Pharmaceutical Research, Vol. 26, No. 5, May 2009 (© 2008)
DOI: 10.1007/s11095-008-9730-0

Research Paper

Transcorneal Permeation of L- and D-Aspartate Ester Prodrugs of Acyclovir: Delineation of Passive Diffusion *Versus* Transporter Involvement

Soumyajit Majumdar,[1,2,4] Tushar Hingorani,[1] Ramesh Srirangam,[1] Rama Sarma Gadepalli,[3] John M. Rimoldi,[2,3] and Michael A. Repka[1,2]

Received April 30, 2008; accepted September 12, 2008; published online October 7, 2008

Purpose. The aim of this study was to evaluate the contribution of amino acid transporters in the transcorneal permeation of the aspartate (Asp) ester acyclovir (ACV) prodrug.
Methods. Physicochemical characterization, solubility and stability of acyclovir L-aspartate (L-Asp-ACV) and acyclovir D-aspartate (D-Asp-ACV) were studied. Transcorneal permeability was evaluated across excised rabbit cornea.
Results. Solubility of L-Asp-ACV and D-Asp-ACV were about twofold higher than that of ACV. The prodrugs demonstrated greater stability under acidic conditions. Calculated pK_a and logP values for both prodrugs were identical. Transcorneal permeability of L-Asp-ACV $\left(12.1 \pm 1.48 \times 10^{-6} cm/s\right)$ was fourfold higher than D-Asp-ACV $\left(3.12 \pm 0.36 \times 10^{-6} cm/s\right)$ and ACV $\left(3.25 \pm 0.56 \times 10^{-6} cm/s\right)$. ACV generation during the transport process was minimal. L-Asp-ACV transport was sodium and energy dependent but was not inhibited by glutamic acid. Addition of BCH, a specific $B^{0,+}$ and L amino acid transporter inhibitor, decreased transcorneal L-Asp-ACV permeability to $2.66 \pm 0.21 \times 10^{-6} cm/s$. L-Asp-ACV and D-Asp-ACV did not demonstrate significant difference in stability in ocular tissue homogenates.
Conclusion. The results demonstrate that enhanced transport of L-Asp-ACV is as a result of corneal transporter involvement (probably amino acid transporter $B^{0,+}$) and not as a result of changes in physicochemical properties due to prodrug derivatization (permeability of D-Asp-ACV and ACV were not significantly different).

KEY WORDS: amino acid transporter; Asp-ACV; cornea; permeability; prodrug.

INTRODUCTION

A major challenge in the treatment of anterior segment ocular infections, with topically applied hydrophilic therapeutic agents, is restricted transcorneal penetration, a major pathway for drug absorption into the anterior segment of the eye. The cornea is a complex tissue consisting of an outer lipophilic epithelium, followed by a hydrophilic stroma and an endothelial layer. Tight junction proteins, expressed on the corneal epithelial cells, act as the primary barrier to the diffusion of hydrophilic molecules (1,2). Other factors such as loss of drug due to lacrimal and conjunctival drainage, increased tear flow and short contact duration also limit absorption of hydrophilic compounds into the ocular tissues, following topical instillation.

Corneal infections, if not treated aggressively, may lead to corneal scarring and permanent vision loss. With respect to infections affecting primarily the superficial corneal epithelium, topical instillation of hydrophilic drug molecules can be a viable treatment strategy. However, for infections located in the deeper layers of the corneal tissue, e.g. the stromal layer, therapy using topically applied hydrophilic compounds is difficult since now the drug has to traverse the corneal epithelium in order to reach the site of infection. Herpetic keratitis is a perfect example of this.

Herpetic keratitis, a corneal infection caused by herpes simplex virus type I (HSV-1), is a major cause of blindness (3). Herpetic keratitis may be superficial, affecting only the corneal epithelial cells, or may affect the deeper layers of the cornea, a condition known as stromal keratitis. Topical trifluridine, vidarabine, idoxuridine and acyclovir (ACV) are currently employed in the treatment of epithelial keratitis (4,5). ACV is a hydrophilic antiviral nucleoside analog that is extremely effective against HSV-1 induced corneal keratitis (6). ACV cannot be formulated as eye drops, since it has a saturation solubility of 1.2 mg/mL in water, and is thus marketed as a 3% ointment (7). Although ACV is significantly less toxic than the other agents, its therapeutic utility is limited to superficial

Electronic supplementary material The online version of this article (doi:10.1007/s11095-008-9730-0) contains supplementary material, which is available to authorized users.

[1] Department of Pharmaceutics, School of Pharmacy, The University of Mississippi, Faser Hall, University, Mississippi 38677, USA.
[2] Research Institute of Pharmaceutical Sciences, The University of Mississippi, University, Mississippi 38677, USA.
[3] Department of Medicinal Chemistry, School of Pharmacy, The University of Mississippi, University, Mississippi 38677, USA.
[4] To whom correspondence should be addressed. (e-mail: majumso@olemiss.edu)

corneal keratitis (8,9). This is due to poor corneal permeability as a result of limited paracellular diffusion (ACV being a hydrophilic molecule) across the epithelial tight junctions. Oral ACV has been demonstrated to be useful in the treatment of systemic as well as ocular HSV infections (10, 11). However, systemic delivery of hydrophilic therapeutic agents for corneal infections is not a very attractive strategy because of nonspecific systemic exposure and the existence of the blood–ocular barriers which restrict diffusion from the systemic circulation into the ocular tissues.

Search for strategies that could improve transcorneal penetration of hydrophilic molecules is thus, currently, an active research area. A number of approaches have been investigated in an attempt to increase corneal permeability of topically instilled ACV, for instance. These include formulation approaches such as ointments, liposomes, collagen shields and nanospheres (12–14). Additionally, attempts to increase transcorneal passive diffusion via the disruption of the membrane structure using penetration enhancers has also been investigated (15). However, possibility of corneal tissue damage is a major concern with the use of penetration enhancers. Moreover, although penetration enhancers improve permeability they do not affect intrinsic solubility.

Currently, the most attractive and non-intrusive strategy for enhancing transcorneal permeability of hydrophilic agents, appears to be transporter targeted prodrug design (16). Transporters are proteins that transfer nutrients across cellular membranes (17). These transporters may be targeted, through rational prodrug design, to enhance permeability of the parent molecules (18–22). Although hydrophilic compounds are ideal for prodrug derivatization, this approach has been demonstrated to be useful for lipophilic compounds also (23). Amino acid and peptide transporters are considered to be the most favorable for drug targeting, as these transporters accept a wide range of substrates and can tolerate significant structural modification to their substrates (24). Additionally, these transporters have higher capacities, making them attractive targets for drug delivery.

Whereas the utility of the corneal peptide transporters, for enhancing transcorneal permeation of peptide transporter targeted ACV ester prodrugs, has been clearly demonstrated (9,18,22), the information on the corneal amino acid transporters remains unclear. The only study that investigates transport of a corneal amino acid transporter targeted prodrug, ACV-glutamate ester (L-Glu-ACV), indicates that although the prodrug is a substrate of the amino acid transporter B^{0+}, and is transported by it, permeability of the prodrug does not seem to differ significantly from that of ACV (24). These results are somewhat confusing since the study also shows that in the presence of a specific inhibitor of B^{0+}, 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic-acid (BCH), an almost 75% decrease in transcorneal permeability occurs. This suggests that there is near complete absence of passive transcorneal diffusion of the prodrug, L-Glu-ACV.

