Pharmacokinetics of Novel Dipeptide Ester Prodrugs of Acyclovir Following Oral Administration: Intestinal absorption and Liver Metabolism.

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Running Title: Oral Absorption of Dipeptide Prodrugs of ACV

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Abbreviations: Gly-Sar, glycylsarcosine; hPEPT1, human intestinal peptide transporter; ACV, acyclovir; VACV, valacyclovir; VVACV, valine-valine acyclovir; GVACV, glycine-valine acyclovir; GYACV, glycine-tyrosine acyclovir; VYACV, valine-tyrosine acyclovir.
ABSTRACT

The amino acid prodrug of acyclovir (ACV), valacyclovir (VACV) is an effective antiherpetic drug. Systemic availability of ACV in humans is 3-5 times higher after oral administration of VACV. Enhanced bioavailability of VACV has been attributed to its carrier mediated intestinal absorption, via hPEPT1 peptide transporter followed by rapid and complete conversion to ACV. An earlier report suggested that the dipeptide ester prodrugs of ACV possess high affinity towards the intestinal oligopeptide transporter hPEPT1 and therefore appear to be promising candidates in the treatment of oral herpes virus infections. In the present study we have examined the bioavailability of a series of dipeptide prodrugs of ACV following oral administration in Sprague-Dawley rats with cannulated jugular and portal veins. The area under plasma-concentration time curves (AUC) expressed as min µg ml⁻¹ for total concentration of VACV (208.4 ± 41.2) and the dipeptide prodrugs, Gly-Val-ACV (GVACV) (416.1 ± 140.9); Val-Val-ACV (VVACV) (147.7 ± 89.3); Val-Tyr-ACV (VYACV) (180.7 ± 81.2) were significantly higher than that of ACV (21.2 ± 5.2) upon intestinal absorption. Interestingly the bioavailability of ACV after administration of GVACV was approximately 2 fold higher than VACV. There was significant metabolism by hepatic first pass effect of the dipeptide prodrugs as evident by the higher levels of ACV obtained after systemic absorption as compared to intestinal absorption of GVACV and VVACV. The dipeptide prodrugs of ACV exhibited higher systemic availability of regenerated ACV upon oral administration and thus appear to be promising drug candidates in treatment of genital herpes infections.
Recently peptide transporters, PepT1 and PepT2 are perhaps the drug transporters that have captured the most 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; Ganapathy and Leibach, 1986; Dantzig and Bergin, 1990; Ganapathy et al., 1995; Hidalgo et al., 1995; Hu et al., 1995; Liang et al., 1995; Tanaka et al., 1998; Ogihara et al., 1999; Zhu et al., 2000). 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 (ACE) and renin inhibitors (Dantzig and Bergin, 1990; Inui et al., 1992; Hashimoto et al., 1994; Han et al., 1998a; Kiss et al., 2000). Even compounds without a peptide bond such as δ-amino levulinic acid (Temple et al., 1998) and ω-amino fatty acid (Doring et al., 1998) are known to be absorbed with the help of these transporters. A surprisingly diverse substrate specificity of the peptide transporters has been utilized very successfully to improve the bioavailability of the nucleoside analogs acyclovir (Steingrimsdottir et al., 2000) and zidovudine (AZT) by designing 5’- amino ester prodrugs (Han et al., 1998b). A third peptide transporter, peptide/histidine transporter (PHT1), translocates histidine and small peptides with high affinity and in a proton gradient dependent manner (Yamashita et al., 1997). Although the presence of the PHT1 has been confirmed in brain and eye, it is not found in intestine or kidney and shows little homology (<20%) with PepT1 and PepT2. The exact physiological role of this transporter is yet to be ascertained. Strategies have been employed to design prodrugs of various poorly absorbed drugs targeted towards
receptors/transporters in order to improve systemic bioavailability (Lupia et al., 1993; Weller et al., 1993; Guo and Lee, 1999; Sakaeda et al., 2001; Anand et al., 2002; Manfredini et al., 2002).

Valacyclovir (VACV) is such a prodrug, which is derived from acyclovir (ACV) by esterifying 3′ hydroxyl group of ACV with L-Valine. Acyclovir, an antiviral nucleoside possesses activity against human herpesviruses. Owing to its limited bioavailability ACV has shown moderate antiviral efficacy following oral administration (Steingrimsdottir et al., 2000). After oral administration, valacyclovir VACV is rapidly absorbed from the gastrointestinal tract and is converted to acyclovir and L-valine by first-pass intestinal and/or hepatic metabolism. VACV has been reported to increase the oral bioavailability of acyclovir by three to five fold in humans (Beauchamp et al., 1992; Lupia et al., 1993; Weller et al., 1993). Enhanced oral absorption (Balimane et al., 1998; de Vrueh et al., 1998; Han et al., 1998b) of acyclovir after administration of valacyclovir has been attributed to the hPEPT1 mediated intestinal translocation of the amino acid prodrug. Recently a protein with significant hydrolytic activity towards valacyclovir was identified and purified from Caco-2 cells and its identity with the Biphenyl hydrolase-like (BPHL) protein, previously cloned from human breast cancer tissue was established (Kim et al., 2003). The high expression of BPHL in the human intestine, liver and kidney (Puente and Lopez-Otin, 1995) suggests an important role for BPHL in the activation of VACV in human tissues.

