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Synthesis of analogs of L-valacyclovir and determination of their substrate activity for the oligopeptide transporter in Caco-2 cells

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Abstract

L-Valacyclovir, a prodrug of acyclovir, is a substrate for the peptide transporter (PepT1) in the intestinal mucosa, which accounts for its higher than expected oral bioavailability. The substrate activity of L-valacyclovir for PepT1 is surprising, particularly when one considers that the molecule has the structural features of a nucleoside rather than a peptide. In an attempt to better understand the structure–transport relationships (STR) for the interactions of L-valacyclovir with PepT1, analogs of this molecule with structural changes in the guanine moiety were synthesized and their substrate activity for PepT1 in Caco-2 cell monolayers was determined. The analogs synthesized include those that had the guanine moiety of L-valacyclovir substituted with purine, benzimidazole, and 7-azaindole. All three analogs (purine, benzimidazole, and 7-azaindole) exhibited affinity for PepT1 in binding studies, but only the purine analog (as the L-valine ester) showed PepT1-associated transcellular transport across Caco-2 cell monolayers. The benzimidazole and 7-azaindole analogs (as their L-valine esters) were rapidly metabolized by esterase when applied to the apical surface of Caco-2 cells, which probably explains their low penetration as the intact prodrugs via PepT1.

Keywords: L-Valacyclovir; PepT1; Caco-2 cells; Structure–transport relationships

1. Introduction

The oligopeptide transporter PepT1 is localized in the small intestine and is involved in the absorption of di- and tri-peptides and many peptidomimetics (Walker et al., 1996; Adibi, 1997; Dantzig, 1997). Furthermore, several non-peptidic compounds have been shown to be substrates for PepT1 (Han et al., 1998; Temple et al., 1998). Bailey et al. (2000) have suggested a substrate template for PepT1 based on known structure–transport relationship (STR) for this transporter. This template suggests four key binding sites on PepT1 for the different structural features of a substrate: a strong charge–charge interaction binding site for an N-terminal amino group; a hydrogen bond to the carbonyl group of the first peptide bond; a hydrophobic pocket for binding the side chain of an amino acid; and one or two carboxylate binding sites.

L-Valacyclovir is an orally active prodrug of the antiviral drug acyclovir (Beauchamp et al., 1992; Crooks and Murray, 1994). The oral absorption of this hydrophilic prodrug appears to be mediated by its substrate activity for PepT1 (Ganapathy et al., 1998; Sinko and Balimane, 1998; de Vrueh et al., 1998). While the L-valine ester group of L-valacyclovir provides an amino group and a carbonyl group as recognition sites for PepT1, the presence of a guanine moiety in this molecule makes it more closely resemble a nucleoside than a di- or tri-peptide. This may suggest that the guanine moiety of L-valacyclovir resembles the structural features of the amino acid side chain and/or the C-terminal carboxylate of a di- or tri-peptide, the other recognition sites suggested by Bailey et al. (2000) as being important in binding to PepT1.

In this study, a series of L-valacyclovir and acyclovir analogs with structural alterations in the guanine moiety

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were synthesized in an attempt to elucidate its role (if any) in binding to PepT1. The substrate activity of these L-valacyclovir and acyclovir analogs for PepT1 and a nucleoside transporter were then examined in Caco-2 cells, an in vitro model of the intestinal mucosa (Hidalgo et al., 1989).

2. Materials and methods

2.1. Materials

Starting materials for synthesis, solvents for HPLC, and all other reagents employed were purchased from Aldrich (Milwaukee, WI, USA) or Acros Organics distributed by Fisher Scientific (Houston, TX, USA) and were used as received. L-Valacyclovir was obtained as a gift from GlaxoSmithKline. Caco-2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from JRH Biosciences (Lenexa, KS, USA). Heat-inactivated fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA, USA). L-Glutamine solution, penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) solution, and non-essential amino acids solution (NEAA, 10 mM in 0.85% saline) were purchased from Life Technologies (Grand Island, NY, USA). Rat-tail collagen (type I) was obtained from Collaborative Biomedical/Becton Dickenson (Bedford, MA, USA). Earle's balanced salt solution (EBSS), Hanks' balanced salt solution (HBSS), GlyPro, carnosine, acyclovir, uridine, inosine, thymidine, dipyridamole, and phloridzin were purchased from Sigma (St. Louis, MO, USA). [^{3}H]Carnosine (5 mCi/ml) was obtained from Moravek Biochemicals (Brea, CA, USA) and [^{3}H]uridine (5 mCi/ml) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA).

2.2. Methods

2.2.1. Synthesis of L-valacyclovir analogs

All reactions involving air-sensitive reagents were performed under argon. All glassware was flame-dried prior to use. The crude products were purified using flash chromatography with 70–230 mesh size silica gel purchased from Merck (West Point, PA, USA). ^{1}H NMR spectra were recorded on a 400-MHz instrument (Bruker DRX-400). Chemical shifts are expressed in parts per million (δ) relative to the internal standard tetramethylsilane (TMS). Positive fast-atom-bombardment mass spectrometry (FAB-MS) was carried out using a Xenon gun on the matrices thioglycerol/glycerol (TG/G), polyethylene glycol 300/glycerol (PEG300/G), and nitrobenzyl alcohol (NBA) as specified below. The mass spectra were recorded on a ZAB HS mass spectrometer (VG Analytical, Manchester, UK) in the Department of Chemistry at The University of Kansas.

2.2.1.1. Synthesis of 2-(9-purinemethoxy)ethyl-L-valinate (**2b**)

2.2.1.1.1. 9-(2-*Benzoyloxyethoxymethyl*)-*purine* To a stirred solution of lithium diisopropylamide (LDA) (4.16 ml, 8.33 mmol) in dry *N*,*N*-dimethylformamide (DMF) (30 ml) was added a solution of purine (1.00 g, 8.33 mmol) in DMF (20 ml) at 0 °C under argon. After 5 min, 2-(chloromethoxy)ethyl benzoate (1.47 ml, 8.33 mmol) was added, and the reaction mixture was stirred for 26 h at room temperature. The solution was quenched with water (20 ml) and the compound was extracted with ethyl acetate (EtOAc) (3×50 ml). The organic phases collected from the extraction were combined, dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. The crude product was purified by flash chromatography eluting with EtOAc to yield a yellow oil (699 mg, 28%) which solidified upon drying in vacuo. ^{1}H NMR ($CDCl_3$) δ 9.15 (1H, s, H-4), 9.00 (1H, s, H-2), 8.25 (1H, s, H-8), 7.95 (2H, d, J=8.0 Hz, H-1′), 7.54 (1H, t, J=7.5 Hz, H-3′), 7.42 (2H, t, J=7.7 Hz, H-2′), 5.77 (2H, s, OCH_2N), 4.46 (2H, t, J=4.7 Hz, CH_2O), 3.93 (2H, t, J=4.7 Hz, CH_2O); FAB-MS (in TG/G) m/z: 299.1 ($M+H^+$), calculated molecular weight: 298.1.

2.2.1.1.2. 9-(2-*Hydroxyethoxymethyl*)-*purine* (**2a**) 9-(2-Benzoyloxyethoxymethyl)-purine (625 mg, 2.09 mmol) was stirred in 40% aq. methylamine (60 ml) for 1.5 h at room temperature. After evaporation of the solvent, the residue was purified by flash chromatography eluting with EtOAc–MeOH (9:1) to yield colorless crystals (112 mg, 28%). ^{1}H NMR (CD_3OD) δ 9.13 (1H, s, H-4), 8.98 (1H, s, H-2), 8.67 (1H, s, H-8), 5.81 (2H, s, OCH_2N), 3.67 (2H, m, CH_2O), 3.65 (2H, m, CH_2O); FAB-MS (vscan in PEG300/Gly) m/z: 195.0882 ($M+H^+$), calculated molecular weight: 194.0803.

2.2.1.1.3. 2-(9-*Purinemethoxy*)*ethyl-N-Boc-L-valinate* To a stirred solution of 9-(2-hydroxyethoxymethyl)-purine (50 mg, 0.26 mmol) in DMF–dichloromethane (1:2, 9 ml) was added *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDAC) (109 mg, 0.57 mmol), 4-(dimethylamino)pyridine (DMAP) (32 mg, 0.26 mmol), and *N*-Boc-L-valine (84 mg, 0.39 mmol) under argon. The solution was stirred at 80 °C for 40 h. After evaporation of the solvent, the residue was purified by flash chromatography eluting with EtOAc to yield a clear oil (63 mg, 62%). ^{1}H NMR ($CDCl_3$) δ 9.20 (1H, s, H-4), 9.04 (1H, s, H-2), 8.28 (1H, s, H-8), 5.75 (1H, s, OCH_2N), 5.05 (1H, d, J=8.2 Hz, NH), 4.30 (2H, m, CH_2O), 4.22 (1H, m, C<u>H</u>NH), 3.81 (2H, m, CH_2O), 2.08 (1H, m, C<u>H</u>$(CH_3)_2$), 1.45 (9H, s, $C(CH_3)_3$), 0.95 (3H, d, J=6.8 Hz, CH_3), 0.87 (3H, d, J=6.8 Hz, CH_3); FAB-MS (in TG/G) m/z: 394.2 ($M+H^+$), calculated molecular weight: 393.2.

