Interactions of the Dipeptide Ester Prodrugs of Acyclovir with the Intestinal Oligopeptide Transporter: Competitive Inhibition of Glycylsarcosine Transport in Human Intestinal Cell Line-Caco-2

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ABSTRACT
The oligopeptide transporter may be exploited to enhance the absorption of drugs by synthesizing their dipeptide ester prodrugs, which may be recognized as its substrates. Various dipeptide esters of acyclovir (ACV), an antiviral nucleoside analog, were synthesized. Enzymatic hydrolysis and affinity of the prodrugs toward the human intestinal peptide transporter hPEPT1 were studied using the human intestinal Caco-2 cell line. Affinity studies were performed by inhibiting the uptake of [3H]glycylsarcosine by the prodrugs. The uptake of glycylsarcosine was found to be saturable at higher concentrations and was competitively inhibited by the prodrugs of ACV. All prodrugs except Tyr-Gly-ACV demonstrated a higher affinity (1.41–2.12 mM) toward hPEPT1 than cephalexin (8.19–2.99 mM) toward hPEPT1 than cephalexin (8.19–2.99 mM). Two prodrugs, Gly-Val-ACV and Val-Val-ACV, showed comparable affinity to Val-ACV, an amino acid prodrug of ACV recognized by PEPT1/PEPT2. The permeability of Gly-Val-ACV (2.99 ± 0.59 × 10–6 cm/s) across Caco-2 was comparable with that of Val-ACV (3.01 ± 0.21 × 10–6 cm/s) and was significantly inhibited (63%) in presence of glycylsarcosine. The transport of GVACV across Caco-2 was saturable at higher concentrations, and the parameters were calculated as Km 3.16 ± 0.31 mM and Vmax 0.014 ± 0.00058 nmol cm–2 min–1. Overall, the results suggest that the dipeptide prodrugs of ACV have a high affinity toward the intestinal oligopeptide transporter hPEPT1 and therefore seem to be promising candidates in the treatment of ocular and oral herpesvirus infections, because cornea and intestinal epithelia seem to express the oligopeptide transporters.

The human peptide transporter (hPEPT1) displays broad substrate specificity and recognizes dipeptides and tripeptides, but not free amino acids, as its primary substrates. The peptide transporter not only carries nutrients across absorptive cell membranes but also functions in the transport of exogenous compounds that have peptide-like structures. Small dipeptides, angiotensin-converting enzyme inhibitors, and β-lactam antibiotics are known substrates for intestinal PEPT1 (Dantzig and Bergin, 1990; Hashimoto et al., 1994; Ganapathy et al., 1995; Han et al., 1998a; Inui et al., 2000). Strategies have been used to design prodrugs of various poorly absorbed drugs targeted toward receptors/transporters for improved bioavailability (Lupia et al., 1993; Weller et al., 1993; Guo and Lee, 1999; Sakaeda et al., 2001; Anand et al., 2002a; Manfredini et al., 2002).

Valacyclovir (VACV) is such a prodrug, which is derived from acyclovir (ACV) by esterifying 3’-hydroxyl group of ACV with L-valine. Acyclovir, an antiviral nucleoside, possesses activity against human herpesviruses. Owing to its limited bioavailability, the drug has shown moderate antiviral efficacy after oral (Steิงrimsdottir et al., 2000) and topical administration (Sanitato et al., 1984). After oral and topical administration, VACV is rapidly and completely converted in vivo by enzymatic hydrolysis to acyclovir, the active parent drug. VACV has been reported to increase the oral bioavailability of acyclovir 3- to 5-fold in humans (Beauchamp et al., 1992; Lupia et al., 1993; Weller et al., 1993). Enhanced oral (Baliмane et al., 1998; de Vrueh et al., 1998; Han et al., 1998b) and ocular (Anand and Mitra, 2002) absorption of acyclovir after administration of valacyclovir have been attributed to the hPEPT1-mediated translocation of the amino acid prodrug.

ABBREVIATIONS: hPEPT1, human intestinal peptide transporter; VACV, valacyclovir; ACV, acyclovir; HSV, herpes simplex virus; GVACV, glycine-valine acyclovir; Gly-Sar, glycylsarcosine; FBS, fetal bovine serum; VVACV, valine-valine acyclovir; YVACV, tyrosine-valine acyclovir; DPBS, Dulbecco’s phosphate-buffered saline; HPLC, high-performance liquid chromatography.
Valacyclovir has been indicated in the treatment of genital herpes, the incidence of which has increased significantly in the past 20 years (Fleming et al., 1997). Although genital herpes is self-limiting in healthy adults, the disease is painful and distressing, with severe psychosocial impact (Manne and Sandler, 1984; Goldmeier et al., 1988). On the other hand, herpes simplex virus (HSV) keratitis is the leading cause of blindness in the United States (Green and Dunkel, 1985) and the most frequent cause of corneal opacities in developed countries (Easty, 1985). Currently available therapy for HSV keratitis involves the topical instillation of trifluorothymidine, idoxuridine, and vidarabine. However, one of the major problems associated with these drugs is their poor ocular absorption, cytotoxicity, and mutagenicity, restricting their use in long-term treatment. Although the utility of valacyclovir against oral and genital herpes infections is well established and well documented (Fleming et al., 1997), it has not been used for topical application against ocular herpes infection, herpes simplex virus keratitis, probably due to a short half-life of ~72 h in pH 5.6 (Anand et al., 2002b). Various lipophilic prodrugs of nucleoside analog acyclovir have been studied for improved ocular absorption (Hughes and Mitra, 1993) to cure HSV keratitis. These prodrugs, although exhibiting an increase in permeability across the cornea, lacked aqueous solubility, thereby restricting their formulation into 1 to 3% eyedrops.

