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Ocular pharmacokinetics of acyclovir amino acid ester prodrugs in the anterior chamber: Evaluation of their utility in treating ocular HSV infections

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ABSTRACT

Purpose: To evaluate *in vivo* corneal absorption of the amino acid prodrugs of acyclovir (ACV) using a topical well model and microdialysis in rabbits.
Methods: Stability of L-alanine–ACV (AACV), L-serine–ACV (SACV), L-isoleucine–ACV (IACV), γ-glutamate–ACV (EACV) and L-valine–ACV (VACV) prodrugs was evaluated in various ocular tissues. Dose-dependent toxicity of these prodrugs was also examined in rabbit primary corneal epithelial cell culture (rPCEC) using 96-well based cell proliferation assay. *In vivo* ocular bioavailability of these compounds was also evaluated with a combination of topical well infusion and aqueous humor microdialysis techniques.
Results: Among the amino acid ester prodrugs, SACV was most stable in aqueous humor. Enzymatic degradation of EACV was the least compared to all other prodrugs. Cellular toxicity of all the prodrugs was significantly less compared to trifluorothymidine (TFT) at 5 mM. Absorption rate constants of all the compounds were found to be lower than the elimination rate constants. All the prodrugs showed similar terminal elimination rate constants (λ_z). SACV and VACV exhibited approximately two-fold increase in area under the curve (AUC) relative to ACV ($p < 0.05$). C_{last} (concentration at the last time point) of SACV was observed to be 8 ± 2.6 μM in aqueous humor which is two and three times higher than VACV and ACV, respectively.
Conclusions: Amino acid ester prodrugs of ACV were absorbed through the cornea at varying rates (k_a) thereby leading to varying extents (AUC). The amino acid ester prodrug, SACV owing to its enhanced stability, comparable AUC and high concentration at last time point (C_{last}) seems to be a promising candidate for the treatment of ocular HSV infections.

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1. Introduction

Herpes simplex keratitis is the leading cause of blindness in the United States as well as the most frequent cause of corneal opacities in developed countries (Turner et al., 2003). Nucleoside analogs developed initially for the treatment of herpes simplex virus (HSV) infections (HSV keratitis) include trifluorothymidine (TFT), idoxuridine (IDU), and cytosine arabinoside (Ara-A). These compounds were found to be too toxic for systemic use and were, therefore, restricted to topical use for herpetic keratitis (Beers and Berkow, 1999). Acyclovir (ACV), also a nucleoside analog, has shown to be clinically effective against herpes viruses, but due to poor aqueous solubility and low corneal permeability, the drug is not very effective against ocular herpes infections (Hughes and Mitra, 1993).

Chemical approach to designing bioreversible prodrugs can be useful in the optimization of drug absorption properties (Stella et al., 1985). Prodrug strategy has been employed in this work to target the nutrient transporters by covalently linking the drug to the nutrient promoiety. A host of transporters have been discovered in the anterior segment, which could be targeted for drug delivery (Dey et al., 2003a). Transport systems for peptide (Anand and Mitra, 2002), amino acid (Jain-Vakkalagadda et al., 2003, 2004; Katragadda et al., 2005) and nucleoside/nucleobase (Majumdar et al., 2003a,b) have been discovered on the corneal epithelium and utilized for targeted drug delivery in our laboratory. Among nutrient transporters, amino acid transporters are preferred for drug delivery due to their ubiquitous nature and overlapping substrate specificity.

Abbreviations: ASCT1, Na^+-dependent neutral amino acid transporter; B0,+, Na^+-dependent neutral and cationic amino acid transporter; ACV, acyclovir; VACV, valine–acyclovir; SACV, serine–acyclovir; AACV, alanine–acyclovir; EACV, γ-glutamate–acyclovir; IACV, isoleucine–acyclovir; IPBS, isotonic phosphate-buffered saline; k_a, absorption rate constant.

