Cellular Uptake Mechanism of Amino Acid Ester Prodrugs in Caco-2/hPEPT1 Cells Overexpressing a Human Peptide Transporter

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Purpose. This study characterized the cellular uptake mechanism and hydrolysis of the amino acid ester prodrugs of nucleoside antiviral drugs in the transiently transfected Caco-2 cells overexpressing a human intestinal peptide transporter, hPEPT1 (Caco-2/hPEPT1 cells).
Methods. Amino acid ester prodrugs of acyclovir and AZT were synthesized and their apical membrane permeability and hydrolysis were evaluated in Caco-2/hPEPT1 cells. The cellular uptake mechanism of prodrugs was investigated through the competitive inhibition study in Caco-2/hPEPT1 cells.
Results. L-Valyl ester of acyclovir (L-Val-ACV) was approximately ten fold more permeable across the apical membrane than acyclovir and four times more permeable than D-valyl ester of acyclovir (D-Val-ACV). Correspondingly, L-valyl ester of AZT (L- Val-AZT) exhibited three fold higher cellular uptake than AZT. Therefore, amino acid ester prodrugs significantly increased the cellular uptake of the parent drugs and exhibited the D,L-stereoselectivity. Furthermore, prodrugs were rapidly hydrolyzed to the parent drugs by the intracellular hydrolysis, following the apical membrane transport. In the inhibition studies, cephalexin and small dipeptides strongly inhibited the cellular uptake of L-Val-ACV while L-valine had no effect, indicating that the peptide transporter is primarily responsible for the apical membrane transport of L-Val-ACV. In addition, the cellular uptake of L-Val-ACV was five times higher in Caco-2/hPEPT1 cells than the uptake in the untransfected Caco-2 cells, implying the cellular uptake of L-Val-ACV was related to the enhancement of the peptide transport activity in Caco-2/hPEPT1 cells.
Conclusions. Caco-2/hPEPT1 system is an efficient *in vitro* model for the uptake study of peptidyl derivatives. Amino acid ester prodrugs significantly improved the cellular uptake of the parent drugs via peptide transport mechanism and were rapidly converted to the active parent drugs by the intracellular hydrolysis.

KEY WORDS: amino acid ester; Caco-2 cells; cellular uptake; peptide transporter; permeability; prodrugs.

INTRODUCTION

Amino acid ester prodrugs of nucleoside antiviral drugs have been reported to increase oral bioavailability (1) and brain uptake (2) of the parent drugs. For instance, valacyclovir, L-valyl ester prodrug of acyclovir, is rapidly and almost completely converted to acyclovir by the enzymatic hydrolysis after absorption and increases the bioavailability of acyclovir three to five folds in humans (1,3). Carrier-mediated absorption process seemed to be involved in the membrane transport of valacyclovir (3) but so far, it is not determined. If the absorption mechanism of water soluble amino acid ester prodrugs is fully understood, it may provide a rationale in the prodrug design of polar drugs to improve oral drug absorption.

To our surprise, the previous studies in the rat indicated that amino acid ester prodrugs could be recognized as peptidyl derivatives and absorbed by peptide transporters, even though there was no peptide bond in their structures (4). Therefore, in this study, the cellular uptake mechanism of amino acid ester prodrugs was examined focusing on the peptide transporter.

Caco-2 cells are well characterized and currently most prevalent cell culture systems as a suitable *in vitro* model for the intestinal drug transport studies (5). However, Caco-2 cells exhibit the various transport systems including peptide transporters (PEPT1 and HPT1), monocarboxylic acid transporter (MCT1), glucose-fructose transporter (GLUT5) and a nucleoside transporter (NT) (6–8). As a result, it is difficult to clearly distinguish the transporter responsible for the uptake of a test compound. Furthermore, peptide transport activity in Caco-2 cells is rather low and sometimes not enough for the screening of peptidomimetic drugs (9).

The cloning of peptide transporters (10,11) has triggered attempts to develop a cell culture model having an enhanced peptide transport activity that can be used as a screening tool for the peptidyl drug candidates arising from rational drug design (12). Recently, using an adenovirus transfection system, Hsu *et al.* (13) in our group established the transiently transfected Caco-2 cells overexpressing a human intestinal peptide transporter, hPEPT1. This newly established Caco-2/hPEPT1 cell line exhibited the highly enhanced peptide transport activity and has been demonstrated as an efficient *in vitro* model for the uptake study of peptidyl derivatives (13).