A series of novel water-soluble dipeptide ester prodrugs of acyclovir (US Patent Pending) were synthesized previously (Nashed and Mitra, 2003). These compounds were designed to target the peptide transporters on the cornea and intestinal epithelial cells for improved
ocular and oral absorption of acyclovir respectively. Results indicated that the dipeptide ester prodrugs of ACV, exhibited high affinity towards the intestinal oligopeptide transporter and the uptake of these prodrugs was efficiently mediated by hPEPT1 as suggested by significant inhibition of uptake of glycylsarcosine. These prodrugs hydrolyzed readily to regenerate the active parent drug, acyclovir (Anand et al., 2003). In this report we describe pharmacokinetics and the bioavailability of these prodrugs following oral administration in Sprague Dawley rats. The role of intestinal and hepatic first pass effect on metabolism of these dipeptide prodrugs is also discussed. The transport characteristics of these prodrugs across Caco-2 monolayers were compared to that of VACV in order to establish whether these compounds may be transported across enterocytic cell membranes owing to their recognition by the peptide transporter.
MATERIALS AND METHODS

Materials

\[^{3}\text{H}]\] Glycylsarcosine (Gly-Sar; 4Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and \[^{14}\text{C}]\) Mannitol (50mCi/mmol) was supplied by Amersham (Piscataway, NJ). Valacyclovir was a gift from GlaxoSmithKline (RTP, NC). Human colon carcinoma derived Caco-2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The growth medium, Dulbecco’s modified Eagle Medium was obtained from Life Technologies (Grand Island, NY). MEM non-essential amino acids (NEAA), penicillin, streptomycin, sodium bicarbonate, HEPES and unlabeled glycylsarcosine (Gly-Sar) were purchased from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Culture flasks (75 cm\(^2\) growth area) and polyester membranes (pore size 0.4 \(\mu\)m) were procured from Costar (Bedford, MA). The buffer components and solvents were obtained from Fisher Scientific (St. Louis, MO). All the di-peptide prodrugs of acyclovir (Figure 1) were synthesized in our laboratory according to previously published procedures (Nashed and Mitra, 2003). Four dipeptide prodrugs were utilized in this study: VVACV, valine-valine acyclovir; GVACV, glycine-valine acyclovir; GYACV, glycine-tyrosine acyclovir; VYACV, valine-tyrosine acyclovir.

Animals

Portal and jugular vein cannulated male Sprague Dawley rats weighing 200-250g were obtained from Charles River Laboratories (NY). 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 U.S. National Institutes of Health.

**Metabolism studies**

*Plasma Hydrolysis*

Eight hundred microliters of the rat plasma was incubated with 200 µl of 1mM solutions of prodrugs at 37°C in a shaking water bath for the length of the study. Fifty microliter samples were withdrawn at predetermined time intervals and were immediately diluted with 50 µl chilled acetonitrile:methanol (4:5 mixture) to precipitate the proteins and supernatant stored at -80°C until further analysis. Apparent first order rate constants were calculated and corrected for any chemical hydrolysis observed with the control.

*Intestinal Homogenate Studies*

Non-cannulated male Sprague-Dawley rats were euthanized by a lethal injection of sodium pentobarbital through the tail vein. Intestinal segments were isolated and stored at -80°C prior to use. Tissues were homogenized in 5 ml chilled (4°C) DPBS for about 4 min with a tissue homogenizer (Tissue Tearor Model 985-370) in an ice bath. Subsequently the homogenates were centrifuged at 14000 x g for 25 min at 4°C to remove cellular debris and the supernatant was used for hydrolysis studies. Protein content of the supernatant was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard (BioRad protein estimation kit, Hercules, CA). The supernatant was equilibrated at 37°C for about 30 min prior to an experiment. Hydrolysis was initiated by the addition of 0.2 ml of a 1mM prodrug solution to 0.8 ml of
the supernatant. The control consisted of 0.8 ml of DPBS instead of the supernatant. Aliquots (50µl) were withdrawn at appropriate time intervals for up to twenty-four hours. The samples were immediately diluted with 50 µl 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.

**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 at passage 25 from American Type Culture Collection (ATCC) and grown in plastic tissue culture flasks. Conventional culture medium containing DMEM, 10% FBS (heat-inactivated), 1% NEAA, 4mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin, and 14mM HEPES at pH 7.4 was employed according to the protocol established in our laboratory for maintaining the cell line. Upon reaching 80% confluency, cells were removed by Trypsin/EDTA treatment and plated at a density of 100,000 cells/cm² on collagen coated plastic dishes containing clear polyester membranes (0.636 cm², 3.0 µm mean pore size). Cells were then grown in medium containing 10% FBS (heat-inactivated). Caco-2 cells employed in our studies were grown for 21-23 days. [14C] Mannitol transport was determined as a marker of cellular integrity, which was <0.3% per hour in representative cell monolayers. Cell layers exhibiting greater than 0.3% [14C] mannitol penetration were discarded.
Transport studies

Transport experiments were carried out with using side-bi-side™ diffusion cells (type VSC-1, Crown Glass Company Inc.). Prior to an experiment, Caco-2 cell monolayer grown on the clear polyester membranes was washed with DPBS (pH 6.0) and incubated at 37°C. Freshly prepared drug solution (1mM) in DPBS (pH 6.0) was placed in the donor chamber and the receiver chamber was filled with DPBS (pH 7.4). The volumes of donor and receptor chambers were 3ml each. Receiver chamber was sampled at predetermined time intervals and equal volume of fresh DPBS solution was added to maintain sink conditions in receiver chamber. All samples were stored at -80°C until further HPLC analysis. All experiments were performed at least in triplicates at 37°C.

