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Pharmacokinetics of amino acid ester prodrugs of acyclovir after oral administration: Interaction with the transporters on Caco-2 cells☆

Suresh Katragadda, Ritesh Jain, Deep Kwatra, Sudharshan Hariharan, Ashim K. Mitra*

Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 5005 Rockhill Road, Kansas City, MO 64110-2499, USA

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ABSTRACT

In vivo systemic absorption of the amino acid prodrugs of acyclovir (ACV) after oral administration was evaluated in rats. Stability of the prodrugs, L-alanine-ACV (AACV), L-serine-ACV (SACV), L-isoleucine-ACV (IACV), γ-glutamate-ACV (EACV) and L-valine-ACV (VACV) was evaluated in various tissues. Interaction of these prodrugs with the transporters on Caco-2 cells was studied. *In vivo* systemic bioavailability of these prodrugs upon oral administration was evaluated in jugular vein cannulated rats. The amino acid ester prodrugs showed affinity towards various amino acid transporters as well as the peptide transporter on the Caco-2 cells. In terms of stability, EACV was most enzymatically stable compared to other prodrugs especially in liver homogenate. In oral absorption studies, ACV and AACV showed high terminal elimination rate constants (λ_z). SACV and VACV exhibited approximately five-fold increase in area under the curve (AUC) values relative to ACV ($p < 0.05$). $C_{max(T)}$ (maximum concentration) of SACV was observed to be 39 ± 22 μM in plasma which is 2 times better than VACV and 15 times better than ACV. $C_{last(T)}$ (concentration at the last time point) of SACV was observed to be 0.18 ± 0.06 μM in plasma which is two times better than VACV and three times better than ACV. Amino acid ester prodrugs of ACV were absorbed at varying amounts (C_{max}) and eliminated at varying rates (λ_z) thereby leading to varying extents (AUC). The amino acid ester prodrug SACV owing to its enhanced stability, higher AUC and better concentration at last time point seems to be a promising candidate for the oral treatment of herpes infections.

1. Introduction

Intestinal absorption of drugs, nutrients, and other compounds is mediated by uptake transporters expressed at the apical enterocyte membrane. These transporters play a significant role in drug absorption and distribution to organic systems. A host of transporters have been discovered at the brush-border membrane of intestinal epithelium, which can be targeted for drug delivery. Transport systems for peptide (Daniel, 2004), amino acid (Frenhani and Burini, 1999), monocarboxylic acids (Enerson and Drewes, 2003), bile acids (Lack, 1979) and vitamins (Said and Mohammed, 2006) are such carriers that have been discovered on the intestinal epithelium and utilized for targeted drug delivery (Tsuji and Tamai, 1996; Tolle-Sander et al., 2004). Among nutrient transporters, amino acid and peptide transporters are preferred for drug delivery due to their ubiquitous nature and overlapping substrate specificity.

Amino acid transporters have been classified on the basis of their functional differences such as sodium dependence and substrate specificity. In terms of specificity they can be divided into three types namely anionic, cationic and neutral (Christensen, 1990). Small neutral amino acids, are transported predominantly by Na^+-dependent transport system ASC (for Ala-, Ser-, and Cys-preferring), system A (for Ala-preferring) and $B^{0,+}$ (neutral and cationic amino acids preferring) and also by Na^+-independent transport system ASC, LAT (large neutral amino acids preferring) and $b^{0,+}$. Amino acid transport systems, $b^{0,+}$, y^+L and $B^{0,+}$ translocate a wider range of substrates, including cationic and neutral amino acids, differing however in their interactions with inorganic monovalent ions such as Na^+.

Depending upon their affinity or capacity, amino acid transporters have been known to transport not only naturally occurring amino acids but also amino acid-related drug compounds such as L-dopa, a therapeutic agent for Parkinsonism; melphalan

Abbreviations: ASCT1, Na^+ dependent neutral amino acid transporter; $B^{0,+}$, Na^+ dependent neutral and cationic amino acid transporter; LAT, large neutral amino acid transporter; hPEPT1, human intestinal peptide transporter; ACV, acyclovir; VACV, valine-acyclovir; SACV, serine-acyclovir; AACV, alanine-acyclovir; EACV, γ-glutamate-acyclovir; IACV, isoleucine-acyclovir; DPBS, Dulbecco's phosphate-buffered saline; AUC, area under the curve; CL/F, clearance; MRT, mean residence time; P_{app}, apparent permeability; HSV, herpes simplex virus.

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* Corresponding author. Tel.: +1 816 235 1615; fax: +1 816 235 5190.
E-mail address: mitraa@umkc.edu (A.K. Mitra).

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(Goldenberg et al., 1979), an anticancer Phe mustard; triiodothyronine (Blondeau et al., 1993) and thyroxine (Lakshmanan et al., 1990), two thyroid hormones; and gabapentin (Su et al., 1995), an anticonvulsant and valacyclovir (Hatanaka et al., 2004), an antiviral drug. A recent report suggests that the ability of ATB(0,+) to transport valacyclovir is comparable to that of the peptide transporter PEPT1 (Hatanaka et al., 2004).

Peptide transporters (PEPT1 and PEPT2) are perhaps the drug transporters that have captured the most recent attention in drug delivery. Small peptides such as di- and tripeptides are transported by PEPT1 and PEPT2 in intestinal and renal epithelial cells, respectively. Structure, function, mechanism, and substrate specificity of the peptide transporters have been extensively studied (Ganapathy and Leibach, 1982; Dantzig and Bergin, 1990; Ganapathy et al., 1995; Hidalgo et al., 1995; Hu et al., 1995; Liang et al., 1995; Han et al., 1998). Due to their broad substrate specificity, PEPT1 and PEPT2 contribute to the intestinal absorption of several drug compounds such as β-lactam antibiotics, cephalosporins, angiotensin-converting enzyme, and renin inhibitors (Dantzig and Bergin, 1990; Han et al., 1998). These findings suggest that amino acid and peptide transporters can have significant potential as a delivery targets for amino acid-based drugs and prodrugs.