In the present study, utility of the corneal amino acid transporter, $B^{0,+}$, in drug delivery is further evaluated. ACV is used as a model compound whose transcorneal permeability is determined as such and upon targeting the corneal amino acid transporter, B^{0+}, through derivatization of the L-Asp-ACV prodrug. Corneal permeation of D-Asp-ACV is also studied to delineate interaction of the D-amino acid prodrug with the amino acid transporter, B^{0+}, as well as to measure the passive diffusion component of the transcorneal penetration process.

MATERIALS AND METHODS

Materials

Acyclovir was obtained from Hawkins Inc (Minneapolis, USA). Hydroxypropyl beta cyclodextrin (HP-β-CD) and other chemicals were purchased from Sigma (St. Louis, MO, USA). All solvents were of HPLC grade and were obtained from Fisher Scientific (St. Louis, USA).

Synthesis of Acyclovir Prodrugs

The amino acid esters of acyclovir were prepared using standard reactions according to the method of Beauchamp *et al*. (25). Acyclovir (**1**) was treated with the appropriately protected N-CBz and O-Bzl protected aspartic acids (**2L** or **2D**), dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) in anhydrous DMF at ambient temperature, yielding the requisite coupled products **3L** and **3D**. Deprotection of the N-CBz and O-Bz protecting groups was accomplished by treating either **3L** or **3D** with 10% Pd/C in a solvent mix consisting of MeOH, water, THF and 0.5N HCl under a hydrogen atmosphere (1 atm). Workup and crystallization of the resulting products afforded the target amino acid esters **4L** and **4D** as their respective HCl salts. Reactions requiring anhydrous conditions were carried out under an argon atmosphere employing conventional flame-dried glassware protocols. Figure 1 is a schematic representation of the synthetic procedure.

Analysis of the products (**3L/D** and **4L/D**) by LC–MS, NMR, elemental analysis, and optical rotation confirmed their structure and composition. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DXR500 spectrometer and referenced to residual proton or carbon signal of solvent. Optical rotations were performed on a Rudolph Autopol IV polarimeter and data collected at 25°C. Purity of the final products **4L** and **4D** were determined to be >99.9% by LC–MS analysis. LC–MS protocols for the analysis of acyclovir conjugates **4D** and **4L** utilized a Waters Micromass ZQ single quadrupole mass spectrometer coupled to a Waters 2695HPLC, controlled by Mass Lynx 4.0 Software. A reverse-phase C18 column (Phenomenex Prodigy, 5 µm, ODS3, 100 × 4.6 mm) was used with a mobile phase consisting of water (spiked with 0.1% formic acid) and CH_3CN (spiked with 0.1% formic acid) at ambient temperature using a constant flow rate of 0.2 mL/min. The mobile phase gradient of 0% CH_3CN in water was increased linearly to 5% CH_3CN from 0–10 min and then to 25% CH_3CN from 10–15 min. Electrospray ionization (ESI) parameters: capillary voltage, 3.2 kV; cone voltage, 20 V; extractor, 3 V; desolvation temperature, 250°C, desolvation gas flow, 300 L/h, source temperature, 125°C. LC–MS was performed using both TIC and SIR (single ion recording) mode. Diode array detection was also used in a tandem fashion (220–400 nm). Retention times (6.4 min, m/z = 341 (MH+1). Ten microliters of a 100 µg/mL sample was injected for each analysis.

Combustion analysis was performed using a PerkinElmer 2400 Series II CHN Analyzer. Melting points were recorded on a Thomas-Hoover melting point apparatus and are uncorrected.

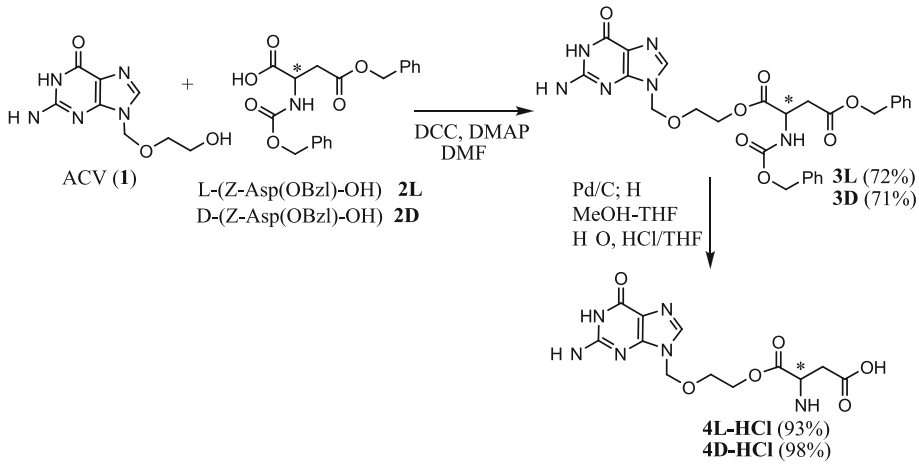

Fig. 1. Schematic representation of synthetic protocol for Asp-ACV conjugates.

Aqueous Solubility Studies

Solubility studies were carried out following standard shake flask method (22). Briefly, excess of L-Asp-ACV or D-Asp-ACV was added to 1 mL vials containing 0.5 mL of water, pre-equilibrated at 25°C. The vials were placed in a shaking water bath, set at 25 ± 0.5°C and 75 rpm, for 24 h. At the end of 24 h, aliquots withdrawn were centrifuged and the supernatant was collected, filtered and analyzed for drug content. Experiments were carried out in duplicate.

Solution Stability Studies

Chemical stability of prodrugs, in aqueous solutions, was examined in hydrochloride (pH 1.2), phthalate (pH 3 and 5) phosphate (pH 7) and borate (pH 9) buffers. Buffers were prepared according to United States Pharmacopoeia (USP) XXX (26). Stability studies were conducted at 25 ± 0.5°C for a period of 7 days. Prodrug stock solutions were prepared in water (1 mg/mL) and used immediately. Aliquots from the stock solutions were added to the buffers in order to achieve a final concentration of 50 μg/mL. Samples were withdrawn at predetermined time points and stored at −80°C until further analysis. Degradation rate constants were calculated from a semi-logarithmic plot of percentage drug remaining as a function of time. Studies were carried out in triplicate.

Solubility as a Function of pH

Buffers, with pH values of 1.2, 3, 5 and 7, were prepared according to USP XXX. Excess of L-Asp-ACV and D-Asp-ACV was then added to 0.5 mL of the buffer (pre-equilibrated to 25 ± 0.5°C) in a shaking water bath, and the studies were terminated at the end of 3 h. The suspension was centrifuged and the supernatant was collected, filtered and analyzed for drug content.

Aqueous Solubility in the Presence of Hydroxypropyl Beta Cyclodextrin

To 0.5 mL of 1% and 5% HP-β-CD solutions, pre equilibrated at 25 ± 0.5°C, excess amount of prodrug was added. Studies were conducted for a period of 24 h. Samples were centrifuged, filtered and analyzed by HPLC.