2.2.1.1.4. 2-(9-*Purinemethoxy*)*ethyl-L-valinate* (**2b**) 2-(9-Purinemethoxy)ethyl-*N*-Boc-L-valinate (50 mg, 0.13

mmol) and 25% trifluoroacetic acid (TFA) in dichloromethane (5 ml) were stirred at room temperature for 3 h. After evaporation, azeotropic distillation, and lyophilization, the product **2b** was obtained as a colorless solid (55 mg, 100%). ^{1}H NMR (CD_3OD) δ 9.18 (1H, s, H-4), 9.02 (1H, s, H-2), 8.70 (1H, s, H-8), 5.82 (2H, s, OCH_2N), 4.46 (2H, m, CH_2O), 4.32 (2H, m, CH_2O), 3.90 (2H, m, CH_2O), 3.87 (1H, m, C<u>H</u>NH), 2.15 (1H, m, C<u>H</u>$(CH_3)_2$), 0.95 (6H, m, 2×CH_3); FAB-MS (vscan in PEG300/G) *m*/*z*: 294.1566 ($M+H^+$), calculated molecular weight: 293.1488.

*2.2.1.2. Synthesis of 2-(1-benzimidazolemethoxy)ethyl-L-valinate (**3b**)*

2.2.1.2.1. 1-*Acetylbenzimidazole* A mixture of benzimidazole (2.00 g, 16.9 mmol), acetic anhydride (35 ml), and acetic acid (50 ml) was heated with stirring at 140 °C for 30 min, and the resulting solution was evaporated to dryness. The product was obtained as orange crystals (2.70 g, 100%). ^{1}H NMR ($CDCl_3$) δ 8.37 (1H, s, CH=N), 8.23 (1H, dd, J_{ortho} = 6.8 Hz, J_{meta} = 2.1 Hz, H-7), 7.80 (1H, dd, J_{ortho} = 6.8 Hz, J_{meta} = 2.3 Hz, H-4), 7.41 (2H, m, H-5 + H-6), 2.74 (3H, s, CH_3); FAB-MS (in G) *m*/*z*: 161.1 ($M+H^+$), calculated molecular weight: 160.1.

2.2.1.2.2. 2-(*Acetoxymethoxy*)*ethyl acetate* Acetyl bromide (50.0 g, 30.1 ml, 0.41 mol) was added dropwise to 1,3-dioxolane (26.6 g, 25.1 ml, 0.36 mol) maintaining the temperature below 5 °C in an ice bath. The reaction mixture was allowed to warm to room temperature and then a solution of sodium acetate (50 g) in acetic acid (200 ml) was added to the mixture at 110 °C. The mixture was further stirred at 110 °C for 1 h. Insoluble material (NaBr) was filtered off. The filtrate was first distilled to remove acetic acid, then the residue was fractionated in vacuo. The fraction boiling at 114–118 °C/10 mm Hg was collected. This yielded 48.6 g (77%) of the product as clear oil. ^{1}H NMR ($CDCl_3$) δ 5.27 (2H, s, OCH_2O), 4.21 (2H, m, OCH_2), 3.81 (2H, m, OCH_2), 2.08 (3H, s, CH_3), 2.06 (3H, s, CH_3); FAB-MS (in DCI-NH_3) *m*/*z*: 194 ($M+NH_4^+$), calculated molecular weight: 176.1.

2.2.1.2.3. 1-(2-*Acetoxyethoxymethyl*)-*benzimidazole* A mixture of 2-(acetoxymethoxy)ethyl acetate (4.40 g, 25 mmol), acetyl benzimidazole (2.00 g, 12 mmol), *p*-toluenesulfonic acid (297 mg, 1.6 mmol), and DMF (25 ml) was stirred at 140 °C for 18 h under argon and then concentrated to dryness. The crude product was purified by flash chromatography eluting with EtOAc/EtOAc–MeOH (4:1) to yield a brown oil (2.14 g, 73%). ^{1}H NMR ($CDCl_3$) δ 7.98 (1H, s, HC=N), 7.82 (1H, m, H-7), 7.53 (1H, m, H-4), 7.33 (2H, m, H-5 + H-6), 5.59 (2H, s, CH_2N), 4.16 (2H, t, *J* = 4.7 Hz, CH_2O), 3.61 (2H, t, *J* = 4.7 Hz, CH_2O), 1.97 (3H, s, CH_3); FAB-MS (in TG/G) *m*/*z*: 235.1 ($M+H^+$), calculated molecular weight: 234.1.

2.2.1.2.4. 1-(2-*Hydroxyethoxymethyl*)-*benzimidazole* (**3-*a***) A mixture of 1-(2-acetoxyethoxymethyl)-benzimidazole (500 mg, 2.12 mol) and NH_4OH (1 N, 50 ml) was stirred at 60 °C for 1.5 h. The resulting solution was neutralized with Amberlite IR-120 at the H^+-form. The solvent was removed in vacuo and the residue was purified by flash chromatography eluting with EtOAc/EtOAc–MeOH (9:1) to give a yellow oil (308 mg, 75%) which crystallized upon drying in vacuo. ^{1}H NMR ($CDCl_3$) δ 8.29 (1H, s, HC=N), 7.68–7.67 (2H, dd, *J* = 8.3 Hz, H-4/H-7), 7.32 (2H, m, H-5 + H-6), 5.71 (2H, s, CH_2N), 3.62 (2H, t, *J* = 4.5 Hz, CH_2O), 3.52 (2H, t, *J* = 4.5 Hz, CH_2O); FAB-MS (in PEG300/G) *m*/*z*: 193.0977 ($M+H^+$), calculated molecular weight: 192.0899.

2.2.1.2.5. 2-(1-*Benzimidazolemethoxy*)*ethyl-N-Boc-L-valinate* 1-(2-Hydroxyethoxymethyl)-benzimidazole (115 mg, 0.60 mmol) was dissolved in DMF–dichloromethane (1:2, 9 ml) by heating. To this solution were added EDAC (252 mg, 0.90 mmol), DMAP (73 mg, 0.60 mmol), and *N*-Boc-L-valine (195 mg, 0.90 mmol) under argon. The mixture was stirred at 80 °C for 18 h. After evaporation of the solvent, water (20 ml) was added and the mixture was extracted with EtOAc (3×20 ml). The organic phases obtained were combined, dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. The crude product was purified by flash chromatography eluting with EtOAc to yield a clear oil (100 mg, 85%). ^{1}H NMR ($CDCl_3$) δ 8.00 (1H, s, HC=N), 7.83 (1H, m, H-7), 7.55 (1H, m, H-4), 7.35 (2H, m, H-5 + H-6), 5.61 (2H, s, CH_2N), 4.99 (1H, d, *J* = 7.4 Hz, NH), 4.27 (2H, t, *J* = 4.4 Hz, CH_2O), 4.20 (1H, m, C<u>H</u>NH), 3.63 (2H, t, *J* = 4.4 Hz, CH_2O), 2.05 (1H, m, C<u>H</u>$(CH_3)_2$), 1.45 (9H, s, $C(CH_3)_3$), 0.92 (3H, d, *J* = 6.4 Hz, CH_3), 0.83 (3H, d, *J* = 6.7 Hz, CH_3); FAB-MS (in TG/G) *m*/*z*: 392.2 ($M+H^+$), calculated molecular weight: 391.2.

2.2.1.2.6. 2-(1-*Benzimidazolemethoxy*)*ethyl-L-valinate* (**3*b***) 2-(1-Benzimidazolemethoxy)ethyl-*N*-Boc-L-valinate (160 mg, 0.41 mmol) was stirred with TFA (25% in dichloromethane, 7.5 ml) at room temperature for 3 h. The reaction mixture was evaporated, and lyophilization gave the product **3b** as colorless crystals (165 mg, 100%). ^{1}H NMR (CD_3OD) δ 9.46 (1H, s, HC=N), 7.98–7.90 (2H, 2×m, H-7 + H-4), 7.68 (2H, m, H-5 + H-6), 5.98 (2H, s, CH_2N), 4.47 (2H, m, CH_2O), 4.37 (2H, m, CH_2O), 3.89 (2H, m, CH_2O), 3.83 (1H, d, *J* = 4.3 Hz, C<u>H</u>NH), 2.16 (1H, m, C<u>H</u>$(CH_3)_3$), 0.99 (3H, d, *J* = 6.4 Hz, CH_3), 0.97 (3H, d, *J* = 6.4 Hz, CH_3); FAB-MS (in PEG300/G) *m*/*z*: 292.1661 ($M+H^+$), calculated molecular weight: 291.1583.