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Depending upon the affinity and/or the capacity, amino acid transporters have been known to transport not only naturally occurring amino acids but also amino acid-related drug compounds such as L-dopa, a therapeutic agent for Parkinsonism; melphalan (Goldenberg et al., 1979), an anticancer Phe mustard; triiodothyronine (Blondeau et al., 1993) and thyroxine (Lakshmanan et al., 1990), two thyroid hormones; and gabapentin (Su et al., 1995), an anticonvulsant and valacyclovir (Hatanaka et al., 2004), an antiviral drug. A recent report suggests that the ability of ATB(0,+) to transport valacyclovir is comparable to that of the peptide transporter PEPT1 (Hatanaka et al., 2004). These findings suggest that amino acid transporters can be utilized as potential delivery targets for amino acid-based drugs and prodrugs.

Trifluorothymidine (TFT), the gold standard for the treatment of ocular herpes simplex virus (HSV) infections, is associated with severe cytotoxicity and mutagenicity in long-term treatments (Beers and Berkow, 1999). In comparison, acyclovir (ACV) exhibits excellent antiviral activity against HSV-1 and -2 and considerably less cytotoxicity due to its selective mechanism of action. However, ACV cannot be formulated into 1–3% eye drops due to its limited solubility (Hughes et al., 1993). One main constraint to topical ocular delivery of ACV in the treatment of HSV-1 keratitis includes poor corneal permeability leading to much lower levels than MIC in the aqueous humor. Amino acid-derivatized prodrugs exhibited excellent solution and enzymatic stability relative to valacyclovir (VACV), a drug of choice for oral and genital herpes infections. The amino acid ester prodrugs also exhibited excellent *in vitro* antiviral efficacy against HSV-1 relative to ACV. Finally, the prodrugs were highly soluble and permeable across the cornea in comparison with ACV (Anand et al., 2004; Katragadda et al., submitted for publication) which can lead to the feasibility of these prodrugs being formulated into 1–3% eye drops. Therefore, these compounds appear to be promising candidates for the treatment of HSV keratitis with stromal involvement.

Topical administration is the preferred mode to treat diseases that affect the anterior chamber of the eye. Unfortunately, the disposition of drugs administered in this manner is not well understood. Several pharmacokinetic models of varying complexity have been proposed to predict absorption and disposition of drugs applied topically to the eye (Lee and Robinson, 1979; Makoid and Robinson, 1979; Miller et al., 1981). Pharmacokinetics of topically applied pilocarpine in the albino rabbit eye has been described using a four-compartment classical model represented by four exponential equations yielding eight equation parameters (Makoid and Robinson, 1979). Another model applied to pilocarpine pharmacokinetics uses a physiological approach (Lee and Robinson, 1979; Miller et al., 1981). However, both modeling approaches are complex with regard to numerical analyses.

Two basic problems in determining anterior chamber kinetics are (i) complexity in k_a determination due to pre-corneal kinetic events and (ii) absorption across the cornea is often slower process than elimination from the eye and an erroneous assignment of slopes is possible. To simplify the approach and correctly estimate ocular absorption rate constant, a "topical infusion" model has been utilized (Eller et al., 1985). In this model, a constant concentration of the drug is maintained over the cornea such that the effect of tear dynamics is minimized and simpler equations can be applied independent of compartment modeling. During constant infusion through the cornea absorption, distribution, and elimination rate constants can be determined independent of the number of the peripheral compartments that are operative. Constant infusion was achieved by the use of a plastic cylindrical well containing the drug solution.