Concentration dependent inhibition of GVACV transport was determined at varying concentrations (0.1-5 mM) of GVACV in presence of 10mM concentration of glycylsarcosine. In order to validate the cell culture system the pH dependent uptake of [³H] Glycylsarcosine was also studied as per the methods reported previously (Anand et al., 2003).

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 (12-18h) with free access to water. Freshly prepared drug solutions in water were administered by oral gavage (0.8ml). Blood samples (200µl) were collected from the jugular and portal veins at predetermined time intervals over a period of 4h. Heparinized saline (200µl) was injected through both the
veins in order to maintain a fairly constant fluid volume. Plasma was immediately separated by centrifugation and then stored at –80°C until further analysis.

Plasma samples were thawed at room temperature and 0.2 ml of methanol was added to 0.2 ml of plasma in an eppendorf tube. The mixture was vortexed for 30sec. and centrifuged at 8960 x g for 10 minutes at 4°C. The supernatant was then separated and an aliquot was directly injected onto the column for HPLC analysis.

Analytical Procedures

All samples were assayed with HPLC. The system was comprised of a Rainin Dynamax Pump SD-200, Rainin Dynamax UV Detector UV-C at 254nm, a HP 1100 series Fluorescence Detector at $ex \lambda = 285$nm, $em \lambda = 370$nm and an Alcott autosampler Model 718 AL HPLC. The column used was a C18 Luna column 4.6 x 250 mm (Phenomenex). The mobile phase consisted of a mixture of buffer and an organic modifier. The percentage of organic phase was varied in order to elute compounds of interest. This method gave rapid and reproducible results. HPLC conditions for the other prodrugs have been reported previously (Anand et al., 2003). The limits of quantification were found to be: ACV, 25ng/ml; VACV, 50ng/ml; GVACV, 100ng/ml; VVACV, 125ng/ml; VYACV, 250ng/ml; YACV, 250ng/ml. The intra and inter day precision (measured by coefficient of variation, CV%) was less than 3% and 5% respectively.

Calculation of Pharmacokinetic Parameters

All relevant pharmacokinetic parameters were calculated using non-compartmental analyses of plasma-time curves following oral administration of ACV, VACV and the
dipeptide prodrugs of ACV using a pharmacokinetic software package Win Nonlin, v2.1
(Pharsight, CA). Maximum plasma concentrations (C\text{max}) were obtained from the plasma-
concentration time curves and the area under the plasma concentration time curves
(AUC\text{0-last} and AUC\text{inf}) were determined by the linear trapezoidal method with
extrapolation. The slopes of the terminal phase of plasma profiles were estimated by log-
linear regression and the terminal rate constant (λ\text{z}) was derived from the slope. The
terminal plasma half-lives were calculated from the equation: t_{1/2} = \frac{0.693}{λ\text{z}}. Clearance
(Cl/F) and Mean Residence Time (MRT) were calculated as ratio of Dose/AUC and
AUMC/AUC respectively. The total concentration parameters were calculated by adding
the concentrations of the administered drug the regenerated intermediates in terms of
ACV.

**Statistical Analysis**

All experiments were conducted at least in triplicate and results are expressed as mean ±
SD. 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).
RESULTS

Metabolism Studies

Plasma

Dipeptide ester prodrugs of ACV exhibited rapid hydrolysis in plasma. VACV exhibited the highest half-life of 346.5 ± 133.3 minutes compared to the dipeptide prodrugs. With GYACV no intact prodrug was observed after one minute sampling. Amongst the dipeptide prodrugs, GVACV generated a half-life of 20.9 ± 5.3 minutes (Table 1). It was observed during the experiments that all the dipeptide prodrugs (except GYACV) cleaved to their amino acid ester conjugate intermediate followed by complete hydrolysis to ACV.

Intestinal Homogenate

The prodrugs hydrolyzed to yield the parent drug ACV in intestinal homogenates. The half-lives of the dipeptide prodrugs GVACV and VVACV were calculated as 17.32 and 24.5 minutes respectively in comparison to 28.8 minutes for VACV. The dipeptide prodrugs VYACV and GYACV rapidly hydrolyzed (no intact prodrug detected after one minute) following incubation with the homogenate (Table 1). GVACV and VVACV (except VYACV and GYACV) cleaved to their amino acid ester intermediate followed by hydrolysis to ACV.
Transport Across Caco-2 Monolayers

Transepithelial transport of 1mM VACV and GVACV has been reported previously (Anand et al., 2003). Therefore in this study transport of 1mM VVACV, GYACV and VYACV was investigated across Caco-2 monolayers. Cumulative amount of drug transported (the sum of the prodrug and the regenerated parent drug) was plotted as a function of time. Apparent permeabilities (P_app) were determined from the linear portion of the cumulative amount transported versus time plot. The apparent permeabilities (P_app) of VACV, GVACV, VVACV, VYACV and GYACV were calculated at pH 6.0 (Table 2).