In the present study, acyclovir and AZT were selected as the different sugar modified purine and pyrimidine nucleoside antiviral drugs and several amino acid ester prodrugs of acyclovir and AZT including valacyclovir were synthesized following the general peptide synthetic pathway. The apical membrane transport mechanism and hydrolysis of amino acid ester prodrugs were investigated in the Caco-2/hPEPT1 cells.

MATERIALS AND METHODS

Materials

Glycyl-[^{3}H]-sarcosine (specific radioactivity, 400 mCi/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA). Acyclovir, Gly-Pro, Gly-Phe, Gly-Sar, cephalexin, L-valine, trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). All tissue culture reagents were obtained from Gibco (Grand Island, NY). Other chemicals were either analytical or HPLC grade.

Synthesis of Prodrugs

Amino acid ester prodrugs were synthesized in the form of trifluoroacetic acid salt by the published procedure of Beauchamp *et al.* (3), and identified by ^{1}H-NMR, ^{13}C-NMR and FAB-MS spectrometers.

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Preparation of Caco-2 /hPEPT1 Cells

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 sodium pyruvate, 1% L-glutamine and penicillin (100 U/ml)/ streptomycin (100 μg/ml). Cells grown in 100 mm tissue culture petri-dishes were passaged every 5 days at a split ratio of 1:5 and all cells were maintained in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C. Cells of passage number 39 to 55 were used throughout this study. For the uptake study, Caco-2 cells were seeded in 6-well culture plates (9.5 cm^2 of growth area) at the density of 10^4 cells/cm^2. At 3 days postseeding, the medium was removed and replaced with 0.25 ml of recombinant adenovirus solution (Multiplicity of infection = 5000 particles/ cell) carrying a human PEPT1 gene (RSV/hPEPT1/SV40 sequence) (13). After 2 hr incubation at 37°C with shaking wells every 15 min, 2.25 ml of medium was added to the each well and incubated at 37°C. The overexpression of hPEPT1 at the protein level, cell viability and transfection efficency after infection were fully characterized as described by Hsu *et al.* (13)

Kinetic Study of Gly-Sar Uptake in Caco-2/hPEPT1 Cells and Caco-2 Cells

At 3 days post-infection, the medium was removed and 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. Gly-Sar solution was prepared at the seven different concentrations (0.01 mM–50 mM) containing ^{3}H-Gly-Sar (0.4 μCi/ml) and 1 ml of each drug solution was added to the cells and 6-well culture plates were agitating on a plate shaker (Maxi rotator, Lab-line Instruments Inc.) at 25°C. At each time point, medium was removed and cells were washed with ice-cold pH 6.0 uptake buffer three times to stop the cellular uptake. One ml of ice-cold Milli-Q water was added and cells were scraped off and transferred into the vials containing 4 ml of the scintillation cocktail. Samples were analyzed by a scintillation counter (Beckman instruments Inc., Model LS-9000) and protein amount of each sample was measured by the method of Lowry *et al.* (14). The same kinetic study was done in untransfected Caco-2 cells.

Uptake Study in Caco-2/hPEPT1 Cells

At 3 days post-infection, the medium was removed and cells were washed twice with pH 6.0 uptake buffer. One ml of each drug solution (1 mM) was added to the cells and 6-well culture plates were agitating on a plate shaker at 25°C. At each time point (5, 15, 30, 45, 60, 120 min), medium was removed and cells were washed three times with ice-cold pH 6.0 uptake buffer. One ml of Milli-Q water was added and cells were scraped off and sonicated for 10 min. Cell lysate was treated with ice-cold trichloroacetic acid (final concentration of 3–5%), vortexed and centrifuged for 5 min at 3000 rpm. After filtration of the supernatant through a membrane filter (0.45 μm), samples were analyzed by HPLC. Protein amount of each sample was determined by the method of Lowry *et al.* (14). The prodrug conversion to its parent drug was determined by the appearance (%) of the parent drug after 30 min incubation at 25°C.

Competitive Inhibition Study on the L-Val-ACV Uptake in Caco-2/hPEPT1 Cells

At 3 days post-infection, the medium was removed and cells were washed twice with pH 6.0 uptake buffer. L-Val-ACV solution (1 mM) was prepared with/without an inhibitor such as cephalexin (10 mM), Gly-Pro (10 mM), Gly-Phe (10 mM), Gly-Sar (10 mM) and L-valine (40 mM). One ml of drug solution was added to the cells and agitating on a plate shaker at 25°C. After 30 min incubation at 25°C, it was following the same procedures as mentioned in the uptake study.

