a** and **6b** blocked 97% of the 5 μM [^{3}H]-Gly-Sar uptake (Figure 4), but only 70% of the current generated by 0.5 mM Gly-Sar (Figure 3B). Thus, **6a** and **6b** seem to be less effectively recognized by the transporter than **2a** and **2b**, but when present at a sufficiently high prodrug:substrate concentration ratio, they are capable of inhibiting Gly-Sar transport entirely. Finally, **6a** and **6b** inhibited [^{3}H]-Gly-Sar uptake more than **3a** and **3b** (Figure 4), further suggesting a preference of the transporter for the **6** isomer pair over the **3** isomers.

hPEPT1-Mediated Transport and Affinity of Peptidomimetic ACV Conjugates. We wished to verify that transport of a prodrug known to be transporting by hPEPT1 could be observed in our *X. laevis* oocyte model system. For this purpose we chose L-Val ACV (**7**)[9,12] and also included a dipeptide analogue of this prodrug, L-Val-L-Val ACV (**8**). First, we compared the currents induced by 10 mM **7** or **8** to those due to 10 mM Gly-Sar, a saturating concentration of the dipeptide (Figure 5) that generates the maximum inward current, I_{max}. Compounds **7** and **8** induced currents equal to 6% and 15% I_{max}, respectively (Figure 5). This demonstrates that **7** and **8** are transported by hPEPT1, and suggests that **8** is transported more efficiently. In the absence of detailed kinetic data, however, it is not possible to determine whether this is due to a higher affinity, a higher turnover rate, or both. The larger signal observed with the dipeptide **8** is consistent with the report of Talluri et al. that the permeability of **8** across a Caco-2 monolayer was

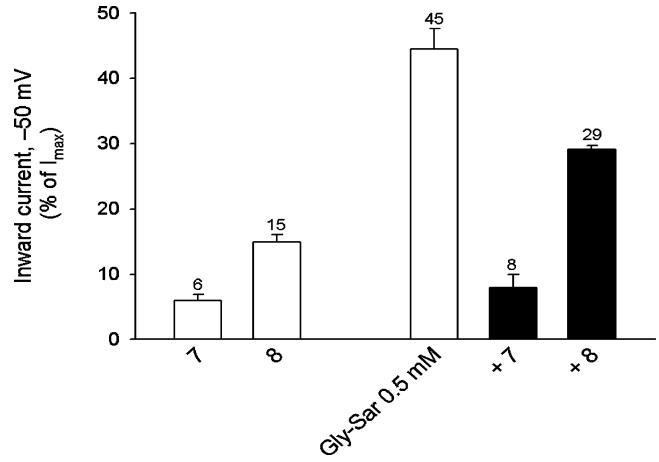


Figure 5. Transport of acyclovir prodrugs by hPEPT1. *X. laevis* oocytes expressing the transporter were superfused with Na^+ buffer at pH 6, and held at −50 mV. White bars: inward currents induced by 10 mM L-Val ACV (**7**), 10 mM L-Val-L-Val ACV (**8**), or 0.5 mM Gly-Sar. Black bars: effect of 10 mM **7** or **8** on the currents due to 0.5 mM Gly-Sar. Data were normalized to the current induced by 10 mM Gly-Sar (I_{max}), 175 $\pm$ 18 nA. Results are shown as mean $\pm$ SEM of at least three oocytes from two different frogs.

approximately 3-fold that of **7**,[24] and is in agreement with our observations with the cHPMPA and cHPMPC prodrugs that suggest preference of the transporter for dipeptide drug conjugates compared to single amino acid analogues.

The effect of ACV conjugates **7** and **8** on Gly-Sar transport was also investigated. Both compounds inhibited Gly-Sar uptake (Figure 4), 94% (**7**) and 87% (**8**), and reduced Gly-Sar currents (Figure 5), 80% (**7**) and 35% (**8**). Inhibition of substrate currents by transported drugs has been observed previously in hPEPT1 and other transporters, such as the renal H^+-coupled oligopeptide cotransporter hPEPT2 and the Na^+/glucose cotransporter hSGLT1.[13,25,26] In these, inhibition is due to the drug being transported at a significantly lower rate than the substrate, which has the effect of slowing down the system and reducing the overall current when cosuperfused with the substrate. The lower inhibitory effects of **8** on Gly-Sar currents (Figure 5, black bars) are consistent with its higher apparent transport rate (Figure 5, white bars), and may be due to the additional amino acid, making it a dipeptide promoiety and giving more points for interaction with the transporter. Therefore, the coadministration of **8** does not interfere with the transport of Gly-Sar as much as that

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(26) Sala-Rabanal, M.; Loo, D. D. F.; Hirayama, B. A.; Wright, E. M. Molecular mechanism of dipeptide and drug transport by the human renal H+/oligopeptide cotransporter hPEPT2. *Am. J. Physiol.* **2008**, *294* (6, Pt.2), F1422–F1432.

of **7**. In turn, **7** is recognized by hPEPT1, but its properties may not be optimal as it only contains one amino acid, which results in a lower transport rate.