In vivo Oral Absorption

Analyses of the metabolites following administration of VACV revealed that the prodrug was rapidly cleaved to the parent drug, ACV. While dipeptide conjugates hydrolyzed to an amino acid ester conjugate which is subsequently cleaved to generate the parent drug, ACV in the case of GVACV and VVACV. After administration of VYACV, the amino acid conjugate intermediate Tyrosine-ACV could not be detected at any time points possibly due to extensive metabolism by intestine and/or plasma. No intact dipeptide prodrug, was visible at any time during the experiment.

Intestinal Absorption

The intestinal absorption of the drugs and prodrugs upon oral administration was determined by sampling the portal vein. The intestinal absorption plasma-concentration time profiles of ACV, VACV, GVACV, VVACV and VYACV are depicted in Figures...
2-6. Figures 2, 3A-5A and 6 depict the total concentration of the drug absorbed in terms of ACV. Pharmacokinetic parameters have been summarized in Table 3. Oral administration of VACV and the dipeptide prodrugs led to an increase in intestinal absorption of ACV as compared to ACV alone. GVACV led to approximately two-fold elevation over VACV, whereas VVACV and VYACV had lower AUC values than VACV. C_max values for total concentration of ACV after administration of ACV, VACV, GVACV, VVACV and VYACV were observed to be 0.89 ± 0.18, 4.53 ± 0.95, 4.07 ± 1.03, 1.43 ± 0.32, 1.64 ± 0.5 µg ml⁻¹ respectively with VACV exhibiting the highest C_max value. Time to reach maximum concentration (T_max) for ACV, VACV, GVACV, VVACV and VYACV did not vary significantly (p<0.05). The mean residence time for regenerated ACV (MRT_(ACV)) for VACV and the dipeptide prodrugs was significantly higher than that of ACV and the highest MRT_(ACV) was observed with VYACV. The mean residence time value of the amino acid conjugate MRT_(AA) of GVACV and VVACV were similar and significantly higher than VACV. The elimination rate constants of regenerated ACV (λ_Z(ACV)) for ACV, VACV, GVACV, VVACV and VYACV were calculated as 0.015 ± 0.001, 0.004 ± 0.0001, 0.005 ± 0.001, 0.01 ± 0.005, 0.008 ± 0.004 minute⁻¹ respectively. Also the elimination rate constants of regenerated amino acid intermediate (λ_Z(AA)) from GVACV and VVACV were observed to be 0.005 ± 0.003, 0.001 ± 0.0005 minute⁻¹ respectively (Table 3). ACV clearance (Cl_(ACV)) (161.3 ± 36.2 ml min⁻¹) following parent drug administration was higher relative to regenerated ACV clearance following administration of VACV (17.6 ± 6.6 ml min⁻¹), GVACV (12.5 ± 5.5 ml min⁻¹), VVACV (15.6 ± 1.1 ml min⁻¹) and VYACV (8.5 ± 2.5 ml min⁻¹). The
clearance of the regenerated VACV following administration of GVACV (31.9 ± 5.3 ml min\(^{-1}\)) was higher compared to VVACV (6.91 ± 0.92 ml min\(^{-1}\)).

**Systemic Absorption**

The systemic absorption of the drugs and prodrugs upon oral administration was determined by sampling the portal vein. The systemic absorption plasma-concentration time profiles of ACV, VACV, GVACV, VVACV and VYACV are depicted in **Figures 2-6**. As mentioned above **Figures 2, 3A-5A and 6** depict the total concentration of the drug absorbed in terms of ACV. The pharmacokinetic parameters obtained following administrations of parent ACV and the prodrugs are listed in **Table 4**. Highest systemic exposure was obtained upon administration of GVACV relative to ACV, VACV, VVACV and VYACV. The AUC obtained after oral administration of GVACV was approximately two fold higher relative to VACV administration. C\(_{\text{max}}\) values for total ACV concentration after administration of ACV, VACV, GVACV, VVACV and VYACV were observed to be 0.83 ± 0.19, 5.44 ± 2.33, 7.2 ± 3.3, 1.27 ± 0.22, 1.85 ± 0.3 µg ml\(^{-1}\) respectively with GVACV exhibiting the highest C\(_{\text{max}}\) value. Time taken to reach maximum concentration (T\(_{\text{max}}\)) for ACV, VACV, GVACV, VVACV and VYACV was not significantly different from each other (p<0.05). AUC’s of the parent drug, ACV obtained after systemic administration of GVACV and VVACV were calculated as 571.68 ± 99.22, 96.8 ± 24.4 min µg ml\(^{-1}\) respectively and were found to be higher than from intestinal absorption 347.8 ± 108.2, 59.04 ± 4.13 min µg ml\(^{-1}\) possibly due to a significant liver metabolism of the amino acid metabolite, VACV. VACV and VYACV did not show such a phenomenon as they rapidly hydrolyzed to yield the parent drug,
ACV. The clearance of ACV (150.5 ± 12.6 ml min⁻¹) following ACV administration was found to be higher compared to clearance of regenerated ACV following administration of VACV (15.5 ± 3.1 ml min⁻¹), GVACV (13.3 ± 3.5 ml min⁻¹), VVACV (13.9 ± 1.36 ml min⁻¹) and VYACV (7.5 ± 0.7 ml min⁻¹). The clearance of the regenerated VACV following administration of GVACV (26.8 ± 4.5 ml min⁻¹) was higher compared to VVACV (12.2 ± 2.6 ml min⁻¹).
DISCUSSION

Although it is not yet possible to cure herpes virus infections, the management of genital herpes infections has improved considerably since the introduction of antiviral drugs in the early 1980s (Balfour, 1999). The incidence of genital herpes infections caused by HSV-1 and 2 has increased significantly in the last 20 years (Fleming et al., 1997).

