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*Rapid Communication*

# 5′-Amino Acid Esters of Antiviral Nucleosides, Acyclovir, and AZT Are Absorbed by the Intestinal PEPT1 Peptide Transporter

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***Purpose.*** General use of nucleoside analogues in the treatment of viral infections and cancer is often limited by poor oral absorption. Valacyclovir, a water soluble amino acid ester prodrug of acyclovir has been reported to increase the oral bioavailability of acyclovir but its absorption mechanism is unknown. This study characterized the intestinal absorption mechanism of 5′-amino acid ester prodrugs of the antiviral drugs and examined the potential of amino acid esters as an effective strategy for improving oral drug absorption.

***Methods.*** Acyclovir (ACV) and Zidovudine (AZT) were selected as the different sugar-modified nucleoside antiviral agents and synthesized to L-valyl esters of ACV and AZT (L-Val-ACV and L-Val-AZT), D-valyl ester of ACV (D-Val-ACV) and glycyl ester of ACV (Gly-ACV). The intestinal absorption mechanism of these 5′-amino acid ester prodrugs was characterized in three different experimental systems; *in situ* rat perfusion model, CHO/hPEPT1 cells and Caco-2 cells.

***Results.*** Testing 5′-amino acid ester prodrugs of acyclovir and AZT, we found that the prodrugs increased the intestinal permeability of the parent nucleoside analogue 3- to 10-fold. The dose- dependent permeation enhancement was selective for the L-amino acid esters. Competitive inhibition studies in rats and in CHO cells transfected with the human peptide transporter, hPEPT1, demonstrated that membrane transport of the prodrugs was mediated predominantly by the PEPT1 $H^+$/dipeptide cotransporter even though these prodrugs did not possess a peptide bond. Finally, transport studies in Caco-2 cells confirmed that the 5′-amino acid ester prodrugs enhanced the transcellular transport of the parent drug.

***Conclusions.*** This study demonstrates that L-amino acid-nucleoside chimeras can serve as prodrugs to enhance intestinal absorption via the PEPT1 transporter, providing a novel strategy for improving oral therapy of nucleoside drugs.

**KEY WORDS:** amino acid ester; PEPT1 transporter; permeability; prodrugs.

## INTRODUCTION

Nucleoside antivirals are polar drugs with low membrane permeability and poor oral absorption (1–3). Sufficient oral bioavailability is essential for broad medical use of drugs. To improve the bioavailability of polar drugs, lipophilic derivatives can serve as prodrugs with enhanced passive membrane permeability. Instability or insolubility of the prodrug in the gastrointestinal tract or slow *in vivo* release of the active drug often limits this approach. An alternate approach exploits membrane transporters in the intestinal epithelial cell that facilitate the transport of polar nutrients, such as amino acid, nucleoside or peptide transporters (4–6). Compared to other transporters, $H^+$/oligopeptide cotransporters have broad substrate specificity, and therefore, represent targets for prodrug design (7,8). For example, we have previously demonstrated that a transporter-targeted prodrug strategy improves the membrane permeability and systemic availability of L-α-methyldopa through peptidyl derivatization (5,9).

Recently, amino acid ester prodrugs of nucleoside analogues have been reported to increase the oral systemic availability of the antiviral, acyclovir. Derivatization with the 5′-L-valyl ester of acyclovir yielded a prodrug (valacyclovir) with three to five times higher systemic availability compared to

