Transport of \( L\)-Valine-Acyclovir Via the Oligopeptide Transporter In The Human Intestinal Cell Line, Caco-2

REMCO L. A. DE VRUEH,¹ PHILIP L. SMITH and CHAO-PIN LEE
Department of Drug Delivery, Pharmaceutical Technologies, Smithkline Beecham Pharmaceuticals, Collegeville, Pennsylvania
Accepted for publication May 12, 1998
This paper is available online at http://www.jpet.org

ABSTRACT
It has been reported that conjugating acyclovir, a potent antiviral with low oral bioavailability, to \( L\)-valine increases its urinary excretion in rats. However, it was also reported that this increase is not found for the \( D\)-valine ester, suggesting that a carrier-mediated mechanism is involved in its intestinal absorption. Therefore, mechanisms involved in the transepithelial transport of \( L\)-valine-acyclovir were investigated using the intestinal cell line, Caco-2, as a model system for the intestinal epithelium. Only the mucosal-to-serosal transport of acyclovir was increased by conjugation with \( L\)-valine (~7-fold), suggesting the involvement of a carrier-mediated mechanism. This conclusion was supported by the finding that this increase was saturable. The mucosal-to-serosal transport of \( L\)-valine-acyclovir could be inhibited by \( \text{l-}\)glycylsarcosine, but not by \( L\)-valine, suggesting the involvement of the dipeptide carrier. Also it was found that \( L\)-valine-acyclovir inhibits the uptake of cephalexin, a substrate for the oligopeptide transporter. Stability of the esters in either the mucosal or serosal bathing solution is more than 90% after completion of the transport study. However, after transport, the receiver solution contained approximately 90% of acyclovir. Based on these findings it was concluded that absorption of the \( L\)-valine ester of acyclovir occurs as a result of uptake by the oligopeptide transporter at the apical cell membrane followed by intracellular hydrolysis of the ester and efflux of acyclovir.

Oral administration is the preferred delivery route for drugs due to the ease of administration and patient compliance. One limitation for oral delivery is poor oral bioavailability of drugs (Lee et al., 1997). The poor oral bioavailability of drugs could be due to unfavorable physiochemical properties (e.g., high molecular weight, charge and low aqueous solubility), instability in the gastrointestinal tract, poor transport across the intestinal epithelium or high first pass metabolism (Humphrey and Ringrose, 1986; Lee et al., 1997; Johnson and Swindell, 1996). Several approaches have been taken to overcome these barriers and possibly improve the oral bioavailability of drugs. For instance, synthesis of analogs with enhanced stability in the gastrointestinal tract and/or enhanced membrane permeability (Bundgaard, 1992), prodrugs with increased absorption (Kahns et al., 1993; Cundy et al., 1994; Obermeier et al., 1996) or inclusion of absorption enhancers in the formulation to enhance drug absorption and oral bioavailability (Swenson and Curatolo, 1992).

Recently, molecules have been rationally designed to target various carriers in the small intestine in an attempt to enhance their oral absorption (Hidalgo and Borchardt, 1990; Hidalgo et al., 1995; Edwards et al., 1996; Smith et al., 1993; Tamura et al., 1996). One limitation for targeting these carriers is the substrate specificity that can be structurally restrictive and stereospecific. Additionally, transport capacities of these transporters may be limited. Nevertheless, studies do show that drugs can be transported across the intestinal epithelium by carrier-mediated mechanisms present in the gastrointestinal tract (Dantzig et al., 1992; Smith et al., 1993; Gochoco et al., 1994).

Acyclovir is an agent used for the treatment of infections caused by herpes viruses. The low oral bioavailability of acyclovir has influenced its dosing frequency and use (de Miranda and Blum, 1983; de Miranda et al., 1981, 1982). It has been reported that the oral bioavailability of acyclovir is highly variable and species dependent ranging from 75.3 ± 1.3% in dogs to 3.7 ± 0.5% in rhesus monkey (de Miranda and Blum, 1983; de Miranda et al., 1981, 1982). Poor water and lipid solubility of acyclovir (solubility in water of 1.3 mg/ml and an octanol to water partition coefficient of 0.018) may contribute to its low gastrointestinal absorption (de Miranda et al., 1981, 1982). The existence of a saturable, carrier-mediated process in the oral absorption of acyclovir by mice, rats and dogs has been proposed based on a decline in the fraction of dose absorbed with increasing doses. However, the
low oral bioavailability observed in human may be a function of poor membrane permeability due to the low partition coefficient of acyclovir.