Although genital herpes is self-limiting in healthy adults, the disease is painful and distressing, with severe psychosocial impact (Manne and Sandler, 1984; Goldmeier et al., 1988). Acyclovir was the first effective antiviral drug approved for widespread 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. Valacyclovir, L-valyl ester prodrug of acyclovir enhances the bioavailability of ACV after oral administration significantly over ACV due to the recognition of VACV by intestinal peptide transporter, hPEPT1 that mediates its transport across intestinal epithelium to blood.

Plasma hydrolysis and intestinal homogenate hydrolysis studies were carried out in order to evaluate the regeneration characteristics of the ACV dipeptide ester prodrugs to the parent drug. All the prodrugs hydrolyzed to regenerate the active parent drug, ACV. The half-lives of the prodrugs ranged from 2.1 to 346.5 minutes (Table 1) in plasma and 17.2 – 28.8 minutes in intestinal homogenates demonstrating varied susceptibility of the prodrugs to the hydrolyzing enzymes. In plasma VACV was found to be the most stable
compound with a half-life of $346.5 \pm 133.3$ minutes and GYACV the least, since no intact prodrug was detected one minute after the beginning of an experiment. Hydrolysis kinetics of the prodrugs in Caco-2 cell suspensions has been previously studied and these prodrugs were also observed to undergo hydrolysis by enterocytic enzymes (Anand et al., 2003) which could limit their bioavailability upon oral administration. The schematic representation of mechanism of bioreversion of VACV and the dipeptide prodrugs to ACV upon \textit{in vitro / in vivo} metabolism has been depicted in Figure 7. GVACV and VVACV are sequentially metabolized to the parent drug, ACV via the amino acid intermediate, whereas VYACV, GYACV and VACV are rapidly metabolized to regenerate the parent drug, ACV.

The dipeptide prodrugs of ACV have been recently studied for their affinity towards hPEPT1 expressed in colon carcinoma cell line, Caco-2. These compounds exhibited high affinity towards the transporter (Anand et al., 2003). In general terms inhibition studies may not be a good predictor for the actual cellular uptake of drug candidates, as the substrates might only bind to the transporter without being translocated by it. Hence the affinity of these prodrugs for hPEPT1 may not be translated into hPEPT1 mediated transepithelial transport and oral delivery. Therefore transport experiments with VACV and the dipeptide prodrugs GVACV, VVACV, VYACV and GYACV were carried our across Caco-2 monolayers at pH 6.0 (Table 2). The permeabilities of VACV and GVACV have been reported previously (Anand et al., 2003).

Oral absorption studies of ACV, VACV and the dipeptide prodrugs GVACV, VVACV and VYACV were carried out in Sprague Dawley rats with cannulated jugular and portal veins. After oral administration, VACV is rapidly absorbed from the GIT and nearly
completely converted to ACV and L-valine by first-pass intestinal and/or hepatic metabolism. A small amount of ACV is converted to inactive metabolites by aldehyde oxidase and by alcohol and aldehyde dehydrogenase. Neither VACV nor ACV is metabolized by cytochrome P450 enzymes. Therefore in order to assess the role of first pass effect due to intestine and liver, intestinal and systemic absorption of the prodrugs was determined by sampling the portal and jugular vein respectively. The samples collected from the portal vein were compared for their parent drug and the metabolite levels with that from the jugular vein in order to establish the role of intestinal and hepatic metabolism.

Upon oral administration of GVACV and VVACV formation of the amino acid intermediate was observed, which was further metabolized to yield ACV. However VYACV rapidly metabolized to ACV as no intact amino acid intermediate metabolite YACV could be detected. However intermediate metabolite VACV formed after administration of GVACV and VVACV underwent significant further metabolism in the liver as evident by higher levels of ACV generated following systemic absorption relative to intestinal absorption (Tables 3 and 4). It is highly likely that the enzyme, BPHL, principally responsible for hydrolysis of VACV is present in the liver as well as intestine (Puente and Lopez-Otin, 1995). AUC’s (TC) obtained after oral administration of VACV and the dipeptide ester prodrugs of ACV were significantly higher (p<0.05) than ACV itself. This increase in bioavailability of ACV upon oral administration has been attributed to the recognition of VACV by intestinal peptide transporter, hPEPT1 that mediates its transport across intestinal epithelium to blood. Also since the dipeptide prodrugs show appreciable affinity towards hPEPT1 (Anand et al., 2003), the oral
absorption of these prodrugs is also believed to be mediated through the intestinal peptide transporter, hPEPT1. GVACV yielded the highest AUC(TC), which is at least two fold higher than VACV following intestinal as well as systemic absorption. Such enhancement in absorption could be attributed to the similar affinity of VACV and GVACV towards hPEPT1 (Anand et al., 2003), which allows it to be absorbed efficiently across the intestinal mucosa. Also upon metabolism of GVACV, VACV is formed, which itself is a substrate of hPEPT1, whereas on the other hand VACV hydrolyses rapidly to form ACV, which is not a substrate of hPEPT1. The same is not the case with VVACV, which hydrolyses to VACV and has a similar AUC(TC) as that of VACV, due to rapid metabolism of VVACV to ACV. As previously reported there is no accumulation of ACV after the administration of VACV at the recommended dosage regimens of 250 mg, 500 mg, and 1 gram of VALTREX® administered 4 times daily for 11 days in volunteers with normal renal function. In our studies the plasma elimination half-life of ACV after administration of VACV ranged from 2.2 hrs-2.8 hrs. upon intestinal and systemic absorption. The plasma clearance of ACV following prodrug administration was significantly reduced (p<0.05) compared to clearance of ACV following administration of ACV alone due to the formation of the ACV upon metabolism of the dipeptide prodrugs as well as the amino acid intermediate.