Animals

Male, albino, New Zealand rabbits weighing between 2 to 2.5 kg were procured from Myrtles' Rabbitry (Thompson Station, TN, USA). Animal experiments conformed to the tenets of Association for Research in Vision and Ophthalmology (ARVO) statement on the Use of Animals in Ophthalmic and Vision Research.

Stability in Tissue Homogenates

Tissue Preparation. Vitreous humor was used as such. Other ocular tissues used in this study were homogenized in ice cold Isotonic Phosphate Buffered Saline (IPBS) using TISSUEMISER (Fisher Scientific, St. Louis, USA). Homogenization was carried out for periods of 30 s, spaced with 1 min intervals, on an ice bath to prevent/minimize enzyme degradation. The homogenate was then centrifuged at 12,500 rpm, for 20 min at 4°C. Protein content in the supernatant was determined according to the method of Bradford (27) and was standardized to 1 mg/mL by diluting with requisite amount of IPBS. The homogenates were used immediately for the enzymatic hydrolysis studies.

Hydrolysis Procedure. Supernatant was equilibrated at 37°C for 30 min. To 0.9 mL of the supernatant, 0.1 mL of 100 μg/mL prodrug stock solution, in IPBS, was added to initiate the stability studies. At predetermined time points, 100 μL aliquots were removed and 100 μL of ice cold 10% glacial acetic acid was immediately added to the samples to precipitate the proteins and arrest the enzymatic degradation process. Prodrug in IPBS was also included as a control in the experimental design to determine chemical hydrolysis rates. These experiments were conducted for a duration of 4 h. Stability in vitreous humor was studied for a period of 8 h. All samples were stored at −80°C until further analysis. At the time of analysis samples were thawed and centrifuged at 12,000 rpm for 10 min and then analyzed by HPLC, as

described under "Analytical Method" section. All experiments were carried out in triplicate.

Corneal Permeation Studies

New Zealand, male, albino, rabbits were anesthetized using ketamine/xylazine administered intramuscularly and euthanized by injecting an overdose of pentobarbital into the marginal ear vein. Eyes were enucleated and were immediately washed with ice cold Dulbecco's Phosphate Buffered Saline (DPBS), pH 7.4, to remove any traces of blood. The corneas were excised immediately; with approximately 1 mm scleral portion remaining adhered to the cornea for ease of mounting. Corneas were mounted between standard, 9 mm, side-by-side diffusion cells (PermeGear Inc., Bethlehem, PA) with the epithelial layer facing the donor side. Temperature was maintained at 34°C during the transport studies with the help of a circulating water bath. Volume of the receiver solution (3.2 mL DPBS) was slightly higher than that of the donor solution (3 mL drug solution) to maintain the natural curvature of the cornea. Drug solution was prepared in DPBS pH 5 ± 0.1 as the prodrugs were stable at this pH for the duration of the experiment (<1% hydrolyzed in 3 h). DPBS pH 7.4 was used as the receiver solution. Contents of both chambers were stirred continuously. Aliquots, 200 µL, were withdrawn at appropriate time intervals and immediately replaced with equal volume of DPBS and stored at −80°C until further analysis.

Na^+ Independent Transport of Prodrugs. To study sodium dependence of the transcorneal L-Asp-ACV permeation mechanism, transport experiments were carried out in Na^+ free medium. Equimolar quantities of choline chloride and K_2HPO_4 were used to replace NaCl and Na_2HPO_4 from the transport medium (DPBS).

Energy Independent Transport of Prodrugs. Corneas were pre-incubated with DPBS, pH 7.4, containing 1 mM Ouabain for 30 min. Ouabain treated corneas were then used for the transport experiments.

Competitive Inhibition Studies. Permeability of the prodrugs was also determined in the presence of the anionic amino acid (L-Glutamic acid, 1 mM) and specific amino acid transporter $B^{0,+}$ inhibitor, BCH (5 mM).

Analytical Method

Samples were analyzed using an HPLC system consisting of Waters 717 plus autosampler, Waters 2475 Multi λ Fluorescence detector (ex λ 270, em λ 380) and Agilent 3395 integrator. Waters C18 Symmetry column, 4.6 × 250 mm, was used. Mobile phase consisted of 15 mM phosphate buffer (pH 2.5) containing 1% acetonitrile. Retention times were 10.5 ± 0.2 min for ACV, 7.4 ± 0.1 min for L-Asp-ACV and 7.5 ± 0.1 min for D-Asp-ACV.

Data Analysis

Flux and permeability calculations were performed as previously reported (9). Data obtained was subjected to statistical analysis using ANOVA. Variance between the groups was checked. A Tukey's HSD was run on all groups. Results were considered statistically significant if p-value was ≤0.05.

RESULTS

Chemical Characterization of the ACV Prodrugs

Structure and composition of L-Asp-ACV and D-Asp-ACV and its hydrochloride salts, synthesized according to the scheme depicted in Fig. 1.The synthetic protocols are outlined below.

(*S*)-1-(2-((2-amino-6-oxo-1H-purin-9(6H)-yl)methoxy)ethyl) 4-benzyl 2-(benzyloxy-carbonylamino)succinate (3L)

Acyclovir (**1**, 500 mg, 2.22 mmol) was dissolved in anhydrous DMF (30 mL) with warming. The resultant solution was cooled to room temperature, and the following were added successively: dimethylaminopyridine (40 mg; 0.30 mmol), *Z*-Asp(OBzl)-OH (**2L**, 1070 mg; 3.0 mmol), dicyclohexylcarbodiimide (780 mg; 3.8 mmol). The progress of the reaction was monitored by LC–MS. The reaction was observed to be complete after 3 days. The heterogeneous mixture was filtered using a sintered glass funnel to remove the urea by-product, and this solid was washed several times with DMF (10 mL). The filtrate was evaporated under reduced pressure at 50°C to remove the majority of DMF. Flash silica gel chromatography of the gummy solid residue (10% $MeOH/CH_2Cl_2$) afforded 900 mg (72%) of **3L** as a white solid. mp. 135–139°C; $[\alpha]_D^{25}$ +10.0 (c = 1.12, $CHCl_3$); LC–MS (ESI+): m/z 587 $(M+Na)^+$; ^{1}H NMR (500 MHz, DMSO-d_6) δ10.65 (s, 1H), 7.85 (d, 1H, J = 8 Hz), 7.81 (s, 1H), 7.34, (bs, 10 H), 6.51 (s, 2H), 5.34 (s, 2H), 5.11 (s, 2H), 5.05 (s, 2H), 4.50 (m, 1H), 4.16 (bs, 2H), 3.65 (s, 2H), 2.85 (m, 1H), 2.77 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.27, 170.17, 157.26, 156.32, 154.40, 151.88, 138.08 (*C*H), 137.23, 136.36, 128.82 (4× C,*C*H)), 128.49 (2× C, *C*H), 128.21 (4× C, *C*H), 117.00, 72.32 (CH_2), 66.76 (CH_2), 66.38 (CH_2), 66.17 (CH_2), 64.30 (CH_2), 50.95 (CH), 36.25 (CH_2).

