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Synthesis, metabolism and cellular permeability of enzymatically stable dipeptide prodrugs of acyclovir

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ABSTRACT

The objective of this study was to synthesize and evaluate novel enzymatically stable dipeptide prodrugs for improved absorption of acyclovir. L-Valine-L-valine-acyclovir (LLACV), L-valine-D-valine-acyclovir (LDACV), D-valine-L-valine-acyclovir (DLACV) and D-valine-D-valine-acyclovir (DDACV) were successfully synthesized. The uptake and transport studies were conducted on a Caco-2 cell line. Buffer stability and metabolism of the prodrugs in Caco-2, rat intestine and liver homogenates were studied.

Structure and purity of the all compounds were confirmed with LC–MS/MS and NMR spectroscopy. Uptake and transport of [^{3}H] glycylsarcosine was inhibited by all prodrugs except DDACV. DLACV and DDACV exhibited no measurable degradation in Caco-2 homogenate. Except DDACV other three prodrugs were hydrolyzed in rat intestine and liver homogenates. The order of permeability across Caco-2 was LDACV > LLACV > DDACV > DLACV. A linear correlation between the amount of prodrug transported and over all permeability of acyclovir was established. This study shows that the incorporation of one D-valine in a dipeptide did not abolish its affinity towards peptide transporters (PEPT). Moreover, it enhanced enzymatic stability of prodrug to a certain extent depending on the position in a dipeptide conjugate. This strategy improved both the cellular permeability and the amount of intact prodrug transported which would enable targeting the nutrient transporters at blood ocular barrier (BOB).

1. Introduction

Infection with herpes simplex virus is the single most frequent cause of corneal opacities in developed countries. Following the initial episode of HSV keratitis, there is approximately a 50% chance of recurrence within 2 years (McGill et al., 1976). This reactivation leads to secondary infection that can cause dendritic keratitis which is the most common cause of corneal opacity in developed countries (Dawson and Togni, 1976). The reoccurrence can only be prevented by comprehensive elimination of virus particles from the body. Continuous suppression rather than intermittent dosing was suggested as the preferred therapeutic intervention.

Genital herpes is also caused by herpes simplex infection, the incidence of which has increased significantly over the past 20 years (Fleming et al., 1997). In United States, the prevalence of this infection has increased by approximately 30% since late 1970's. About 86 million people suffer with genital infection worldwide (Halioua and Malkin, 1999).

Acyclovir was approved as a drug of choice for herpes simplex infections. However, it has limited oral bioavailability, which is primarily due to its poor solubility and permeation across tissues. Various strategies have been investigated to improve the cellular permeability of acyclovir. Transporter targeted delivery appears to be a promising approach in which drug is attached to a ligand (promoiety) which is a substrate for specific nutrient transporter. Valacyclovir (VACV) is a valine ester prodrug of acyclovir which results increase in the oral bioavailability of acyclovir three- to five-fold (Jacobson, 1993; Soul-Lawton et al., 1995; Perry and Faulds, 1996). Such absorption enhancement is due its recognition and translocation of the prodrug by peptide transporters (PEPT). Eventually, a series of novel water-soluble dipeptide ester prodrugs of acyclovir were designed to target the PEPT on intestine as well as cornea (Anand et al., 2003a,b).

Systemic drug delivery (intravenous or oral) is potentially an effective route to treat various ocular disorders. However, drugs administered by this route must cross the blood ocular barriers (BOB) to reach the inner ocular tissues. BOB is comprised of blood aqueous and blood retinal barrier. The transport of hydrophilic molecules like acyclovir and ganciclovir from systemic route is restricted by BOB. Recently, a functionally active PEPT was identified on both blood aqueous and blood retinal barrier (Dias et al., 2002; Atluri et al., 2004). The expression of PEPT can be utilized

Abbreviations: LLACV, L-valine-L-valine-acyclovir; LDACV, L-valine-D-valine-acyclovir; DLACV, D-valine-L-valine-acyclovir; DDACV, D-valine-D-valine-acyclovir.