Strategies have been used to design prodrugs of various poorly absorbed drugs targeted toward receptors/transporters to improve systemic bioavailability (Anand et al., 2004a, 2004b; Steffansen et al., 2004; Tolle-Sander et al., 2004). Valacyclovir (VACV) is such a prodrug that is derived from acyclovir (ACV) by esterifying 3-hydroxyl group of ACV with L-valine. Acyclovir, an antiviral nucleoside, possesses activity against human herpes viruses. Owing to its limited bioavailability, ACV has shown moderate antiviral efficacy after oral administration (Steingrimsdottir et al., 2000). VACV has been reported to increase the oral bioavailability of acyclovir by 3–5-fold in humans (Purifoy et al., 1993; Weller et al., 1993). Enhanced oral absorption of acyclovir after administration of valacyclovir has been attributed to the amino acid ($B^{0,+}$) (Hatanaka et al., 2004) and peptide (PEPT1) (Ganapathy et al., 1998) mediated intestinal translocation.

A series of novel water-soluble amino acid ester prodrugs of acyclovir were synthesized previously (Anand et al., 2004b; Katragadda et al., 2008). These compounds were designed to target the transporters on the cornea and intestinal epithelial cells for improved ocular and oral absorption of acyclovir, respectively. Previous results indicated that the amino acid ester prodrugs of ACV exhibited affinity toward the amino acid transporters, and the uptake of these prodrugs was efficiently mediated by ASCT1 and $B^{0,+}$ (Anand et al., 2004b; Katragadda et al., 2008). In this report, we describe pharmacokinetics of these prodrugs after oral administration in Sprague-Dawley rats. The interaction of these amino acid prodrugs with the transporters on Caco-2 cells is also discussed. The transport of these prodrugs across Caco-2 monolayers was compared with that of VACV to establish the transport of these compounds across enterocytic cell membranes.

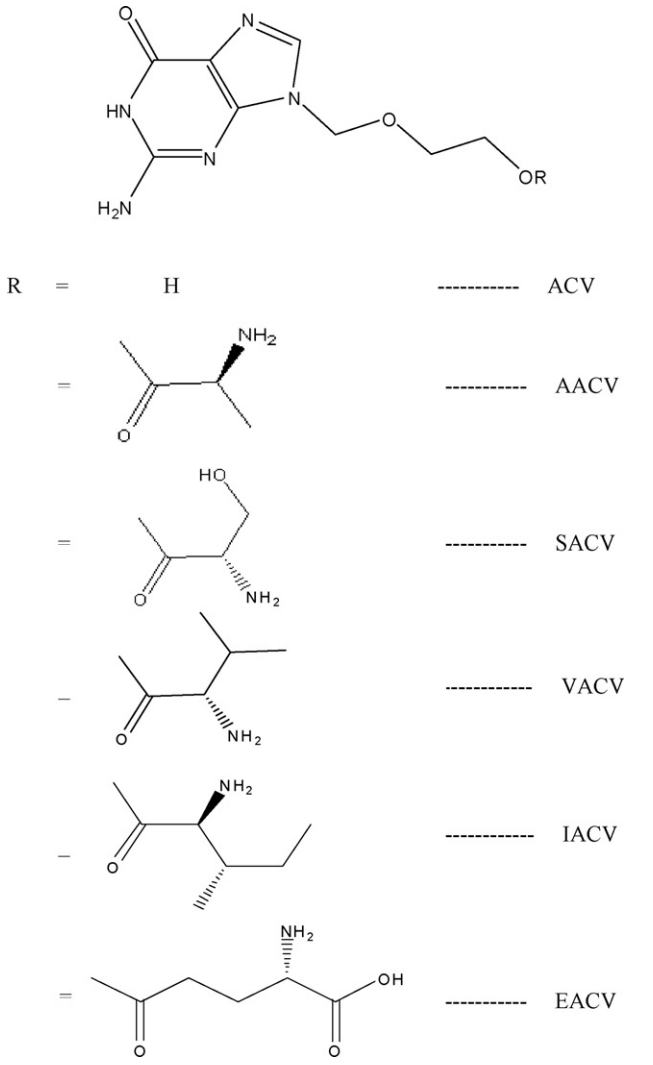

Fig. 1. Structures of amino acid prodrugs of acyclovir.

2. Materials and methods

2.1. Materials

[^{3}H] alanine (66 Ci/mmol), [^{3}H] phenylalanine (50 Ci/mmol), and [^{3}H] arginine (42 Ci/mmol) were obtained from NEN Biochemicals (Boston, MA, USA). [^{3}H]Glycylsarcosine (4 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Valacyclovir was a gift from GlaxoSmithKline (Research Triangle Park, NC). Human colon carcinoma derived Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). 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, and HEPES were purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Culture flasks (75-cm^2 growth area), 12 wells (3.8 cm^2 growth area per well), and polyester transwells (pore size 0.4 μM with diameter of 6.5 mm) were procured from Costar (Bedford, MA). The buffer components and solvents were obtained from Fisher Scientific (St. Louis, MO). All the amino acid prodrugs of acyclovir (Fig. 1) were synthesized in our laboratory according to previously published procedures (Anand et al., 2004b; Katragadda et al., 2008). Four amino acid prodrugs were used in this study: L-alanine-ACV (AACV), L-serine-ACV (SACV), L-isoleucine-ACV (IACV), and γ-glutamate-ACV (EACV).

2.2. Animals

Jugular vein cannulated male Sprague-Dawley rats weighing 200–250 g were obtained from Charles River Laboratories (Wilmington, MA). Animal care and treatment in this investigation was in compliance with the Guide for the Care and Use of Laboratory

Animals as adopted and promulgated by the National Institute of Health.

2.3. Cell culture

All cell cultures were maintained in humidified incubator at 37 °C with a 5% carbon dioxide in air atmosphere. Caco-2 cells were obtained from American Type Culture Collection and grown in plastic tissue culture flasks. The passage number used for this study ranged from 40 to 45. Conventional culture medium containing Dulbecco's modified Eagle's medium, 10% FBS (heat-inactivated), 1% nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 44 mM sodium bicarbonate and 20 mM HEPES at pH 7.4 was used according to the protocol established in our laboratory for maintaining the cell line. Upon reaching 80% confluence, cells were removed by trypsin/EDTA treatment and plated at a density of 200,000 cells/cm^2 on 12-well plates and 65,000 cells/cm^2 on transwells containing clear polyester membranes (1.1 cm^2, 0.4 μm mean pore size). Cells were then grown in medium containing 10% FBS (heat-inactivated). Caco-2 cells used in our studies were grown for 17–20 days.