Another major constraint in the determination of ocular pharmacokinetics is the inaccessibility of ocular fluids such as aqueous and vitreous humors for continuous serial sampling which then leads to the fact of a single rabbit being used for a single time point. A complete pharmacokinetic profile is usually constructed by sacrificing 6–20 rabbits at each time point. Microdialysis has been proven to be beneficial over conventional sampling techniques in determining ocular pharmacokinetics by both reducing the number of subjects and providing statistically robust data. Microdialysis has been applied in aqueous and vitreous drug disposition and delivery studies (Waga et al., 1991, 1999; Hughes and Mitra, 1993; Stempels et al., 1993; Rittenhouse and Pollack, 2000). In this study we have employed microdialysis for sampling the aqueous humor. We conceptualized the combination of the topical well infusion model and aqueous humor microdialysis sampling for precisely predicting ocular absorption of a series of ACV-amino acid ester prodrugs.

In this report, we have determined *in vivo* corneal absorption of the amino acid ester prodrugs utilizing a topical infusion model along with aqueous humor microdialysis in New Zealand White rabbits. Recently in our laboratory, ASCT1 (Na^+-dependent neutral

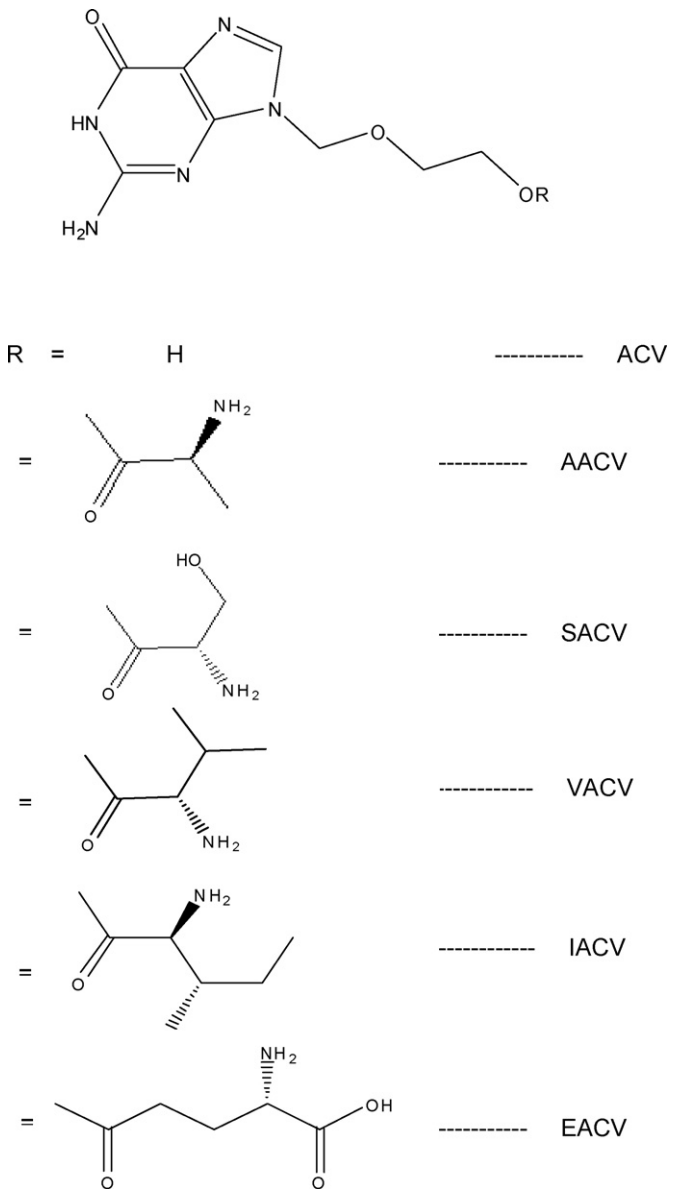

Fig. 1. Structure of acyclovir (ACV) and amino acid ester prodrugs of acyclovir (AACV, SACV, VACV, IACV and EACV).

amino acid transporter), B0,+ (Na^+-dependent neutral and cationic amino acid transporter) were identified on the corneal epithelium and were screened for their utility in transporting amino acid-tethered prodrugs (Anand et al., 2004; Katragadda et al., submitted for publication). The most suitable candidates from the previous *in vitro*/*ex vivo* studies were chosen and their ocular pharmacokinetics was studied (Anand et al., 2004; Katragadda et al., submitted for publication). The aqueous humor kinetics of the amino acid ester prodrugs (AACV, SACV, IACV and EACV) was compared with VACV, which is currently the prodrug indicated for oral and genital herpes infections.