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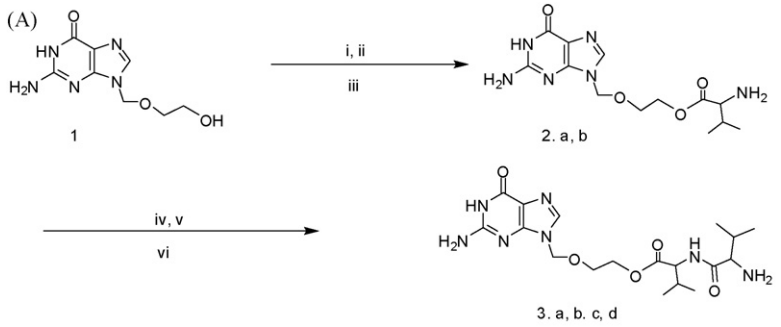

(B)

ACV-D-Val(2a): Yield: 85%, LC/MS(M/z): 325.4, NMR(CD_3OD): δ 0.94-0.96, (m, 6H), 1.04-1.06(m, 1H), 2.12-2.17(m, 1H), 3.77-3.85(m, 3H), 4.23-4.28(m, 1H), 5.45(s, 2H), 7.84(s, 1H)

ACV-D-Val-D-Val(3a): Yield: 83%, LC/MS(M/z): 424.4, NMR(CD_3OD): δ (0.89-0.92(m, 3H), 0.98-1.03(m, 3H), 1.09-1.18(m, 3H), 1.25-1.33 (m, 3H), 2.07-2.16(m, 2H), 3.39-3.44(m, 1H), 3.73-3.75(brs, 1H), 3.79-3.82(m, 2H), 4.22-4.27(m, 2H), 5.51(s, 2H), 8.30(s, 1H)

ACV-D-Val-L-Val(3b): Yield: 92%, LC/MS(M/z): 424.4, NMR(CD_3OD): δ 0.86-0.90 (m, 6H), 1.01-1.06(m, 6H), 2.01-2.10(m, 1H), 2.14-2.19(m, 1H), 3.76-3.78(m, 3H), 4.22-4.24(m, 2H), 4.32-4.33(brs, 1H), 5.47(s, 2H), 7.99(s, 1H)

ACV-L-Val-D-Val(3c): Yield: 86.5%, LC/MS(M/z): 424.4, NMR(CD_3OD): δ 0.86-0.94 (m, 3H), 0.98-1.06(m, 3H), 1.19-1.34(m, 6H), 2.15-2.20(m, 2H), 3.20-3.29(m, 2H), 3.78-3.86(m, 2H), 4.24-4.33(m, 2H), 5.47(s, 2H), 8.11(s, 1H)

Fig. 1. Synthesis and characterization. (A) Synthetic scheme: (i) a, Boc-D-Val-OH; b, Boc-L-Val-OH, DCC, DMF, 0 °C, 1 h. (ii) DMAP, DMF, rt, 48 h. (iii) TFA/DCM (2:1), 0 °C, 2.5 h. (iv) TEA, rt, 10 min. (v) a, Boc-L-Val-OH; b, Boc-D-Val-OH, DCC, DMF, 0 °C, 1 h, rt, 48 h; (vi) TFA/DCM (2:1), 2.5 h. (B) NMR spectra, LC/MS of all the new compounds.

to increase the ocular bioavailability of acyclovir following oral or intravenous administration.

However, effective absorption by the PEPT at BOB requires the prolonged presence of intact prodrug at the site of absorption. VACV as well as the dipeptide prodrugs of acyclovir given orally were found to be rapidly metabolized to parent drug (Soul-Lawton et al., 1995; Anand et al., 2004). As a result, the availability of prodrug to ocular transporters expressed at BOB is extremely limited. Enhanced residence time of intact prodrug in the systemic circulation will facilitate targeting the transporters present on blood ocular barrier after oral or systemic administration. Hence, there is a need for design of prodrugs that are fairly stable in the systemic circulation such that translocation by the nutrient transporters at BOB can be achieved in therapeutic range.

Even though the oral bioavailability of VACV is three- to five-fold higher than acyclovir it is still incomplete (54.5%) possibly due to its premature hydrolysis in intestinal lumen (Granero and Amidon, 2006). Such luminal hydrolysis will minimize the amount of intact prodrug available to the transporter on the intestinal epithelium. Enzymatically stable prodrugs are needed in order to increase the both the oral as well as the ocular bioavailability of acyclovir after oral administration.

Hydrolytic enzymes (peptidases and esterases) responsible for the bioreversion of dipeptide prodrugs are stereospecific and have high affinity for L-isomers. Hence, D-isomers were incorporated into the dipeptide moieties at a definite position to modulate the rate of metabolism of the prodrugs. Previous studies have reported that incorporation of a D-isomer into a dipeptide causes high retention of its affinity towards PEPT and also imparts higher enzymatic stability (Tamura et al., 1996; Steffansen et al., 1999; Friedrichsen et al., 2001).