2.4. Uptake studies

All uptake studies were conducted on Caco-2 cells after 17–20 days seeding. The medium was removed and cells were washed twice with DPBS (pH 7.4). In typical uptake experiments, cells were incubated with substrates ([^{3}H] alanine (7.6 nM); [^{3}H] arginine (11.9 nM); [^{3}H] phenylalanine (10 nM); and [^{3}H] glysar (125 nM)) prepared in DPBS for 30 min. Following incubation, cells were washed three times with ice-cold HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer to terminate the uptake experiment. Then cells were lysed overnight with 1 ml 0.05% (w/v) Triton X-100 in 1N NaOH at room temperature. Aliquots (500 μl) from each well were transferred to scintillation vials containing 5 ml scintillation cocktail (Fisher Scientific, Fairlawn, NJ). Samples were then analyzed by liquid scintillation spectrophotometry using scintillation counter (Beckman Instruments Inc., Model LS-6500) and the rate of uptake was normalized to the protein content of each well. The amount of protein in the cell lysate was measured by BioRad protein estimation kit using bovine serum albumin as the standard (BioRad Protein estimation Kit, Hercules, CA).

2.5. Transport studies

Transport experiments were carried out with 12 well transwells (Costar, Bedford, MA). Before the experiment, Caco-2 cell monolayer grown on the clear polyester membranes was washed with DPBS (pH 7.4) and incubated at 37 °C. Freshly prepared drug solution (1 mM) in DPBS (pH 7.4) was placed in the donor chamber and the receiver chamber was filled with DPBS (pH 7.4). The volumes of donor and receiver chambers were 0.5 and 1.5 ml each, respectively. Receiver chamber was sampled at predetermined time intervals and an equal volume of fresh DPBS solution was added to maintain sink conditions. All samples were stored at −80 °C until further HPLC analysis. All experiments were performed at least in triplicates at 37 °C.

2.6. Preparation of homogenates

2.6.1. Caco-2 homogenate

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). Cells were homogenized in 2 ml of chilled (4 °C) DPBS for about 10 min with a mechanical homogenizer in an ice bath. Subsequently, the homogenates were centrifuged at 12,500 rpm for 25 min at 4 °C to remove cellular debris, and the supernatant was used for hydrolysis studies.

2.6.2. Plasma

Noncannulated male Sprague-Dawley rats were anesthetized by administering ketamine (50 mg/kg) and xylazine (5 mg/kg) subcutaneously and subsequently euthanized by puncturing the heart. Blood was collected from the heart. Plasma was immediately separated from the blood by centrifugation at 6500 rpm for 10 min to remove the debris and then the supernatant (plasma) was stored at −80 °C until the hydrolysis studies were conducted.

2.6.3. Intestinal and liver homogenate

Noncannulated male Sprague-Dawley rats were euthanized by a lethal injection of sodium pentobarbital through the tail vein. Intestinal segments and liver were isolated and stored at −80 °C before use. Tissues were homogenized in 5 ml of chilled (4 °C) DPBS for about 4 min with a tissue homogenizer (Tissue Tearor model 985-370; Biospec Products Inc., Bartlesville, OK) in an ice bath. Subsequently, the homogenates were centrifuged at 12,500 rpm for 25 min at 4 °C to remove cellular debris and the supernatant was used for hydrolysis studies.

2.7. Metabolism studies

The supernatant obtained from the above described procedure was equilibrated at 37 °C for about 15 min before an experiment. Supernatant (0.8 ml) was incubated with 0.2 ml of 1 mM solutions of prodrugs at 37 °C in a shaking water bath for the length of the study. The control consisted of 0.8 ml of DPBS instead of the supernatant. Aliquots (50 μl) were withdrawn at appropriate time intervals for up to 24 h. The samples were immediately diluted with 50 μl of chilled acetonitrile/methanol (4:5 mixture) to precipitate the proteins and stored at −80 °C until further analysis. Apparent first order rate constants were calculated and corrected for any chemical hydrolysis observed with the control. The protein content of the supernatant was determined by the method of Bradford (1976) using bovine serum albumin as the standard (protein estimation kit; BioRad, Hercules, CA).

2.8. In vivo studies with Sprague-Dawley rats

Oral absorption studies of ACV and its prodrugs were carried out at an equivalent dose of 20.0 mg/kg. Animals were fasted overnight (12–18 h) with free access to water. Freshly prepared drug solutions in water were administered by oral gavage (0.8 ml). Blood samples (200 μl) were collected from the jugular vein at predetermined time intervals over a period of 5 h. Heparinized saline (200 μl) was injected through the vein to maintain a fairly constant fluid volume. Plasma was immediately separated by centrifugation and then stored at −80 °C until further analysis.

2.9. Sample preparation

Using a simple protein precipitation method ACV and its prodrugs were extracted from rat plasma. Plasma samples were thawed at room temperature and 0.2 ml of acetonitrile was added to 0.1 ml of plasma and 0.01 ml of internal standard (GCV 0.5 μg/ml) in a 1.5 ml centrifuge tube. The sample was then vortexed vigorously for 1 min and the samples were allowed to rest for 10 min at room temperature prior to centrifugation. Samples were centrifuged at approximately 12,500 rpm for 30 min at 4 °C. The supernatant was

then separated and evaporated using Speedvac (SAVANT Instruments, Inc., Holbrook, NY). The dry residues were dissolved in 100 μl of water (0.1% formic acid) and then the sample was vortexed for 2 min and centrifuged at 12,500 rpm for 15 min at 4 °C. Calibration standards were prepared by spiking plasma with the standard solutions. The supernatant was analyzed using a LC/MS/MS.

2.10. Analytical procedures

2.10.1. HPLC

The HPLC system was comprised of a Rainin Dynamax Pump SD-200 and a Rainin Dynamax UV Detector UV-C at 254 nm. The column used was a C18 Luna column 4.6 mm × 250 mm (Phenomenex, Torrance, CA). Mobile phase consisted of a mixture of buffer and an organic modifier. The percentage of organic phase was varied in order to elute the compounds of interest. This method gave rapid and reproducible results. HPLC conditions for the various compounds have been described previously (Anand et al., 2004b; Katragadda et al., 2008). Intra- and interday precision (measured by coefficient of variation, CV%) was less than 3 and 5%, respectively.