HPLC Assay

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

Estimate of Permeability in Caco-2/hPEPT1 Cells

Permeability coefficients (P_{app}) were calculated from the linear portion of an uptake versus time plot using the following equation:

$$P_{app} = \frac{1}{AC_0} \cdot \frac{dm}{dt}$$

where A is diffusion area (cm^2), C_o is the initial concentration (μmol/ml), and dm/dt is the initial uptake rate (μmol/min).

Statistical differences between two means were evaluated using t-test assuming unequal variance.

Determination of K_m and V_{max}

K_m and V_{max} of Gly-Sar uptake were determined using SigmaPlot program (Jandel Scientific, San Rafael, CA) for the non-linear regression to

$$V = \frac{V_{max} \cdot C}{(K_m + C)} + K_d \cdot C$$

where $K_d \cdot C$ represents a diffusional uptake rate.

RESULTS

Kinetic Study of the Gly-Sar Uptake in Caco-2 and Caco-2/hPEPT1 Cells

Kinetic parameters of Gly-Sar uptake were determined in both Caco-2 and Caco-2/hPEPT1 cells. K_m and V_{max} were obtained as 0.7 ± 0.17 mM, 62.5 ± 5.0 nmol/hr/mg·protein in Caco-2 cells (Mean ± SD, n = 3) and 2.1 ± 0.5 mM, 833.6 ± 80.5 nmol/hr/mg·protein in Caco-2/hPEPT1 cells (Mean ± SD, n = 6), respectively. Both cell lines exhibited small passive diffusional uptake and K_d values (nmol/hr/mg protein/mM) were 5.5 ± 0.25 in Caco-2/hPEPT1 cells (Mean ± SD, n = 6) and 11.1 ± 1.21 in Caco-2 cells (Mean ± SD, n = 3). The V_{max} determined in Caco-2/hPEPT1 cells was approximately

fifteen fold higher than V_{max} in Caco-2 cells. For the negative control, the cells were infected with Ad.RSVlacZ (which did not carry hPEPT1) and no significant difference was observed in Gly-Sar uptake after infection (13). Therefore, the kinetic study of Gly-Sar uptake indicated that transfected Caco-2/hPEPT1 cells significantly enhanced the peptide transport activity while retaining the substrate specificity.

Uptake Study in Caco-2/hPEPT1 Cells

Cellular uptake of L-Val-ACV was evaluated in both Caco-2 and Caco-2/hPEPT1 cells. Initial uptake rate (pmol/min/cm^2) of L-Val-ACV was 13.85 ± 3.24 in Caco-2 cells (Mean ± SD, n = 4) and 63.75 ± 9.24 in Caco-2/hPEPT1 cells (Mean ± SD, n = 6). The cellular uptake of L-Val-ACV increased five folds after overexpression of hPEPT1 transporter, whereas the uptake of parent drugs was not significantly changed after transfection (data not shown). Thus, Caco-2/hPEPT1 cells would be an efficient model to evaluate the cellular uptake mechanism of our prodrugs targeting the hPEPT1 transporter.

The stability of amino acid ester prodrugs was determined in both the donor solution and inside of the cells after 30 min incubation (Table I and Fig. 1). The hydrolysis of amino acid ester prodrugs was below 10% in the supernatant, while it was above 95% inside the cells except D-Val-ACV which was relatively stable against the enzymatic hydrolysis. These data indicated that L-amino acid ester prodrugs are rapidly converted to the parent drugs by the intracellular hydrolysis following the apical membrane transport. Therefore, in the present study, the total uptake of prodrugs into the cells were determined by adding the amount of parent drugs converted from prodrugs to the amount of intact prodrugs inside cells.

Apparent cellular uptake permeabilities (P_{app}) of acyclovir and its amino acid ester prodrugs were determined in Caco-2/hPEPT1 cells from the linear portion of time versus uptake curve (Fig. 2). The estimated P_{app} values were: acyclovir; (1.66 ± 0.215) × 10^{-7} cm/sec, L-Val-ACV; (17.0 ± 2.42) × 10^{-7} cm/sec, D-Val-ACV; (3.86 ± 0.82) × 10^{-7} cm/sec, and Gly-ACV; (2.10 ± 0.262) × 10^{-7} cm/sec, respectively (Fig. 3). L-Val-ACV was approximately ten fold more permeable across the apical membrane than its parent drug, acyclovir and four times more permeable than its D-isomer, D-Val-ACV.