A series of novel water-soluble dipeptide ester prodrugs of acyclovir (U.S. patent pending) were thus synthesized (Y. E. Nashed, B. S. Anand, and A. K. Mitra, manuscript submitted for publication) to target the peptide transporter on the cornea and intestinal epithelial cells for improved ocular and oral bioavailability of acyclovir, respectively. In comparison with VACV, the dipeptide prodrugs exhibited increased solution stability in the pH range studied with no measurable degradation in pH 5.6 during a 7-day experiment, thereby rendering the aqueous formulation to be stable for a period of 2 to 3 years (Anand et al., 2002b). These prodrugs once transported across or influxed into the target cells would undergo chemical as well as enzymatic hydrolysis to release the active parent drug, ACV. In this report, we have discussed the application of these prodrugs for improved oral bioavailability by assessing their hydrolysis and affinity to target the peptide transporter on the cornea and intestinal epithelial cells (type VSC-1; Crown Glass Company, Inc., Somerville, NJ) and 12-well tissue culture treated plastic plates. Cells were then grown in medium containing 10% FBS (heat-inactivated). Caco-2 cells used in our studies were grown for 21 to 23 days. [14C]Mannitol transport was determined as a marker of cellular integrity, which was <0.3% per hour in representative cell monolayers.

Metabolism Studies in Cell Suspensions

Confluent Caco-2 cells, grown in tissue culture flasks were isolated with the aid of mechanical scraper and washed thrice with Dulbecco’s phosphate-buffered saline (DPBS). The cells were resuspended in DPBS, pH 7.4, at a concentration of 1.0 \times 10^6 cells/ml, and 800 \mu l of the cell suspension was incubated with 200 \mu l of 1 mM solutions of prodrugs at 37°C in a shaking water bath for the length of the study. Hundred-microliter samples were withdrawn at predetermined time intervals, and the sample was purified by precipitating the cellular proteins into the organic solvent mixture and stored at -80°C until further analysis. The protein content of the cell suspension was determined by the method of Bradford (1976) using bovine serum albumin as the standard (protein estimation kit; BioRad, Hercules, CA). Apparent first order rate constants were calculated and corrected for any chemical hydrolysis observed with the control.

Transport Studies

Transport experiments were done using side-by-side diffusion cells (type VSC-1; Crown Glass Company, Inc., Somerville, NJ) and Transwell inserts. Before the experiment with Gly-Sar, Caco-2 cell monolayers grown on the clear polyester membranes and Transwell inserts were washed with DPBS (pH 6.0) and incubated at 37°C. Freshly prepared drug solutions in DPBS (pH 6.0) was placed in the donor chamber and the receiver chamber was filled with DPBS. The volumes of donor and receptor chambers were 3 ml each for side-by-side diffusion cells and 0.5 and 1.5 ml, respectively, for Transwell

Materials and Methods

Materials

[^3H]Glycylsarcosine (Gly-Sar; 4 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and [14C]mannitol (50 mCi/mmol) was supplied by Amersham Biosciences Inc. (Piscataway, NJ). Valacyclovir was a gift from GlaxoSmithKline, (Research Triangle Park, NC). Human colon carcinoma derived Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). The growth medium, Dulbecco’s modified Eagle’s medium, was obtained from Invitrogen (Carlsbad, CA). Minimal essential medium nonessential amino acids, penicillin, streptomycin, sodium bicarbonate, HEPES, unlabeled Gly-Sar, and cephalexin were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). Culture flasks (75-cm² growth area), polystyrene Transwells (pore size 0.4 \mu m with diameter of 6.5 mm) and polystyrene membranes (pore size 0.4 \mu m) were procured from Costar (Cambridge, MA). The buffer components and solvents were obtained from Fisher Scientific Co. (Fair Lawn, NJ). All the dipeptide prodrugs of acyclovir (Fig. 1) were custom synthesized in our laboratory (Y. E. Nashed, B. S. Anand, and A. K. Mitra, manuscript submitted for publication). The dipeptide prodrugs used for this study were valine-valine acyclovir (VVACV), tyrosine-glycine acyclovir (YGACV), glycine-valine acyclovir (GVACV), glycine-tyrosine acyclovir (GYACV), valine-tyrosine acyclovir (YVACV), and tyrosine-valine acyclovir (YVACV).
inserts. Sampling from the receiver chamber was done up to a period of 3 h at time intervals of 15, 30, 45, 60, 90, 120, 150, and 180 min, and fresh DPBS solution was replaced to maintain sink conditions in receiver chamber. The samples were stored at −80°C until analyzed by HPLC. All experiments were performed at 37°C. Transport studies with the prodrugs were also carried out using side-by-side diffusion cells. The pH-dependent transport of VACV and GVACV was assessed at pHs 6.0 and 7.4 at a concentration of 1 mM. Transport inhibition experiments of prodrugs with Gly-Sar were carried out at pH 6.0 because it has been reported as the pH of maximal transport for the prototypical oligopeptide transporter substrate Gly-Sar (Guo et al., 1999). Concentration-dependent transport of GVACV was also determined at varying concentrations (0.1–10 mM) and Michaelis-Menten parameters $K_m$ and $J_{max}$ were calculated.