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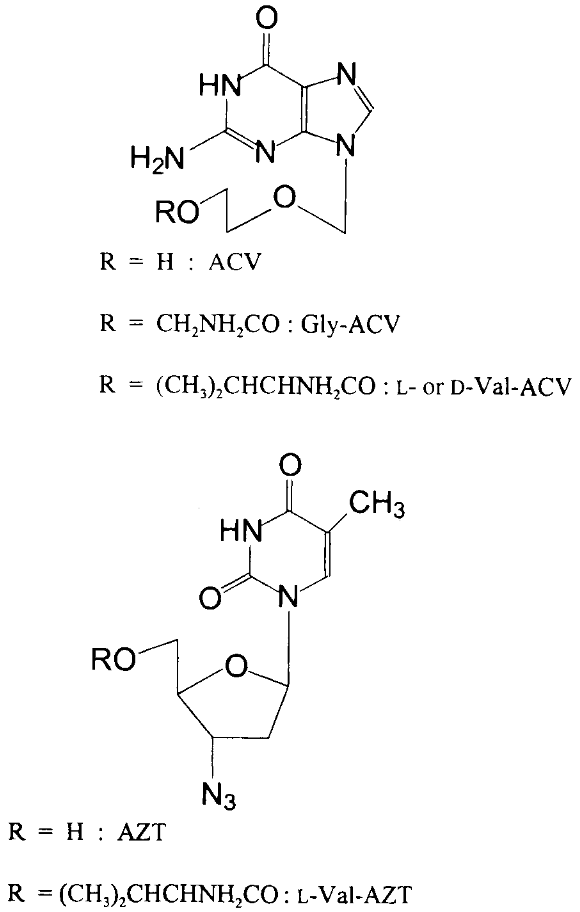
acyclovir in humans (10). Because the prodrug, valacyclovir is at least as polar as the parent, due to the protonated α-amino group at physiological pH, carrier mediated transport has been suggested to play a role in absorption (11). However, the membrane transport mechanism has not been determined.

In this report, we have synthesized and determined the membrane transport characteristics of several D- and L-amino acid esters of acyclovir and AZT including valacyclovir (Fig. 1) in three experimental systems such as rats, CHO/hPEPT1 cells and Caco-2 cells.

## MATERIALS AND METHODS

### Materials

Acyclovir, AZT, L-valine, D-valine, glycine, N-Boc-L-valine, N-Boc-D-valine, N-Boc-glycine, N,N-dimethylformamide, N,N-dicyclohexylcarbodiimide, 4-Dimethylamino-pyridine, Gly-Gly, Gly-Pro, Gly-Phe, Gly-Sar, cephalexin, cephradine, trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). $^{14}$C-PEG 4000 (61.2mCi/mmol) was provided from Amersham (Arlington heights, IL) and $^{14}$C-mannitol (49.3 mCi/mmol) was purchased from New England Nuclear Products (Boston, MA). $^{3}$H-Gly-Sar (400 mCi/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA). Other chemicals were either analytical or HPLC grade.



R = H : ACV

R = $CH_2NH_2CO$ : Gly-ACV

R = $(CH_3)_2CHCHNH_2CO$ : L- or D-Val-ACV

R = H : AZT

R = $(CH_3)_2CHCHNH_2CO$ : L-Val-AZT

**Fig. 1.** Structures of prodrugs.

### Animals

Male Sprague Dawley rats from Charles River, 250–300 g were used.

### Cell Cultures

CHO/hPEPT1 cells were constructed as described by Covitz *et al.* (12) and cultured in F-12 nutrition mixture (HAM) containing 10% FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml) and 200 μg/ml G418. Caco-2 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1 mM Na-pyruvate, 1% L-glutamine and penicillin (100 U/ml)/streptomycin (100 μg/ml). All cells were maintained in an atmosphere of 5% $CO_2$ and 90% relative humidity at 37°C.

### Methods

*Synthesis of Prodrugs*

5′-amino acid ester prodrugs of acyclovir and AZT were synthesized by the published procedures of Beauchamp *et al.* (11), and identified by $^{1}$H-NMR, $^{13}$C-NMR and FAB-MS spectrometers.

In Situ *Rat Perfusion Study*

The perfusion solution consisted of pH 6.5 Mes buffer, a substrate or a substrate with inhibitors, PEG 4000 and a trace amount of $^{14}$C-PEG 4000 as a water flux marker. The osmolarity of perfusion solution was adjusted to 300 ± 5 mosm/kg water. Rats were fasted with free access to water for approximately 18 hours before the experiment and anesthetized with i.m. injection of 50% urethane in water (1.5 g/kg). During the experiment, the rats were kept on the heating slide to maintain their normal temperature. The abdomen was opened and 10 cm segment of jejunum was selected for the cannulation of both ends. After cannulation, the isolated segment was rinsed with isotonic saline and covered with an isotonic saline-wet gauze. Drug solution was perfused through the intestinal segment with the flow rate of 0.192 ml/min and steady state was usually achieved in 30 min from the start of perfusion. After reaching steady state, perfusates were collected every 10 min for an hour. In the end of experiment, the intestinal segments were quickly excised to measure the length and rats were sacrificed with overdose of anesthetics.