Attempts to increase the oral bioavailability of acyclovir include formulation and “prodrug” approaches (Beauchamp et al., 1992; Park et al., 1992; Colla et al., 1983; Bando et al., 1994; Shao et al., 1994). Recently, it was reported that oral administration of amino acid ester prodrugs of acyclovir increases urinary excretion of acyclovir in rats (Burnette and de Miranda, 1994; Beauchamp and Krenitsky, 1993; Beauchamp et al., 1992). No prodrug could be detected in the urine, indicating that these esters are subjected to extensive in vivo hydrolysis (Beauchamp and Krenitsky, 1993; Beauchamp et al., 1992). Among the amino acid esters evaluated, oral administration of L-val-acv produced the greatest increase in urinary excretion of acyclovir (~63%) although oral administration of D-val-acv resulted in an urinary excretion of only ~7% (Beauchamp and Krenitsky, 1993; Beauchamp et al., 1992). This stereospecific absorption suggests that a carrier-mediated mechanism may be involved in the oral absorption of L-val-acv in rats.

The mechanisms involved in transepithelial transport of L-val-acv were investigated employing the intestinal cell line, Caco-2. The Caco-2 cells have been shown to exhibit characteristics of both small and large intestine and to express carrier-mediated transport processes for amino acids, sugars, dipeptides, bile acids and nucleosides (Thwaites et al., 1993; Hu and Borchardt, 1992; Blais et al., 1987; Hidalgo and Borchardt, 1990; Hidalgo et al., 1995).

Materials and Methods

Chemicals. Acyclovir (9-(2-hydroxyethoxymethyl)guanin), t-cephalexin, L-valine, Gly-Sar were purchased from Sigma Chemical Co. (St. Louis, MO). L- and D-Chz-valine were obtained from Bachem Bioscience Inc. (Philadelphia, PA). [3H]Cephalexin (49.3 mCi/mmol) was purchased from New England Nuclear Products (Boston, MA). All other chemicals were from commercial suppliers or as described previously (Eddy et al., 1995; Gochoco et al., 1994).

Synthesis of valine esters of acyclovir. D- and L-val-acv were synthesized as described by Beauchamp et al. (1992) with the following modifications. Synthesis was performed on a 0.9-mmol scale and all amounts and volumes were adjusted accordingly. Recovery of L-val-acv and D-val-acv were 0.22 g (70%) and 0.24 g (75%), respectively. Chemical structures and purity were confirmed by 1H NMR, mass spectrometry, elemental analysis and high-performance liquid chromatography.

Cell culture. Caco-2 cells were grown in 75-cm² T-flasks in a 5% CO₂/95% air atmosphere at 37°C. The culture medium consisted of DMEM, containing 10% fetal calf serum, 1% NEAA, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin. Cells were harvested with 0.25% (w/v) trypsin-1 mM EDTA. For uptake studies, cells were grown as confluent monolayers on collagen-coated 0.4-μm pore-Transwell filters (6.5 mm diameter, Costar, Cambridge, MA). For the permeability experiments, 0.1 ml of a cell suspension, containing 3 to 4 × 10⁶ cells/ml, was seeded onto rat tail collagen-coated snapwells (2 piece Snapwell Transwell plates, 0.4-μm pore size, 12 mm diameter; Costar). For collagen coating, a 5 mg/ml aqueous solution of rat tail collagen was diluted 4-fold with 70% ethanol. A total of 100 μl of this solution was spread onto the well and the solvent was evaporated under UV light overnight. Mucosal and serosal chamber volumes were maintained at 0.4 and 3.5 ml, respectively. The culture medium was replaced on alternate days. Permeability experiments were performed between 17 and 21 days after seeding, when the monolayers had reached confluency.