**Uptake Studies**

In typical uptake experiments, cell monolayers were incubated with the prodrug solutions prepared in DPBS (pH 6.0) for 10 min, except for time-course studies. The concentration-dependent uptake of glycylsarcosine was studied using [3H]Gly-Sar along with varied concentrations (0.25–20 mM) of unlabeled Gly-Sar (pH 6.0). For affinity studies, prodrugs (10 mM) were incubated along with radiolabeled and unlabeled Gly-Sar for 10 min. For Dixon plots and dose-response studies, [3H]Gly-Sar was incubated along with increasing concentrations (0.25–20 mM) of unlabeled Gly-Sar and Val-Val-ACV. After incubation, the cell monolayers were washed three times with ice-cold HEPES buffer to terminate the uptake experiment. After the washings, cells were lysed overnight using 1 ml 0.1% (w/v) Triton X-100 in 0.3 N NaOH at room temperature. Aliquots (500 μl) from each well were then transferred to scintillation vials containing 5 ml of scintillation cocktail (Fisher Scientific Co.). Samples were then analyzed by the liquid scintillation spectrophotometry using scintillation counter (model LS-6500; Beckman Coulter, Inc., Fullerton, CA) and the rate of uptake was normalized to the protein content of each well. The amount of protein in the cell lysate

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**Fig. 1.** Structures of dipeptide prodrugs of acyclovir.
was measured by the protein estimation kit using bovine serum albumin as standard (Bio-Rad).

**Analytical Procedures**

All samples were assayed using HPLC. The system comprised of a Rainin Dynamax pump SD-200; Rainin Dynamax UV detector UV-C at 254 nm; a Hewlett Packard 1100 Series fluorescence detector at excitation \( \lambda = 285 \text{ nm} \), emission \( \lambda = 370 \text{ nm} \); and an Alcott autosampler (model 718 AL HPLC). The column used was a C18 Luna column (4.6 \( \times \) 250 mm; Phenomenex, Torrance, CA). The mobile phase consisted of a mixture of buffer and an organic modifier. The percentage of organic phase was varied to elute compounds of interest. This method gave rapid and reproducible results. HPLC conditions for the various compounds have been summarized in Table 1.

**Data Analysis**

**Permeability Measurements across Caco-2 Monolayers.** Steady-state fluxes were determined from the slope of the cumulative amount of drug transported versus time graph and expressed per unit of cross-sectional surface area of the membrane as described by eq. 1. The cumulative amount of drug transported is the sum of the receptor cell prodrug and the regenerated drug.

\[
\text{Flux}(J) = \frac{d(dM/dt)}{A}
\]

where \( M \) is the cumulative amount of drug transported and \( A \) is the cross-sectional surface area exposed to permeant. Caco-2 membrane permeabilities are determined by normalizing the steady-state flux to the donor concentration, \( C_d \), according to eq. 2.

\[
\text{Permeability}(P_{\text{app}}) = \frac{\text{Flux}}{C_d}
\]

**Affinity Calculations.** The concentration-dependent uptake of \([3H]\)glycylsarcosine was fitted to the modified Michaelis-Menten equation described in eq. 3.

\[
V = \frac{V_{\text{max}} \cdot C}{K_m + C} + K_d \cdot C
\]

Equation 3 takes into account both the carrier-mediated process (as described by the classical Michaelis-Menten equation) and the nonsaturable passive diffusion process. \( V \) represents the total rate of uptake. \( V_{\text{max}} \) is the maximum rate of uptake for the carrier-mediated process, \( K_m \) is the permeant concentration where half the maximal rate is reached, and \( K_d \) is the rate constant for the nonsaturable diffusion component. Concentration-dependent transport of GVCAV was fitted to the classical Michaelis-Menten equation, which takes into account only the saturable component. \( K_m \), \( V_{\text{max}} \), and \( K_d \) of uptake of \([3H]\)glycylsarcosine and transport of GVACV were determined using a nonlinear least-squares regression analysis program (KaleidaGraph, version 3.09; Synergy Software, Reading, PA).