*2.2.1.3. Synthesis of 2-(7-azaindolemethoxy)ethyl-L-valinate (**4b**)*

2.2.1.3.1. 1-(2-*Benzoyloxyethoxymethyl*)-7-*azaindole* To a stirred suspension of sodium hydride (NaH) (60% in mineral oil, 339 mg, 8.46 mmol) in dry DMF (30 ml) was added a solution of 7-azaindole (1.00 g, 8.46 mmol) in DMF (20 ml) at 0 °C under argon. After 5 min, 2-(chloromethoxy)ethyl benzoate (1.49 ml, 8.46 mmol) was added and the reaction mixture was stirred at room

temperature for 14 h. The solution was quenched with water (20 ml) and the solvent was evaporated. The product was dissolved in water (20 ml), extracted with EtOAc (3×50 ml), and purified by flash chromatography eluting with petroleum ether (PE)–EtOAc (from 9:1 to 4:1) to give a clear oil (1.18 g, 47%). ^{1}H NMR ($CDCl_3$) δ 8.33 (1H, dd, J=4.7 Hz, J=1.5 Hz, H-4), 7.98 (2H, dd, J=7.0 Hz, J=1.4 Hz, H-1′), 7.90 (1H, dd, J=7.8 Hz, J=1.7 Hz, H-6), 7.54 (2H, dt, J=7.4 Hz, J=1.3 Hz, H-3′), 7.41 (2H, t, J=7.6 Hz, H-2′), 7.35 (1H, d, J=3.6 Hz, H-2), 7.10 (1H, dd, J=7.8 Hz, J=4.7 Hz, H-5), 6.51 (1H, d, J=3.6 Hz, H-3), 5.78 (2H, s, OCH_2N), 4.39 (2H, t, J=4.7 Hz, CH_2O), 3.81 (2H, t, J=4.7 Hz, CH_2O); FAB-MS (in TG/G) m/z: 297.1 ($M+H^+$), calculated molecular weight: 296.1.

2.2.1.3.2. 1-(2-*Hydroxyethoxymethyl*)-7-*azaindole* (**4*a***) 1-(2-Benzoyloxyethoxymethyl)-7-azaindole (1.10 g, 3.7 mmol) was stirred in 40% aq. methylamine (110 ml) at room temperature for 1.5 h. After evaporation of the solvent, the residue was purified by flash chromatography eluting with PE–EtOAc (from 1:1 to 1:2 to pure EtOAc). The product was obtained as a clear oil (490 mg, 77%). ^{1}H NMR ($CDCl_3$) δ 8.27 (1H, dd, J=4.8 Hz, J=1.5 Hz, H-4), 8.02 (2H, dd, J=7.8 Hz, J=1.5 Hz, H-6), 7.53 (1H, d, J=3.7 Hz, H-2), 7.16 (1H, dd, J=7.8 Hz, J=4.8 Hz, H-5), 6.57 (1H, d, J=3.7 Hz, H-3), 5.74 (2H, s, OCH_2N), 3.60 (2H, t, J=4.8 Hz, CH_2O), 3.54 (2H, t, J=4.9 Hz, CH_2O); FAB-MS (in PEG300/G) m/z: 193.0977 ($M+H^+$), calculated molecular weight: 192.0899.

2.2.1.3.3. 2-[1-(7-*Azaindolemethoxy*)]*ethyl-N-Boc-*L*-valinate* To a stirred solution of 1-(2-hydroxyethoxymethyl)-7-azaindole (290 mg, 1.51 mmol) in DMF–dichloromethane (1:2, 15 ml) were added EDAC (636 mg, 3.32 mmol), DMAP (184 mg, 1.51 mmol), and *N*-Boc-L-valine (492 mg, 2.26 mmol) under argon. The solution was stirred at 80 °C for 16 h. After evaporation of the solvent, the residue was purified by flash chromatography eluting with PE–EtOAc (1:1) to yield 543 mg (92%) of the product as a clear oil. ^{1}H NMR ($CDCl_3$) δ 8.32 (1H, d, J=4.7 Hz, H-4), 7.91 (1H, d, J=7.7 Hz, H-6), 7.33 (1H, d, J=3.5 Hz, H-2), 7.10 (1H, dd, J=7.7 Hz, J=4.7 Hz, H-5), 6.53 (1H, d, J=3.5 Hz, H-3), 5.73 (1H, s, OCH_2N), 5.04 (1H, d, J=8.4 Hz, NH), 4.23 (1H, m, C<u>H</u>NH), 4.20 (2H, m, CH_2O), 3.67 (2H, m, CH_2O), 2.05 (1H, m, C<u>H</u>$(CH_3)_2$), 1.43 (9H, s, $C(CH_3)_3$), 0.91 (3H, d, J=6.8 Hz, CH_3), 0.82 (3H, d, J=6.8 Hz, CH_3); FAB-MS (in G) m/z: 392.2 ($M+H^+$), calculated molecular weight: 391.2.

2.2.1.3.4. 2-[1-(7-*Azaindolemethoxy*)]*ethyl-*L*-valinate* (**4*b***) 2-[1-(7-Azaindolemethoxy)]ethyl - *N* - Boc-L-valinate (500 mg, 1.27 mmol) was stirred with TFA (25% in dichloromethane, 20 ml) at room temperature for 3 h and then subjected to evaporation, azeotropic distillation, and lyophilization. The product **4b** was obtained as a yellow oil (520 mg, 100%). ^{1}H NMR (CD_3OD) δ 8.31 (1H, dd, J=5.0 Hz, J=1.3 Hz, H-4), 8.15 (1H, dd, J=7.9 Hz, J=1.4 Hz, H-6), 7.58 (1H, d, J=3.6 Hz, H-2), 7.26 (1H, dd, J=7.9 Hz, J=5.0 Hz, H-5), 6.65 (1H, d, J=3.7 Hz, H-3), 5.75 (2H, s, OCH_2N), 4.42 (2H, m, CH_2O), 4.27 (2H, m, CH_2O), 3.82 (1H, d, J=4.5 Hz, C<u>H</u>NH), 3.76 (2H, m, CH_2O), 2.15 (1H, m, C<u>H</u>$(CH_3)_2$), 0.95 (3H, d, J=6.4 Hz, CH_3), 0.94 (3H, d, J=6.4 Hz, CH_3); FAB-MS (in PEG300/G) m/z: 292.1661 ($M+H^+$), calculated molecular weight: 291.1583.

2.2.2. Characterization of physicochemical properties

The calculated partition coefficients in octanol/water (cLogP) for acyclovir, L-valacyclovir, and other analogues were calculated using a commercial product ACDlabs (version 4.1) developed by Advanced Chemistry Development, Canada.

2.2.3. Caco-2 cell cultures

Caco-2 cells were maintained under culture conditions (in 150-cm^2 T-flasks at 37 °C, 5% CO_2/95% air atmosphere with 95% humidity) as described by Gao et al. (2000). The culture medium consisted of DMEM, 10% FBS, 1% NEAA, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells used for experimentation were seeded at a density of 1×10^5 cells/cm^2 either on 24-well clusters or polycarbonate membranes of Transwells® coated with rat tail collagen and grown for 21–28 days following the procedure described previously (Gao et al., 2000). All cells used for the current studies were between passages 24 and 50. [^{14}C]Mannitol flux assays were performed on each batch of Caco-2 cell monolayers grown on Transwell® membranes (n=3). Cell monolayers with apparent permeability coefficients (P_{app}) of [^{14}C]mannitol $<3\times10^{-7}$ cm/s were considered to be appropriate for transport studies.

2.2.4. Uptake and transport properties of L*-valacyclovir analogs*

2.2.4.1. Uptake studies

Uptake experiments were performed in triplicate using Caco-2 cells grown in 24-well clusters at 37 °C in EBSS (pH 6.0). Prior to the initiation of the uptake experiments, the culture medium was removed and the cells were rinsed three times followed by 20-min equilibration with HBSS (pH 7.4). After removal of HBSS, uptake of radiolabeled compounds was initiated by adding 150 μl of 'assay solution' to the culture wells. For [^{3}H]carnosine uptake studies, the 'assay solution' contained [^{3}H]L-carnosine (0.1 mM, 1 μCi/ml) without or with the L-valacyclovir analog (10 mM) in EBSS. For [^{3}H]uridine uptake studies, the 'assay solution' contained [^{3}H]uridine (0.1 mM, 1 μCi/ml) without or with the L-valacyclovir analog (10 mM) in EBSS. Aliquots (20 μl) of each 'assay solution' were taken prior to initiation of the experiment to determine the total radioactivity added to each well. Cellular uptake was terminated after a 20-min incubation for [^{3}H]carnosine experiments or after a 10-s incubation for [^{3}H]uridine

experiments by removing the 'assay solution' from the culture wells followed by rinsing with 3×1.5 ml ice-cold HBSS. The cells in each well were then lysed with 0.3 M NaOH (300 μl) and transferred to scintillation vials. Radioactivity associated with the cells was determined by liquid scintillation counting.