2. Materials and methods

2.1. Materials

VACV was a gift from GlaxoSmithKline Inc., Research Triangle Park, NC. The amino acid prodrugs namely L-alanine–ACV (AACV), L-serine–ACV (SACV), γ-glutamate–ACV (EACV) and L-isoleucine–ACV (IACV) were synthesized in our laboratory (Fig. 1). Ninety-six-well plates were purchased from Costar (Bedford, MA). Trifluorothymidine ophthalmic solution (1%) was obtained from Falcon Pharmaceuticals (Fort Worth, TX). Ketamine HCl was supplied by Fort Dodge animal health and Xylazine by Bayer animal health. Nembutal Sodium was purchased from Abbott laboratories (Chicago, IL). Linear microdialysis probes (MD-2000, 0.32 mm × 10 mm, polyacrylo nitrile membrane and 0.22 mm tubing) employed for aqueous humor sampling were procured from Bioanalytical Systems (West Lafayette, IN). Microinjection pump (CMA/100) for perfusing the isotonic buffer saline was obtained from CMA/Microdialysis (Acton, MA). Topical wells were custom made by Hansen Ophthalmic Development Corporation (Iowa City, IA) according to special instructions (Fig. 2A). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO). The solvents were of HPLC grade and obtained from Fisher Scientific Company (St. Louis, MO).

2.2. Animals

New Zealand White male rabbits weighing between 5.0 and 5.5 lb were obtained from Myrtle's Rabbitry (Thompson Station, TN). Animal care and treatment in this investigation was in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

2.3. Prodrugs stability in perfusate buffer

The perfusate made of isotonic phosphate buffer saline (IPBS) was prepared at pH 7.4. Stock solution of the prodrugs (1 mM) was prepared in IPBS buffer and used immediately. Aliquots (9.8 ml) were placed in screw-capped vials and allowed to equilibrate at 34 °C. Prodrugs stock solution (0.2 ml) was subsequently added. The vials were placed in a constant shaker bath set at 34 °C and 60 rpm. Samples (0.1 ml) were collected at appropriate time intervals for up to 96 h and were immediately stored at −80 °C until further analysis. All experiments were conducted at least in triplicate.

2.4. Prodrugs stability in ocular tissue homogenates

The hydrolysis of the prodrugs in ocular tissue homogenates was carried out as described previously (Dias et al., 2002). The method is described briefly in the following sections.

2.4.1. Preparation of ocular tissues

New Zealand White male rabbits were utilized for this study. Animals were euthanized by a lethal injection of sodium pentobarbital (50 mg/kg) through the marginal ear vein. Each eye was immediately enucleated, and the ocular surface was rinsed with