*Inhibition Study on the Gly-Sar Uptake in CHO/hPEPT1 Cells*

It was performed as described previously by Covitz *et al.* (12). Cells were seeded in the 12-well culture plates (3.8 cm$^2$ growth area, Falcon, Franklin Lakes, NJ) at the density of $10^5$ cells/cm$^2$. At 2 days postseeding, the cells were washed twice with pH 6.0 uptake buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 1 mM $NaH_2PO_4$, 5 mM D-glucose and 5 mM MES. Each inhibitor solution (0.01 mM–50 mM) containing $^{3}$H-Gly-Sar (20 μM, 0.4 μCi/ml) was added to each well and incubated for 30 min at 25°C. Cells were washed with ice-cold pH 6.0 uptake buffer three times to stop the cellular uptake. One ml of 1.5% ice-cold Triton X solution was added to each well and incubated for 30 min at 25°C. Cells were scraped off and transferred into the vials containing 4 ml of the scintillation cocktail. Samples were analyzed by scintillation counter (Beckman LS-9000 counter, Beckman Instruments, Fullerton, CA) with automatic quenched correction and protein amount of each sample was measured by the method of Lowry *et al.* (13).

### *Transport Studies in Caco-2 Cells*

Cell suspension (0.1 ml), containing 3–4 × $10^5$ cells/ml, was seeded onto rat tail collagen-coated snapwells (2 piece Snapwell™ Transwell plates, 0.4 μm pore size, 12 mm diameter, Costar, Cambridge, MA). For collagen coating, 5 mg/ml aqueous solution of rat tail collagen was diluted 4 fold with 70% ethanol. One hundred μL of this solution was spread onto the well and the solvent was evaporated under UV light. Mucosal and serosal chamber volumes were maintained at 0.4 and 3.5 ml respectively. The culture medium was replaced in every other day. Transport studies were performed between 17 and 21 days after seeding. For competition studies, inhibitors (20 mM) were added to the mucosal reservoir, while glucose (8 mM) and mannitol (12 mM) were added to the basolateral reservoir to maintain osmolarity. 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 pH 5.4, 50 mM ammonium acetate buffer 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, $^{14}$C-mannitol (2.5 μCi/chamber) (14). Intact cell monolayers exhibit transepithelial resistance greater than 250 ohm/cm$^2$ and $^{14}$C-mannitol permeability less than 0.006 cm/h. Monolayers with a mannitol permeability greater than 0.006 cm/h were not included in the results.

## ANALYSIS

### HPLC Assay

A high performance liquid chromatography (HPLC) system consists of a pump (Waters, Model 510, Milford, MA), an automatic injector (Waters, Wisp model 712, Milford, MA), a reversed phase column (Ultrasphere, 5μ, C-18, 4.6 × 250 mm, Beckman), and a UV detector (Waters, 990 Photodiode Array Detector, Milford, MA) at 254 nm. The pump, data acquisition and integration were controlled by a Water Baseline 810 Software Package (Dynamic Solutions, CA). The mobile phase was pH 3.5 ammonium formate buffer containing 3–8% acetonitrile and the flow rate was 1 ml/min.

### Data Analysis

### *(A)* In Situ *Rat Perfusion Study*

A modified boundary layer model was used to estimate the wall permeability ($P_w$) at steady state (15). The water flux was evaluated using Eq. (1) for each perfusate sample to retain the assumption that there is no radial convection during perfusion.

$$\%\text{ of water flux} = [(A_i - A_f) / (A_f \times L)] \times 100 \qquad (1)$$

where $A_i$ and $A_f$ are the initial and final amount per minute of $^{14}$C-PEG 4000, respectively and L is the perfused intestinal length. The perfusate samples with water flux below ±1%/cm were used to estimate $P_w$. The effective permeability ($P_{eff}$) and aqueous permeability ($P_{aq}$) were estimated using Eqs. (2) and (3), respectively.

$$P_{eff} = Q(1 - C_m/C_0) / 2\Pi RL \qquad (2)$$

$$P_{aq} = (ARGz^{1/3} / D)^{-1} \qquad (3)$$

where Q: flow rate, $C_m$: outlet concentration, $C_0$: inlet concentration, R: radius of the intestine, L: perfused intestinal length and D: diffusion coefficient. The Graetz number, Gz, is defined in Eq. (4) and A is obtained by Eq. (5).

$$Gz = \Pi DL / 2Q \qquad (4)$$

$$\begin{array}{ll} 0.004 \le Gz < 0.01 & A = 10Gz + 1.01 \\ 0.01 \le Gz < 0.03 & A = 4.5Gz + 1.065 \\ 0.03 \le Gz & A = 2.5Gz + 1.125 \end{array} \qquad (5)$$

Finally, $P_w$ was estimated by the following equation:

$$1/P_w = 1/P_{eff} - 1/P_{aq} \qquad (6)$$

The statistical differences between the $P_w$ values of prodrugs with/without inhibitors were tested using two sample t-test assuming unequal variances.