2.2.4.2. Transport experiments

The transcellular transport studies were performed in triplicate as described previously by our laboratory (Gao et al., 2000). Briefly, 1.5 ml of the assay solution containing the L-valacyclovir analog in EBSS (pH 6.0) was applied to the donor compartment on the apical (AP) side of the Caco-2 cell monolayers grown on Transwell® membranes and 2.6 ml of HBSS (pH 7.4) was added to the receiver compartment on the basolateral (BL) side of the Caco-2 cell monolayers. Aliquots were taken from both sides (200 μl from receiver side, 20 μl from donor side) at various times up to 180 min. The same volume of HBSS was replenished on the receiver side after each sampling. Samples were then transferred into ice-cold HPLC vials containing 100 μl of 0.05 N HCl to minimize enzymatic degradation and stored at −80 °C until analysis by HPLC.

2.2.5. HPLC analysis

HPLC analysis was performed using a Shimadzu gradient system (Shimadzu, Tokyo, Japan) consisting of two LC-10A pumps, an SCP-6 controller, and an SPD-10A UV detector coupled with a PC computer. Chromatographic separation of samples was carried out on a Vydac C_{18} reversed-phase column (5 μM, 300 Å, 250 mm L×4.6 mm I.D.). Gradient elution was performed at a flow rate of 1.0 ml/min from 0 to 10% acetonitrile in water containing 0.1% trifluoroacetic acid as an ion-pairing agent. The UV detection was performed at 254 nm. The temperature was maintained at 4 °C during sample analysis.

2.2.6. Data analysis

The % uptake of radiolabeled compounds ([^{3}H]carnosine or [^{3}H]uridine) was calculated using Eq. (1):

$$\%\ \text{Uptake} = (Q_{\text{retained}}/Q_{\text{total}}) \times 100 \quad (1)$$

where Q_{retained} is the radioactivity (dpm) retained in the cell monolayer and Q_{total} is the total radioactivity (dpm) added to each well.

Comparisons were made between control cell monolayers, which were incubated with radiolabeled compound alone, and the cell monolayers that were incubated with a L-valacyclovir analog or a known inhibitor of PepT1 (GlyPro) or inhibitors of the nucleoside transporter (dipyridamole and phloridzin). The uptake of the radiolabeled compound in control cell monolayers was considered as 100%. If test compounds reduced the uptake of the radiolabeled compound below control values, this result was interpreted as reflecting the binding of the test compound to the transporter.

The apparent permeability coefficients (P_{app}) for the L-valacyclovir analogs were calculated using Eq. (2):

$$P_{\text{app}} = (\Delta Q/\Delta t)/(A \times C_0), \quad (2)$$

where $\Delta Q/\Delta t$ is the mass appearance rate of a test compound in the receiver solution (mmol/s), which is the slope of the linear portion of the graph when the cumulative amount of a test compound transported is plotted against time (Gao et al., 2000); A is the surface area of the cell monolayers (cm^2); and C_0 is the initial concentration of the test compound on the donor side ($mmol/cm^3$).

2.2.7. Statistical analysis

All data are expressed as means±S.D. Statistical analysis was performed using a paired Student's *t*-test. A P value ≤ 0.05 is considered to be statistically significant.

2.2.8. Aqueous stability studies

The aqueous stability of L-valacyclovir analogs was determined by HPLC after storage in EBSS (pH 6.0) at −18 °C for 16 days or at 37 °C for 2 h.

3. Results

3.1. Synthesis of L-valacyclovir analogs

To examine the role of the guanine moiety of L-valacyclovir in binding to PepT1, a number of analogs lacking possible hydrogen bonding sites were synthesized (Fig. 1). Substitution of guanine with purine resulted in compound **2b**, which lacks the most obvious hydrogen-donating (the amino group) and hydrogen-accepting (the carbonyl oxygen) groups. In the benzimidazole analog **3b** and the 7-azaindole analog **4b**, two additional possible hydrogen bonding sites were removed.

To synthesize these L-valacyclovir analogs (**2b**–**4b**), we initially needed to attach the 2-hydroxyethoxy methyl moiety to the respective heteroaromatic system. Attachment of the 2-hydroxyethoxy methyl moieties to benzimidazole was performed according to the procedures used to synthesize acyclovir (Matsumoto et al., 1988). Benzimidazole was acetylated followed by reaction with 2-(acetoxymethoxy)ethyl acetate and cleavage of the acetyl group with ammonium hydroxide. This synthetic scheme was optimized using larger amounts of catalyst, higher temperature, and longer reaction time than those described by Matsumoto et al. (1988) (see Section 2.2.1.2 for details). A similar approach was attempted with purine, but selective acetylation of N-9 was not possible. Instead, a literature procedure using the N-9-alkyl substituted purinesulfinamides (Hanna et al., 1994) was adopted, and selective N-9 deprotonation was performed with lithium

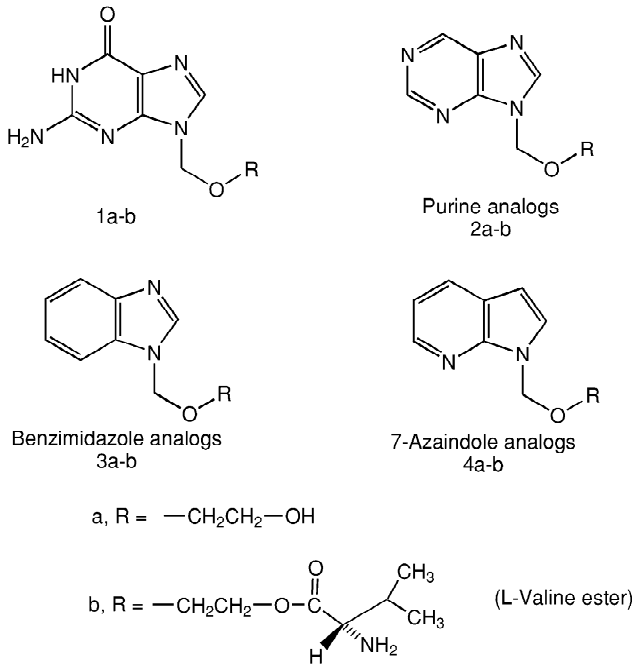

Fig. 1. Analogs of acyclovir (**1a**) and L-valacyclovir (**1b**).

diisopropylamide in dimethyl formamide followed by addition of 2-(chloromethoxy)ethyl benzoate and cleavage of the benzoyl group with methylamine. Substitution at the N-9 position of the side chain was verified by a heteronuclear multiple bond correlation experiment, which showed coupling from the NCH_2O carbon to C-8 and C-6. Finally, selective introduction of the 2-hydroxyethoxy methyl moiety in the N-1 position of 7-azaindole was performed by deprotonation with NaH, addition of 2-(chloromethoxy)ethyl benzoate, and deprotection with methylamine (Beauchamp et al., 1985).

After introduction of the 2-hydroxyethoxy methyl moiety, the hydroxy group was coupled to *N*-Boc-protected L-valine using EDAC as coupling reagent. One equivalent of DMAP was necessary to obtain a reasonable yield. The Boc-group was removed with 25% trifluoroacetic acid in dichloromethane.

The purine (**2b**), benzimidazole (**3b**), and 7-azaindole (**4b**) L-valacyclovir analogs were all stable during synthesis and upon storage. Chiral HPLC was performed on the L-Val-benzimidazole analog **3b** to exclude the possibility of racemization during synthesis. The results of these experiments confirmed that no significant racemization had taken place (data not shown).

The lipophilic characteristics of the nucleoside analogs of L-valacyclovir and acyclovir were estimated based on cLogP values as shown in Tables 1 and 3. Reducing the hydrogen-bonding sites in the guanine moiety tends to increase the hydrophobicity of these molecules. The order of hydrophobicity of L-valacyclovir and its analogs (**1b**–**4b**) is: **4b** > **3b** ≫ **2b** ≅ **1b**. Acyclovir and its analogs (**1a**–**4a**) show an order similar to their L-valine ester prodrugs (**4a** > **3a** ≫ **2a** ≅ **1a**), but with a decrease in the values of cLogP by ~2 logarithms.

Table 1
Apparent permeability coefficients (P_{app}) of L-valacyclovir (**1b**) and its analogs **2b**–**4b** in the AP-to-BL direction across Caco-2 cell monolayers in the absence or presence of GlyPro[a] and their calculated partition coefficients in octanol/water (cLogP)

Compound	cLogP[b]	$P_{app} \times 10^6$ (cm/s)[c]		
		−GlyPro	+GlyPro	
1b	0.04	4.55 (±0.25)	0.75 (±0.19)	$P<0.01$
2b	−0.05	3.0 (±0.22)	1.68 (±0.16)	$P<0.01$
3b	2.01	25.1 (±0.06)	21.0 (±1.36)	$P<0.05$
4b	2.84	30.2 (±1.94)	26.1 (±2.06)	$P<0.05$

[a] Transport experiments of L-valacyclovir and its analogs (0.5 mM) were conducted in the presence (5 mM) or absence of GlyPro at 37 °C in EBSS as described in the Materials and methods section. Results are the mean±S.D. of three separate experiments.