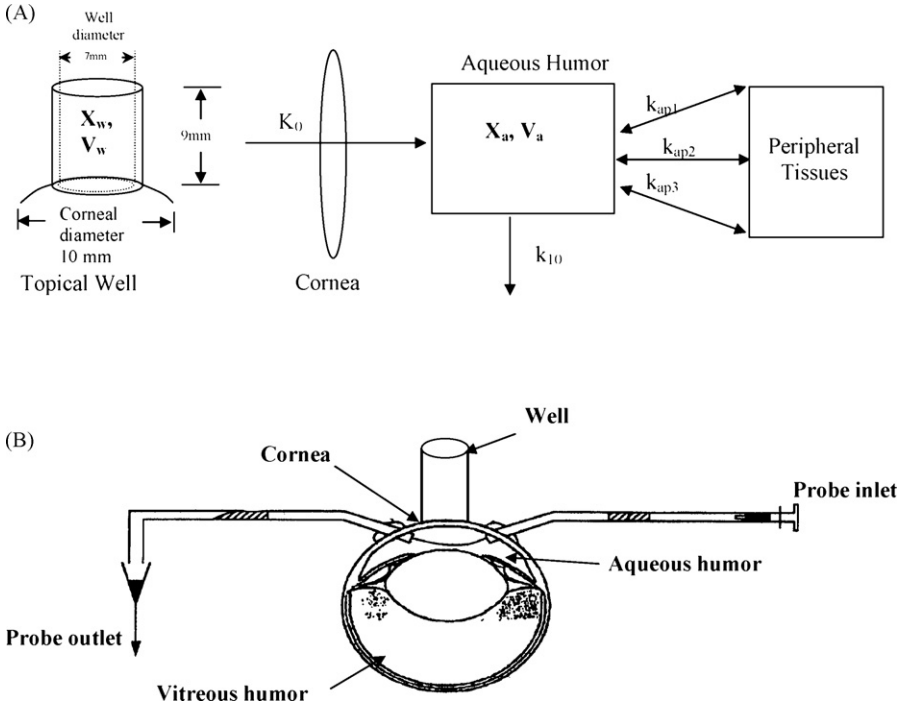

Fig. 2. (A) Schematic model representing the absorption barriers and elimination pathways when drug is administered at a constant rate to the surface of the cornea. The dimensions of the plastic cylindrical well are also specified. Elimination was assumed to be primarily through the central (aqueous humor) compartment. From aqueous humor, drug may reversibly distribute to peripheral tissues. (B) Schematic representation of the well model in anterior chamber ocular microdialysis.

ice-cold, pH 7.4, isotonic phosphate buffer saline (IPBS) to remove any trace of blood. Aqueous humor was aspirated using a 27-gauge needle attached to a 1-ml tuberculin syringe. Cornea, lens and iris ciliary body were sequentially removed after cutting along the scleral–limbus junction. Aqueous humor and tissue samples were stored at −80 °C prior to use. The tissues were homogenized in 5 ml chilled (4 °C) IPBS for about 4 min with a tissue homogenizer (Tissue Tearor, Model 985-370; Dremel Multipro, Racine, WI) in an ice bath. Subsequently, the homogenates and aqueous humor were centrifuged separately at 12,500 rpm for 25 min at 4 °C to remove cellular debris, and the supernatant was used for hydrolysis studies. Protein content of each supernatant was determined with a BioRad assay using bovine serum albumin as the standard.

2.4.2. Tissue hydrolysis

The supernatant was equilibrated at 34 °C for about 30 min prior to an experiment. Hydrolysis was initiated by the addition of 0.2 ml of a 1 mM prodrug solution to 0.8 ml of the supernatant. The control consisted of 0.8 ml of IPBS instead of the supernatant. Aliquots (50 μl) were withdrawn at appropriate time intervals for up to 48 h. The samples were immediately diluted with 50 μl chilled methanol to quench the reaction and stored at −80 °C until further analysis. Subsequently, the samples were thawed and centrifuged at 10,000 rpm for 10 min prior to analysis by HPLC for the intact ester prodrug and the regenerated parent drug, acyclovir. Apparent first-order rate constants were calculated and corrected for any chemical hydrolysis observed with the control.

2.5. Cell proliferation assay

Cell proliferation assay was performed to examine the toxicity of AACV, SACV, EACV and IACV in comparison with ACV, TFT, and VACV. The commercial assay used (CellTiter 96 AQ_{ueous} Non-radioactive Cell Proliferation Assay Kit; Promega, Madison, WI) constitutes a colorimetric method for determining the number of proliferating cells in culture. The studies were performed on primary corneal epithelial cell cultures. The procedure for preparing primary corneal epithelial cell culture was reported previously from our laboratory (Dey et al., 2003b).