In conclusion oral administration of dipeptide ester prodrugs of ACV led to an increase in intestinal and systemic absorption of ACV as compared to direct administration of ACV. The dipeptide prodrugs of ACV except VYACV are rapidly metabolized to the amino acid intermediate metabolite, VACV due to intestinal first pass effect. Despite their rapid metabolism the dipeptide prodrugs are efficiently absorbed by the intestinal peptide transporter.
transporter, hPEPT1 leading to an increase in intestinal absorption of ACV relative to oral ACV itself. Therefore, the dipeptide prodrugs of ACV particularly GVACV may provide a significant therapeutic advantage in the treatment of oral and genital HSV infections and may be considered a considerable improvement over VACV.
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Figures

Figure 1 Structures of dipeptide prodrugs of acyclovir.

Figure 2 Plasma-concentration time profile of ACV following (■) Intestinal and (◊) Systemic absorption of Acyclovir. Errors bars represent the S.D.’s (N=6).

Figure 3 A) Plasma-concentration time profile of ACV following (■) Intestinal and (◊) Systemic absorption of VACV. B) Plasma concentration time curves of intact VACV after (▲) intestinal and (△) systemic absorption and regenerated ACV upon (■) intestinal and (□) systemic absorption. Errors bars represent the S.D.’s (N=6).

Figure 4 A) Plasma-concentration time profile of ACV following (■) Intestinal and (◊) Systemic absorption of GVACV. B) Plasma concentration time curves of regenerated VACV after (▲) intestinal and (△) systemic absorption and regenerated ACV upon (■) intestinal and (□) systemic absorption. Errors bars represent the S.D.’s (N=6).

Figure 5 Plasma-concentration time profile of ACV following (■) Intestinal and (◊) Systemic absorption of VVACV. B) Plasma concentration time curves of regenerated VACV after (▲) intestinal and (△) systemic absorption and regenerated ACV upon (■) intestinal and (□) systemic absorption. Errors bars represent the S.D.’s (N=6).

Figure 6 Plasma-concentration time profile of ACV following (■) Intestinal and (◊) Systemic absorption of VYACV. Errors bars represent the S.D.’s (N=6).

Figure 7 Mechanism of bioreversion (in vitro and in vivo metabolism) of Val-ACV and ACV dipeptide ester prodrugs, Gly-Val-ACV, Val-Val-ACV, Val-Tyr-ACV to Acyclovir. Gly-Val-ACV and Val-Val-ACV are sequentially hydrolyzed via Val-ACV to yield the parent drug ACV, where as Val-Tyr-ACV is rapidly hydrolyzed to ACV without the formation of the intermediate amino acid metabolite.
Table 1. Enzymatic stability of ACV prodrugs in Rat Plasma and Intestinal Homogenates

<table>
<thead>
<tr>
<th>Drug</th>
<th>Plasma$^a$</th>
<th>Intestinal Segments$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{obs}^c$</td>
<td>$t_{1/2}^d$</td>
</tr>
<tr>
<td></td>
<td>$(\text{min}^{-1})$</td>
<td>$(\text{min.})$</td>
</tr>
<tr>
<td>VACV</td>
<td>0.002 ± 0.001</td>
<td>346.5 ± 133.3</td>
</tr>
<tr>
<td>GVACV</td>
<td>0.034 ± 0.009</td>
<td>20.9 ± 5.3</td>
</tr>
<tr>
<td>VVACV</td>
<td>0.13 ± 0.009</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>VYACV</td>
<td>0.53 ± 0.48</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>GYACV</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. (n=3-6)

$^a$ measured using rat plasma

$^b$ measured using intestinal segments

$^c$ First order rate constant

$^d$ half life

* No intact prodrug detected after one minute
Table 2 Transport of Dipeptide ester prodrugs of ACV (1mM) across Caco-2 cell monolayers at pH 6.0.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Permeability, $P_{app}$ *10^6 cm/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val-ACV a</td>
<td>3.01 ± 0.21</td>
</tr>
<tr>
<td>Gly-Val-ACV a</td>
<td>2.89 ± 0.59</td>
</tr>
<tr>
<td>Val-Val-ACV</td>
<td>1.96 ± 0.14</td>
</tr>
<tr>
<td>Val-Tyr-ACV</td>
<td>0.71 ± 0.47</td>
</tr>
<tr>
<td>Gly-Tyr-ACV</td>
<td>2.09 ± 0.41</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. (n=3-6)

a Ref:(Anand et al., 2003)
Table 3 Pharmacokinetic Parameters following intestinal absorption