On the other hand, L-Val-AZT and AZT were rapidly saturated in the cellular uptake (data not shown). Without radiolabeled compounds, it was difficult to measure the linearity of the initial uptake due to the detection limit of HPLC assay. Therefore, the uptake amounts of L-Val-AZT and AZT were compared at the steady state. After 30 min incubation, the cellular uptake (nmol/mg protein, Mean ± SD, n = 3) was obtained as 8.75 ± 1.03 for L-Val-AZT and 2.86 ± 0.15 for AZT, respectively. Consequently, L-Val-AZT exhibited three times higher cellular uptake than its parent drug, AZT.

Table I. Stability of Amino Acid Ester Prodrugs During the Uptake Study in Caco-2/hPEPT1 Cells (Mean ± SD, n = 3)

	% of prodrug reconversion[a]	
	The supernatant	Inside of cells
L-Val-ACV	0.6 ± 0.08	96.9 ± 1.89
D-Val-ACV	0.3 ± 0.002	46.5 ± 7.77
L-Val-AZT	8.4 ± 2.2	100

[a] This was determined by the appearance (%) of the parent drug after 30 min incubation at 25°C.

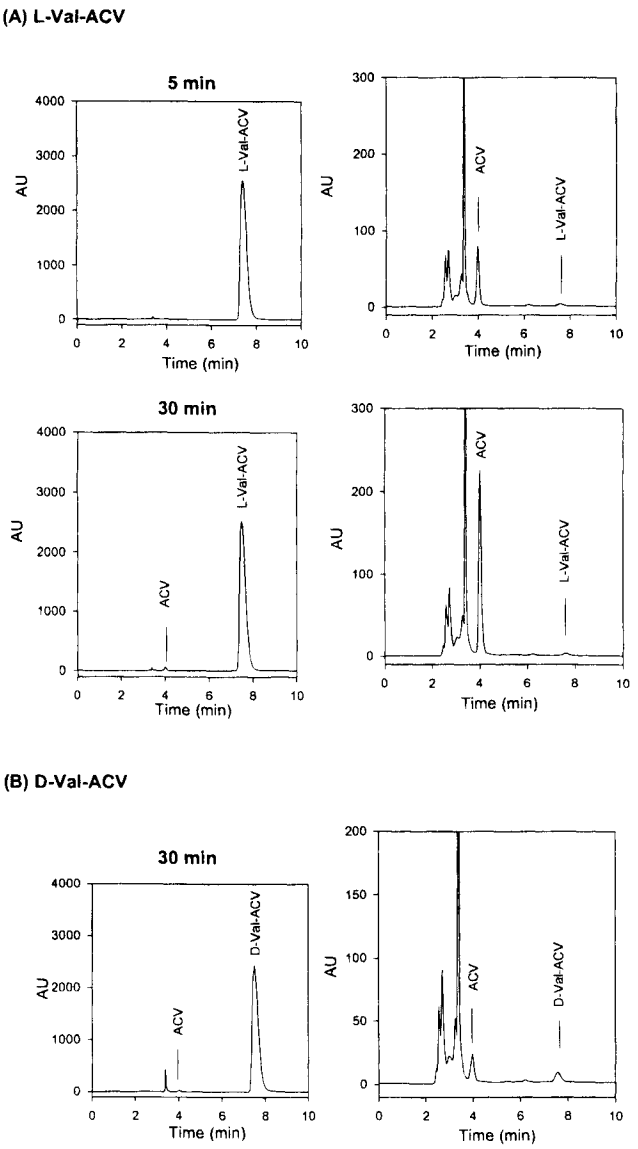

Fig. 1. High performance liquid chromatograms demonstrating the hydrolysis of prodrugs during the uptake study in Caco-2/hPEPT1 cells: left panels—the supernatant, right panels—inside of cells.

Competitive Inhibition Study on the L-Val-ACV Uptake in Caco-2/hPEPT1 Cells

The cellular uptake of L-Val-ACV was evaluated in the presence of several inhibitors to further characterize the absorption mechanism. Small dipeptides (Gly-Sar, Gly-Pro, Gly-Phe) and cephalexin, which are known as the substrates of a PEPT1 transporter, significantly reduced the cellular uptake of L-Val-ACV, but the amino acid, L-valine had no inhibition effect (Fig. 4), indicating the peptide carrier mediated transport mechanism of amino acid ester prodrugs.