Cells were seeded onto 96-well plates for the cell proliferation assay. Solutions of TFT, ACV, AACV, SACV, IACV, VACV, and EACV at different concentrations (0.2–5 mM) were prepared in the culture medium, and 100 μl of a given drug solution was added to the wells. Cells were incubated with the drug solution in a humidified 5% CO_2 atmosphere for a period of 48 h to evaluate the cytotoxic effect of the drugs. Positive control experiments consisted of cells incubated with culture medium without drugs, and the negative control consisted of wells without cells filled with culture medium without drugs. Cell proliferation in the presence of various concentrations of the drugs tested was calculated as a percentage of the positive control (without drug) at each time point. The values were corrected using the negative control (without cells). Color determination was measured at 485 nm (reference at 590 nm) using a 96-well microtiter plate reader (SpectraFluor Plus; Tecan, Maennedorf, Switzerland).

2.6. In vivo absorption experiments

2.6.1. Probe implantation

Aqueous humor sampling to assess the ocular absorption of the amino acid prodrugs was carried out using microdialysis. Animals were anesthetized prior to the surgery by administering ketamine (50 mg/kg) and xylazine (5 mg/kg) intramuscularly. Pupils were dilated by topical instillation of 1% tropicamide prior to probe implantation. A linear microdialysis probe was implanted in the anterior chamber using a 25G needle. It was inserted across the cornea without causing any damage to iris-ciliary body and the outlet of linear probe was placed into the needle at bevel edge. The needle was then slowly withdrawn such that the probe remained fixed in the anterior chamber (Fig. 2A). The probe was perfused with IPBS at a flow rate of 3 μl/min with the help of a microinjection pump. Animals were kept under anesthesia throughout an experiment with ketamine HCl and xylazine given intramuscularly every 40 min. After probe implantation, the animals were allowed to stabilize for 2 h. This duration has been shown to be sufficient for the restoration of intraocular pressure and replenishment of the aqueous humor lost during probe implantation (Macha and Mitra, 2001).

2.6.2. Microdialysis

Subsequent to probe implantation and recovery of the animal, the eyelids of the rabbits were mechanically retracted with Colibri retractors. The plastic well (Hansen Ophthalmic Development Corporation, Iowa City, IA) was then placed on the cornea with the help of a surgical adhesive. Care was taken to avoid contact with the entry and exit ports of the aqueous humor microdialysis probe (Fig. 2A). Subsequent to placing the well, the animals were allowed to stabilize for another 45 min to maintain proper intraocular pressure. After this time period, 200 μl of IPBS-containing drug/prodrug was added to the well at time zero and samples were collected at pre-determined time points through probe outlet. Constant infusion was allowed for a period of 120 min. Following this period, the drug solution was aspirated off from the well and subsequently removed. The corneal surface was washed clean with a few drops of distilled water. Samples were collected every 20 min throughout the infusion and post-infusion phases over a period of 8 h. At the end of the experiment, euthanasia was performed under deep anesthesia with an intravenous injection of sodium pentobarbital through the marginal ear vein. Samples were analyzed by HPLC.

2.7. Retrodialysis

Retrodialysis, also called as reverse microdialysis was performed by replacing the perfusate buffer (IPBS 7.4) with a known concentration of drug solution (ACV 10 μg/ml). Theory behind retrodialysis is based on the dynamic equilibrium principle where the amount of drug lost to the surrounding tissue at the sample site equals the amount of drug gained from the surrounding tissue. Microdialysis probes were perfused with the drug solution at a flow rate of 3 μl/min for 12 h to check the probe integrity and also for variation in the profile. The profile of ACV in the probe perfusate was constant indicating no measurable loss in probe integrity (Fig. 3).