In this work, a series of stereoisomeric valine–valine based dipeptide ester prodrugs of acyclovir were designed. The prodrugs include LLACV, LDACV, DLACV and DDACV. They were evaluated for their ability to interact with PEPT, enzymatic stability and permeability across Caco-2. Emphasis was placed on mechanistic details about the effect of rate of prodrug hydrolysis on cellular permeability.

The interaction and transport studies were conducted on Caco-2. This cell line is known to express the human di/tripeptide transporter hPEPT1. This model cell line has been employed to characterize peptidomimetics and other peptide substrates that were recognized by the peptide transporter (Nielsen et al., 2001; Anand et al., 2003b).

2. Materials and methods

2.1. Materials

[^{3}H] Glycylsarcosine (Gly-Sar-4 Ci/mmol) was procured 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 (Manassas, VA). Phospholipon^R 90G was obtained as a gift from American Lecithin Company. The growth medium, Dulbecco's modified Eagle's medium, was obtained from Invitrogen (Carlsbad, CA). Minimal essential medium, non-essential amino acids, penicillin, streptomycin, sodium bicarbonate, HEPES, unlabelled Gly-Sar and cephalexin were purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). Culture flasks (75-cm^2 growth area) and polyester transwells (pore size 0.3 μm with diameter of 6.5 mm) were procured from Costar (Cambridge, MA). The buffer components and solvents were obtained from Fisher Scientific Co. (Fair Lawn, NJ). All the dipeptide prodrugs of acyclovir were synthesized in our laboratory. All other chemicals were purchased from

Sigma chemical company and were used without further purification.

2.2. Synthesis

The prodrugs LLACV, LDACV, DLACV and DDACV were synthesized according to our previously published procedure with slight modifications (Nashed and Mitra, 2003). The synthesis scheme is shown in Fig. 1A. The products were purified by silica gel column chromatography and the final products were obtained by deprotection using TFA. The compounds were recrystallized from cold diethyl ether. The reaction progress was monitored by TLC and LC/MS/MS. The structures of the intermediate and final compounds were confirmed by ^{1}H NMR spectroscopy and mass spectra analysis.

^{1}H NMR was carried out using a Varian-400 MHz NMR spectrometer. Chemical shifts (δ) were expressed in parts per million (ppm) relative to the NMR solvent signal (CD3OD, 3.31 ppm for proton) using tetra methyl silane as an internal standard. Mass analysis was carried using a hybrid triple quadrupole linear ion trap mass spectrometer (Q trap LC/MS/MS spectrometer, Applied Biosystems) under enhanced mass (EMS) mode.

2.3. Cell culture

Caco-2 cell line between passages 28 and 38 was utilized for the study. All cultures were maintained in humidified incubator at 37 °C with 5% carbon dioxide in air atmosphere. The culture medium containing Dulbecco's modified Eagle's medium, 10% FBS (heat-inactivated), 1% non-essential amino acids, 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 14 mM HEPES at pH 7.4 was added according to the protocol established in our laboratory for maintaining the cell line. At 80% confluency, cells were removed by treating with trypsin/EDTA and then plated at a density of 100,000 cells/cm^2 on collagen-coated transwell (polycarbonate membranes) and 12-well tissue culture plastic plates for transport and uptake studies, respectively. The culture medium was replaced every alternate day over 1-week period and daily thereafter. The monolayers grown for 21–23 days postseeding were utilized for all uptake and transport studies.

2.4. Uptake and transport studies

Uptake and transport studies across Caco-2 were conducted according to standard protocol for 15 min and 3 h, respectively (Udata et al., 2003). Uptake and transport of 0.25 uCi/ml of [^{3}H] GS was determined in the presence and absence (controls) of all prodrugs to delineate their interactions with PEPT. For all the interaction studies, 10 mM concentrations of prodrugs and Gly-Sar were used. One mM concentrations of prodrugs were used for the transport studies. Permeability of all the prodrugs across Caco-2 was determined from the transport data. Since PEPT is a proton-dependent transporter, all the studies were conducted with a pH gradient of 6.0 on the donor and 7.4 on the receiver side. For the transport studies, the integrity of cell layer was ensured with [^{14}C] mannitol transport.