[b] cLogP from ACD (version 4.1).

[c] All the permeability coefficients for the 'prodrugs', L-valacyclovir (**1b**) and its analogs (**2b**–**4b**) listed in this table were the 'apparent' values based only on the appearance of the 'drugs' in their hydrolyzed forms (**1a**–**4a**) on the basolateral side of Caco-2 monolayers.

*3.2. Inhibitory effect of L-valacyclovir (**1b**) and analogs **2b**–**4b** on carnosine uptake in Caco-2 cell monolayers*

The affinities of PepT1 in Caco-2 cell monolayers for the L-valacyclovir analogs were estimated by measuring their ability to inhibit the uptake of [^{3}H]carnosine (0.1 mM), a known substrate for this oligopeptide transporter. As shown in Fig. 2, L-valacyclovir (**1b**) and its purine (**2b**), benzimidazole (**3b**), and azaindole (**4b**) analogs (all at 10 mM) significantly inhibited [^{3}H]carnosine uptake. The extent of inhibition (=75%) was comparable to that produced by GlyPro (10 mM), which is a known inhibitor of PepT1. Acyclovir (**1a**), however, showed very little effect on [^{3}H]carnosine uptake at a concentration of 5 mM. One percent DMSO, which was used to solubilize acyclovir, had no effect on [^{3}H]carnosine uptake (data not shown). The acyclovir analogs **2a**, **3a**, and **4a** at 10 mM also showed no inhibitory effect on [^{3}H]carnosine uptake in the Caco-2 cells (Fig. 2).

*3.3. Transport of L-valacyclovir (**1b**) and its analogs **2b**–**4b** across Caco-2 cell monolayers*

In order to determine PepT1-mediated transport activity for L-valacyclovir analogs **2b**–**4b**, the AP-to-BL flux of these prodrugs across Caco-2 cell monolayers was determined in the absence or presence of GlyPro, a known inhibitor of PepT1. For comparative purposes, the transport of L-valacyclovir (**1b**) itself across Caco-2 cell monolayers was also investigated. The results of these experiments are presented in Fig. 3. Since the valine ester bonds of L-valacylovir (**1b**) and its analogs (**2b**–**4b**) can potentially be hydrolyzed by esterases upon exposure to the AP side of

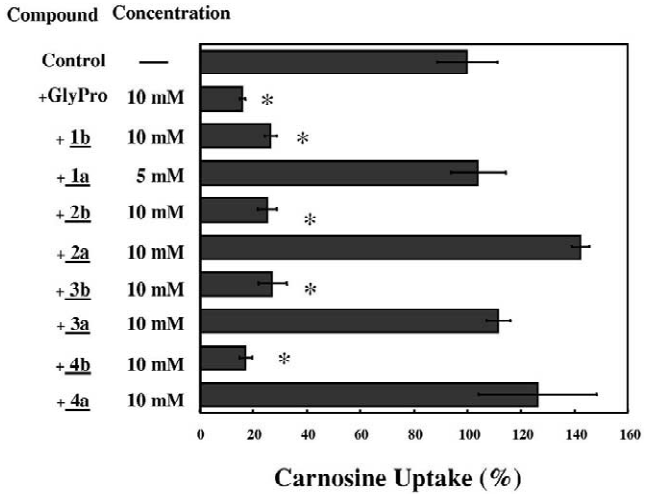

Fig. 2. Effects of acyclovir and L-valacyclovir analogs on [^{3}H]carnosine uptake in Caco-2 cells. [^{3}H]Carnosine (0.1 mM, 5 mCi/ml) uptake into Caco-2 cell monolayers was studied in the presence or absence of GlyPro, acyclovir (**1a**) and its analogs **2a**–**4a**, and L-valacyclovir (**1b**) and its analogs **2b**–**4b** at the indicated concentrations. Incubations were conducted for 20 min at 37 °C in EBSS as described in the Materials and methods section. Results are the means±S.D. of quadruplicate determinations from one of two independent experiments. The asterisks (*) indicate that the reduction from the control experiment (no addition) was statistically significant ($P<0.05$) according to paired Student's *t*-test.

Caco-2 cell monolayers, HPLC analytical methods were developed that permit the detection of both the 'prodrugs' (**1b**–**4b**) and the 'drugs' (**1a**–**4a**). As shown in Fig. 3, incubation of L-valacyclovir and its analogs (**1b**–**4b**) on the AP side of Caco-2 cell monolayers resulted in a time-dependent appearance of acyclovir (**1a**) (panel A) and its analogs (**2a**–**4a**) (panels B–D), respectively, on the BL side of the cell monolayers. No detectable levels of the 'prodrugs' (**1b**–**4b**) were observed on the BL side of these cell monolayers. The amount of **1a** appearing on the BL side after application of **1b** to the AP side exhibited a short lag time (~20 min) followed by a linear appearance of **1a**. At 180 min the level of **1a** on the BL side reached ~12% of the initial amount of **1b** that was added on the AP side of the cell monolayer. The appearance of acyclovir (**1a**) on the BL side of the cell monolayer was significantly reduced (from 12 to 2%) when GlyPro, a known inhibitor of PepT1, was included along with L-valacyclovir (**1b**) on the AP side (Fig. 3, panel A). When the purine analog **2b** was applied to the AP side of the Caco-2 cell monolayers, the appearance of **2a** on the BL side was also linear with time (9% at 180 min) and partially inhibited by inclusion of GlyPro on the AP side (Fig. 3, panel B). However, the profiles for appearance of **3a** (from **3b**) and **4a** (from **4b**) on the BL side of the Caco-2 cell monolayers were very different from those observed for **1a** (from **1b**) and **2a** (from **2b**) in three respects. First, the levels of **3a** (Fig. 3, panel C, 50%) and **4a** (Fig. 3, panel D, 42%) appearing on the BL side of the cell monolayers after application of **3b** and **4b**, respectively, to the AP side were significantly higher than that of **1a** (Fig. 3, panel A, 12%) and **2a** (Fig. 3, panel B, 9%). Secondly, the appearance of **3a** (Fig. 3, panel C) and **4a** (Fig. 3, panel D) on the BL side of the cell monolayers was not linear with time but instead appeared to reach steady-state levels after ~100 min of incubation. Thirdly, the levels of **3a** (Fig. 3, panel C) and **4a** (Fig. 3, panel D) appearing on the BL side were only slightly affected (from 50 to 42% in **3a** and 42 to 39% in **4a**) by inclusion of GlyPro with **3b** and **4b**, respectively, on the AP side of the Caco-2 cell monolayers. Table 1 shows a summary of the P_{app} values of all compounds tested. The differences between the P_{app} values in the presence and the absence of GlyPro are statistically significant (**1b** and **2b**, $P<0.01$; **3b** and **4b**, $P<0.05$).

*3.4. Stability of L-valacyclovir (**1b**) and its analogs **2b**–**4b** upon exposure to the AP side of Caco-2 cell monolayers*

During the transport experiments described above, the stabilities of L-valacyclovir (**1b**) and its analogs **2b**–**4b** on the AP side of Caco-2 cell monolayers were monitored. Although the purine (**2b**), benzimidazole (**3b**), and 7-azaindole (**4b**) analogs were all stable in EBSS (pH 6.0) and HBSS (pH 7.4) at 37 °C up to 2 h (data not shown), exposure of these analogs in EBSS (pH 6.0) to the AP side of Caco-2 cells resulted in their rapid hydrolysis to the corresponding acyclovir analogs **2a**–**4a** (Table 2). Such a rapid hydrolysis did not occur when L-valacyclovir and its analogs **2b**–**4b** were directly added to the HBSS on the BL side of Caco-2 cell monolayers (data not shown). It is interesting to compare the stability of analogs **2b**–**4b** to that of L-valacyclovir (**1b**) (Table 2). The order of stability was **1b** > **2b** ≫ **3b** ≫ **4b** when these compounds were incubated in EBSS (pH 6.0) on the AP side of Caco-2 cell monolayers.

*3.5. Transport of acyclovir (**1a**) and its analogs (**2a**–**4a**) across Caco-2 cell monolayers*

To better understand the 'apparent' transport characteristics of L-valacyclovir (**1b**) and its analogs **2b**–**4b** (Fig. 3), we determined the transport characteristics of acyclovir (**1a**) and its analogs (**2a**–**4a**) in the AP-to-BL direction across Caco-2 cell monolayers. Consistent with their reported oral bioavailability (de Miranda and Blum, 1983), the P_{app} value for acyclovir (**1a**) (Table 3) was found to be significantly lower than that for L-valacyclovir (**1b**) (Table 1). In contrast, the values for the acyclovir analogs **2a**–**4a** (Table 3) were significantly greater than the P_{app} values for their L-valine esters **2b**–**4b** (Table 1). The relative P_{app} values for the benzimidazole and azaindole analogs of acyclovir (**4a** = **3a** > **2a** ≫ **1a**) are consistent with their lipophilic characteristics as estimated by cLogP values (Table 3).