**Statistical Analysis**

All experiments were conducted at least in triplicate and the results are expressed as mean ± S.D. except in the case of Michaelis-Menten parameters \( K_m \), \( V_{\text{max}} \), \( K_d \), and the affinities \( K_i \), where the values are presented as mean ± S.E. Student’s \( t \) test was used to detect statistical significance between the affinities of the prodrugs and VACV and \( p < 0.05 \) was considered to be statistically significant. Statistical significance was also tested by \( t \) test between the affinities of the prodrugs and cephalaxin. Statistical comparisons between the

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>Composition of Aqueous Phase (( \text{pH} 2.5 ))</th>
<th>Composition of Organic Phase</th>
<th>Mobile Phase Aq:Org</th>
<th>Retention Times*&lt;sup&gt;a,b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV</td>
<td>25 mM NH(_4)H(_2)PO(_4)</td>
<td>Acetonitrile</td>
<td>98:2</td>
<td>8.8</td>
</tr>
<tr>
<td>VACV</td>
<td>25 mM NH(_4)H(_2)PO(_4)</td>
<td>Acetonitrile</td>
<td>95:5</td>
<td>5.2</td>
</tr>
<tr>
<td>YVACV</td>
<td>25 mM NH(_4)H(_2)PO(_4)</td>
<td>Acetonitrile</td>
<td>94:6</td>
<td>3.9</td>
</tr>
<tr>
<td>GVACV</td>
<td>25 mM NH(_4)H(_2)PO(_4)</td>
<td>Acetonitrile</td>
<td>95:5</td>
<td>5.2</td>
</tr>
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<td>5.2</td>
</tr>
</tbody>
</table>

* UV detection at \( \lambda_{\text{max}} = 254 \text{ nm} \).
*<sup>b</sup> Fluorescence detection at \( \lambda = 285 \text{ nm} \); emission \( \lambda = 370 \text{ nm} \).
*<sup>c</sup> Retention times noted when separately injected onto the column and not as a function of hydrolysis.
affinities of various prodrugs were performed using the analysis of variance (SPSS for Windows, release 10.0.7; SPSS, Inc., Chicago, IL).

Results

Time- and Concentration-Dependent Uptake of $[^3H]$Glycylsarcosine. Time course of Gly-Sar uptake (Fig. 2) was linear up to 20 min. As seen in Fig. 2, inset, the inhibition of $[^3H]$Gly-Sar uptake was significant after 10 min. Based on these results 10-min uptake was performed for all the experiments to maximize the radioactivity uptake and also to allow for a significant inhibition within the linear region of the uptake. Figure 3 demonstrates the uptake of glycylsarcosine as a function of concentration ($K_m$ of 1.63 ± 0.25 mM, $V_{max}$ of 0.078 ± 0.005 μmol min$^{-1}$ mg$^{-1}$). The Caco-2 cell uptake of glycylsarcosine was found to comprise a nonsaturable component ($K_d$ of 12.2 ± 0.57 μl min$^{-1}$ mg$^{-1}$), which was deducted from the total uptake to calculate the saturable uptake. The Woolf-Augustinsson-Hofstee transformation of the data from the concentration-dependent uptake of $[^3H]$Gly-Sar resulted in a biphasic transport process (Fig. 3, inset) for the mixed uptake (transport-mediated and the passive diffusion component). However, a linear Woolf-Augustinsson-Hofstee plot ($R^2 = 0.89$) resulted when only the saturable uptake component was plotted (data not shown). Therefore, the kinetics of Gly-Sar uptake matched a single, saturable carrier model along with the linear diffusion component.

Caco-2 Metabolism Studies. The prodrugs hydrolyzed to yield the parent drug ACV in Caco-2 homogenates. The percentage remaining of the intact prodrugs after a 10-min period ranged from 38 to 97%. The prodrugs hydrolyzed to the active parent drug, ACV. The dipeptide prodrug GGACV was rapidly hydrolyzed (no intact drug detected within 1 min) after incubation with the cell suspension and was therefore not used for further inhibition experiments (Table 2).

Uptake Experiments. All the prodrugs at a concentration of 10 mM were found to significantly inhibit the uptake of Gly-Sar. The amino acid prodrugs tyrosine-ACV and glycine-ACV and the parent drug acyclovir alone did not inhibit the uptake of $[^3H]$Gly-Sar, whereas unlabeled glycylsarcosine and the dipeptide val-val significantly inhibited ($p < 0.05$) the uptake of $[^3H]$Gly-Sar (Fig. 4). Lineweaver-Burk transformations of the Michaelis-Menten data showed that the prodrugs inhibited the uptake of glycylsarcosine in a competitive manner. $K_i$ values of the prodrugs except YGACV (Table 3) were higher than that of cephalaxin ($p < 0.05$). The IC$_{50}$ values of Gly-Sar and VVACV from dose-response curves (Fig. 5) were estimated by fitting the data to the nonlinear equation $E/E_0 = 1/1 + ([I]/IC_{50})$ and were found to be 2.78 ± 0.34 and 3.46 ± 0.21 mM, respectively. $K_i$ values were calculated by the method of Cheng and Prusoff (1973) and were estimated to be 2.49 and 3.18 mM for Gly-Sar and VVACV, respectively. $K_i$ values for Gly-Sar and VVACV calculated by different approaches were in agreement (Table 3).