2.10.2. LC/MS/MS

The analysis of the plasma samples was performed using a triple quadrupole mass spectrometer with electrospray ionization (ESI) on a turbo ionspray source (API 2000; Applied Biosystems, Foster City, CA, USA) coupled to a liquid chromatography system (Agilent HP1100, Agilent Technology Inc., Palo Alto, CA, USA) and C18-column 100 × 2.0 mm (Phenomenex, Torrance, CA). The mobile phase consisted of 25% methanol and 75% water with 0.1% formic acid, and was delivered at a flow rate of 0.15 ml/min. The sample volume injected was 50 μl and the analysis time was 5–6 min. Multiple reaction monitoring (MRM) mode was utilized to detect the compound of interest. The mass spectrometer was operated in the positive-ion detection mode at collision energy of 18 V. The turbo ionspray temperature was optimized at 150 °C. Nitrogen gas was used to obtain collision-induced detection (CID). Ions were detected by monitoring the decay of the m/z 225 precursor ion to the m/z 152 product ion for acyclovir, the decay of the m/z 256 precursor ion to the m/z 152 product ion for ganciclovir, internal standard (IS). The typical ion source parameters were, declustering potential (DP) 36 V; collision energy (CE) 18 eV; entrance potential (EP) 6 V; and collision cell exit potential (CXP) 4 V. Peak areas for all components were automatically integrated by using Analyst™ software and peak-area ratios (area of analytes to area of IS) were plotted vs. concentration by weighted linear regression (1/concentration). The analytical data resulted from prodrugs with MRM method show a significant linearity which extends to picomolar range. This method gave rapid and reproducible results. The limits of quantification were found to be ACV 1 ng/ml; AACV 5 ng/ml; SACV 5 ng/ml; EACV 5 ng/ml; IACV 5 ng/ml and VACV 5 ng/ml. Intra- and interday precision (measured by coefficient of variation, CV%) was less than 3 and 5%, respectively.

2.11. Permeability measurements

Steady state fluxes (SSF) were determined from the slope of the cumulative amount of drug transported vs. time plot and expressed per unit of Caco-2 cell surface area as described by Eq. (1). The cumulative amount of drug transported is considered as the sum of the receptor cell prodrug and regenerated drug.

$$\text{Flux}\,(J) = \left(\frac{dM/dt}{A}\right) \quad (1)$$

M is the cumulative amount of drug transported and A is the Caco-2 cell surface area exposed to permeant. Caco-2 cell membrane permeabilities are determined by normalizing the SSF to the donor concentration, C_d according to Eq. (2).

$$\text{permeability}\,(P_{app}) = \frac{\text{flux}}{C_d} \quad (2)$$

2.12. Statistical analysis

All experiments were conducted at least in triplicate and results are expressed as mean ± S.D. Student's t-test was used to detect statistical significance between the parameters of the prodrugs and ACV and $p < 0.05$ was considered to be statistically significant. Statistical comparisons between the parameters of the prodrugs were performed using the analysis of variance (SPSS for Windows, release 10.0.7; SPSS Inc., Chicago, IL).

All relevant pharmacokinetic parameters were calculated using noncompartmental analyses of plasma-time curves after oral administration of ACV and the amino acid prodrugs of ACV using a pharmacokinetic software package WinNonlin, version 2.1 (Pharsight, Mountain View, CA). Data Fit was examined by observing R^2, correlation or coefficient of variance (CV), weighted residuals and predicted vs. observed values. Maximum plasma concentrations (C_{max}) and area under the plasma concentration time curves (AUC_{0-last} and AUC_{0-inf}) were obtained from the plasma-concentration time profiles using noncompartmental analysis. The slopes of the terminal phase of plasma profiles were estimated by log-linear regression and the terminal rate constant (λ_z) was derived from the slope. The terminal plasma half-lives were calculated from the equation: $t_{1/2} = 0.693/\lambda_z$. The total concentration (TC) parameters were calculated by adding the concentrations of the administered prodrug (PD) and the regenerated ACV.

3. Results

3.1. Uptake of [^{3}H] alanine in the presence of prodrugs

[^{3}H] Alanine (model substrate for ASC system) uptake was carried out in the presence of 5 mM alanine, AACV, SACV, EACV, IACV, and VACV. [^{3}H] alanine uptake was significantly inhibited in the presence of 5 mM alanine and AACV (Table 1).

3.2. Uptake of [^{3}H] arginine in the presence of prodrugs

[^{3}H] Arginine (model substrate for $B^{0,+}$ system) uptake was carried out in the presence of 5 mM arginine, AACV, SACV, EACV, IACV, and VACV. [^{3}H] Arginine uptake was significantly inhibited in the presence of 5 mM arginine and partially inhibited in the presence of 5 mM IACV (Table 1).

3.3. Uptake of [^{3}H] phenylalanine in the presence of prodrugs

[^{3}H] Phenylalanine (model substrate for LAT system) uptake was performed in the presence of 5 mM phenylalanine, AACV, SACV, EACV, IACV, and VACV. [^{3}H] Phenylalanine uptake was significantly inhibited in the presence of 5 mM phenylalanine and IACV (Table 1).

3.4. Uptake of [^{3}H] glysar in the presence of prodrugs

[^{3}H] Glysar (model substrate for peptide transporter system) uptake was performed in the presence of 5 mM glysar, AACV, SACV, EACV, IACV, and VACV. [^{3}H] Glysar uptake was significantly inhibited in the presence of 5 mM glysar, SACV and partially inhibited in the presence of 5 mM IACV and VACV (Table 1).

Table 1
Uptake of [^{3}H] L-alanine (7.6 nM), [^{3}H] L-arginine (11.9 nM), [^{3}H] L-phenylalanine (10 nM) and [^{3}H] glysar (125 nM) by Caco-2 in the presence of corresponding nonradioactive substrate and prodrugs

Units = fraction uptake of control per mg of protein	[^{3}H] Alanine	[^{3}H] Arginine	[^{3}H] Phenylalanine	[^{3}H] Glysar
Control	6.58 ± 0.75	7.81 ± 1.23	16.27 ± 2.66	2.38 ± 0.25
Corresponding nonradioactive substrate[a]	2.63 ± 0.67*	3.60 ± 0.37*	2.21 ± 0.16*	0.45 ± 0.04*
AACV[b]	1.90 ± 0.29*	6.50 ± 1.17	9.22 ± 2.11	2.49 ± 0.32
SACV[b]	7.64 ± 0.79	8.44 ± 0.67	12.72 ± 1.12	0.35 ± 0.05*
IACV[b]	4.93 ± 0.75	5.05 ± 0.67*	4.06 ± 0.33*	0.70 ± 0.06*
EACV[b]	6.59 ± 1.65	8.54 ± 0.24	14.58 ± 2.60	2.18 ± 0.09
VACV[b]	6.25 ± 1.40	8.50 ± 1.17	12.92 ± 0.17	0.87 ± 0.1*

Values are mean ± S.D. (n = 4–8).
[a] Uptake of radioactive substrate in the presence of corresponding nonradioactive competitive substrate (5 mM).
[b] Uptake of radioactive substrate in the presence of prodrugs (5 mM).
* Indicates $p < 0.05$.