Since the P_{app} values for the more lipophilic acyclovir

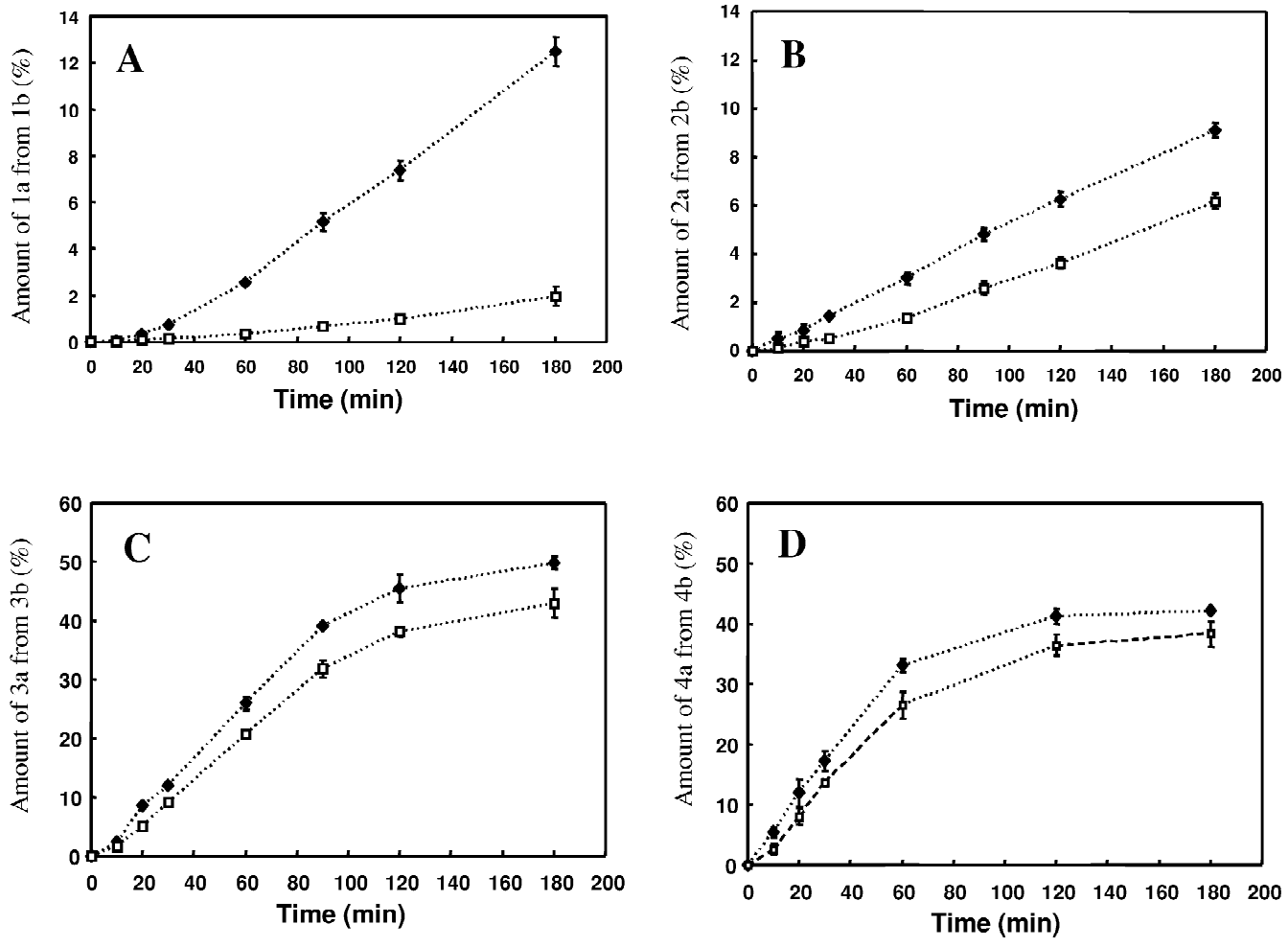

Fig. 3. Time course of the appearance of acyclovir (**1a**) or its analogs **2a**–**4a** on the BL side when L-valacyclovir (**1b**) or its analogs **2b**–**4b** were applied on the AP side of Caco-2 cell monolayers. Experiments were done in the presence (□) or absence (♦) of 5 mM GlyPro using a 0.5-mM concentration of L-valacyclovir (**1b**) or its analogs **2b**–**4b** at 37 °C in EBSS. Results are the means±S.D. of three separate experiments. Panel A: % acyclovir (**1a**) from L-valacyclovir (**1b**); panel B: % **2a** from **2b**; panel C: % **3a** from **3b**; panel D: % **4a** from **4b**.

analogs **3a** and **4a** were quite high, we conducted experiments to show that their permeation across Caco-2 cells was not mediated by a transporter system. One possibility is that the acyclovir analogs **2a**–**4a** are themselves substrates for PepT1. However, this possibility can be ruled out because inclusion of GlyPro has no effect on the transport of **2a**–**4a** in the AP-to-BL direction across Caco-2 cell monolayers (Table 3).

Another possibility is that the acyclovir analogs **2a**–**4a** are substrates for a Na^+-independent nucleoside transporter that has been reported by Ward and Tse (1999) to be present in Caco-2 cells. To evaluate this possibility an

Table 2
Time course of disappearance of L-valacyclovir (**1b**) and its analogs **2b**–**4b** and the appearance of acyclovir (**1a**) and its analogs **2a**–**4a** on the AP side of Caco-2 cells

Time (min)	% Of total **1b**–**4b** (0.5 mM) added to AP side[a]							
	1b	**1a**	**2b**	**2a**	**3b**	**3a**	**4b**	**4a**
0	100	0	100	0	100	0	100	0
30	84	2	99	0.5	60	27	23	46
60	82	6	84	2.0	25	43	0	49
120	76	16	55	3.7	5	41	–	38
180	60	26	32	5.4	2	38	–	39

[a] The amounts of L-valacyclovir (**1b**) and its analogs (**2b**–**4b**) and acyclovir (**1a**) and its analogs (**2a**–**4a**) on the AP side of Caco-2 cell monolayers were monitored with times up to 180 min. The incubation medium consisted of EBSS (pH 6.0) as described in the Materials and methods section. Results are expressed as the percentage of the total amounts of L-valacyclovir (**1b**) and its analogs (**2b**–**4b**) added to the AP incubation mixture.

Table 3
Apparent permeability coefficients (P_{app}) of acyclovir (**1a**) and its analogs **2a**–**4a** in the AP-to-BL direction across Caco-2 cell monolayers in the absence or presence of GlyPro and their calculated partition coefficients in octanol/water (cLogP)

Compound	$P_{app} \times 10^6$ (cm/s)[a]		cLogP[b]
	−GlyPro	+GlyPro	
1a	0.43 (±0.01)	–	−1.76
2a	9.17 (±0.79)	9.42 (±0.96)	−1.85
3a	39.8 (±0.06)	42.1 (±1.5)	0.21
4a	47.9 (±0.79)	44.4 (±2.0)	1.37

[a] Transport experiments of acyclovir (**1a**) and its analogs **2a**–**4a** (0.5 mM) were conducted in the presence (5 mM) or absence of GlyPro at 37 °C in EBSS (pH 6.0) as described in the Materials and methods section. Results are the mean±S.D. of three separate experiments.
[b] cLogP from ACD (version 4.1).

assay for the nucleoside transporter using [^{3}H]uridine was developed. The uptake of [^{3}H]uridine into Caco-2 cells from the AP side is time-dependent, reaching steady-state at 4 min. The uptake of [^{3}H]uridine into Caco-2 cells can be inhibited by dipyridamole and phloridzin, known inhibitors of the nucleoside transporters (Plagemann and Aran, 1990), and by a purine (inosine) and a pyrimidine (thymidine) nucleoside (Fig. 4). Acyclovir (**1a**) does not inhibit the uptake of [^{3}H]uridine, whereas its analogs **2a**–**4a** exhibit inhibitory activities comparable to those of dipyridamole and phloridzin, inosine and thymidine on [^{3}H]uridine uptake. The order of potency for inhibition of [^{3}H]uridine uptake was **4a** ≫ **3a** = **2a** ≫ **1a**. These results

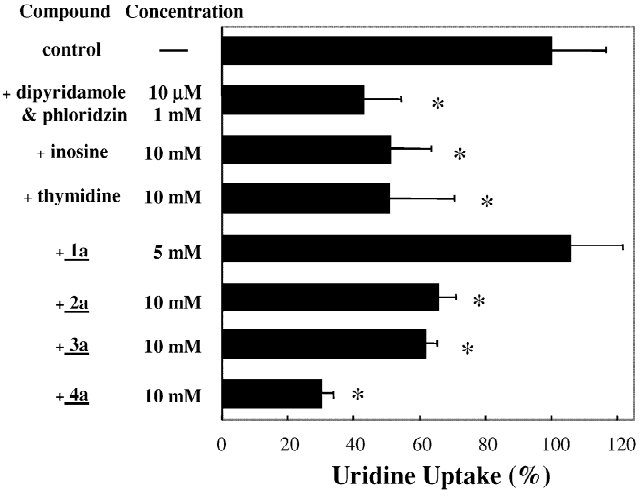

Fig. 4. Effect of known inhibitors of the nucleoside transporter (dipyridamole and phloridzin), nucleosides (inosine and thymidine) and acyclovir (**1a**) and its analogs **2a**–**4a** on uptake of [^{3}H]uridine in Caco-2 cell monolayers. [^{3}H]Uridine (0.1 mM, 5 mCi/ml) uptake into Caco-2 cell monolayers was studied in the absence or presence of these potential inhibitors at the concentrations indicated. Incubations were conducted for 10 s at 37 °C in EBSS as described in the Materials and methods section. Results are the means±S.D. of quadruplicate determinations from one of two independent experiments. The asterisks (*) indicate that the reduction from the control experiment (without addition) was statistically significant ($P < 0.05$) according to paired Student's *t*-test.