Transport Experiments. The transport of $[^3H]$Gly-Sar in presence of 10 mM VACV, VVACV, and VYACV was also studied using Transwell inserts. The dipeptide prodrugs of

Fig. 2. Time-dependent cellular uptake of $[^3H]$glycylsarcosine: ○, $[^3H]$Gly-Sar alone; ■, $[^3H]$Gly-Sar with 5 mM unlabeled Gly-Sar. Inset, comparison of uptake of $[^3H]$Gly-Sar in presence of 5 mM Gly-Sar at various time intervals.
ACV significantly inhibited (p < 0.05) the transepithelial transport of Gly-Sar (Fig. 6). VACV, VYACV, and VYACV had a similar effect on the inhibition of the transport of Gly-Sar. The transepithelial transport of 1 mM VACV and GVACV was also studied across Caco-2 monolayers. Cumulative amount of drug transported (the sum of the prodrug and the regenerated parent drug) was plotted as a function of time (Fig. 7). Apparent permeabilities \( P_{\text{app}} \) were determined from the linear portion of the cumulative amount versus time plot. The results indicated that the permeabilities of VACV (5.67 \( \pm \) 1.13 \( \times \) 10\(^{-6}\) cm/s) and GVACV (5.23 \( \pm \) 0.57 \( \times \) 10\(^{-6}\) cm/s) at pH 7.4 across Caco-2 monolayers were comparable. Moreover, the transport of VACV and GVACV was found to be pH-dependent with a \( P_{\text{app}} \) of 3.01 \( \pm \) 0.21 \( \times \) 10\(^{-6}\) cm/s at pH 6.0 compared with 5.67 \( \pm \) 1.13 \( \times \) 10\(^{-6}\) cm/s at pH 7.4 for VACV and a \( P_{\text{app}} \) of 2.99 \( \pm \) 0.59 \( \times \) 10\(^{-6}\) cm/s at pH 6.0 compared with 5.23 \( \pm \) 0.57 \( \times \) 10\(^{-6}\) cm/s at pH 7.4 for GVACV (Fig. 8). Similar results for uptake and transport of VACV in Chinese hamster ovary cells (Guo et al., 1999) and intact rabbit cornea (Anand and Mitra, 2002), respectively, have been reported, wherein the pH of maximum transport was found to be 7.4. Also the transport of VACV and GVACV was significantly inhibited in the presence of 10 mM concentration of Gly-Sar (Table 4). The inhibition in transport of VACV (47% inhibition) and GVACV (63% inhibition) in presence of Gly-Sar indicates the involvement of the oligopeptide transporter in the absorption of the amino acid and the dipeptide prodrug of acyclovir. The concentration-dependent transport of GVACV comprised a saturable component with a \( K_m \) of 3.16 \( \pm \) 0.31 mM and \( V_{\text{max}} \) of 0.014 \( \pm \) 0.00058 nmol cm\(^{-2}\) min\(^{-1}\) (Fig. 9). Transformation of the data from the transport of GCACV resulted in a Woolf-Augustinsson-Hofstee plot (\( R^2 = 0.935 \)) (Fig. 9, inset). The kinetics of GVACV transport matched a single, saturable carrier model.

**Table 2**

<table>
<thead>
<tr>
<th>Drug</th>
<th>( 10^5 \times k_{\text{obs}} )</th>
<th>( t^{1/2} )</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( \text{min}^{-1} \text{mg}^{-1} \text{protein} )</td>
<td>min</td>
</tr>
<tr>
<td>Val-ACV</td>
<td>0.56 ( \pm ) 0.038</td>
<td>123.7 ( \pm ) 8.3</td>
</tr>
<tr>
<td>Tyr-Val-ACV</td>
<td>10.1 ( \pm ) 0.46</td>
<td>6.9 ( \pm ) 0.3</td>
</tr>
<tr>
<td>Tyr-Gly-ACV</td>
<td>5.23 ( \pm ) 0.58</td>
<td>13.3 ( \pm ) 1.5</td>
</tr>
<tr>
<td>Gly-Tyr-ACV</td>
<td>3.21 ( \pm ) 0.46</td>
<td>21.5 ( \pm ) 3.2</td>
</tr>
<tr>
<td>Val-Val-ACV</td>
<td>2.14 ( \pm ) 0.44</td>
<td>35.2 ( \pm ) 6.9</td>
</tr>
<tr>
<td>Gly-Val-ACV</td>
<td>0.637 ( \pm ) 0.01</td>
<td>108.1 ( \pm ) 2.4</td>
</tr>
<tr>
<td>Val-Tyr-ACV</td>
<td>0.275 ( \pm ) 0.03</td>
<td>250.4 ( \pm ) 27.8</td>
</tr>
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</table>

* Measured using Caco-2 cell suspension at pH 7.4.
* No intact drug detected during the experiment.