Elemental analysis calculated for $C_{27}H_{28}N_6O_8$ C, 57.44; H, 5.00; N, 14.89. Found C, 57.36; H, 4.88, N, 14.85.

(*R*)-1-(2-((2-amino-6-oxo-1H-purin-9(6H)-yl)methoxy)ethyl) 4-benzyl 2-(benzyloxy-carbonylamino)succinate (3D)

The same procedure was repeated for the D-isomer using **2D**. Yield: 890 mg (71%) white powder, m.p. 126–128°C; $[\alpha]_D^{25}$ −9.2; (c = 1.02, $CHCl_3$), LC–MS (ESI+): m/z 587 $(M+Na)^+$; Elemental analysis calculated for $C_{27}H_{28}N_6O_8$ C, 57.44; H, 5.00; N, 14.89. Found C, 57.40; H, 4.91, N, 14.78.

(*S*)-3-amino-4-(2-((2-amino-6-oxo-1H-purin-9(6H)-yl) methoxy)ethoxy)-4-oxobutanoic acid-HCl (4*L*-HCl)

Compound **3L** (850 mg) was dissolved in methanol (60 mL). To this solution, THF (17 mL), water (17 mL), and 0.5N HCl (3 mL) were added successively while stirring, followed by 10% Pd-C (140 mg). The reaction mixture was purged under vacuum, and a balloon of H_2 gas was used to

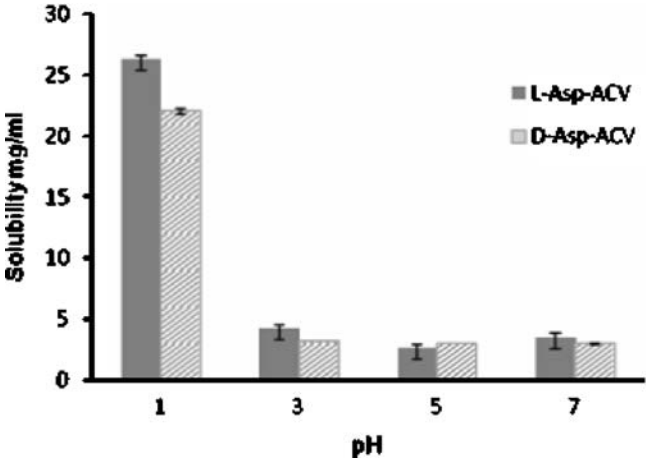

Fig. 2. Solubility of L-Asp-ACV and D-Asp-ACV at 25°C as a function of pH. Results are depicted as mean±SD (n=3).

blanket the reaction at RT. After stirring the reaction for 2.5 h, the catalyst was filtered using gravity filtration and a double-layer of filter paper. The catalyst was washed with methanol, the filtrate evaporated and the residue dried under high vacuum to remove remaining solvent. The residue was dissolved in a minimum amount of water and filtered using a 1.5 μM filter. Absolute ethanol was added and the final product precipitated as a powder, which was filtered and washed with cold ethanol and dried under high vacuum overnight to yield **4L-HCl** as a white solid (500 mg; 93% yield) mp. 168–172°C; $[\alpha]_D^{25}$ +3.08 (c = 0.53, H_2O); LC-MS (ESI+): m/z 341 $(M+H)^+$; ^{1}H NMR (500 MHz, DMSO-d_6) δ10.90 (bs, 1H), 8.61 (bs, 3H), 7.86 (s, 1H), 6.74 (s, 2H), 5.37 (s, 2H), 4.26 (m, 3H), 3.70 (m, 2H), 2.93 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.91, 170.02, 157.29, 154.65, 151.87, 138.08 (*C*H), 116.87, 72.36 (CH_2), 66.61 (CH_2), 64.87 (CH_2), 49.59 (*C*H), 35.68 (CH_2). Elemental analysis calculated for $C_{12}H_{17}ClN_6O_6$: C, 38.26; H, 4.55; N, 22.31. Found: C, 38.27; H, 4.89; N, 22.15. (**4L-HCl**)

(*R*)-3-amino-4-(2-((2-amino-6-oxo-1H-purin-9(6H)-yl) methoxy)ethoxy)-4-oxobutanoic acid-HCl (4*D*-HCl)

White powder, 98% yield. mp. 148–154°C; $[\alpha]_D^{25}$ −2.57 (c = 0.53, H_2O); LC-MS (ESI+): m/z 341 $(M+H)^+$. Elemental analysis calculated for $C_{12}H_{19}ClN_6O_7$ C, 38.63; H, 4.85; N, 21.29. Found: C, 36.63; H, 4.64; N, 21.05. (**4D-HCl** × 1 H_2O).

Aqueous Solubility

These studies were carried out at 25°C in a reciprocating water bath. Aqueous solubility of the prodrugs was observed to be 3.84 ± 0.01 mg/mL (10.21 ± 0.15 mmol/mL) and 3.85 ± 0.15 mg/mL (10.21 ± 0.4 mmol/mL) for L-Asp-ACV and D-Asp-ACV, respectively. Aqueous solubility of ACV, under similar experimental conditions, was observed to be 1.2 ± 0.07 mg/mL (5.3 ± 0.31 mmol/mL). Thus, a two fold increase in the aqueous solubility of ACV is achieved.

In the pH dependent solubility studies, aqueous solubility of the prodrugs was highest at pH 1.2, whereas it remained almost constant at the other pH values studied (Fig. 2). The pH dependent solubility studies were carried out for a period of 3 h since drug degradation was rapid at higher pH values.

HP-β-CD had no effect on the aqueous solubility of either of the prodrugs, indicating lack of complex formation between the prodrugs and HP-β-CD.

Chemical Stability

Aqueous hydrolysis rate constants of L-Asp-ACV and D-Asp-ACV were determined in various buffers. The loss of prodrugs exhibited an apparent first order degradation mechanism. Hydrolysis in the alkaline pH range was observed to be much faster than that in the acidic pH buffers, indicating susceptibility of the prodrugs to base catalyzed hydrolysis (Fig. 3). Half-lives of L-Asp-ACV in pH 1.2, 3 and 5 buffers were 627 ± 31, 371 ± 16 and 262 ± 4 h, respectively. In pH 7 and 9, the half-lives decreased to 32 ± 0.4 and 5.6 ± 0.2 h, respectively. Degradation rate–pH profile of D-Asp-ACV was observed to be similar to that of L-Asp-ACV (Fig. 3).

LogP and pK_a

Predicted values of LogP and pK_a were obtained using the ACD/I-Lab Web service (ACD/Log P 8.02) and (ACD/pKa 8.03), respectively. Predicted LogP value for both L-Asp-ACV and D-Asp-ACV was −1.56. The prodrugs were found to be as hydrophilic as the parent drug ACV (logP = −1.59, calculated using ACD/I-Lab software) (28). Prodrugs were observed to elute earlier, in comparison to ACV, through a C18, 250 mm length HPLC column.

pK_a values obtained for both stereoisomers were also the same; 2.11 (OH of aspartate), 4.66 (N of pentane ring) and 7.97(NH of aspartate).