Table 2
Permeability values of ACV, AACV, SACV, EACV, IACV, and VACV across Caco-2 cell monolayer

	Permeability ($\times 10^6$) (cm/s)
ACV	1.5 ± 0.2
AACV	2.04 ± 0.18
SACV	4.66 ± 0.37
EACV	1.56 ± 0.13
IACV	2.08 ± 0.11
VACV	4.43 ± 0.56

Values are mean ± S.D. (n = 3–6).

3.5. Transport across Caco-2 monolayers

Transport of 1 mM ACV, AACV, SACV, EACV, IACV and VACV was investigated across Caco-2 monolayers. P_{app} values were determined from the linear portion of the cumulative amount transported vs. time plot. The P_{app} values of ACV, AACV, SACV, EACV, IACV and VACV were calculated at pH 7.4 (Table 2).

3.6. Metabolism studies

3.6.1. Caco-2 homogenate

The half-lives of the amino acid prodrugs SACV and IACV were calculated as 6.1 and 6.9 h, respectively, in comparison with 1.6 and 1.15 h for VACV and AACV, respectively. The amino acid ester prodrugs AACV and VACV are rapidly hydrolyzed after incubation with the homogenate as compared to other prodrugs (Table 3). EACV showed moderate hydrolysis in Caco-2 homogenate.

3.6.2. Plasma

Amino acid ester prodrugs of ACV exhibited appreciable stability in plasma. VACV exhibited the highest half-life of 226 ± 67 h compared with other amino acid prodrugs. AACV rapidly hydrolyzed after incubation with plasma compared to other prodrugs. Among the amino acid prodrugs, SACV generated a half-life of 195 ± 57 h (Table 3). It was observed during the experiments that all the amino acid prodrugs (except AACV) showed intact prodrug followed by hydrolysis to ACV.

3.6.3. Intestinal homogenate

The prodrugs hydrolyzed to yield the parent drug ACV in intestinal homogenates. The half-lives of the amino acid prodrugs EACV and SACV were calculated as 8.2 and 2.1 h, respectively, in comparison with 1.3 h for IACV. The amino acid ester prodrugs VACV and AACV rapidly hydrolyzed to ACV (36 and 6 min, respectively) after incubation with the intestinal homogenate (Table 3).

3.6.4. Liver homogenate

The prodrugs were rapidly and completely hydrolyzed to yield the parent drug ACV in liver homogenates. EACV showed exceptional stability in liver homogenate compared to other prodrugs. The half-life of the amino acid prodrug EACV was calculated as 223 h, where as other remaining prodrugs were completely hydrolyzed before 0.5 h. The amino acid ester prodrug AACV was rapidly hydrolyzed (no intact prodrug detected after 1 min) after incubation with the homogenate (Table 3).

3.6.5. In vivo oral absorption

Analyses of the metabolites after administration of amino acid prodrugs revealed that the prodrugs were rapidly cleaved to the parent drug, ACV. After administration of AACV, the intact AACV could not be detected at any time point, possibly due to extensive metabolism by intestine and/or liver. Remaining prodrugs showed intact prodrug for the entire duration of the experiment in very small amounts compared with the regenerated ACV.

3.6.6. Total concentration

In vivo oral absorption profiles of all the prodrugs were analyzed by the total concentration (prodrug and regenerated ACV) (Fig. 2) and by the individual profiles as intact prodrug (Fig. 3) and regenerated ACV (Fig. 4). In this section we will be discussing the total concentration profiles of ACV and its prodrugs. The systemic absorption of the drugs and prodrugs upon oral administration

Table 3
In vitro enzymatic stability of ACV prodrugs in Caco-2, rat plasma, intestine and liver homogenates

	AACV		SACV		EACV		VACV		IACV	
	Rate ($k^a \times 10^3$)	Half life (h)	Rate ($k^a \times 10^3$)	Half life (h)	Rate ($k^a \times 10^3$)	Half life (h)	Rate ($k^a \times 10^3$)	Half life (h)	Rate ($k^a \times 10^3$)	Half life (h)
Caco-2	10.1 ± 0.6	1.15 ± 0.07	2.1 ± 0.6	6.1 ± 2.2	2.82 ± 0.54	4.2 ± 0.7	11.9 ± 0.7	1.6 ± 0.15	1.8 ± 0.6	6.9 ± 1.9
Plasma	2.45 ± 0.35	4.8 ± 0.7	0.06 ± 0.02	195 ± 57	0.28 ± 0.04	42.4 ± 6.1	0.05 ± 0.02	226 ± 67	0.08 ± 0.006	139 ± 9.2
Intestine	105 ± 2.7	0.11 ± 0.002	5.7 ± 0.7	2.1 ± 0.2	1.42 ± 0.08	8.2 ± 0.5	18.5 ± 0.7	0.6 ± 0.02	8.8 ± 0.5	1.32 ± 0.07
Liver	–	[b]	27 ± 3	0.44 ± 0.06	0.05 ± 0.002	223 ± 8.8	163 ± 9	0.07 ± 0.004	165 ± 0.9	0.07 ± 0.004

Values are mean ± S.D. (n = 3–6).
[a] min^{-1} mg $protein^{-1}$.
[b] No intact prodrug detected after 1 min.

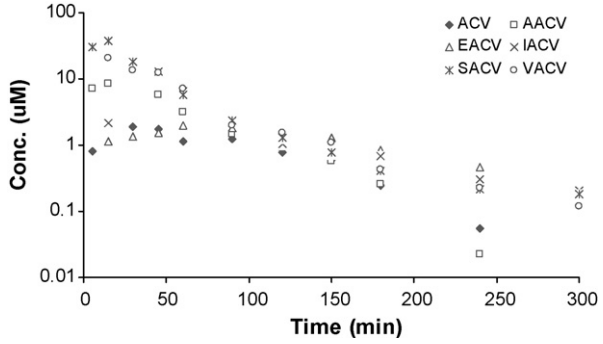

Fig. 2. Plasma-concentration time profile of total concentration of ACV upon oral administration of (♦) ACV, (□) AACV, (✳) SACV, (×) IACV, (△) EACV, and (○) VACV.