**Discussion**

Strategies such as targeting a specific molecule toward a transporter/receptor by designing prodrugs may be used to improve tissue bioavailability. The prodrugs are themselves biologically inactive and therefore it is essential for these prodrugs to yield the active parent drug upon administration as soon as their goals are achieved. A series of acyclovir dipeptide ester prodrugs (U.S. patent pending) were synthe-
sized in our laboratory and their affinities for intestinal peptide transporter hPEPT1 were evaluated. According to a previous report, the dipeptide prodrugs of ACV have shown excellent efficacy against herpes viruses. Also, these prodrugs exhibit excellent solution stability compared with valacyclovir and less cytotoxicity compared with trifluorothymidine and acyclovir itself. These dipeptide prodrugs can be formulated into 1 to 3% solution of eye drops, are capable of being recognized by the oligopeptide transporter, and therefore seem to be promising drug candidates in the treatment of HSV keratitis, a clinical manifestation not adequately treated by the current therapy (Anand et al., 2002b).

The Caco-2 cell suspension hydrolysis studies were carried out to evaluate the regeneration characteristics of the prodrugs to the parent drug. All the prodrugs hydrolyzed to regenerate the active parent drug, ACV. The half-lives of the prodrugs ranged from 6.92 to 250.4 min (Table 2), demonstrating varied susceptibility of the prodrugs to the intestinal cellular enzymes.

Lineweaver-Burk transformations of the uptake of Gly-Sar in presence of various prodrugs were of a competitive type, revealing that the prodrugs shared a common enzyme site as that of Gly-Sar. Therefore, it can be speculated that these prodrugs would be transported by the peptide transporter. The \( V_{\text{max}} \) values of Gly-Sar in presence of all the prodrugs did not change compared with control, whereas the \( K_m \) values were different (Table 5), confirming that the dipeptide prodrugs inhibited the uptake of Gly-Sar in a competitive manner.

The affinities of the prodrugs were evaluated as a measure of inhibition of uptake of \([\text{3H}]\)Gly-Sar across Caco-2 cells (Fig. 4). Cephalexin, a \( \beta \)-lactam antibiotic, has been reported to be a substrate for PEPT1 with a \( K_i \) of 10.9 ± 1.31 mM (Sawada et al., 1999). In our studies, we calculated the \( K_i \) of cephalexin to be close to ~8 mM (Table 3), which is in agreement with the previous reports. Also the calculated value of \( K_i \) for VACV (~1.41 mM) toward PEPT1 from our studies (Table 3) was very similar to the reported value of 2.7 mM (Nielsen et al., 2001). The \( K_i \) values of the dipeptide prodrugs were compared with VACV and cephalexin. Cephalexin and VACV indicated the lower limit and the higher limit of affinity for the prodrugs, respectively.

The affinities, \( K_i \), of all the prodrugs (except YGACV) were significantly higher (\( p < 0.05 \)) than that of cephalexin. The affinities of VACV, GVACV, and VVACV were significantly higher (lower \( K_i \)) than VYACV, GYACV, YVACV, and YGACV. Moreover the \( K_i \) values of all the prodrugs were significantly different (\( p < 0.05 \)) from that of YGACV, indicating a higher affinity for hPEPT1 than YGACV (Table 3). Recently, Bailey et al. (2000) proposed a general template for

### Table 3

| Affinities of various prodrugs of acyclovir and cephalexin toward peptide transporter on the human intestinal Caco-2 cell line |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Michaelis Menten | Lineweaver Burk | Dixon           | Dose Response   |
| \( K_i \)        | \( mM \)         |                 |                 |                 |
| Val-ACV          | 1.41 ± 0.56\( ^a \) | 1.61 ± 0.15     | 2.51 ± 0.29     | 3.18 ± 0.18     |
| Gly-Val-ACV      | 1.42 ± 0.24\( ^a \) | 1.82 ± 0.34     | 3.27 ± 0.71     |                 |
| Val-Val-ACV      | 1.94 ± 0.49\( ^a \) | 1.89 ± 0.22     |                 |                 |
| Val-Tyr-ACV      | 2.97 ± 0.34\( ^a,b \) | 3.94 ± 0.61     |                 |                 |
| Gly-Tyr-ACV      | 3.56 ± 1.98\( ^a,b \) | 4.11 ± 0.32     |                 |                 |
| Tyr-Val-ACV      | 4.96 ± 0.97\( ^a,b \) | 8.23 ± 0.43     |                 |                 |
| Tyr-Gly-ACV      | 7.99 ± 0.54\( b \) | 9.97 ± 0.92     |                 |                 |
| Cephalexin       | 8.19 ± 2.12\( b \) |                 |                 |                 |
| Glycylsarcosine  | 2.35 ± 0.67      |                 | 2.49 ± 0.13     |                 |