Transport studies. Transport studies were conducted as described previously (Gochoco et al., 1994; Hidalgo et al., 1993). For competition studies inhibitors (20 mM final concentration) were added to the mucosal reservoir and glucose (8 mM) plus mannitol (12 mM) were added to the basolateral reservoir to maintain osmolality. After cells had been equilibrated for 1 hr, samples were taken from the donor solution (25 μl) and the receiver solution (1 ml) at specified times. Samples taken from the receiver solution were replaced with an equal volume of the appropriate bathing solution whereas samples from the donor solution were not replaced. Samples taken from the donor solution and receiver solution were acidified immediately with 1 ml of 50 mM ammonium acetate buffer pH 5.4 and 25 μl of 1 M acetic acid, respectively. Epithelial integrity of cell monolayers was assessed throughout experiments by monitoring transepithelial resistance and flux of the paracellular marker, [14C]mannitol (2.5 μCi/chamber) (Swaan et al., 1994; Marks et al., 1991). Intact cell monolayers exhibit a transepithelial resistance greater than 250 ohm/cm² and a [14C]mannitol permeability less than 0.006 cm/hr. Monolayers with a mannitol permeability of more than 0.006 cm/hr were not included in the results.

Inhibition of cephalexin uptake. The effect of acyclovir and its valine esters on [3H]cephalexin cellular accumulation was studied as described previously (Eddy et al., 1995). Briefly, cells were incubated with 100 μM [3H]cephalexin (0.2 μCi/well) and various concentrations of inhibitor in the apical bathing solution (pH 6.0). Basolateral pH was maintained at 7.4. After a 15-min incubation at 37°C, uptake was stopped by adding ice cold Hanks’ balanced salt solution, pH 7.4, and cells were washed three times. Filters and cells were solubilized in 10 ml scintillation liquid and radioactivity determined by liquid scintillation spectrometry. Cellular accumulation is expressed as the mean (nmol·min⁻¹·mg protein⁻¹) of triplicate determinations. Total protein content of cells cultured on polycarbonate filters for 18 to 25 days was previously determined to be 0.425 mg protein/cm² (Gochoco et al., 1994).

Sample analysis. High-performance liquid chromatography was performed using a Waters-HPLC system equipped with an Ultrasphere C-18, 5 μm column (25 cm × 4.6 mm I.D.). Elution was performed with 5.9% acetonitrile in 50 mM ammonium acetate buffer, pH 5.4 at a flow rate of 1 ml/min. Detection was performed using a Waters model 484 UV detector (Waters Associates, Milford, WA) and monitored at 250 nm. Radioactivity was determined in an LS6000SC Liquid Scintillation Spectrometer (Beckman, Fullerton, CA). Counts per minute (cpm) were converted to disintegration per min (dpm).

Data analysis. Transport rates of acyclovir and its valine esters were calculated as described previously (Grass and Sweetana, 1988) and are expressed as nmol/hr·cm². Permeabilities Pₐ₃ (cm/hr) were calculated using the following equation:

\[ P_{\text{app}} = \frac{V_{\text{r}}}{A \cdot C_{\text{r}}} \cdot \frac{dC_{\text{r}}}{dt} \]

where \( V_{\text{r}} \) is the receiver volume, \( A \) is the surface area of the exposed tissue, \( C_{\text{r}} \) is the concentration of the donor solution and \( dC_{\text{r}}/dt \) is the rate of concentration change in the receiver solution (Burton et al., 1993). For D- and L-val-acv transport studies both the esters and acyclovir were quantified in the receiver solution and summed for calculating transport rates and permeability. The rates of Cephalexin uptake in Caco-2 cells were calculated using the following equation:

\[ \text{Uptake rate} = \frac{RA_{\text{cell}}}{RA_{\text{spec}}} \cdot t_{\text{inc}} \cdot AP \]

where \( RA_{\text{cell}} \) is the amount of radioactivity found in cells (dpm), \( RA_{\text{spec}} \) is the specific radioactivity (dpm/pmol), \( t_{\text{inc}} \) is the incubation...
time (15 min), and AP is the amount of cell protein (0.1409 mg). Uptake rates are expressed as pmol/min · mg protein. All values are presented as mean and S.E.M. of values from at least triplicate determinations.

**Statistical analysis.** Statistical analysis was performed using unpaired Student’s t test. The results were considered to be significant when P < .05.

**Results**

**Transport of acyclovir and its valine esters.** Both the mucosal(m)-to-serosal(s) and the s-to-m fluxes for acyclovir and its valine esters were determined in Caco-2 cell monolayers. Acyclovir, D- and L-val-acv all have similar and low s-to-m transport rates (table 1). For D-val-acv, there is no significant difference between the m-to-s and s-to-m fluxes. The m-to-s flux of acyclovir is slightly less than its s-to-m flux. The m-to-s flux of the L-val-acv is approximately 7-fold more than those of D-val-acv and acyclovir.