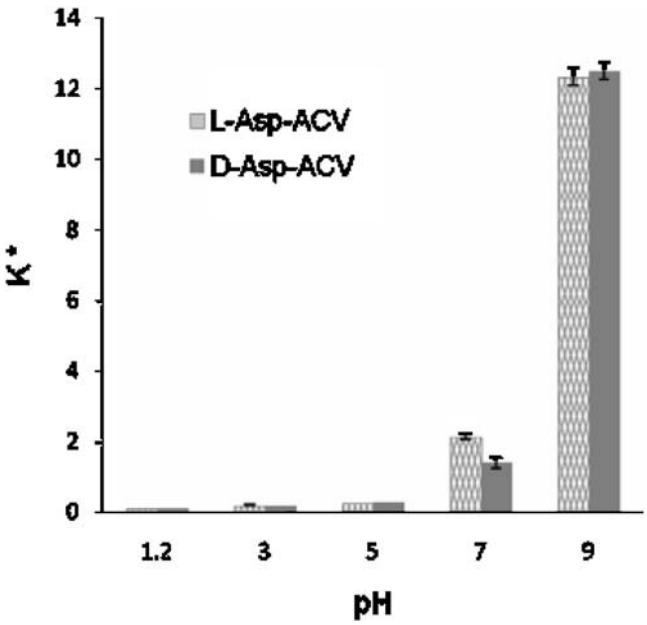

Fig. 3. Apparent first order hydrolysis rate constants (k^*) ×10^2, h^{-1} of L-Asp-ACV and D-Asp-ACV at 25°C as a function of pH. Results are depicted as mean±SD (n=3).

Enzymatic Stability

Tissue homogenates were prepared in IPBS pH 7.4. The observed apparent degradation rate is a summation of chemical and enzymatic degradation rates. Aqueous hydrolysis rate constants, obtained from the degradation rate of the control (prodrug in IPBS), were subtracted from the overall observed rate constant to estimate rate constant for the enzyme mediated hydrolytic process. Rate constants were obtained from log concentration remaining *versus* time plots. Table I depicts the enzyme mediated degradation rate constants, and half-lives, of the prodrugs in corneal, iris-ciliary body and retinal pigmented epithelium homogenates (1 mg/mL protein concentration). Overall observed degradation rate constants have been reported for the degradation of the prodrugs in vitreous humor (Table 1). Degradation rate in vitreous humor was studied to determine enzymatic degradation of the prodrugs in the ocular tissues. This data may be useful in future for back-of-the eye drug delivery strategies.

Corneal Permeation Studies

The pH of the donor solution was adjusted to 5 to minimize degradation of the prodrugs, L-Asp-ACV and D-Asp-ACV. Preliminary studies demonstrated that more than 99% of the prodrug remained intact in the donor solution (pH 5) at the end of 3 h. Thus, majority of the drug available for permeation across the cornea would be in the form of the prodrugs. L-Asp-ACV and D-Asp-ACV are relatively rapidly hydrolyzed in DPBS pH 7.4 ($t_{1/2}$: 5.1 ± 0.1 h and 5.6 ± 0.2 h for L-Asp-ACV and D-Asp-ACV respectively). Consequently, generation of ACV in the receiver solution samples is observed (Fig. 4) due to chemical hydrolysis in buffer and enzymatic hydrolysis in the cornea. Permeability values were calculated on the basis of total ACV, i.e. summation of the concentration of ACV as such and ACV in the form of the prodrug, in the receiver solution. Corneal permeability of L-Asp-ACV was observed to be significantly higher than that of D-Asp-ACV and ACV (Fig. 5). There was no statistically significant difference between the transcorneal permeability coefficients of ACV and D-Asp-ACV.

Corneal permeation of L-Asp-ACV in the presence of 1 mM ouabain, a Na/K ATPase inhibitor, was studied to determine the energy dependence of the transport process. Permeability of L-Asp-ACV decreased significantly from $12.1 \pm 1.47 \times 10^{-6} cm.s^{-1}$ to $3.78 \pm 0.56 \times 10^{-6} cm.s^{-1}$ when the corneas were pretreated with ouabain.

L-Asp-ACV permeability across excised rabbit corneas also decreased drastically, to $2.66 \pm 0.21 \times 10^{-6} cm.s^{-1}$, in the presence of BCH, a specific inhibitor of the amino acid transporter $B^{0,+}$ (Fig. 6). Permeability also decreased significantly when the transport studies were carried out in a sodium free medium. In the absence of sodium, transcorneal permeation coefficient of L-Asp-ACV was observed to be $3.39 \pm 0.22 \times 10^{-6} cm.s^{-1}$, an almost 80% decrease (Fig. 6). On the other hand, glutamic acid, which bears a net negative charge at pH5, failed to inhibit the transport of L-Asp-ACV (Fig. 6).

Transcorneal permeability of D-Asp-ACV and permeability of L-Asp-ACV in the presence of BCH, ouabain or sodium free medium did not differ statistically from each other (Figs. 5 and 6).

DISCUSSION

Overcoming the barriers to transcorneal diffusion of hydrophilic therapeutic moieties is a major challenge faced by ophthalmic drug delivery scientists. In recent years, transporter targeted drug delivery has shown tremendous promise as a strategy to enhance transmembrane permeability of hydrophilic compounds, otherwise diffusion limited by epithelial tight junctions. The amino acid transporters, LAT1, $B^{0,+}$ and ASC1, have been recently identified on the rabbit and/or human cornea (29,30). The fact that, ACV prodrugs and other model conjugates, targeted towards these transporters, interact with the corneal amino acid transporters has already been demonstrated. For example, Vakkalagadda *et al.* (30) reported decreased transport of [^{3}H]-L-Arginine, a substrate of $B^{0,+}$, in the presence of L-Glu-ACV and phenylalanine-ACV. However, only one report so far investigates corneal permeability of amino acid transporter targeted prodrugs and the involvement of transporters in the process (24). In their study, although the authors show that L-Glu-ACV is a substrate of the amino acid transporter $B^{0,+}$, corneal permeability of L-Glu-ACV and ACV did not differ significantly from each other. Corneal permeability of the amino acid prodrug was $5.32 \pm 0.69 \times 10^{-6} cm.s^{-1}$ (mean ± SD), whereas that of the parent drug ACV was $4.24 \pm 1.41 \times 10^{-6} cms^{-1}$ (24). Furthermore, in the presence of BCH, a specific inhibitor of $B^{0,+}$, transcorneal permeability decreased to 75% of that of L-Glu-ACV alone. This indicates that the contribution of passive diffusion to the overall corneal permeability of L-Asp-ACV is less than 25% of the corneal permeability of the parent drug ACV. The report by Anand *et al.* (24) thus suggests that a change in structural configuration of ACV, as a result of prodrug derivatization, leads to decreased passive diffusion.