Table 4
Apparent permeability coefficients (P_{app}) of acyclovir analogs **2a**–**4a** in the AP-to-BL direction across Caco-2 cell monolayers in the absence and presence of nucleoside transporter inhibitors

Compound	$P_{app} \times 10^6$ (cm/s)[a]	
	−Inhibitors	+Inhibitors
2a	12.8 (±0.21)	10.2 (±0.73)
3a	65.3 (±2.28)	56.9 (±1.16)
4a	82.5 (±6.24)	77.6 (±8.8)

[a] Transport studies with acyclovir analogs **2a**–**4a** (0.5 mM) were conducted in the presence or absence of 10 μM dipyridamole and 1 mM phloridzin at 37 °C in EBSS (pH 6.0) as described in the Materials and methods section. Results are the mean±S.D. of three separate experiments.

suggest that acyclovir analogs **2a**–**4a** may be substrates for the nucleoside transporter, which could then be in part involved in their transcellular transport across Caco-2 cell monolayers. However, when the transcellular transport characteristics of the acyclovir analogs **2a**–**4a** were determined in the presence of dipyridamole and phloridzin, these inhibitors of the nucleoside transporter had no effect on their permeation (Table 4).

4. Discussion

L-Valacyclovir (**1b**) was designed as a prodrug of acyclovir (**1a**) in an attempt to improve the oral bioavailability of this antiviral agent (Beauchamp et al., 1992; Crooks and Murray, 1994). In subsequent studies, several laboratories (Ganapathy et al., 1998; Sinko and Balimane, 1998; de Vrueh et al., 1998) demonstrated that L-valacyclovir (**1b**) was a substrate for PepT1 in the intestinal mucosa, which provided a rational explanation for the good oral bioavailability of this hydrophilic prodrug. In retrospect, L-valacyclovir (**1b**) is an example of an almost 'perfect' prodrug for enhancing oral absorption. First, L-valacyclovir (**1b**) is stable in the lumen of the intestine (Sinko and Balimane, 1998), and it is relatively stable to the enzymes on the surface of intestinal mucosal cells (e.g. Caco-2 cells; Table 2). Secondly, it is a substrate for PepT1, which mediates its uptake into intestinal mucosal cells (e.g. Caco-2 cells; Figs. 2 and 3). Thirdly, it appears to be a substrate for intracellular esterases that catalyze the bioconversion of L-valacyclovir (**1b**) to acyclovir (**1a**). Finally, acyclovir (**1a**), not L-valacyclovir (**1b**), appears in blood (Soul-Lawton et al., 1995) and on the BL side of intestinal mucosal cells (e.g. Caco-2 cells; Fig. 3). These transport and metabolic stability characteristics of L-valacyclovir (**1b**) are summarized in Fig. 5 (panel A).

In an attempt to better understand the role of the guanine moiety in the binding of L-valacyclovir to PepT1, we synthesized in this study a series of analogs having different heteroaromatic rings (purine, benzimidazole, and 7-azaindole) replacing the guanine moiety. The biological

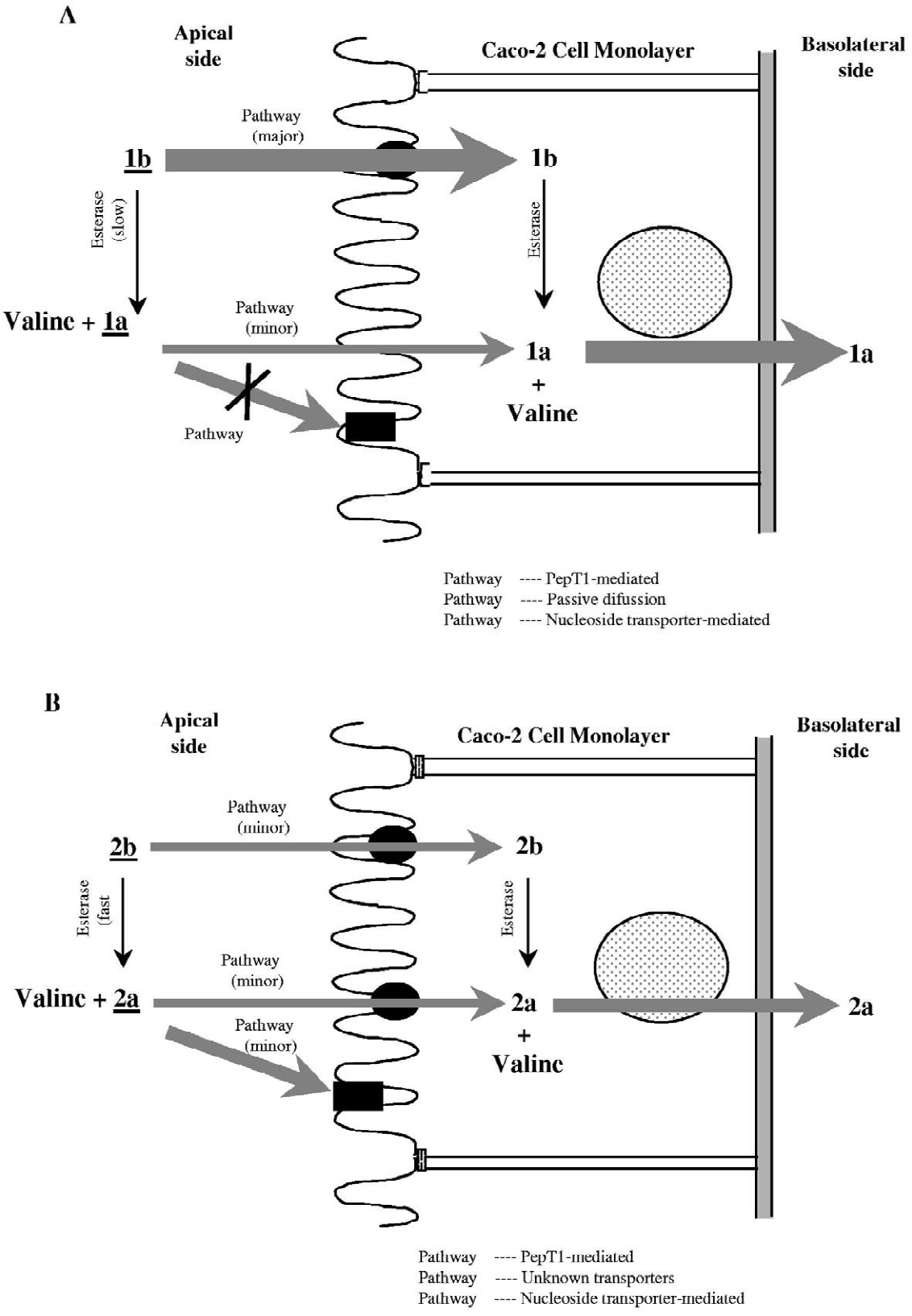

Fig. 5. Model for the transport and metabolic stability characteristics of L-valacyclovir (**1b**) and its analogs (**2b**–**4b**).