### *(B) Inhibition Study in CHO/hPEPT1 Cells*

In this study, $IC_{50}$ is defined as the drug concentration to show the 50% inhibition of Gly-Sar uptake. As described by De Lean *et al.* (16), it was determined from non-linear regression of dose-response curve by the Sigma Plot program (Jandel Scientific., San Rafael, CA) using the equation $f = (a - d)/(1 + (x/c)^b) + d$, where a and d represent the maximum and minimum uptake, respectively, x is the inhibitor concentration and b is the slope factor.

### *(C) Transport Study in Caco-2 Cells*

Transport rates of acyclovir and its valine esters were calculated as described previously (17) and are expressed as nmol/h · cm$^2$. Apparent permeability ($P_{app}$) were calculated using the following equation:

$$P_{app} = \frac{V_r}{A \cdot C_o} \cdot \frac{dC_r}{dt}$$

where $V_r$ is the receiver volume, A is the surface area of the exposed tissue, $C_o$ is the concentration of the donor solution and $dC_r/dt$ is the rate of concentration change in the receiver solution.

## RESULTS

### In Situ *Perfusion Study in Rats*

As shown in Fig. 2, the intestinal membrane permeability studies indicate that the L-valyl ester of acyclovir (L-Val-ACV)

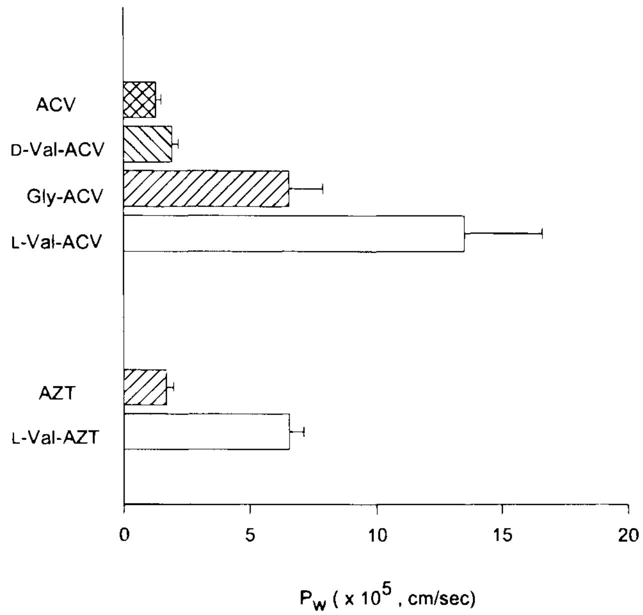


**Fig. 2.** Intestinal membrane permeabilities of amino acid ester prodrugs and their parent drugs in rats (0.01 mM, Mean ± SE). ACV and AZT: n = 6, the others: n = 4.

is approximately ten-fold more permeable across the membrane than the parent drug and its D-isomer, D-Val-ACV. The glycyl ester prodrug (Gly-ACV) exhibited a lower, but still significant, four-fold enhancement. These results supported the findings by Beauchamp *et al.* (11) that L-valine may have the optimal combination of chain length and branching at the beta carbon of the amino acid for the intestinal absorption. The L-valyl ester of AZT exhibited a three-fold enhancement in permeability, indicating that the nucleoside component can also be variable. These results establish stereoselectivity with respect to the amino acid residue and demonstrate that the putative transporter can recognize various amino acid progroups. The membrane permeabilities of the prodrugs were also evaluated at different concentrations. The permeabilities decreased significantly when the concentration increased from 0.01 mM to 1 mM in all cases (Fig. 3), further supporting a carrier-mediated transport mechanism, whereas the parent drugs are transported by passive diffusion (18,19).