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ACV</th>
<th>VACV</th>
<th>GVACV</th>
<th>VVACV</th>
<th>VYACV</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{AUC}_{(0-\text{last})} \text{ (TC)} ) (min (\mu g \text{ ml}^{-1}))</td>
<td>21.2 ± 5.2</td>
<td>208.4 ± 41.2</td>
<td>416.1 ± 140.9</td>
<td>147.7 ± 89.3</td>
<td>180.7 ± 81.2</td>
</tr>
<tr>
<td>(\text{AUC}_{\text{inf}} \text{ (TC)} ) (min (\mu g \text{ ml}^{-1}))</td>
<td>27.9 ± 6.1</td>
<td>237.9 ± 51.8</td>
<td>598.7 ± 164.1</td>
<td>266.1 ± 34.3</td>
<td>282.2 ± 59.7</td>
</tr>
<tr>
<td>(C_{\text{max}} \text{ (TC)} ) ((\mu g \text{ ml}^{-1}))</td>
<td>0.89 ± 0.18</td>
<td>4.53 ± 0.95</td>
<td>4.07 ± 1.03</td>
<td>1.43 ± 0.32</td>
<td>1.66 ± 0.6</td>
</tr>
<tr>
<td>(T_{\text{max}} \text{ (TC)} ) (min)</td>
<td>24.0 ± 8.9</td>
<td>22.5 ± 5.0</td>
<td>21.4 ± 5.77</td>
<td>15.1 ± 5.7</td>
<td>15.0 ± 5.77</td>
</tr>
<tr>
<td>(C_{\text{last}} \text{ (TC)} ) ((\mu g \text{ ml}^{-1}))</td>
<td>0.05 ± 0.01</td>
<td>0.3 ± 0.003</td>
<td>0.81 ± 0.12</td>
<td>0.54 ± 0.4</td>
<td>0.52 ± 0.12</td>
</tr>
<tr>
<td>(\text{AUC}_{(0-\text{t})} \text{ (ACV)} ) (min (\mu g \text{ ml}^{-1}))</td>
<td>21.2 ± 5.2</td>
<td>246.2 ± 82.2</td>
<td>347.8 ± 108.2</td>
<td>59.04 ± 4.13</td>
<td>180.7 ± 81.2</td>
</tr>
<tr>
<td>(\text{AUC}_{(0-\text{t})} \text{ (AA)} ) (min (\mu g \text{ ml}^{-1}))</td>
<td>_</td>
<td>19.07 ± 2.5</td>
<td>143.6 ± 51.4</td>
<td>100.8 ± 9.7</td>
<td>_</td>
</tr>
<tr>
<td>(C_{\text{max}} \text{ (ACV)} ) ((\mu g \text{ ml}^{-1}))</td>
<td>0.89 ± 0.18</td>
<td>4.86 ± 1.51</td>
<td>4.06 ± 1.44</td>
<td>1.11 ± 0.38</td>
<td>1.66 ± 0.6</td>
</tr>
<tr>
<td>(C_{\text{max}} \text{ (AA)} ) ((\mu g \text{ ml}^{-1}))</td>
<td>_</td>
<td>0.41 ± 0.2</td>
<td>0.99 ± 0.54</td>
<td>0.54 ± 0.14</td>
<td>_</td>
</tr>
<tr>
<td>(\text{Cl/F} \text{ (ACV)} ) (ml min(^{-1}))</td>
<td>161.3 ± 36.2</td>
<td>17.6 ± 6.6</td>
<td>12.5 ± 5.5</td>
<td>15.6 ± 1.12</td>
<td>8.5 ± 2.5</td>
</tr>
<tr>
<td>(\text{Cl/F} \text{ (AA)} ) (ml min(^{-1}))</td>
<td>_</td>
<td>_</td>
<td>31.9 ± 5.3</td>
<td>6.91 ± 0.92</td>
<td>_</td>
</tr>
<tr>
<td>(\text{MRT} \text{ (ACV)} ) (min.)</td>
<td>44.8 ± 5.6</td>
<td>62.6 ± 6.6</td>
<td>64.1 ± 5.35</td>
<td>69.2 ± 3.9</td>
<td>94.7 ± 3.3</td>
</tr>
<tr>
<td>(\lambda_{Z} \text{ (ACV)} ) (min(^{-1}))</td>
<td>0.015 ± 0.001</td>
<td>0.004 ± 0.0001</td>
<td>0.005 ± 0.001</td>
<td>0.01 ± 0.005</td>
<td>0.008 ± 0.004</td>
</tr>
<tr>
<td>(\text{MRT} \text{ (AA)} ) (min.)</td>
<td>_</td>
<td>12.6 ± 2.5</td>
<td>110.9 ± 18.7</td>
<td>129.1 ± 9.5</td>
<td>_</td>
</tr>
<tr>
<td>(\lambda_{Z} \text{ (AA)} ) (min(^{-1}))</td>
<td>_</td>
<td>_</td>
<td>0.005 ± 0.003</td>
<td>0.001 ± 0.0005</td>
<td>_</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. (n=3-6);
TC- Total concentration in terms of ACV; ACV- Acyclovir; AA- amino acid intermediate.
Table 4 Pharmacokinetic Parameters following systemic absorption