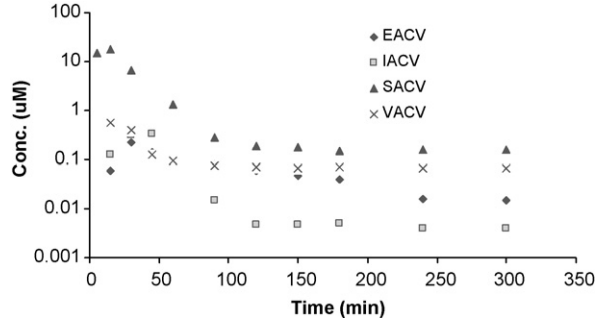

Fig. 3. Plasma-concentration time profile of intact prodrug upon oral administration of (▲) SACV, (■) IACV, (♦) EACV, and (×) VACV.

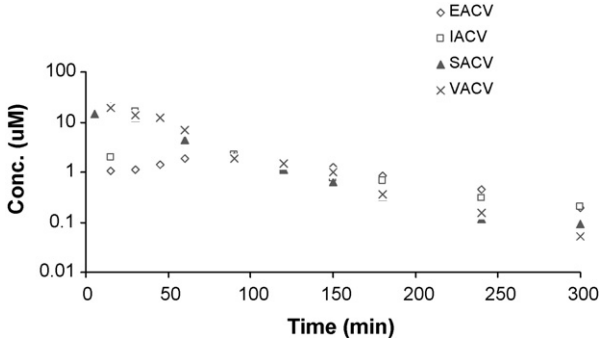

Fig. 4. Plasma-concentration time profile of regenerated ACV upon oral administration of (▲) SACV, (□) IACV, (◊) EACV, and (×) VACV.

was determined by sampling from the jugular vein. The systemic absorption of total plasma-concentration of ACV vs. time profiles of ACV, AACV, SACV, EACV, IACV and VACV are depicted in Fig. 2. Pharmacokinetic parameters have been summarized in Table 4. Oral administration of amino acid prodrugs led to an increase

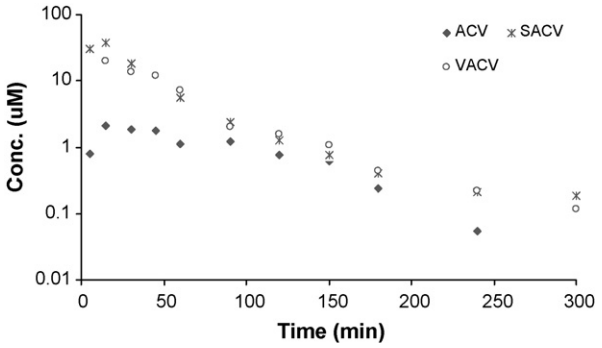

Fig. 5. Plasma-concentration time profile of total concentration of ACV upon oral administration of (♦) ACV, (✳) SACV, and (○) VACV.

in intestinal absorption of ACV compared with ACV alone. SACV and VACV led to approximately five-fold elevation of area under the curve ($AUC_{inf(T)}$) over ACV (Fig. 5), whereas IACV and AACV increased $AUC_{inf(T)}$ by three- and two-folds, respectively. $C_{max(T)}$ values for total concentration of ACV after administration of ACV, AACV, SACV, EACV, IACV and VACV were observed to be 2.3 ± 0.3, 12.1 ± 1.8, 39 ± 22, 2.5 ± 0.1, 20 ± 5 and 22 ± 0.3 μM, respectively, with SACV exhibiting the highest maximum concentration (C_{max}). Time to reach maximum concentration ($T_{max(T)}$) for ACV, AACV, SACV, IACV and VACV did not vary significantly ($p < 0.05$) except EACV. The mean residence time for total concentration of EACV [$MRT_{last(T)}$] was higher than that of ACV. The elimination rate constants of total concentration of ACV ($\lambda_{z(T)}$) for ACV, AACV, SACV, IACV, EACV, and VACV were calculated as 0.04 ± 0.006, 0.04 ± 0.004, 0.01 ± 0.002, 0.01 ± 0.002, 0.01 ± 0.00003 and 0.02 ± 0.002 min^{-1}, respectively indicating ACV and AACV had high elimination rate and then followed was VACV. $C_{last(T)}$ (concentration at the last time point) of SACV observed to be 0.18 ± 0.06 μM in plasma which is two times better than VACV and three times better than ACV.

3.6.7. Intact prodrug

Pharmacokinetic parameters of intact SACV, EACV, IACV and VACV upon their oral administration (Fig. 3) have been listed in Table 5A. The systemic absorption of intact prodrug plasma-concentration time profiles of SACV, EACV, IACV and VACV are depicted in Fig. 3. As mentioned above intact prodrug of AACV was not observed in plasma indicating extensive intestine/hepatic metabolism. Highest systemic exposure was obtained upon administration of SACV relative to EACV, IACV and VACV. The AUC obtained after oral administration ($AUC_{inf(PD)}$) of SACV was approximately 25-fold higher relative to EACV, IACV and VACV administration. C_{max} values for intact prodrug concentration ($C_{max(PD)}$) after administration of SACV, EACV, IACV and VACV were observed to be 18 ± 12, 0.46 ± 0.15, 0.23 ± 0.06, and 0.56 ± 0.2 μM, respectively, with SACV exhibiting the highest C_{max} value ($p < 0.05$). C_{last} of SACV ($C_{last(PD)}$)

Table 4
Pharmacokinetic parameters for systemic absorption of amino acid prodrugs of ACV

Parameter	ACV	AACV	SACV	IACV	EACV	VACV
$\lambda_{z(T)}$ (1/min)	0.04 ± 0.006	0.04 ± 0.004	0.01 ± 0.002	0.01 ± 0.002	0.01 ± 0.0003	0.02 ± 0.002
$T_{max(T)}$ (min)	19 ± 4	20 ± 8.6	14 ± 5	34 ± 4	80 ± 9	20 ± 4.3
$C_{max(T)}$ (μM)	2.3 ± 0.3	12.1 ± 1.8	39 ± 2	20 ± 5	2.5 ± 0.1	22 ± 0.3
$C_{last(T)}$ (μM)	0.06 ± 0.04	0.03 ± 0.01	0.18 ± 0.06	0.2 ± 0.01	0.2 ± 0.02	0.12 ± 0.008
$AUC_{inf(T)}$ (min μM)	204 ± 33	524 ± 28	1466 ± 173	815 ± 104	345 ± 40	1032 ± 130
$MRT_{last(T)}$ (min)	72 ± 6.4	47 ± 3	51 ± 10	64 ± 2	115 ± 5	49 ± 2

Values presented herewith are for total concentration. Values are mean ± S.E. ($n = 4$–6). *$p < 0.05$ compared to ACV. *T*: total concentration in terms of ACV; MRT: mean residence time; AUC: area under curve; C_{max}: maximum concentration, T_{max}: time to reach maximum concentration, λ_z: terminal elimination rate constant, C_{last}: concentration at the last time point.