Although the amino acid ester prodrugs of nucleosides are structurally distinct from dipeptides, we suspected the involvement of the main intestinal $H^+$/dipeptide cotransporter, PEPT1. Studies with competitive inhibitors of the intestinal peptide transporter, cephalexin and various dipeptides, significantly reduced the permeability of L-valyl ester of acyclovir, while the free amino acid, L-valine, had no effect (Fig. 4). Furthermore, the L-valyl ester of AZT and glylcyl ester of acyclovir strongly inhibited the transport of the L-valyl ester of acyclovir, indicating that prodrugs compete each other for a common intestinal absorption pathway. These results suggested that the PEPT1 peptide transporter is primarily responsible for the transport of these amino acid ester prodrugs across the apical membrane of the intestinal epithelial cell.

### *Inhibition Study in CHO/hPEPT1 Cells*

Peptide carrier mediated membrane transport of the amino acid esters was confirmed by competitive uptake studies in the stably transfected Chinese Hamster Ovary (CHO) cells overexpressing the hPEPT1 transporter. As shown in Fig. 5, all amino acid ester prodrugs significantly inhibited the uptake of a standard peptide substrate Gly-Sar, indicating the strong competition for a hPEPT1 transporter. $IC_{50}$ values of the amino acid ester prodrugs (Table I) were similar to that of Gly-Sar reported by Covitz et al. (12), but lower than those for cephradine and enalapril, known substrates of the peptide transporter (20,21). Thus, these non-peptide amino acid-nucleoside esters display surprisingly good affinity for the hPEPT1 transporter. In addition, $IC_{50}$ of D-Val-ACV was two to three fold higher than L-Val-ACV, implying that L-configuration is more favorable for the binding to the hPEPT1 transporter.

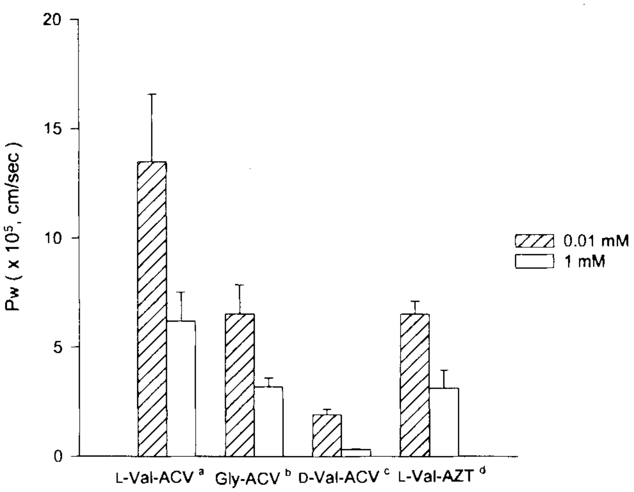


**Fig. 3.** Concentration dependence of the intestinal membrane permeability of the prodrugs in rats (Mean ± SE). a: n = 6 (0.01 mM), n = 4 (1 mM), b: n = 4, c: n = 4 (0.01 mM), n = 3 (1 mM), d: n = 6 (0.01 mM), n = 4 (1 mM).

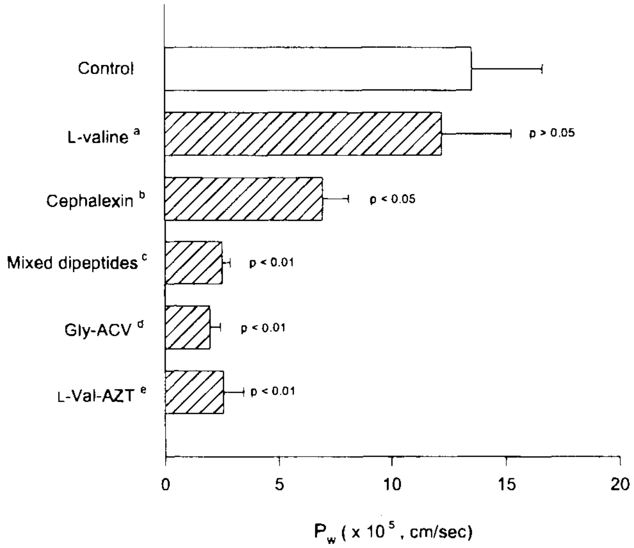


**Fig. 4.** Competitive inhibition on the intestinal absorption of L-Val-ACV in rats (0.01 mM, Mean ± SE). a 40 mM, b: 5 mM, c: 8 mM Gly-Gly, 4 mM Gly-Phe, 4 mM Gly-Pro, 4 mM Gly-Sar, d: 4 mM, e: 4 mM. All experiments were done with n = 4 except control (n = 6).