**Concentration dependency of L-val-acv transport.** As seen in figure 1, the m-to-s flux of L-val-acv is concentration dependent and nonlinear. The s-to-m flux of L-val-acv is a liner function of L-val-acv concentration.

**Inhibition of L-val-acv transport.** The transport of L-val-acv across Caco-2 cell monolayers in the presence of L-valine or Gly-Sar was examined. From the results presented in table 2, it can be seen that L-valine (20 mM) did not inhibit L-val-acv (0.8 mM) transport although Gly-Sar (20 mM) significantly reduced L-val-acv (0.8 mM) transport.

**Inhibition of cephalixin uptake into Caco-2 cells.** The uptake of cephalixin, a substrate for the intestinal oligopeptide transporter, in the presence or absence of acyclovir, D- and L-val-acv was determined. Data in table 3 illustrate that L-val-acv at a concentration of 0.5 mM has an inhibitory effect on [3H]cephalexin uptake comparable to that of 25 mM cephalixin. Neither D-val-acv nor acyclovir at similar concentrations showed any inhibitory effect. At 20 mM concentration, L-val-acv inhibited cephalixin uptake by 88%.

**pH dependence of L-val-acv transport.** The effect of varying pH of the mucosal bathing solution on the transport of L-val-acv across Caco-2 monolayers was examined. The data in table 4 suggest that the introduction of a pH-gradient did not increase the transport of L-val-acv as would be expected.

**Stability of acyclovir valine esters.** Stability of the acyclovir esters in either the mucosal or serosal solution was determined. Table 5 shows that more than 90% of L-val-acv remained intact after 150-min incubation in the mucosal or serosal bathing solution.

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>M-to-s Permeability (cm/hr)</th>
<th>S-to-m Permeability (cm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclovir</td>
<td>0.0013 ± 0.0002</td>
<td>0.0039 ± 0.0001^a^</td>
</tr>
<tr>
<td>L-val-acv</td>
<td>0.0197 ± 0.0028</td>
<td>0.0032 ± 0.0005^a^</td>
</tr>
<tr>
<td>D-val-acv</td>
<td>0.0024 ± 0.0006</td>
<td>0.0032 ± 0.0006</td>
</tr>
</tbody>
</table>

Transport studies were performed with 0.8 mM compound present on the donor side. Both bathing solutions were buffered at pH 7.4. The calculated fluxes were normalized for their respective concentrations and expressed as the permeability in cm/h. Results are the mean ± S.E.M. of at least three determinations.

^a^ Significantly different from the m-to-s permeability at P < .05.

**Discussion**

It was reported previously that oligopeptides and peptido-mimetics can be translocated across the small intestinal epithelium via interaction with the proton-dependent intestinal oligopeptide transporter (Smith et al., 1993). The driving forces involved in this translocation process are the combined concentration gradients for protons and substrate. This
translocation process is thus pH dependent, unidirectional (from m-to-s only) and saturable. The m-to-s flux of acyclovir is similar to the m-to-s flux of mannitol in Caco-2 cell monolayers. This low m-to-s flux of acyclovir in Caco-2 cell monolayers suggests that the low oral bioavailability of acyclovir in human is due to its limited absorption in the gastrointestinal tract. Additionally, the similar m-to-s and s-to-m transport rates shown in table 1 suggest that passive diffusion through the intestinal epithelium is the major transport route for acyclovir and D-val-acev. Conversely, the significantly higher m-to-s transport of L-val-acev compared to its s-to-m transport in Caco-2 cell monolayers suggests that L-val-acev is transported across the intestinal epithelium via a carrier-mediated mechanism. Further support for this conclusion is provided by the findings shown in figure 1 that the m-to-s transport of L-val-acev is a saturable function of concentration while the s-to-m flux is a linear function of concentration.

Based on its structure, L-val-acev may interact with and be transported by either an amino acid transporter or by the oligopeptide transporter (Smith et al., 1993; Beauchamp et al., 1992; Dantzig and Bergin, 1990; Thwaites et al., 1993). It has been described that, in addition to dipeptides, a wide range of structurally unrelated molecules show affinity and/or transport by the oligopeptide carrier (Kramer et al., 1990; Dantzig and Bergin, 1990; Hu et al., 1989). Involvement of these carriers were investigated through competition studies, using L-valine or Gly-Sar, a biologically stable dipeptide, as inhibitors. When 20 mM L-Val was added to the mucosal bathing solution, the m-to-s transport of L-val-acev was not significantly altered (table 2). However, mucosal addition of Gly-Sar (20 mM) reduced L-val-acev transport by approximately 65% (table 2).