Table 5A
Pharmacokinetic parameters for systemic absorption of amino acid prodrugs of ACV

Parameter	SACV	IACV	EACV	VACV
$C_{max(PD)}$ (μM)	18 ± 12	0.46 ± 0.15	0.23 ± 0.06	0.56 ± 0.2
$C_{last(PD)}$ (μM)	0.15 ± 0.01	0.004 ± 0.0004	0.015 ± 0.0012	0.06 ± 0.0006
$AUC_{last(PD)}$ (min μM)	547 ± 347	15 ± 4	17 ± 1	34 ± 6
$AUC_{inf(PD)}$ (min μM)	573 ± 339	16 ± 4	18 ± 1	37 ± 6

Values presented herewith are for intact prodrug. Values are mean ± S.E. (n = 4–6). PD: concentration of prodrug; AUC: area under curve; C_{max}: maximum concentration; C_{last}: concentration at the last time point.

was observed to be 0.15 ± 0.01 (M in plasma which is two times better than VACV.

3.6.8. Parent drug

Pharmacokinetic parameters of regenerated ACV upon oral administration of SACV, EACV, IACV and VACV (Fig. 4) have been listed in Table 5B. In contrast to intact prodrug, AUC values of the regenerated ACV ($AUC_{inf(ACV)}$) from SACV, IACV and VACV were similar. Lower systemic exposure of regenerated ACV was obtained upon administration of EACV relative to SACV, IACV and VACV. The AUC obtained after oral administration of EACV was approximately three-fold lesser relative to SACV, IACV and VACV administration. C_{max} values for regenerated ACV concentration ($C_{max(ACV)}$) after administration of SACV, EACV, IACV and VACV were observed to be 21 ± 10, 2.3 ± 0.2, 19 ± 5, and 22 ± 0.5 μM, respectively, with EACV exhibiting the lowest C_{max} value ($p < 0.05$). C_{last} ($C_{last(ACV)}$) value of EACV and IACV (0.2 ± 0.01 (M) was better than VACV and SACV and the reason may be these prodrugs degrade faster than VACV and SACV in plasma resulting in more regenerated ACV at the last time point.

4. Discussion

The incidence of genital herpes infections caused by HSV-1 and HSV-2 has increased significantly in the past 20 years (Fleming et al., 1997). Since the introduction of antiviral drugs in the early 1980s (Balfour, 1999), the management of genital herpes infections has improved considerably although it is not yet possible to cure herpes virus infections. 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). Acyclovir was the first effective antiviral drug approved for use and is still extensively prescribed, particularly in the treatment of immunocompetent patients with genital HSV disease (Perry and Faulds, 1996). Although acyclovir is a well tolerated and effective antiviral drug, its bioavailability after oral administration is low. As a result, up to five times administration per day is often necessary for the management of genital HSV disease. In this study amino acid prodrugs of acyclovir were studied to improve the bioavailability of acyclovir thereby improving the therapy.

The amino acid ester prodrugs of ACV have been studied for their affinity toward amino acid and peptide transporters expressed in colon carcinoma cell line, Caco-2. These compounds (especially IACV) exhibited affinity toward various transporters by inhibiting various model substrates. In general terms, inhibition studies may not be a good predictor for the actual cellular transport of drug candidates, because the substrates might only bind to the transporter without being translocated by it. Hence, the affinity of these prodrugs may not be translated into transepithelial transport and oral delivery. Therefore, transport experiments with the amino acid prodrugs AACV, SACV, EACV, IACV, and VACV were carried out across Caco-2 monolayers at pH 7.4 (Table 2). The above argument was proved in case of IACV, despite exhibiting inhibition in uptake studies, the transport of IACV did not improve much. To delineate the mechanistic details further studies are needed.

Table 5B
Pharmacokinetic parameters for systemic absorption of amino acid prodrugs of ACV

Parameter	SACV	IACV	EACV	VACV
$C_{max(ACV)}$ (μM)	21 ± 10	19 ± 5	2.3 ± 0.2	22 ± 0.5
$C_{last(ACV)}$ (μM)	0.09 ± 0.02	0.2 ± 0.01	0.2 ± 0.03	0.05 ± 0.008
$AUC_{last(ACV)}$ (min μM)	897 ± 321	779 ± 106	297 ± 45	991 ± 126
$AUC_{inf(ACV)}$ (min μM)	903 ± 322	800 ± 103	313 ± 47	994 ± 126

Values presented herewith are for regenerated ACV from prodrug. Values are mean ± S.E. (n = 4–6). ACV: concentration of generated parent drug; AUC: area under curve; C_{max}: maximum concentration; C_{last}: concentration at the last time point.

Caco-2, plasma, intestinal and liver homogenate hydrolysis studies were carried out to evaluate the hydrolysis characteristics of ACV amino acid ester prodrugs. Hydrolysis kinetics of the prodrugs in Caco-2 cell suspensions indicated that these prodrugs can (AACV, VACV) undergo hydrolysis by enterocytic enzymes, which could limit their bioavailability upon oral administration. The rank order stability of the prodrugs (except EACV) seem to follow a trend as more enzymatically active tissues (liver) caused more hydrolysis compared to less active tissues (plasma). Intestine exhibited more hydrolysis of the prodrugs compared to Caco-2 homogenate confirming improved and varied enzymatic activity. In plasma, VACV and SACV were found to be the most stable compounds with a half-life of 195 ± 57 and 226 ± 67 h, respectively. These *in vitro* studies further suggest that upon oral administration, hydrolysis of the prodrugs by intestinal/hepatic system will be extensive than by systemic circulation.

In liver, EACV was found to be the most stable compound with a half-life of 223 ± 8.8 h and AACV the least, because no intact prodrug was detected 1 min after the beginning of an experiment (Table 3). The less enzymatic liability of EACV can be explained by the lack of amino terminus near the ester bond (hydrolysis site) which is formed between γ-carboxy terminus of glutamic acid and hydroxyl group of ACV. Further studies were needed to confirm the importance of amino terminus in facilitating the enzymatic hydrolysis of the ester bond (Fig. 1).