2.5. Aqueous stability

Chemical stability of LDACV, DLACV and DDACV was determined in Dulbecco's phosphate buffer at pH 5.0, 6.0 and 7.4 using 1 mM concentration of each prodrug. The pH of all the buffers was adjusted with a calibrated pH meter before initiating a study. Experiments were conducted at 37 °C for 1-week period. 9 ml of buffer was taken in a screw capped vials and equilibrated using a constant shaker water bath set at 37 °C and 60 rpm. To the buffer 1 ml of prodrug solution was added to initiate the study. Aliquots (100 μl) were taken at predetermined time intervals and injected into HPLC for analysis.

2.6. Metabolism studies

Confluent Caco-2 cells grown in tissue culture flasks were washed three times with DPBS (pH 7.4). They were scrapped into ice-cold DPBS pH 7.4 and homogenized with a glass homogenizer. The homogenate was centrifuged and the supernatant was collected. The amount of protein in the supernatant was estimated by the method of Bradford (1976) with bovine serum albumin as the standard (protein estimation kit; Bio-Rad, Hercules, CA). For intestinal and liver homogenate studies, tissues were obtained from Sprague–Dawley male rats. Animals were euthanized with sodium pentobarbital and tissues were cleaned with cold DPBS pH 7.4 and homogenized with a tissue homogenizer (Tissue tearer model 985-370).

An aliquot, 800 μl of the supernatant was incubated in a shaking water bath set at 37 °C and 60 rpm. Subsequently, 200 μl of a 1 mM prodrug solution was added to initiate the metabolic activity. Samples (50 μl) were withdrawn at appropriate time intervals into microcentrifuge tubes prefilled with 50 μl of ice-cold organic mixture containing acetonitrile and methanol (3:4) to precipitate the cellular proteins and stop the reaction. The samples were stored at −80 °C until further analysis. For the HPLC analysis, the samples were thawed and centrifuged to remove any precipitated protein. The supernatant was injected into HPLC. The apparent first-order rate constants were calculated from the slope of log prodrug concentration vs. time plot and corrected for any chemical hydrolysis observed with the control.

The hydrolysis of the prodrugs was also studied in Fasted state Simulated Intestinal fluid (FaSSIF). The composition of FaSSIF was published elsewhere (Dressman et al., 1998; Ingels et al., 2004).

2.7. Analysis

All the uptake samples were analyzed with a liquid scintillation counter (Beckman Instruments Inc., Model LS-6500) and rate of uptake was normalized to the protein content of each well. The amount of protein in the cell lysate was measured by Bio-Rad protein estimation kit using bovine serum albumin as standard (Bio-Rad protein estimation kit, Hercules, CA).

All non-radioactive samples were analyzed with HPLC. The system comprised of a Rainin Dynamax Pump SD-200 and a HP 1100 series fluorescence detector set at an excitation λ of 285 nm and an emission λ of 360 nm. C18 Luna 4.6 mm × 250 mm (Phenomenex) and C8 Luna 4.6 mm × 250 mm columns were used. The mobile phase consisted of a mixture of 25 mM potassium phosphate buffer with varying percentages of acetonotrile depending on the prodrug to be detected. The flow rate was maintained 1 ml/min. The method was validated with parameters such as selectivity, precision and accuracy. The accuracy was determined by checking the closeness of mean test results obtained by this method to the true concentration of the analyte. The precision was measured by replicate analysis of standards with known concentrations.

2.8. Permeability calculations

The flux and the permeability values were calculated according to Fick's first law of diffusion. The flux was obtained by dividing the slope of cumulative amount of drug/prodrug transported vs. time

plot with cross-sectional area of transwell.

$$\text{flux}(J) = \frac{(dM/dT)}{A}, \quad A, \text{area of the transwell.} \tag{1}$$

The permeability was calculated by dividing the flux with the concentration of donor.

$$P = \frac{\text{flux}(J)}{\text{donor concentration (Cd)}} \tag{2}$$

2.9. Statistical analysis

Student's *t*-test was used to determine the statistical difference between two sets. $p < 0.05$ was considered as statistically significant. All the experiments were performed at least in triplicates.

3. Results

3.1. Synthesis

The synthetic scheme is provided in Fig. 1A. The yield, mass and NMR spectra for all the compounds are given in Fig. 1B.

3.2. HPLC analysis

Blank samples were obtained from appropriate biological matrix (Caco-2, liver and intestine). No interfering peaks were observed and thus the selectivity was ensured. The mean value obtained for accuracy measurements was within 15% of the actual value. The R.S.D. was less than 3% for all the concentrations tested for precision.