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ACV</th>
<th>VACV</th>
<th>GVACV</th>
<th>VVACV</th>
<th>VYACV</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(0-last) (TC) (min µg ml(^{-1}))</td>
<td>20.4 ± 3.8</td>
<td>237.1 ± 64.6</td>
<td>633.8 ± 115.5</td>
<td>157.3 ± 74.2</td>
<td>225.7 ± 26.4</td>
</tr>
<tr>
<td>AUC(_{\text{inf}}) (TC) (min µg ml(^{-1}))</td>
<td>29.9 ± 4.2</td>
<td>288.6 ± 59.4</td>
<td>900.7 ± 74.8</td>
<td>258.2 ± 37.5</td>
<td>290.9 ± 38.7</td>
</tr>
<tr>
<td>C(_{\text{max}}) (TC) (µg ml(^{-1}))</td>
<td>0.83 ± 0.19</td>
<td>5.44 ± 2.33</td>
<td>7.2 ± 3.3</td>
<td>1.27 ± 0.22</td>
<td>1.85 ± 0.31</td>
</tr>
<tr>
<td>T(_{\text{max}}) (TC) (min)</td>
<td>24.0 ± 5.4</td>
<td>20.0</td>
<td>22.2 ± 8.3</td>
<td>15.0 ± 10.0</td>
<td>15.0 ± 10.0</td>
</tr>
<tr>
<td>C(_{\text{last}}) (TC) (µg ml(^{-1}))</td>
<td>0.06 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>1.1 ± 0.21</td>
<td>0.42 ± 0.26</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td>AUC(_{(0-t)}) (ACV) (min µg ml(^{-1}))</td>
<td>20.47 ± 3.8</td>
<td>232.53 ± 66.04</td>
<td>571.68 ± 99.22</td>
<td>96.8 ± 24.4</td>
<td>226.72 ± 26.72</td>
</tr>
<tr>
<td>AUC(_{(0-t)}) (AA) (min µg ml(^{-1}))</td>
<td>_</td>
<td>15.02 ± 0.48</td>
<td>133.1 ± 48.2</td>
<td>95.8 ± 1.1</td>
<td>_</td>
</tr>
<tr>
<td>C(_{\text{max}}) (ACV) (µg ml(^{-1}))</td>
<td>0.83 ± 0.19</td>
<td>5.30 ± 2.38</td>
<td>6.9 ± 1.50</td>
<td>0.82 ± 0.39</td>
<td>1.85 ± 0.31</td>
</tr>
<tr>
<td>C(_{\text{max}}) (AA) (µg ml(^{-1}))</td>
<td>_</td>
<td>1.08 ± 0.01</td>
<td>1.30 ± 0.89</td>
<td>0.45 ± 0.01</td>
<td>_</td>
</tr>
<tr>
<td>Cl/F (ACV) (ml min(^{-1}))</td>
<td>150.5 ± 12.6</td>
<td>15.5 ± 3.1</td>
<td>13.3 ± 3.5</td>
<td>13.9 ± 1.36</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td>Cl/F (AA) (ml min(^{-1}))</td>
<td>_</td>
<td>_</td>
<td>26.8 ± 4.5</td>
<td>12.2 ± 2.6</td>
<td>_</td>
</tr>
<tr>
<td>MRT(_{(ACV)}) (min.)</td>
<td>45.82 ± 5.31</td>
<td>60.36 ± 8.76</td>
<td>79.61 ± 10.64</td>
<td>50.98 ± 18.21</td>
<td>102.4 ± 5.02</td>
</tr>
<tr>
<td>λ(_{Z}) (ACV) (min(^{-1}))</td>
<td>0.017 ± 0.007</td>
<td>0.0052 ± 0.001</td>
<td>0.006 ± 0.001</td>
<td>0.009 ± 0.001</td>
<td>0.0072 ± 0.003</td>
</tr>
<tr>
<td>MRT(_{(AA)}) (min.)</td>
<td>_</td>
<td>12.8 ± 0.16</td>
<td>73.2 ± 28.9</td>
<td>126.1 ± 3.21</td>
<td>_</td>
</tr>
<tr>
<td>λ(_{Z}) (AA) (min(^{-1}))</td>
<td>_</td>
<td>_</td>
<td>0.006 ± 0.002</td>
<td>0.005 ± 0.001</td>
<td>_</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. (n=3-6);
TC- Total concentration in terms of ACV; ACV- Acyclovir; AA- amino acid intermediate. C\(_{\text{max}}\) (AA) peak concentration of amino acid intermediate; C\(_{\text{max}}\) (ACV) peak concentration of ACV; MRT\(_{AA}\) (min.)- mean residence time of amino acid intermediate; MRT\(_{ACV}\) (min.)- mean residence time of ACV.
<table>
<thead>
<tr>
<th>Prodrug</th>
<th>R</th>
<th>Prodrug</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>VACV</td>
<td></td>
<td>VVACV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VYACV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GVACV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GYACV</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1
Figure 2

![Graph showing concentration (mcg/ml) against time (min.) for systemic and intestinal absorption.](image)

- **Systemic Absorption**
- **Intestinal Absorption**

Figure 2
Figure 3
Figure 4

A
- Systemic Absorption
- Intestinal Absorption

B
- Systemic-VACV
- Systemic-ACV
- Intestinal-VACV
- Intestinal-ACV

Figure 4
Figure 5

A
- Systemic Absorption
- Intestinal Absorption

B
- Systemic-VACV
- Systemic-ACV
- Intestinal-VACV
- Intestinal-ACV
Figure 6

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 7

- Gly-Val-ACV
- Val-Val-ACV
- Val-ACV, Val-Tyr-ACV, Gly-Tyr-ACV
- Val-ACV

Dipeptidases

Esterases

Biphenyl hydrolase-like (BPHL) protein

BPHL, Esterases

ACV