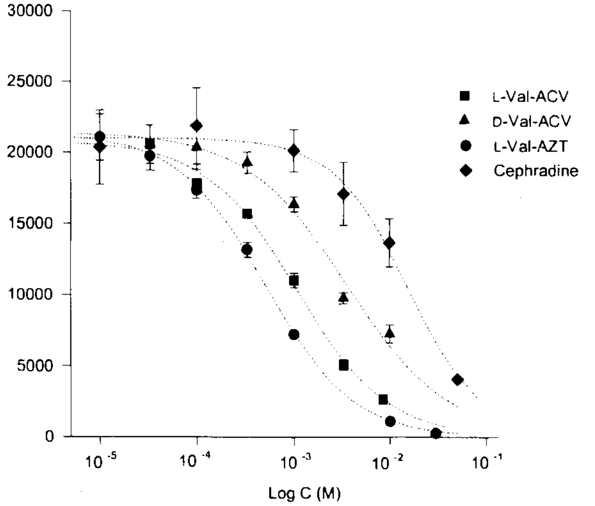


**Fig. 5.** Inhibition of Gly-Sar uptake in CHO/hPEPT1 cells. Log C represents the inhibitor concentration.

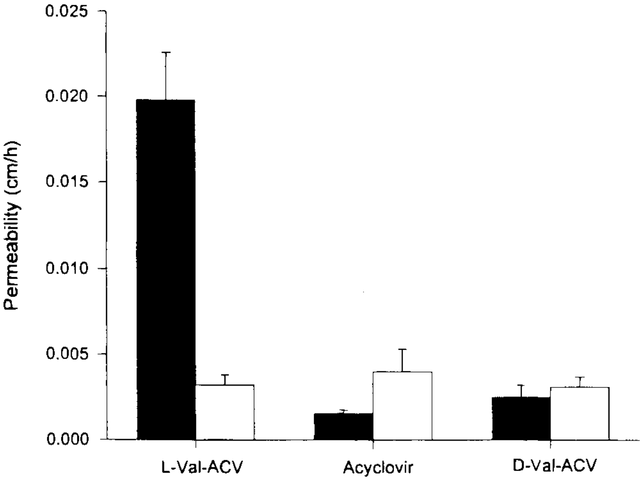


**Fig. 6.** Mucosal(m)-to-serosal(s) (filled bars) and s-to-m fluxes (open bars) of acyclovir and its valine esters across Caco-2 cell monolayer at 37°C. Transport studies were performed with 0.8 mM drug solution in the donor side. Both donor and receiver compartment solutions were buffered at pH 7.4. The fluxes were normalized for their respective concentrations and expressed as the permeability in cm/hr. Mean ± SE, n = 3.

### *Transport Study in Caco-2 Cells*

Both the mucosal(m)-to-serosal(s) and the s-to-m fluxes for acyclovir and its valine esters were determined in Caco-2 cells. The m-to-s flux of L-Val-ACV is seven fold higher than acyclovir or the corresponding D-ester, whereas acyclovir, D- and L-Val-ACV, all have similar and low s-to-m transport rates (Fig. 6). Moreover, the m-to-s flux of L-Val-ACV is saturable but s-to-m flux of L-Val-ACV is a linear function of the concentration (Fig. 7). These results suggest that L-Val-ACV is transported across the intestinal epithelium via a carrier-mediated mechanism while passive diffusion is the major transport route for acyclovir. The $K_m$ and $V_{max}$ values for the m-to-s flux of L-Val-ACV were determined as 292 ± 55 μM and 15.7 ± 1.3 nmol/h · cm$^2$. The m-to-s flux of L-Val-ACV was sensitive to competitive inhibition by small dipeptides and cephalexin (data not shown) as observed in rat jejunum and in transfected CHO cells. Finally, more than 90% of the drug on the receiver compartment was in the form of ACV rather than the prodrug, indicating prodrug hydrolysis during cellular transport.