Oral absorption studies of ACV and the amino acid prodrugs AACV, SACV, EACV, IACV and VACV were carried out in Sprague-Dawley rats with cannulated jugular vein. After oral administration, AACV is rapidly and completely hydrolyzed by both intestinal and hepatic enzymes as no intact AACV could be detected in the systemic circulation. Upon oral administration of other prodrugs, intact prodrug was observed for the entire duration of the experiment in varying amounts. However these prodrugs, after being absorbed from the gastrointestinal tract, underwent extensive first pass intestinal and/or hepatic metabolism. This was evident by higher levels of ACV generated compared to intact prodrug in systemic circulation (Tables 5A and 5B).

$AUC_{inf(T)}$ values obtained after oral administration of the amino acid ester prodrugs of ACV were significantly higher ($p < 0.05$) than ACV itself. This increase in bioavailability of ACV upon oral administration can be attributed to the interaction of these prodrugs with intestinal amino acid transporters (ASC and $B^{0,+}$) and peptide transporter (PEPT1) that mediates their transport across intestinal

epithelium to blood (Table 1). SACV and VACV yielded the highest $AUC_{inf(T)}$, which is at least five-fold higher than ACV after systemic absorption (Fig. 5). C_{max} value of SACV was 2 times better than VACV and 15 times better than ACV. $C_{last(T)}$ values obtained after administration of SACV, EACV and IACV are three times better than the $C_{last(T)}$ value obtained after administration of ACV. The same is not the case with VACV which may be due to rapid metabolism of VACV to ACV (Table 3). As previously reported, there is high accumulation of ACV after the administration of VACV at the recommended dosage regimens of 250 mg, 500 mg, and 1 g of VALTREX (valacyclovir hydrochloride) administered four times daily for 11 days in volunteers with normal renal function (Weller et al., 1993).

Even though EACV did not result in appreciable increase in $AUC_{inf(T)}$ and $C_{max(T)}$ values, the notable difference in $T_{max(T)}$, $C_{last(T)}$ and $MRT_{last(T)}$ values can be attributed to slower absorption due to less lipophilic nature ($T_{max(T)}$) and higher enzymatic stability ($C_{last(T)}$, $MRT_{last(T)}$) of EACV compared to other prodrugs (Table 4, Fig. 2). In our studies, the plasma elimination half-life of ACV after administration of ACV and AACV ranged from 15 to 20 min upon systemic absorption. The reason for similar elimination pattern for AACV and ACV was due to the fact that only regenerated ACV from AACV was observed in the plasma. The plasma elimination half-life of total ACV after administration of VACV ranged from 30 to 40 min whereas SACV, IACV and EACV had a half-life of 60–90 min. Higher elimination rate of VACV compared to SACV and EACV again can be attributed to the enzymatic liability of the drug.

One of the notable differences observed between intact prodrug profiles of prodrugs was C_{max} ($C_{max(PD)}$), C_{last} ($C_{last(PD)}$) and AUC ($AUC_{inf(PD)}$) values of SACV were significantly higher compared to all other prodrugs (Table 5A, Fig. 3). The reason can be the combination of factors given below. It had higher absorption ($C_{max(PD)}$), higher stability and lower elimination rate of SACV ($\lambda_{(T)}$). Even though EACV exhibits comparable elimination rate and higher stability than SACV, the limiting factor for lower AUC of EACV should be lower absorption ($C_{max(PD)}$). This was again confirmed by the regenerated ACV profiles of prodrugs as AUC and C_{max} values of all the prodrugs ($AUC_{inf(ACV)}$) were similar except EACV (Table 5B, Fig. 4).

IACV seem to possess affinity towards peptide transporter (PEPT1) and also with various amino acid transporters (Table 1). Interestingly SACV also seem to interact with peptide transporter (PEPT1) on Caco-2 cells. In terms of enzymatic stability, the prodrugs can be ranked in a descending order as EACV ≫ SACV > IACV > VACV > AACV and in lipophilicity scale the compounds can be ranked as IACV > VACV > AACV > SACV > EACV. Besides stability and lipophilicity, ionization state of the prodrug also can limit the permeability/bioavailability of a prodrug as reported with VACV (Balimane and Sinko, 2000). Further studies need to be performed to confirm the effect of ionization on the permeability of other prodrugs. AACV being a highly unstable and EACV being highly hydrophilic seem to limit their bioavailability. So the improved bioavailability of other prodrugs may be the result of interplay of the factors given above.

In conclusion, oral administration of amino acid ester prodrugs of ACV led to an increase in systemic absorption of ACV compared with direct administration of ACV. There seems to be a very good correlation between the permeability of these prodrugs across Caco-2 cell monolayer and AUC values after oral administration confirming that Caco-2 cell line can be a very good screening model to predict the oral absorption of amino acid ester prodrugs (Fig. 6). The amino acid prodrugs of ACV except EACV are rapidly metabolized to the regenerated ACV due to intestinal/hepatic first pass effect. Despite their rapid metabolism, the amino acid prodrugs are efficiently absorbed by the intestinal amino acid and peptide transporters, leading to an increase in intestinal absorption of ACV relative to ACV administered as such. High C_{last} value of SACV can improve the therapy by maintaining higher systemic concentrations of drug for longer periods thereby requiring lower dosage or lower frequency of dosing. Therefore, the amino acid prodrugs of ACV particularly SACV owing to its enhanced stability, enhanced AUC and high concentration at last time point (C_{last}) seems to be a promising candidate for the treatment of oral herpes infections.

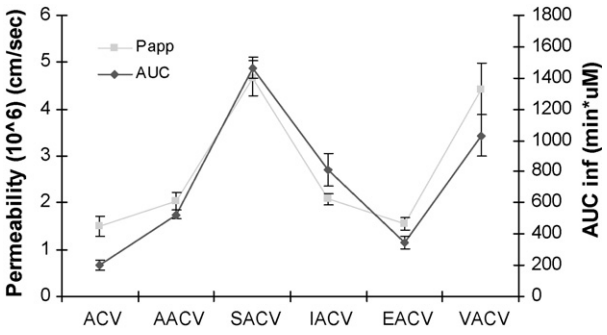

Fig. 6. Correlation between apparent permeability (P_{app}) across Caco-2 cells and area under the curve ($AUC_{inf(T)}$) obtained after oral administration of ACV and its amino acid ester prodrugs. Errors bars represent S.D. ($n = 6$).

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