3.3. Aqueous stability

All the prodrugs showed excellent aqueous stability. The chemical stability of LLACV was studied earlier (Anand et al., 2003b). In that study, it was observed that the dipeptide prodrugs were relatively more stable at acidic pH than at neutral and alkaline pH. Among the prodrugs studied, only LDACV showed significant degradation at pH 8.5 with a first-order rate constant of $0.011 \pm 0.0004\,h^{-1}$. With other prodrugs, no hydrolysis was observed at any pH during the time course of a study. This study demonstrates that these prodrugs were highly stable and can be stored for longer periods at slightly acidic pH.

3.4. Interactions with PEPT

All the prodrugs except DDACV inhibited both the uptake and transport of [^{3}H] GS into Caco-2 indicating their ability to interact with PEPT (Figs. 2 and 3). Among all the prodrugs studied, DLACV caused the most inhibition. In the transport studies, DDACV was not included as it did not inhibit the uptake of [^{3}H] GS. Interaction of LLACV with PEPT was already reported from our laboratory (Anand et al., 2003b). In that study, it was observed that 10 mM of LLACV had significantly inhibited the transport of [^{3}H] GS across Caco-2 demonstrating its ability to interact with PEPT.

3.5. Hydrolysis of prodrugs in cell and tissue homogenates

Hydrolysis of all the prodrugs was studied in FaSSIF, Caco-2, intestinal and liver homogenates. The dipeptides with a D-valine at terminal position were less susceptible to enzymatic hydrolysis. A compound with two D-valine moieties, DDACV was found to be extremely stable as no metabolism was detected in any of the cell/tissue homogenates studied. Except LLACV no other prodrug

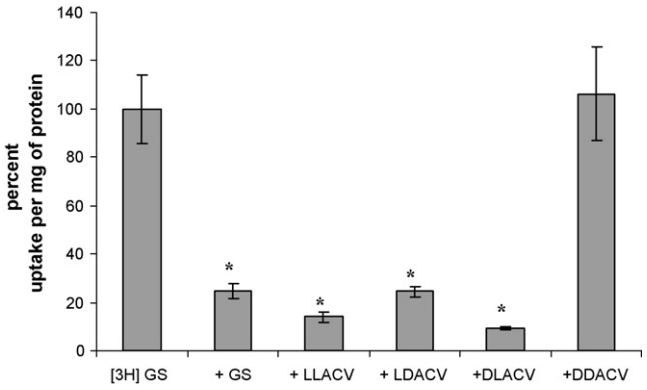

Fig. 2. Percent uptake of [^{3}H] GS by Caco-2 in presence of all prodrugs. Each value is expressed as percent uptake relative to control ([^{3}H] GS alone). Asterisks (*) represent significant difference from the control ($p \leq 0.05$).

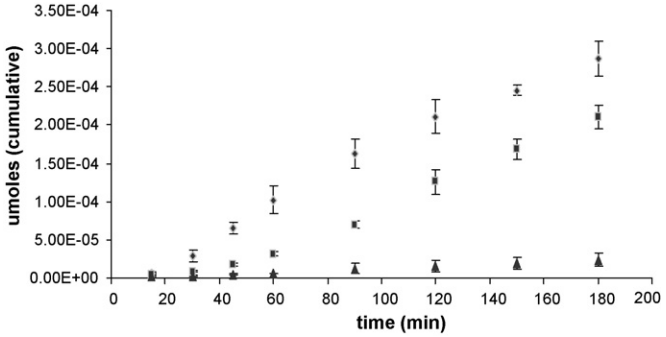

Fig. 3. Transport of [^{3}H] GS in presence of 1 mM LDACV and DLACV across Caco-2. Each value is represented as mean ± S.D. ($n = 4$) (♦, [^{3}H GS]; ■, [^{3}H GS] + LDACV; ▲, [^{3}H GS] + DLACV).

was hydrolyzed in FaSSIF (Fig. 4). In Caco-2 homogenate, the first-order rate constants for LLACV and LDACV degradation are reported (Table 1), however, no degradation of DLACV was observed. The bioconversion pathway of LDACV was followed in Caco-2 homogenate (Fig. 5). LDACV bioconversion involves the hydrolysis of the peptide bond resulting in formation of amino acid conjugate DACV. Esterases can subsequently cleave DACV ester bond to parent drug ACV. A small amount of ACV might have been also formed directly from the dipeptide (Fig. 5).