\( ^a \) \( p < 0.05 \) compared with cephalexin.
\( ^b \) \( p < 0.05 \) compared with VACV.
substrate specificity for peptide transporter PEPT1. According to this model, which identifies 10 key features for substrate binding to PEPT1, the side chain of the dipeptides may interact with the hydrophobic pocket in the PEPT1 protein. All dipeptide prodrugs studied here differ from each other by the amino acid residues attached to the parent drug, ACV (Fig. 1). A comparative account of hydrophobicity scales of various amino acids has been reported previously (Janin, 1979; Kyte and Doolittle, 1982). According to both scales, the order of hydrophobicity decreases from valine to glycine to tyrosine. Although speculative, we can suggest that the high affinity observed for VACV, GVACV, and VVACV could be due to the high and moderate hydrophobicity of the valine and glycine moiety, respectively, with probably a major contribution from the valine moiety. On the other hand, the moderate affinity of prodrugs with valine-tyrosine, glycine-tyrosine, and tyrosine-valine as the amino acid residues in comparison with VACV, VVACV, and GVACV could be explained by the rank order of hydrophobicity values of the three amino acid residues, i.e., valine > glycine > tyrosine and also due to a less favorable orientation of the molecule due to the presence of the tyrosine residue. The low affinity observed for YGACV can be explained by the short half-life for YGACV and the presence of two amino acids, glycine and tyrosine, having lower hydrophobicity values than valine. These observations need to be ultimately confirmed by structure activity relationships.

From studies on the in vitro antiviral efficacy against HSV-1 and HSV-2, it was observed that VVACV was more effective than ACV, VACV, and also the other dipeptide prodrugs, thereby making it the most effective prodrug against ocular HSV keratitis (Anand et al., 2002b). Therefore, the mechanism of inhibition of Gly-Sar by VVACV was further examined by dose-response curve (Fig. 5) and Dixon plots. Dose-response curve (Fig. 5) and Dixon plot for radiolabeled

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**Fig. 5.** A, dose-response curve of inhibition of cellular uptake of [3H]glycylsarcosine by unlabeled glycylsarcosine (●) and Val-Val-ACV (○).
Gly-Sar in presence of unlabeled Gly-Sar were also studied to compare the affinities of Gly-Sar and VVACV. $K_i$ of VVACV calculated from all the approaches (Table 3) was in agreement and was best estimated to be 2.38 mM, which was very similar to the calculated $K_i$ value of 2.42 mM approximately for Gly-Sar (Table 3). It was observed during the hydrolysis studies that the dipeptide prodrugs cleaved to the parent drug via the formation of the amino acid intermediate. Therefore, the uptake of Gly-Sar in presence of the amino acid prodrugs YACV and GACV and ACV was also studied. It was observed that ACV, YACV, and GACV did not inhibit the uptake of Gly-Sar (Fig. 4). Therefore, it can be concluded that the dipeptide prodrugs cleaving to YACV or GACV inhibited the uptake of Gly-Sar, owing to the interaction of the intact prodrug with hPEPT1. In addition to the uptake inhibition experiments, transport of [$^3$H]Gly-Sar in presence of VACV, VYACV, and VYACV was also studied. VYACV was chosen as it showed the longest half-life of 250.4 min (Table 1). Results indicated that VYACV caused a similar inhibition of

**TABLE 4**

Transport of 1 mM concentration of VACV and GVACV across Caco-2 in the presence of 10 mM glycylsarcosine

<table>
<thead>
<tr>
<th></th>
<th>Permeability, $P_{app}$ $10^6$ cm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Sar (control)</td>
<td>Val-ACV  $3.01 \pm 0.21 \times 10^{-6}$</td>
</tr>
<tr>
<td>Gly-Val-ACV</td>
<td>2.99 $\pm$ 0.59 $\times 10^{-6}$</td>
</tr>
</tbody>
</table>

* $p < 0.05$ compared with $P_{app}$ of VACV.

**Fig. 7.** A, permeation profile of the prodrug-l-Val-ACV across Caco-2 as a function of time: ▲, regenerated parent drug ACV; ■, intact l-Val-ACV; and ◆, cumulative amount of l-Val-ACV ($R^2 = 0.99$). B, permeation profile of the prodrug Gly-Val-ACV across Caco-2 as a function of time: ▲, regenerated parent drug ACV; ■, Val-ACV; ◆, intact GVACV; and ◆, cumulative amount of Gly-Val-ACV ($R^2 = 0.98$). Values are mean ± S.D. ($n = 3$).

**Fig. 8.** Permeability of 1 mM VACV and GVACV as a function of pH. *, $p < 0.05$ compared with VACV and GVACV permeabilities at pH 6.0. Values are mean ± S.D. ($n = 3$).
transport as VACV and VVACV (Fig. 6), suggesting that the dipeptide prodrugs of ACV interact with hPEPT1.