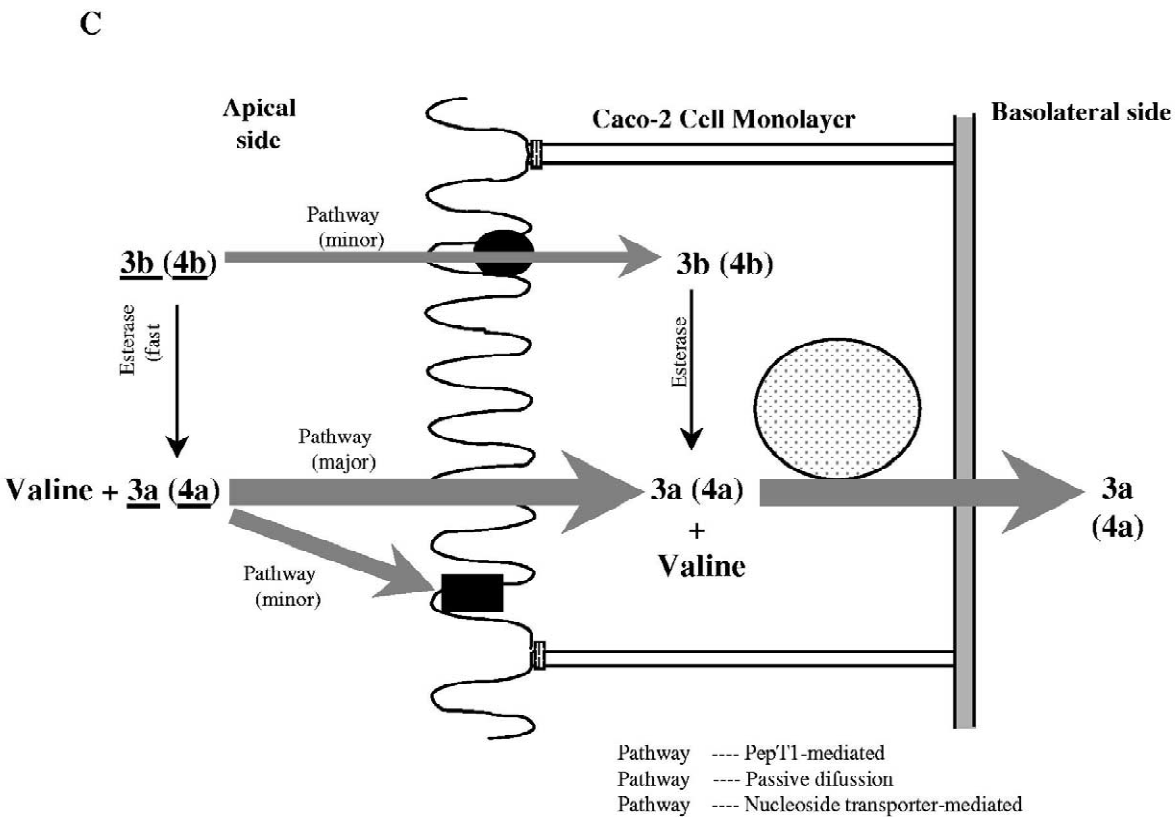

Fig. 5. (*continued*)

properties of the purine (**2a**–**b**), benzimidazole (**3a**–**b**), and 7-azaindole (**4a**–**b**) analogs were characterized and the pathways by which they permeate across Caco-2 cell monolayers were elucidated.

The data shown in Fig. 2 clearly show that all of the L-valine esters (**2b**–**4b**) of the acyclovir analogs (**2a**–**4a**) bind to PepT1. The data presented in Fig. 2 also clearly show that acyclovir (**1a**) and its analogs **2a**–**4a** do not bind to this transporter. The purine (**2b**), benzimidazole (**3b**), and 7-azaindole (**4b**) analogs of L-valacyclovir (**1b**) were designed to have reduced sites for potential hydrogen-bonding interactions. It is also noteworthy that these heteroaromatic moieties in **2b**–**4b** exhibited quite different lipophilicity characteristics (order of hydrophobicity: **4a**≅**3a**>>**2a**>**1a**; Table 3). The PepT1 binding data shown in Fig. 2 would suggest that the heteroaromatic moieties in L-valacyclovir (**1b**) and its analogs **2b**–**4b** are not contributing significantly to their binding to this transporter. These results would suggest that the primary recognition sites for PepT1 are the amino group and the carbonyl group provided by the valine. Finally, it is important to mention that simply binding the analogs **2b**–**4b** to PepT1 (Fig. 2) does not ensure that these prodrugs will be as effective at delivering the acyclovir analogs **2a**–**4a** as L-valacyclovir (**1b**) is at delivering acyclovir (**1a**) across a cell culture model (Caco-2 cells) of the intestinal mucosa. As described above, L-valacyclovir (**1b**) has many other characteristics (e.g. luminal stability, intracellular instability) that allow it to effectively deliver acyclovir (**1a**) across the intestinal mucosa.

The transport characteristics of the purine analog **2b** are similar to those of L-valacyclovir (**1b**), whereas the transport characteristics of the benzimidazole (**3b**) and 7-azaindole (**4b**) analogs are very different. The purine analog **2b** delivers the acyclovir analog **2a** to the BL side of a Caco-2 monolayer in a time-dependent manner that is partially inhibited by GlyPro. However, the total amount of **2a** delivered to the BL side after application of **2b** to the AP side of the cell monolayer is less than that observed with acyclovir (**1a**) delivered from L-valacyclovir (**1b**). This may suggest that the purine analog **2b** is a poorer substrate for PepT1; thus, transport of the purine analog **2b** by PepT1 is less efficient than that of L-valacyclovir (**1b**) and/or that the bioconversion of the **2b** to **2a** intracellularly is less efficient than the bioconversion of L-valacyclovir (**1b**) to acyclovir (**1a**). The latter possibility may explain the low recovery of **2a** on the BL side of the cell monolayers when total mass balance on both sides were compared, i.e. the purine analog **2b** may be trapped in the cell monolayers. It is also worth noting that, in the presence of GlyPro, the purine analog **2b** delivers more **2a** than L-valacyclovir (**1b**) delivers acyclovir (**1a**). This would suggest that the permeation of analog **2a** across the Caco-2 monolayers is greater than acyclovir (**1a**), which

was confirmed in separate studies (Table 3). The purine analog **2a** was shown to have affinity for the nucleoside transporter in Caco-2 cells (Fig. 4), which might contribute in part to its higher permeation across Caco-2 cell monolayers than that of acyclovir (**1a**). However, inclusion of nucleoside transporter inhibitors seemed to have limited effect on permeation of **2a** across Caco-2 cell monolayers. Based on its cLogP value, **2a** seems to be hydrophilic rather than lipophilic and transcellular passive diffusion is unlikely for **2a**. These observations suggest that transport of **2a** across cell monolayers may also involve a transporter system in the Caco-2 cells that has not yet been identified. In summary, the purine analog **2b** appears to deliver **2a** across Caco-2 cell monolayer by a PepT1-dependent mechanism mediating the uptake of **2b** (pathway a, Fig. 5B) and a nucleoside transporter-dependent mechanism mediating the uptake of **2a** (pathway c, Fig. 5B). In addition, a third but unknown transporter system may also be involved in transport of **2a** formed by hydrolysis of **2b** on the AP surface of Caco-2 cells.

The transport characteristics of the benzimidazole (**3b**) and 7-azaindole (**4b**) analogs are similar to each other but very different from that of L-valacyclovir (**1b**). Analogs **3b** and **4b** deliver their acyclovir analogs **3a** and **4a**, respectively, across Caco-2 cell monolayers in a time-dependent manner that rapidly reaches steady-state and is only marginally affected by the inclusion of GlyPro on the AP side of the monolayers (Fig. 3C,D). The total amounts of **3a** and **4a** that ultimately appear on the BL side of the cell monolayers when **3b** and **4b** are applied to the AP side are significantly greater than the amounts of acyclovir (**1a**) delivered from L-valacyclovir (**1b**). This enhanced delivery of **3a** and **4a** can be attributed to the instability of **3b** and **4b** to esterases on the AP side of Caco-2 cells (Table 2) and the excellent permeation characteristics of **3a** and **4a** across Caco-2 cell monolayers (Table 3). Like **2a**, the acyclovir analogs **3a** and **4a** also appear to be substrates for the nucleoside transporter (Fig. 5), which may also contribute to this enhanced permeation of **3a** and **4a** across Caco-2 cell monolayers. In summary, the benzimidazole (**3b**) and 7-azaindole (**4b**) analogs appear to deliver **3a** and **4a**, respectively, across Caco-2 cell monolayers primarily via their rapid hydrolysis by esterases on AP side of Caco-2 cells and then the rapid passive diffusion of **3a** and **4a** across the cell monolayers (Fig. 5C). There may also be a small contribution by a PepT1-dependent mechanism mediating the uptake of **3b** and **4b** (pathway a, Fig. 5C) and/or a nucleoside transporter-dependent mechanism mediating the uptake of **3a** and **4a** (pathway c, Fig. 5C), but these appear to be minor pathways.

In conclusion, the results presented in this study indicate that the guanine moiety of L-valacyclovir does not contribute significantly to its recognition at PepT1, and the primary recognition sites of this molecule for PepT1 are the amino group and the carbonyl group provided by the valine ester of L-valacyclovir. However, the potential hydrogen-bonding sites in the guanine moiety of L-valacyclovir may contribute to its substrate activity for PepT1. Reducing these hydrogen-bonding sites in the guanine moiety of L-valacyclovir may affect the transport efficiency of the molecule via PepT1. On the other hand, the potential hydrogen-bonding sites in the guanine moiety of acyclovir play a significant role in the permeation characteristics of this molecule across Caco-2 cell monolayers. Reducing these potential hydrogen-bonding sites in the guanine moiety of acyclovir as shown in a series of acyclovir analogs in this study greatly enhances the permeation of these molecules across Caco-2 cell monolayers due to their increased lipophilicity as well as the substrate activity of these molecules for the nucleoside transporter in Caco-2 cells.

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