In both intestine and liver homogenates, LLACV disappeared in less than 10 min. The degradation first-order rate constants as well as the half lives of all the prodrugs in intestinal and liver homogenates are summarized in Tables 2 and 3. The order of enzymatic stability was DDACV > DLACV > LDACV > LLACV. For all the

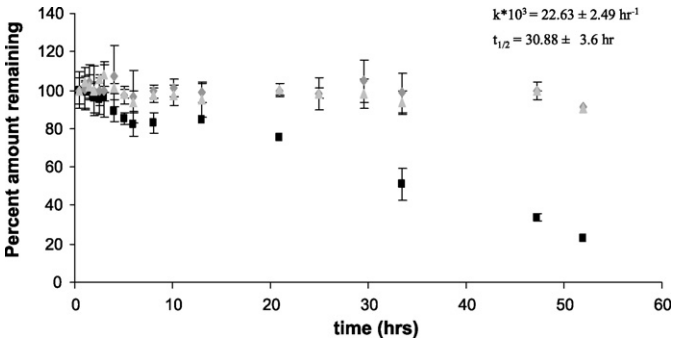

Fig. 4. Rate of degradation of LLACV, LDACV and DLACV in FaSSIF. Each value is represented as mean ± S.D. ($n = 3$) (▲, LDACV; ▼, DLACV; ■, LLACV).

Table 1
Stability in Caco-2 homogenate—first-order degradation rate constants and half lives of all prodrugs

Drug	$K \times 10^3$ (h^{-1})	$t_{1/2}$ (h)
LLACV	92.23 ± 4.79	7.52 ± 0.40
LDACV	13.33 ± 1.96	52.80 ± 8.42
DLACV	[a]	[a]
DDACV	[a]	[a]
D-Val-ACV	29.86 ± 4.71	23.56 ± 3.42

[a] No degradation during the time of study. Each value is represented as mean ± S.D. ($n = 3$).

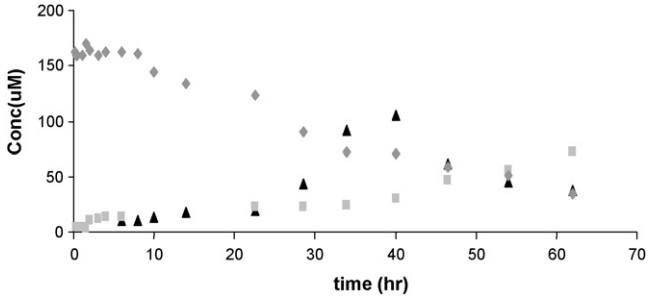

Fig. 5. Bioconversion pathway of LDACV in Caco-2 homogenate (♦, LDACV; ▲, D-Val-ACV; ■, acyclovir).

prodrugs the rate of hydrolysis was higher in liver relative to intestine.

3.6. Permeability studies

From the transport data cumulative amount (sum of prodrug, the regenerated aminoacid metabolite and the parent drug) was plotted with time. The apparent permeabilities (Papp) of all the prodrugs across Caco-2 were calculated from the linear portion of the plot according to Eq. (1). All the prodrugs except DLACV enhanced acyclovir permeability. Among them, LDACV was the most permeable (Fig. 6). The value was two times of L-Val-ACV and LLACV. DLACV has the lowest permeability of all. The order of the apparent permeability across Caco-2 was LDACV > LLACV > L-Val-ACV > DDACV > DLACV.

Table 2
Stability in rat intestinal homogenate—first-order degradation rate constants and half lives of all prodrugs

Drug	$K \times 10^3$ (h^{-1})	$t_{1/2}$ (h)
LLACV	–	<0.08
LDACV	688.23 ± 48.68	1.01 ± 0.07
DLACV	110.06 ± 4.52	6.27 ± 0.25
DDACV	[a]	[a]

[a] Represents no degradation during the time of study. Symbol "–" represents the whole prodrug degraded in less than first sampling time point (5 min). Each value is represented as mean ± S.D. ($n = 3$).

Table 3
Stability in rat liver homogenate—first-order degradation rate constants and half lives of all prodrugs

Drug	$K \times 10^3$ (h^{-1})	$t_{1/2}$ (h)
LLACV	–	<0.08
LDACV	1388.23 ± 64.24	0.49 ± 0.02
DLACV	245.06 ± 11.25	2.82 ± 0.18
DDACV	[a]	[a]

[a] Represents no degradation during the time of study. Symbol "–" represents the whole prodrug degraded in less than first sampling time point (5 min). Each value is represented as mean ± S.D. ($n = 3$).