The objective of this study was to further investigate the utility of amino acid transporter $B^{0,+}$ in transcorneal drug

Table I. Apparent First Order Rate Constants (k^*) $\times 10^3$, min^{-1} and Half-lives ($t_{1/2}$, min) of L-Asp-ACV and D-Asp-ACV in Ocular Tissue Homogenates

Prodrug		Cornea	Iris-ciliary	Vitreous humor	Retina/choroid
L-Asp-ACV	k^*	2.4±0.1	5.2±0.2	4.2±0.1	1.35±0.1
	t 1/2	289.7±12.2	134.1±5.1	165.6±3.9	51.6±1.6
D-Asp-ACV	k^*	3.1±0.2	5±0.4	5±0.5	0.1±0.01
	t 1/2	223.8±12.6	139.4±10.1	140.6±15.1	1,197.2±88.1

Results are depicted as mean±SD (n=3)

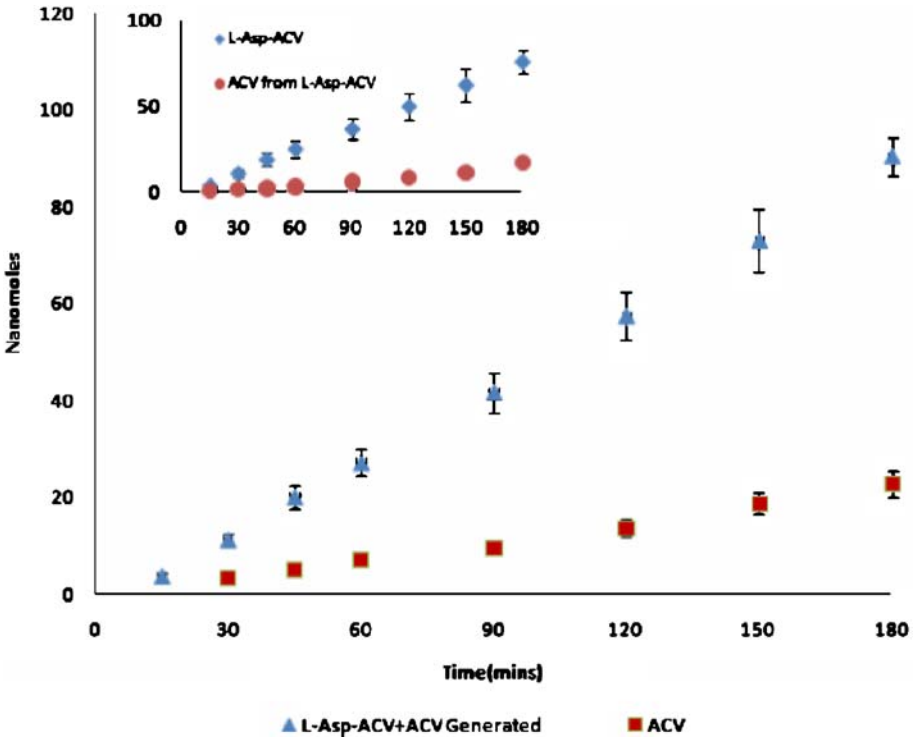

Fig. 4. Transport of L-Asp-ACV (in terms of total ACV) and ACV across isolated rabbit corneas, at 34°C, as a function of time. Insert represents total amount of the intact prodrug and ACV generated during the permeation studies with L-Asp-ACV. Results are depicted as mean±SE (n=4).

delivery. Earlier studies have demonstrated that acyclovir-L-aspartate β-ester is a substrate of the amino acid transporter $B^{0,+}$ expressed on other mammalian tissues. Yang *et al.* (31) had hypothesized the involvement of a carrier system for the transport of acyclovir-L-aspartate β-ester across the nasal mucosa. Hatanaka *et al.*(21) also proved that acyclovir-L-aspartate β-ester is transported by the amino acid transporter $B^{0,+}$. The authors used *Xenopus laevis oocytes* to illustrate transport of prodrugs by $B^{0,+}$.

In the present study, ACV and its aspartate ester prodrugs were used as the model compounds to study transcorneal permeation. The purpose of these studies were two-fold: first, to investigate whether the aspartate ester is indeed a substrate of the corneal amino acid transporter $B^{0,+}$ and second, to investigate whether the structural modifications in ACV lead to a drastic decrease in passive diffusion component, as observed for L-Glu-ACV by Anand *et al.* (24).

Solubility of the hydrochloride salts of L-Asp-ACV were observed to be twofold greater than that of the parent drug ACV. Although significantly higher than ACV, the solubility values are surprisingly lower compared to the solubility values reported for some of the other mono- and di-amino acid prodrugs studied (18,19). A possible reason for this observation could be the existence of intra-molecular bonding

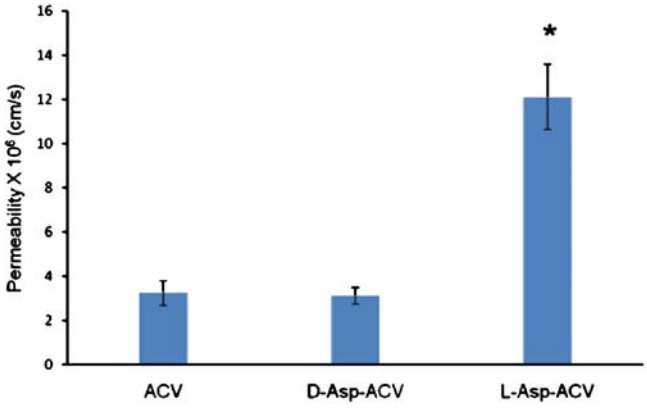

Fig. 5. Transcorneal permeability of acyclovir, L-Asp-ACV and D-Asp-ACV across isolated rabbit corneas, at 34°C, as a function of time. Permeability of L-Asp-ACV and D-Asp-ACV are in terms of total ACV. Values represent a mean±SE (n=4). *p<0.05.

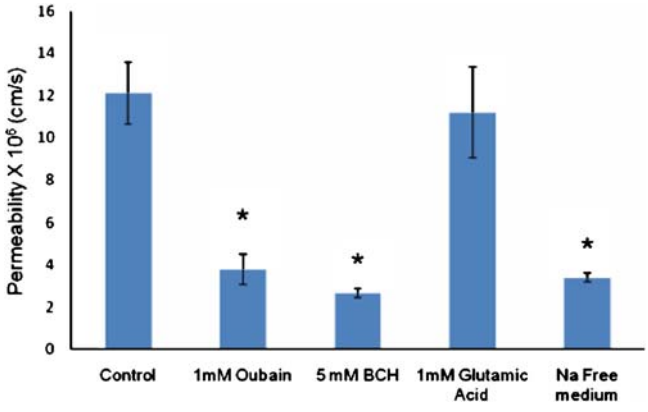

Fig. 6. Transcorneal permeability of L-Asp-ACV alone (control) and in the presence of ouabain (1 mM) and glutamic acid (1 mM), BCH (5 mM) and sodium free medium, across isolated rabbit corneas, at 34°C, as a function of time. Permeability values are in terms of total ACV. Results are depicted as mean±SE (n=3). *p<0.05.

in solution. It is notable, however, that at pH 1.2, corresponding to the pK_a of the free carboxyl group on the aspartate(2.11), solubility of the prodrug is almost tenfold higher than that of ACV. Solubility values for the β-aspartate and glutamate ACV esters have not been reported in the earlier studies reported in the literature.