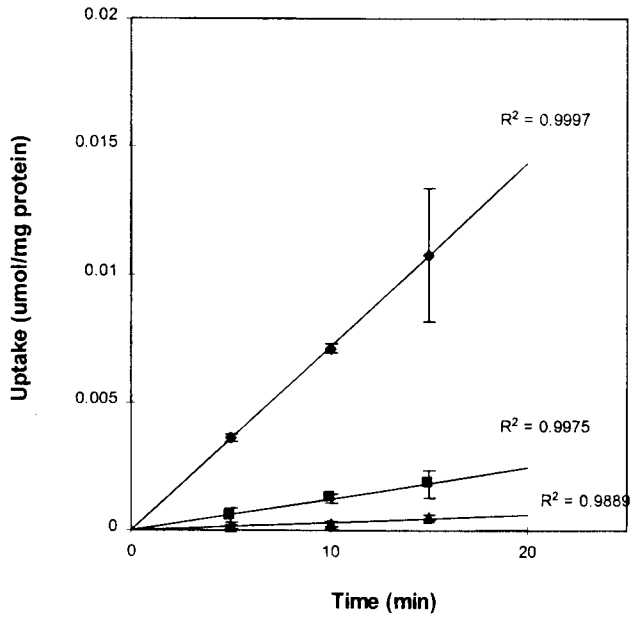

Fig. 2. Time course of cellular uptake of acyclovir prodrugs in Caco-2/hPEPT1 cells (Mean ± SD, n = 3): (◆); L-Val-ACV, (■); D-Val-ACV, (▲); Gly-ACV.

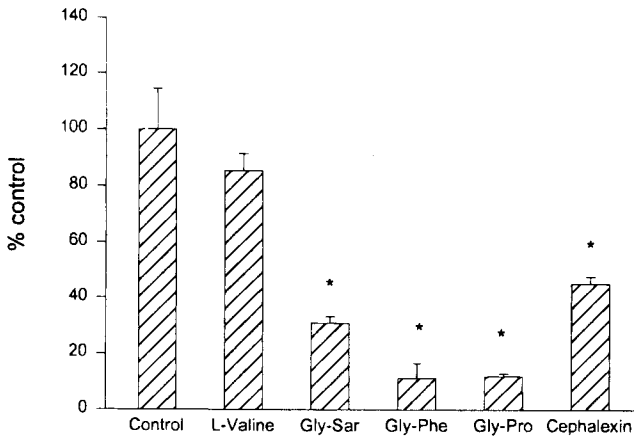

Fig. 4. Inhibition study on the uptake of L-Val-ACV in Caco-2/hPEPT1 cells (Mean ± SD, n = 6 except L-valine (n = 5)): (*); $p < 0.01$.

DISCUSSION

Due to the broad substrate specificity, peptide transporters can be a potential target in the prodrug design to improve oral drug absorption of a polar drug. For example, the membrane permeability of the polar α-methyl dopa was significantly improved through peptidyl derivatives which were water soluble but well absorbed via a peptide transporter (15). However, it had not been established whether amino acid esters also could be applied to the peptide transporter-targeted prodrug strategy until our previous rat study suggested the absorption mechanism of amino acid ester prodrugs (4). Therefore, if we can confirm the cellular uptake of amino acid ester prodrugs to be mediated by a peptide transporter, even though there is no peptide bond in the structures, it will have a great impact on the prodrug design and targeting the peptide transporter.

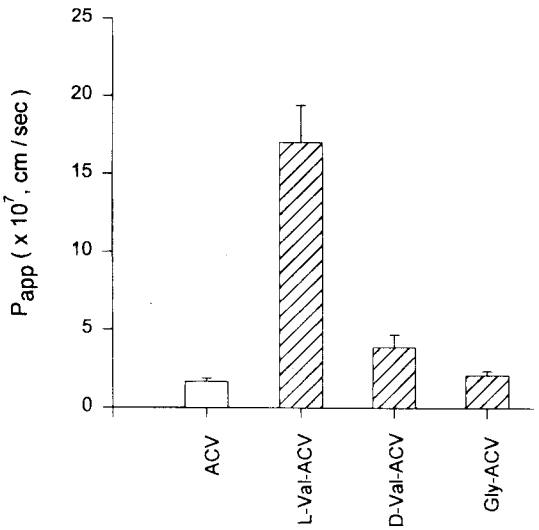

Fig. 3. Permeability of acyclovir and its amino acid ester prodrugs in Caco-2/hPEPT1 cells (Mean ± SD, n = 6).