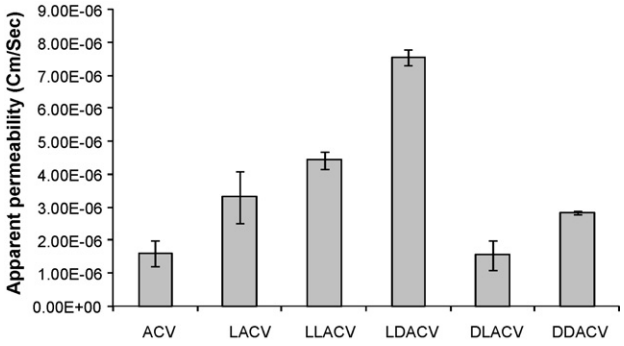

Fig. 6. Apparent permeability of all the prodrugs across Caco-2. Each value is represented as mean ± S.D. ($n = 4$).

3.7. Correlation between amount of prodrug transported and apparent permeability

The total amount of prodrug (dipeptide and amino acid conjugate) transported across the cell layer varied depending on the rate of bioconversion during their translocation across Caco-2 cells. The highest amount of prodrug was observed in the receiver chamber with LDACV followed by LLACV, DDACV and DLACV. With DLACV and DDACV about 90% of the drug transported was intact dipeptide conjugate which is probably due to its high excellent enzymatic stability in Caco-2 homogenate. An excellent linear correlation between the amount of intact prodrug transported and the overall apparent permeability of the prodrug was noted (Fig. 7).

4. Discussion

The concept of modulating the enzymatic hydrolysis rate of prodrugs and its implications in drug delivery have not been exploited to any large extent. Novel enzymatically stable dipeptide conjugates of acyclovir were developed and the Effect of enzymatic stability of prodrug on cellular permeability was explained. Results indicate that incorporation of one D-amino acid into a dipeptide does not abolish its affinity towards PEPT. Moreover, it confers high stability against metabolizing enzymes which could result in higher cellular permeability.

In this study, a series of Val–Val diastereomers of acyclovir were synthesized and the importance of enzymatic stability in determining the cellular permeability was evaluated. Interaction studies have shown that all the prodrugs except DDACV have retained their affinity towards PEPT with varying degrees of affinity (Figs. 2 and 3). The order of their affinity was DLACV > LLACV > LDACV. Incorpora-

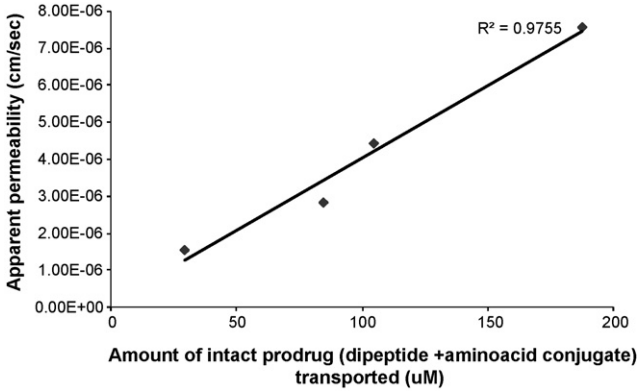

Fig. 7. Correlation between apparent permeability and amount of intact prodrug (dipeptide and amino acid conjugate) transported across Caco-2.

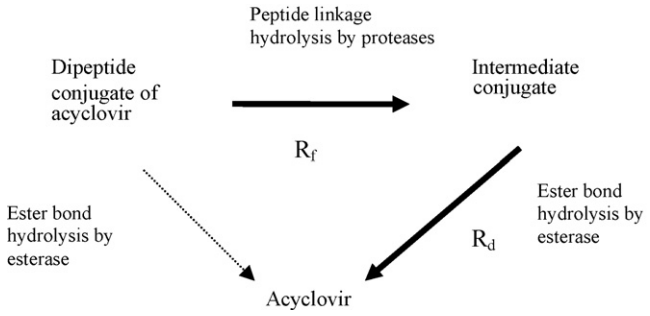

Fig. 8. Enzymatic hydrolysis mechanism of dipeptide prodrugs of acyclovir.