Taken together, the results from three different experimental systems were very consistent and summarized as follows. First, amino acid ester prodrugs significantly (three to ten folds) increased the membrane permeability of their parent drugs. Second, L-configuration of amino acid showed more favorable membrane transport than D-configuration. Third, the intestinal absorption of amino acid ester prodrugs is PEPT1 transporter-mediated, even though there is no peptide bond in their structures.

**Table I.** Inhibition of Gly-Sar Uptake in CHO/hPEPT1 Cells

| Compounds | IC$_{50}$(mM)[a] |
|---|---|
| L-Val-ACV | 1.1 ± 0.3 |
| D-Val-ACV | 3.4 ± 0.7 |
| Gly-ACV | 1.4 ± 0.3 |
| L-Val-AZT | 0.4 ± 0.03 |
| Cephradine | 15 ± 2 |
| Enalapril | 4.5 ± 0.7 |

[a] Mean ± SE from two independent studies and each study has n = 3.

## DISCUSSION

In the present study, 5′-amino acid ester prodrugs of acyclovir and AZT exhibited an even higher affinity to the intestinal PEPT1 transporter than the cephradine and enalapril, although they do not contain either a peptide bond or free

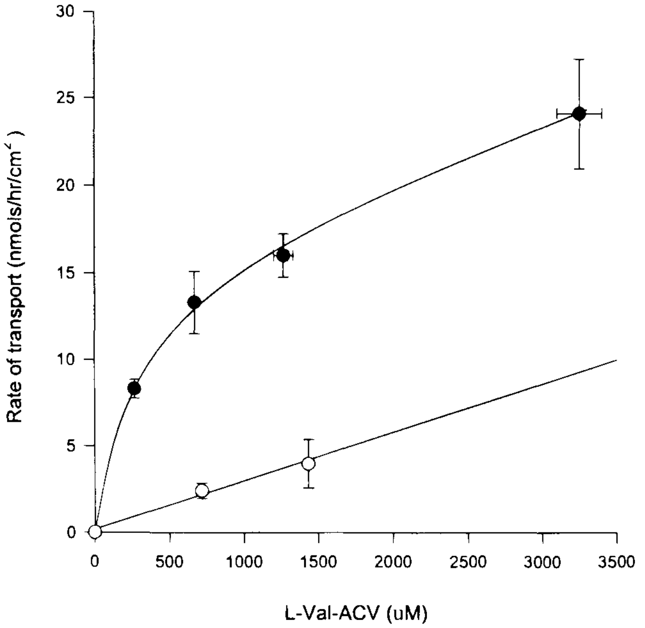


**Fig. 7.** Concentration dependence of the membrane transport of L-Val-ACV in Caco-2 cells. Closed symbols represent m-to-s flux and open symbols the s-to-m flux at 37°C. Both curves had a coefficient of correlation greater than 0.99. Mean ± SE, n = 3.

carboxyl group. The amino acid-nucleoside chimeras represent the first set of substrates for this carrier that have an N-terminal free α-amino group but do not contain a peptide bond and absent a C-terminal free carboxyl group. Wide structural variations are known to be allowed at the N-terminal α-amino group side of di- or tri-peptide substrates for the hPEPT1 transporter. However, substitution of the C-terminal free carboxyl group greatly influences affinity to the transporter and only a few cases are known where blocking of this group still permits recognition by the transporter (22–24). Our results here indicate that the structure-transport relationship for the PEPT1 peptide transporter is even more broad than previously suspected (7,8,25). The fact that a peptide bond is not needed for transport is particularly important for this prodrug strategy, since amides are usually too stable for facile *in vivo* release of the active component (26).

The increase in intestinal membrane permeability of these nucleoside prodrugs, combined with hydrolysis to the active drug, is expected to improve absorption and systemic availability in humans by 2- to 4-fold, as judged from correlation between fraction-dose absorbed and rat and human intestinal membrane permeability (27). Thus, amino acid ester prodrugs of the polar nucleoside antiviral agents can significantly improve the oral absorption and systemic availability through peptide-carrier mediated transport and cellular hydrolysis. This study establishes a new strategy for oral prodrug design, targeting polar, non-peptide prodrugs to the intestinal epithelial brush border membrane peptide transporter. Given the surprisingly broad substrate specificity of this transporter, combined with rapid epithelial cell hydrolysis of the ester prodrugs during transport, this new approach to prodrug design may be useful for improving the absorption and systemic availability of other polar antiviral and cancer chemotherapeutic agents.

## ACKNOWLEDGMENTS

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