These results implicate the oligopeptide transporter as the carrier involved in absorption of L-val-acev. Further support for this conclusion is provided by uptake studies with cephalexin, a substrate for the oligopeptide transporter (Dantzig and Bergin, 1990; Eddy et al., 1995; Hidalgo et al., 1993; Gochoco et al., 1994). As shown in table 3, L-val-acev at a concentration of 0.5 mM has an inhibitory effect on [3H]cephalexin uptake comparable to that of 25 mM cephalixin, whereas both D-val-acev and acyclovir at similar concentrations as L-val-acev do not show any inhibitory effect. At 20 mM concentration, L-val-acev inhibited cephalixin uptake by approximately 88%.

This study clearly demonstrates that transport of the amino acid ester of acyclovir, L-val-acev, occurs by a carrier-mediated mechanism. The findings that the transport of L-val-acev can be inhibited by the dipeptide Gly-Sar and that L-val-acev inhibits the uptake of cephalixin, a molecule previously shown to be transported by the oligopeptide transporter in Caco-2 cells support the conclusion that absorption of this prodrug occurs as a result of uptake by the dipeptide carrier at the apical cell membrane (Dantzig and Bergin, 1990; Eddy et al., 1995; Hidalgo et al., 1993; Gochoco et al., 1994).

Transport by the oligopeptide transporter is proposed to be dependent on a pH gradient (Smith et al., 1993). However, the introduction of a pH-gradient did not increase the transport of L-val-acev as would be expected (table 4). No significant differences were observed in mannitol flux indicating that the monolayers was not altered by changes in apical pH. Lack of pH dependence of L-val-acev transport may result from use of a saturable substrate concentration. Accordingly, pH-dependent transport by the oligopeptide transporter would only be observed at low substrate concentrations and would become indistinct at saturating concentrations. Alternatively, the lack of pH dependence of L-val-acev transport may indicate that uptake of L-val-acev by the oligopeptide transporter in the apical cell membrane is not the rate limiting step for its transport. Similar results were reported in the transport studies of stereoisomers of dipeptide Val-Val across the Caco-2 cells. The cellular uptakes of these dipeptides did not correlate with their transepithelial transport. The efflux of these dipeptides across the basolateral membrane of Caco-2 cell monolayer appeared to be the rate limiting step for their transepithelial transport (Tamura et al., 1996). The involvement of multiple transporters may be another reason for the lack of pH-dependence of L-val-acev transport in Caco-2 cells. Cook and coworkers (1977) have recently reported a significant decrease in Caco-2 cell permeability to L-val-acev in the presence of quinidine (0.2 mM), an inhibitor of organic cation transporter. L-val-acev transport across Caco-2 cell monolayers was also inhibited by cepharidine. These results suggest the involvement of multiple transporters for the transport of L-val-acev across Caco-2 cell monolayers.

Stability of the esters in either the mucosal or serosal bathing solution is more than 90% after completion of the transport study (table 5). However, after m-to-s transport the receiver side contained approximately 90% of acyclovir. These results indicate that ester hydrolysis occurs within the epithelial cells. Therefore, the transport mechanism for L-val-acev across Caco-2 cells involves interaction and uptake of L-val-acev by the oligopeptide transporter, ester hydrolysis within the epithelial cells followed by efflux of acyclovir.

To our knowledge, this is the first demonstration of a molecule lacking a peptide bond that is transported by the intestinal oligopeptide transporter. These results indicate that there is a range of molecules that can be designed for uptake by the oligopeptide transporter thus providing a strategy for designing molecules with enhanced oral bioavailability.
Acknowledgments

The technical assistance and advice of Priya Eddy, Dave Chiosone and Fred Ryan are gratefully acknowledged. We also thank Siobhan Derrickson. We especially want to thank the Department of Synthetic Chemistry for the synthesis of [3H]cephalexin and the Department of Analytical, Physical and Structural Chemistry for the analysis of the valine esters of acyclovir.

References