Conclusion

In summary, several peptidomimetic prodrugs incorporating different promoieties were synthesized and evaluated for their ability to be transported actively by hPEPT1, using tracer uptake and electrophysiology in *Xenopus* oocytes overexpressing the transporter. Under the conditions studied, none of the cHPMPC conjugates **2**−**4** evoked a current, indicating that there is no detectable hPEPT1-mediated transport of these compounds. However, they all inhibited the transport of hPEPT1 substrate Gly-Sar, suggesting that they are recognized by the transporter. At 10 mM, compounds **2a**, **2b**, and **4** blocked 93−100% of the current due to 0.5 mM Gly-Sar, whereas **2c** and **2d** were weaker inhibitors. This suggests that, although these compounds are not hPEPT1 substrates, they may bind to the transporter with low to high affinity, depending on the stereochemistry at the terminal amino acid, but without much sensitivity to the position of the drug-linked serine within the dipeptide conjugate. The single amino acid cHPMPC conjugates **3a** and **3b** showed low inhibitory potency and little stereospecificity, a pattern reproduced with the analogous cHPMPA conjugates **5** and **6a**,**b**. Thus affinity for the transporter also appears to be promoted by the presence of a dipeptide in the conjugate, which is consistent with the known specificity of hPEPT1. The ability of the transporter model to detect prodrug hPEPT1 transport activity was verified using the known hPEPT1 substrate valacyclovir (**7**) and its dipeptide analogue, L-Val-L-Val acyclovir (**8**). Both compounds were transported in our model system, but **8** exhibited a higher transport rate, which may be attributed to its dipeptide promoiety. The results suggest the L-amino-terminal prodrugs **2a**, **2b** and **4** are stereospecifically recognized by hPEPT1, but are not transported due to the steric and/or polar structural properties of the linked cHPMPC drug cargo. Similar results were obtained with three single amino acid conjugates of cHPMPA (**5**, **6a** and **6b**).

It is interesting that the point of attachment of the large drug side chain within the dipeptide did not observably affect the potent affinity of the L-Val-L-Ser prodrug conjugates. It should be noted that the nucleoside base (G) in valacyclovir is larger than that in our HPMPC and HPMPA prodrugs; the sugar-mimicking moiety is smaller and is linked differently to the amino acid, via esterification of the terminal hydroxy group of the drug to the valine carboxylic acid, instead of by formation of a phosphonic ester bond with a serine side chain, as in our compounds. The molecular rigidity introduced by the presence of the cyclic phosphonate ring may also affect transport by hPEPT1, but because the compounds were tested in diastereomeric pairs (racemic at the chiral phosphorus), the absence of a signal indicating proton-coupled transport implies that this stereochemistry does not play a decisive role. Additional studies using acyclic analogues are needed to determine the effect the ring has on transport.

Table 1. Calculated log *D* values at pHs 6.5 and 7.4[a]

	log *D*	
compound	pH 6.5	pH 7.4
1	−3.52	−3.52
2	−1.31	−1.31
3	−1.32	−0.99
4	−1.43	−1.43
5	−1.21	−0.88
6	−1.97	−1.64
7	−1.59	−0.92
8	−2.20	−1.37

[a] Values calculated using Marvin Sketch v. 5.2.2.

Thus, the enhanced absorption of prodrug **2a** relative to the parent drug cannot be explained by active transport mediated by hPEPT1, although the prodrug is bound and inhibits Gly-Sar transport, and although this interaction with the transporter exhibits specificities—dipeptide over single amino acid, N-terminal residue in L-configuration—similar to those displayed by known substrates. This raises the question whether some other active transporter, perhaps unknown, could be responsible. To assess the alternative of passive transport, a qualitative[27] estimate of permeability can be made by comparing the log *D* values of the drugs and prodrugs studied here (Table 1). Keeping in mind the limitations of this and other single parameter permeability estimators,[27] it can be seen that the prodrugs have log *D* values that are about 2 logs more positive than HPMPC, and similar to those of valacyclovir and its dipeptide derivative, which are actively transported by hPEPT1. Further work will be necessary to establish whether an as yet unknown active process augments passive transport of prodrugs such as **2a**, and whether replacement of HPMPC or HPMPA by a different drug cargo would allow the peptidomimetic to not merely bind, but also be transported by, hPEPT1.

Abbreviations Used

HPMPC, cidofovir; cHPMPC, cyclic cidofovir; HPMPA, 9-[(2*S*)-3-hydroxy-2-phosphonomethoxypropyl]adenine; ACV, acyclovir; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DMAP, *N*,*N*-dimethylaminopyridine; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; HOBt, 1-hydroxybenzotriazole; SAR, structure−activity relationship; STR, structure−transport relationship; TBAF, tetrabutylammonium fluoride; TCP, tritylchloride polystyrene resin; TEA, triethylamine; TFA, trifluoroacetic acid.

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Acknowledgment. This work was supported by National Institutes of Health Grants AI061457 and AI056864 to C.E.M. L.W.P. was a 2007−2008 Stauffer Fellow and a 2008−2009 WiSE Merit Fellow. The authors wish to thank Professor Ernest M. Wright (UCLA Department of Physiology) for kindly making it possible to conduct the voltage-clamped *X. laevis* oocyte assays in his laboratory, and for helpful advice.

Supporting Information Available: ^{1}H NMR spectra of the intermediates: compounds **9a**, **9b**, **10**, **11a**−**d**, **12a**, **12b**, **13**, **14**, **15a**, and **15b**. ^{1}H and ^{31}P NMR spectra of final products: **2a**−**d**, **3a** and **3b**, **4**, **5**, **6a**, **6b**, **17a**, and **17b**. ^{1}H and ^{13}C NMR spectra of compound **8**. LC−MS traces for the target compounds: **2a**, **2d**, **3a**, **4**, **5**, **8**, **17a**, and **17b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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