L-Asp-ACV and D-Asp-ACV exhibited a typical ester hydrolysis rate–pH profile with rapid base mediated hydrolysis occurring at the higher pH values. The prodrugs were relatively stable in an acidic medium. The results are consistent with earlier reports investigating chemical stability of the ACV ester (alkyl and amino acid promoieties) prodrugs (24,32). Buffer strength was observed to affect the rate of degradation (data not presented) and may be one of the reasons that half-life of acyclovir-L-aspartate β-ester in pH 7.4 buffer (50 mM) at 25°C as determined by Yang *et al.* (31) was greater than that observed in this study, where the buffer concentration was 200 mM. Other factors such as intramolecular bonding, salt form and change in packing arrangements could also contribute to the decreased stability of L-Asp-ACV.

Enzymatic hydrolysis rates of L-Asp-ACV and D-Asp-ACV, in corneal and iris ciliary body homogenates and vitreous humor appear to be almost similar. However, in the retina/choroid homogenates, enzymatic hydrolysis of L-Asp-ACV was significantly faster compared to that of D-Asp-ACV. This probably indicates differences in tissue distribution of concerned esterases. Interestingly, results from this study as well as other reports, suggest that the aspartate ester (both β- and α-) is considerably more stable in the ocular tissue homogenates compared to other amino acid ester ACV prodrugs studied (24). This is possibly as a result of increased chain length of promoieties and low concentrations of appropriate esters (e.g. butyrylesterases) (33). There are several reports in the literature that demonstrate the effect of chain length on ocular tissue esterase activity (33–35).

Corneal permeability of L-Asp-ACV, in terms of total ACV reaching the receiver side, was significantly higher than that of D-Asp-ACV and of ACV, suggesting transporter involvement. The results indicate that transcorneal penetration of L-Asp-ACV is mediated by an energy and sodium dependent transporter, as evident from drastically reduced permeation rates in the presence of ouabain and in the absence of sodium in the transport medium. The above data, along with the observation that corneal permeability of L-Asp-ACV was reduced in the presence of BCH, a specific inhibitor of $B^{0,+}$, but was unaffected by the anionic amino acid glutamic acid, strongly suggests that L-Asp-ACV is translocated across the corneal epithelium by the amino acid transporter $B^{0,+}$.The difference in transcorneal permeability of ACV $\left(5.55 \pm 0.71 \times 10^{-6} cms^{-1}\right)$ and ACV in the presence of BCH 5 mM $\left(4.43 \pm 0.17 \times 10^{-6} cms^{-1}\right)$, ouabain 1 mM $\left(5.38 \pm 0.2 \times 10^{-6} cms^{-1}\right)$ and glutamic acid 1 mM $\left(4.51 \pm 0.18 \times 10^{-6} cms^{-1}\right)$ was not found to be statistically significant ($p > 0.05$). This demonstrates that these compounds do not affect corneal integrity under the experimental conditions employed. It is interesting to note that when the transporter was inhibited, corneal permeability decreased to values similar to that of ACV. This is in sharp contrast to the results observed for L-Glu-ACV (24) and also suggests the involvement of a single transporter in the transcorneal translocation process of L-Asp-ACV.

Amino acid transporter $B^{0,+}$, in addition to neutral and cationic L amino acids, is also known to transport D amino acids (21). However, in this study, significant difference was not observed between the permeability coefficients of D-Asp-ACV and ACV. Moreover, as discussed in the above paragraph, corneal permeability of L-Asp-ACV in the presence of BCH decreased to values similar to that of ACV and thus that of D-Asp-ACV. These results thus indicate that D-Asp-ACV is not a substrate of the amino acid transporter $B^{0,+}$. Importantly, the results also demonstrate that the structural modifications to ACV, as a result of the aspartate ester prodrug derivatization, do not diminish the passive diffusion component of the transcorneal permeation process.

In conclusion, L-Asp-ACV is a substrate of the corneal $B^{0,+}$, and produces a fourfold increase in transcorneal permeability of ACV. Structural modification of ACV, as a result of aspartate ester derivatization, does not decrease the transcorneal passive diffusion component. Thus, corneal amino acid transporter $B^{0,+}$ targeted prodrug derivatization is a viable option for enhancing corneal permeability of hydrophilic therapeutic agents.

ACKNOWLEDGEMENT

This project was supported by NIH grant numbers P20RR021929, from the National Center for Research Resources, and EY018426-01 from the National Eye Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

REFERENCES

1. I. P. Kaur, and R. Smitha. Penetration enhancers and ocular bioadhesives: two new avenues for ophthalmic drug delivery. *Drug Dev. Ind. Pharm.* **28**:353–369 (2002). doi:10.1081/DDC-120002997.
2. E. Mannermaa, K. S. Vellonen, and A. Urtti. Drug transport in corneal epithelium and blood–retina barrier: emerging role of transporters in ocular pharmacokinetics. *Adv. Drug Deliv. Rev.* **58**:1136–1163 (2006). doi:10.1016/j.addr.2006.07.024.
3. D. A. Jabs. Acyclovir for recurrent herpes simplex virus ocular disease. *N. Engl. J. Med.* **339**:340–341 (1998). doi:10.1056/NEJM199807303390510.
4. S. Guess, D. U. Stone, and J. Chodosh. Evidence-based treatment of herpes simplex virus keratitis: a systematic review. *Ocul. Surf.* **5**:240–250 (2007).
5. K. R. Wilhelmus. The treatment of herpes simplex virus epithelial keratitis. *Trans. Am. Ophthalmol. Soc.* **98**:505–532 (2000).
6. K. R. Wilhelmus. Therapeutic interventions for herpes simplex virus epithelial keratitis. *Cochrane Database Syst. Rev.* CD002898 (2007).
7. P. Chetoni, S. Rossi, S. Burgalassi, D. Monti, S. Mariotti, and M. F. Saettone. Comparison of liposome-encapsulated acyclovir with acyclovir ointment: ocular pharmacokinetics in rabbits. *J Ocul Pharmacol Ther.* **20**:169–177 (2004). doi:10.1089/108076804773710849.
8. S. Ghosh, V. Jhanji, E. Lamoureux, H. R. Taylor, and R. B. Vajpayee. Acyclovir therapy in prevention of recurrent herpetic