Since Hsu *et al.* (13) in our group newly established the transiently transfected Caco-2 cells overexpressing a hPEPT1 transporter, Caco-2/hPEPT1 cells were considered a more efficient *in vitro* model for the uptake study of our prodrugs than the untransfected Caco-2 cells. Before examining our prodrugs, the functional expression level of a peptide transporter in Caco-2/hPEPT1 cells was evaluated through the kinetic study of Gly-Sar uptake. Compared with Kd which indicates the contribution of passive diffusion, V_{max}/K_m was approximately 35 fold higher in Caco-2/hPEPT1 cells and 17 fold higher in Caco-2 cells, demonstrating that Gly-Sar uptake was predominantly carrier-mediated in both cell lines, particularly in Caco-2/hPEPT1 cells. V_{max}/K_m was approximately four fold higher in Caco-2/hPEPT1 cells compared with that in Caco-2 cells, indicating that Caco-2/hPEPT1 cells significantly enhanced the capacity of peptide transport system while retaining the substrate specificity. In addition, the cellular uptake of L-Val-ACV was five folds higher in Caco-2/hPEPT1 cells than that in Caco-2 cells. Thus, Caco-2/hPEPT1 cells were selected as an efficient model for the characterization of the cellular uptake mechanism of amino acid ester prodrugs.

Prodrugs are designed to overcome the undesirable properties of drugs and prodrug itself is (usually) biologically inactive. Therefore, prodrugs should be converted to the parent drugs *in vivo* to have drug efficacy as soon as their goals are achieved. The HPLC assay on the supernatant above the cell monolayer as well as inside cells revealed that following the membrane transport, the prodrugs were rapidly converted to their parent drugs by the intracellular hydrolysis with the L-isomer exhibiting a faster reconversion than D-isomer (Table I and Fig. 1). Therefore, amino acid ester prodrugs satisfy a critical requirement that prodrugs should be converted to the parent drugs to recover the drug efficacy as soon as they overcome the absorption problem of the parent drug.

The cellular uptake of amino acid ester prodrugs was compared with that of the parent drugs in Caco-2/hPEPT1 cells to examine how effectively the prodrugs improve the cellular uptake of the parent drugs. The cellular uptake of acyclovir and AZT was three to ten fold increased by the amino acid

ester prodrugs. Furthermore, L-configuration of amino acid is more suitable for the prodrug strategy, since L-Val-ACV showed much higher membrane permeability as well as faster reconversion to acyclovir than D-Val-ACV. These results provide an explanation on the findings of Beauchamp *et al.* (3) that L-Val-ACV increased the bioavailability of acyclovir three fold in rats but D-Val-ACV did not. In addition, the significant difference of permeability between L-Val-ACV and Gly-ACV in the uptake study (Fig. 3) strongly support the suggestion by Beauchamp *et al.* (3) 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.

In the competitive inhibition studies, the substrates of a peptide transporter such as small dipeptides and cephalexin competed with L-Val-ACV for the cellular uptake, while amino acid, L-valine, had no inhibition effect (Fig. 4). These results strongly suggest the peptide carrier mediated membrane transport of amino acid ester prodrugs, while their parent drugs were transported by the passive diffusion (8,16). The involvement of a peptide transporter in the cellular uptake of amino acid ester prodrugs was also supported by the significantly increased cellular uptake of L-Val-ACV following the enhancement of a peptide transport activity in the Caco-2/hPEPT1 cells.

Further study is needed to make clear whether other transporters (e.g., nucleoside transporters and nucleobase transporters) are also involved in the cellular uptake of prodrugs, although the peptide transport system appeared to be the primary absorption pathway of amino acid ester prodrugs. In addition, for the general application of amino acid esters to the peptide transporter-targeted prodrug strategy, structure-transport relationship should be thoroughly investigated with the large structural variation of prodrugs in future work.

In conclusion, the results of our present studies can be summarized as follows. First, amino acid ester prodrugs significantly improve the cellular uptake of the parent drugs via peptide transport mechanism, though there is no peptide bond in their structures. Second, L-configuration of amino acid allows more favorable cellular uptake and faster prodrug-drug reconversion. Third, amino acid ester prodrugs are rapidly converted to the active parent drugs by the intracellular hydrolysis. Consequently, our findings in the present study provide far more flexibility in the structural modification targeting a peptide transporter to improve oral absorption of poorly permeable drugs.

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