Generally speaking, inhibition studies may not be a good predictor for the actual cellular uptake of drug candidates, because the substrates might only bind to the transporter without being translocated. Hence, the affinity of these prodrugs for hPEPT1 may not be translated into hPEPT1 mediated transepithelial transport and oral delivery. Therefore, transport experiments with VACV and GVACV in absence and presence of Gly-Sar were carried out. Once the prodrug traverses the apical membrane, the transport across the basolateral membrane might be mediated by another peptide transporter present on the surface of the basolateral membrane, which might be distinct from the one on the apical membrane. The transport of GVACV was compared with that of VACV because VACV has been shown to be transported across Caco-2 by the intestinal peptide transporter (Han et al., 1998b). GVACV was chosen for these studies because it showed a comparable affinity to that of VACV and had a half-life of approximately 108 min. The transport of VACV and GVACV was found to be pH-dependent (Fig. 8), which is consistent with the report on the interaction of positively charged dipeptides with PEPT1 (Temple et al., 1996; Amasheh et al., 1997; Lister et al., 1997). VACV and GVACV have three pK\textsubscript{a} values (1.90, 7.47, and 9.43) and exist as a mixture of cationic and neutral species at pH 7.4, which is the pH of maximal transport among the range studied. Similar findings have been reported wherein the transport of VACV was maximum at pH 7.4 due to the presence of a mixture of cationic and neutral species at pH 7.4 (Guo et al., 1999; Anand and Mitra, 2002). The permeability (at pH 6.0) of GVACV, $2.99 \pm 0.59 \times 10^{-6}$ cm/s, was similar to a permeability of $3.01 \pm 0.21 \times 10^{-6}$ cm/s for VACV (Fig. 8). Inhibition of transport of both VACV and GVACV by Gly-Sar lends direct support to the probable interaction of the newly syn-

![Graph](https://via.placeholder.com/150)

**Fig. 9.** Concentration-dependent transport of GVACV across Caco-2 ($R^2 = 0.996$). Inset, Woolf-Augustinsson-Hofstee linear transformation ($R^2 = 0.935$) of the data (fluxes of GVACV, $J$ (moles per square centimeter per minute) versus fluxes of GVACV/concentration of GVACV, $J/S$). Values are mean ± S.D. ($n = 4–6$).

**TABLE 5** Comparisons of kinetic parameters for glycylsarcosine

<table>
<thead>
<tr>
<th>Effect of dipeptide prodrugs on the inhibition of uptake of glycylsarcosine. Values are mean ± S.E. ($n = 3–6$).</th>
<th>$V_{max}$ (mol/min/mg)</th>
<th>$K_{m}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycylsarcosine parameters</td>
<td>Lineweaver Burk</td>
<td>0.086 ± 0.019</td>
</tr>
<tr>
<td></td>
<td>Eadie Hofstee</td>
<td>0.071 ± 0.009</td>
</tr>
<tr>
<td>Gly-Sar (with drugs) parameters</td>
<td>Lineweaver Burk</td>
<td>0.081 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>+ Val-ACV</td>
<td>0.095 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>+ Gly-Val-ACV</td>
<td>0.099 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>+ Val-Tyr-ACV</td>
<td>0.092 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>+ Gly-Tyr-ACV</td>
<td>0.094 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>+ Tyr-Val-ACV</td>
<td>0.091 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>+ Tyr-Gly-ACV</td>
<td>0.087 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>+ Cephalexin</td>
<td>0.101 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$ Control.

$^b p < 0.05$ compared with control.
thethesized dipeptide prodrugs with the oligopeptide transporter.

In addition to the inhibition data the concentration-dependent transport of GVACV was found to be saturable at higher concentrations ($K_m$ of 3.16 ± 0.31 mM; $V_{max}$ of 0.014 ± 0.00058 nmol cm$^{-2}$ min$^{-1}$) (Fig. 9). Transformation of the data resulted in a linear Woolf-Augustinsson-Hofstee plot ($R^2 = 0.935$) (Fig. 9, inset) and therefore the kinetics of GVACV transport matched a single, saturable carrier model. The $K_i$ and $K_m$ of GVACV were found to be very similar further confirming the sharing of the same binding site on the transporter.

In conclusion, the results of the present study indicate that the dipeptide prodrugs of ACV, a poorly absorbed antiviral nucleoside, exhibit high affinity toward the intestinal oligopeptide transporter. The uptake of these prodrugs was efficiently mediated by hPEPT1 because they significantly inhibit the uptake of glycylsarcosine. These prodrugs hydrolyze readily to regenerate the active parent drug, acyclovir, thereby fulfilling the basic requirement of a prodrug. These prodrugs owing to their high affinity, excellent solution stability, and in vitro antiviral activity against herpes infections are promising drug candidates against oral and ocular herpes infections.

Acknowledgments

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References


Affinities of Dipeptide Prodrugs of Acyclovir to hPEPT1