keratitis following penetrating keratoplasty. *Am. J. Ophthalmol.* **145**:198–202 (2008). doi:10.1016/j.ajo.2007.10.005.
9. S. Majumdar, Y. E. Nashed, K. Patel, R. Jain, M. Itahashi, D. M. Neumann, J. M. Hill, and A. K. Mitra. Dipeptide monoester ganciclovir prodrugs for treating HSV-1-induced corneal epithelial and stromal keratitis: *in vitro* and *in vivo* evaluations. *J. Ocul. Pharmacol. Ther.* **21**:463–474 (2005). doi:10.1089/jop.2005.21.463.
10. S. Y. Lee. Herpes simplex virus ocular infections. *Drugs Today (Barc)*. **34**:241–249 (1998). doi:10.1358/dot.1998.34.3.485179.
11. A. Simmons. Clinical manifestations and treatment considerations of herpes simplex virus infection. *J. Infect Dis.* **186**(Suppl 1): S71–S77 (2002). doi:10.1086/342967.
12. M. Fresta, G. Fontana, C. Bucolo, G. Cavallaro, G. Giammona, and G. Puglisi. Ocular tolerability and *in vivo* bioavailability of poly (ethylene glycol) (PEG)-coated polyethyl-2-cyanoacrylate nanosphere-encapsulated acyclovir. *J. Pharm. Sci.* **90**:288–97 (2001). doi:10.1002/1520-6017(200103)90:3<288::AID-JPS4>3.0.CO;2-5.
13. S. L. Law, K. J. Huang, and C. H. Chiang. Acyclovir-containing liposomes for potential ocular delivery. Corneal penetration and absorption. *J. Control Release*. **63**:135–140 (2000). doi:10.1016/S0168-3659(99)00192-3.
14. B. Wang, W. Teng, and X. Han. An experimental study of a new route for acyclovir administration for anti-keratitis therapeutic treatment. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*. **14**:379–381 (2000).
15. S. Majumdar, K. Hippalgaonkar, and M. A. Repka. Effect of chitosan, benzalkonium chloride and ethylenediaminetetraacetic acid on permeation of acyclovir across isolated rabbit cornea. *Int. J. Pharm.* **348**:175–178 (2008). doi:10.1016/j.ijpharm.2007.08.017.
16. S. Majumdar, S. Duvvuri, and A. K. Mitra. Membrane transporter/receptor-targeted prodrug design: strategies for human and veterinary drug development. *Adv. Drug Deliv. Rev.* **56**:1437–1452 (2004). doi:10.1016/j.addr.2004.02.006.
17. M. Palacin, R. Estevez, J. Bertran, and A. Zorzano. Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol. Rev.* **78**:969–1054 (1998).
18. B. Anand, Y. Nashed, and A. Mitra. Novel dipeptide prodrugs of acyclovir for ocular herpes infections: Bioreversion, antiviral activity and transport across rabbit cornea. *Curr Eye Res*. **26**:151–163 (2003). doi:10.1076/ceyr.26.3.151.14893.
19. B. S. Anand, and A. K. Mitra. Mechanism of corneal permeation of L-valyl ester of acyclovir: targeting the oligopeptide transporter on the rabbit cornea. *Pharm. Res.* **19**:1194–1202 (2002). doi:10.1023/A:1019806411610.
20. M. E. Ganapathy, and V. Ganapathy. Amino acid transporter ATB0,+ as a delivery system for drugs and prodrugs. *Curr. Drug Targets Immune. Endocr. Metabol. Disord.* **5**:357–364 (2005). doi:10.2174/156800805774912953.
21. T. Hatanaka, M. Haramura, Y. J. Fei, S. Miyauchi, C. C. Bridges, P. S. Ganapathy, S. B. Smith, V. Ganapathy, and M. E. Ganapathy. Transport of amino acid-based prodrugs by the Na+- and Cl(−) -coupled amino acid transporter ATB0,+ and expression of the transporter in tissues amenable for drug delivery. *J. Pharmacol. Exp. Ther.* **308**:1138–1147 (2004). doi:10.1124/jpet.103.057109.
22. K. Patel, S. Trivedi, S. Luo, X. Zhu, D. Pal, E. R. Kern, and A. K. Mitra. Synthesis, physicochemical properties and antiviral activities of ester prodrugs of ganciclovir. *Int. J. Pharm.* **305**:75–89 (2005). doi:10.1016/j.ijpharm.2005.08.024.
23. C. Santos, J. Morais, L. Gouveia, E. de Clercq, C. Pannecouque, C. U. Nielsen, B. Steffansen, R. Moreira, and P. Gomes. Dipeptide derivatives of AZT: synthesis, chemical stability, activation in human plasma, hPEPT1 affinity, and antiviral activity. *ChemMedChem*. **3**:970–978 (2008).
24. B. S. Anand, S. Katragadda, Y. E. Nashed, and A. K. Mitra. Amino acid prodrugs of acyclovir as possible antiviral agents against ocular HSV-1 infections: interactions with the neutral and cationic amino acid transporter on the corneal epithelium. *Curr. Eye Res.* **29**:153–166 (2004). doi:10.1080/02713680490504614.
25. L. M. Beauchamp, G. F. Orr, P. de Miranda, T. Burnette, and T. A. Krenitsky. Amino acid ester prodrugs of acyclovir. *Antivir. Chem. Chemother.* **3**:157–164 (1992).
26. United States Pharmacopeial Commission. The United States Pharmacopoeia XXX, NF XXV, United States Pharmacopeial Commission, Inc., Rockville, MD, 2006, 806–807.
27. M. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976). doi:10.1016/0003-2697(76)90527-3.
28. B. Garre, K. Shebany, A. Gryspeerdt, K. Baert, K. van der Meulen, H. Nauwynck, P. Deprez, P. De Backer, and S. Croubels. Pharmacokinetics of acyclovir after intravenous infusion of acyclovir and after oral administration of acyclovir and its prodrug valacyclovir in healthy adult horses. *Antimicrob. Agents Chemother.* **51**:4308–4314 (2007). doi:10.1128/AAC.00116-07.
29. B. Jain-Vakkalagadda, S. Dey, D. Pal, and A. K. Mitra. Identification and functional characterization of a Na+-independent large neutral amino acid transporter, LAT1, in human and rabbit cornea. *Invest. Ophthalmol. Vis. Sci.* **44**:2919–2927 (2003). doi:10.1167/iovs.02-0907.
30. B. Jain-Vakkalagadda, D. Pal, S. Gunda, Y. Nashed, V. Ganapathy, and A. K. Mitra. Identification of a Na+-dependent cationic and neutral amino acid transporter, B(0,+), in human and rabbit cornea. *Mol. Pharm.* **1**:338–346 (2004). doi:10.1021/mp0499499.
31. C. Yang, H. Gao, and A. K. Mitra. Chemical stability, enzymatic hydrolysis, and nasal uptake of amino acid ester prodrugs of acyclovir. *J. Pharm. Sci.* **90**:617–624 (2001). doi::10.1002/1520-6017(200105)90:5<617::AID-JPS1018>3.0.CO;2-5.
32. H. Bundgaard, E. Jensen, and E. Falch. Water-soluble, solution-stable, and biolabile N-substituted (aminomethyl)benzoate ester prodrugs of acyclovir. *Pharm. Res*. **8**:1087–1093 (1991). doi:10.1023/A:1015837931256.
33. C. G. Jordan. How an increase in the carbon chain length of the ester moiety affects the stability of a homologous series of oxprenolol esters in the presence of biological enzymes. *J. Pharm. Sci.* **87**:880–885 (1998). doi:10.1021/js970280p.
34. S. C. Chang, and V. H. Lee. Influence of chain length on the *in vitro* hydrolysis of model ester prodrugs by ocular esterases. *Curr. Eye Res.* **2**:651–656 (1982). doi:10.3109/02713688209019993.
35. D. S. Chien, H. Sasaki, H. Bundgaard, A. Buur, and V. H. Lee. Role of enzymatic lability in the corneal and conjunctival penetration of timolol ester prodrugs in the pigmented rabbit. *Pharm. Res.* **8**:728–733 (1991). doi:10.1023/A:1015845916293.