tion of two D-isomers completely abolished their affinity towards PEPT. These results are in conjunction with the study done by Tamura et al. where only the dipeptide moiety was studied (Tamura et al., 1996). Among all the prodrugs studied only LLACV and LDACV hydrolyzed in Caco-2 homogenate and LDACV was relatively more stable of the two compounds. Thus, the incorporation of D-amino acid had increased the metabolic stability of the dipeptide conjugate. The hydrolysis mechanism of dipeptide prodrugs of acyclovir was explained before (Anand et al., 2003a,b; Katragadda et al., 2006). The peptidases hydrolyze the dipeptide bond generating the amino acid ester of acyclovir which will be further hydrolyzed by esterase producing acyclovir. Formation of acyclovir directly from dipeptide is also possible albeit to a small extent (Fig. 8). LDACV also hydrolyzed initially into D-Val-ACV which was evident by rapid increase in D-Val-ACV concentration and then it gradually decreases as it was further acted upon by esterase and aminopeptidases to release acyclovir (Fig. 5). The enzyme concentration available in Caco-2 homogenate may not be sufficient to hydrolyze all the prodrugs. Hence, the metabolism studies were carried out in rat intestine and liver tissue homogenates. The results indicate that DDACV was the most stable followed by DLACV. Thus, incorporation of two D-valine moieties into a dipeptide moiety can enhance the enzymatic stability but also abolishes to a large extent its affinity towards PEPT. The degradation half lives of dipeptide with one D-valine indicate that dipeptide with a D-isomer attached to L-valine (DLACV) had more metabolic stability than the one attached to acyclovir (LDACV). This concept is further interpreted by permeability studies.

The incomplete bioavailability of L-Val-ACV was attributed to premature hydrolysis in the intestinal fluid (Granero and Amidon, 2006). These results had shown that stereoisomeric prodrugs might be promising candidates for further improvement in oral bioavailability of acyclovir. The dipeptide conjugates may remain intact to a greater extent in the intestine after oral administration and as a result will be recognized by PEPT and possibly get translocated at a higher rate. It would be interesting to study the degradation of these prodrugs in vivo as they will be subjected to combined hydrolysis effect of both intestine and liver (first-pass effect). All the above studies conclude that both metabolism as well as the affinity towards PEPT can be modulated with the incorporation of one D-isomer at a definite position of a dipeptide.

As the ultimate objective of this work is to examine whether this enhanced enzymatic stability could lead to an increase in the cellular permeability of these dipeptide conjugates, transport studies were conducted across Caco-2 cell line. The order of apparent permeability was LDACV > LLACV > DDACV > DLACV (Fig. 6). LDACV had the highest apparent permeability across Caco-2.

The higher permeability of dipeptide prodrugs was due to intact dipeptide as well as the intermediate, i.e. amino acid conjugate formed from the dipeptide conjugate (Katragadda et al., 2006). Hence, both the rate of formation (R_f) and degradation (R_d) of the transient amino acid conjugate will affect the cellular permeability of dipeptide conjugate (Fig. 8). Ideally, to achieve higher permeability R_f should be much higher than R_d. With LLACV the rate of formation was rapid but the generated L-Val-ACV was relatively less stable. In contrast, with DLACV and DDACV the rate of generation of amino acid intermediate was much slower due to their higher enzymatic stability and less than 10% of aminoacid conjugate was observed in the receiver chamber. In case of LDACV, both rate of formation and the stability of D-Val-ACV were optimum ($R_f \gg R_d$) resulting in enhanced permeability across Caco-2. D-Isomer in LDACV stabilizes the ester linkage whereas in DLACV it stabilizes the peptide linkage. As a result, the rate of formation of aminoacid intermediate (D-Val-ACV) was rapid from LDACV compared to DLACV. However, DDACV was highly stable as both ester and peptide linkages become less susceptible to enzymatic hydrolysis. Hence, to achieve higher absorption it would be prudent to stabilize the ester linkage rather than peptide bond in a dipeptide conjugate.

A good correlation was obtained between the amount of prodrug (dipeptide + aminoacid) transported across Caco-2 and the apparent permeability (Fig. 7). The highest amount of amino acid intermediate was also observed with LDACV (data not shown). Just by enhancing the enzymatic stability of the dipeptide conjugate may not lead to higher permeability. The stability should be improved such that the dipeptide will rapidly generate the intermediate amino acid conjugate that is also stable enough to permeate across the cell membrane intact.

These stereoisomeric prodrugs of acyclovir could result in higher oral bioavailability of acyclovir which would be beneficial in the treatment of genital herpes infections. Moreover, the enhanced amount of intact prodrug in the systemic circulation would enable targeting the PEPT expressed at blood ocular barriers which could improve the treatment of herpes keratitis.

In summary, these results have shown that the metabolic stability as well as the cellular permeability can be modulated with the incorporation of a D-isomer of an aminoacid at a definite position into a dipeptide conjugate. This concept can be extended to various other drug molecules, especially for increasing their bioavailability in tissues like eye and brain after oral administration.

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