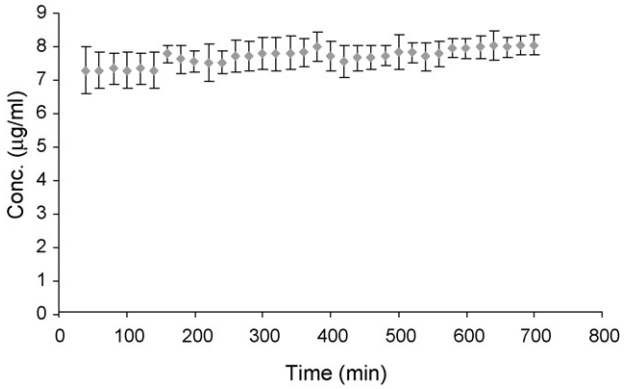

Fig. 3. Aqueous humor concentration–time profile of ACV (◆) upon infusion of ACV as a perfusate.

2.8. In vitro probe calibration

In vitro probe calibration was performed by placing the probe in IPBS, pH 7.4, containing appropriate drug/prodrug of known concentration. The probe was perfused at a flow rate of 3 μl/min with IPBS and the dialysate was collected every 20 min. Relative recovery of a compound was calculated by

$$\text{recovery} = \frac{C_d}{C_s} \quad (1)$$

where, C_d is the dialysate concentration and C_s is the known concentration of drug/prodrug in perfusate. The concentration of the respective drug in aqueous humor during the pharmacokinetic experiment was calculated by dividing the dialysate concentration with *in vitro* recovery.

The recovery of the linear probe was between 23 and 28% for ACV, 28 and 32% for AACV, 17 and 23% for SACV, 31 and 38% for EACV, 20 and 25% for IACV, and 30 and 35% for VACV. There was no significant variation (<1–2%) in the percent recovery of the probes over the experimental time period (Fig. 3). However, an average of the recovery determined before and after the experiment was considered for assessing the probe integrity during an experiment.

2.9. Absorption kinetics

As illustrated schematically in Fig. 2B, K_0 represents the overall input rate into the corneal tissue, X_a is the amount of drug in the aqueous humor, X_w is the amount of drug in the well, V_a is the physiological volume of the aqueous humor, V_w is the volume of drug solution in the well. The constants k_{ap} and k_{pa} are the first-order rate constants for the transfer of the drug/prodrug from the aqueous humor to the peripheral compartments and vice versa and k_{10} represents the overall elimination from the aqueous humor. The subscripts a and p refer to the aqueous and peripheral compartments, respectively.

Assuming sink conditions during initial infusion period (i.e. $X_w \gg X_a$ since $C_w \gg C_a$), the absorption rate constant, k_a can be calculated according to

$$k_a = \frac{(dC_a/dt)_I V_a}{C_w V_w} \quad (2)$$

where, C_a refers to the concentration in aqueous humor, k_a represents corneal first-order absorption rate constant, C_w is the concentration of the drug/prodrug in the well (11.1 mM), V_w denotes the volume of drug/prodrug solution in the well (200 μl), V_a is the physiological volume of aqueous humor (250 μl) which was assumed to be constant during an infusion period; which is further indicated by negligible volume of loss from the corneal well after infusion period. Subscript I refers to the initial rate which can be determined from the initial slope of C_a versus t as determined by aqueous humor microdialysis.

The disposition mean residence time for the drug/prodrugs in the aqueous humor, MRT_d is defined by

$$MRT_d = \frac{AUMC}{AUC} - \frac{t}{2} \quad (3)$$

where, AUMC represents the area (0–∞) under the aqueous humor concentration × time calculations plotted versus time, whereas AUC is the area (0–∞) under the aqueous humor concentration versus time and t is the time of infusion.

The topical infusion method along with Eqs. (2) and (3) permits a rational and reliable determination of ocular pharmacokinetic rate constants whereby absorption, distribution, and elimination can be characterized using noncompartmental analysis eliminating the need for complex compartmental analysis. A detailed explanation for the derivation of the above pharmacokinetic parameters was given by Eller et al. (1985).

2.10. Analytical procedures

All samples were assayed using RP-HPLC. The system consisted of a Rainin Dynamax Pump SD-200, Rainin Dynamax UV Detector at 254 nm and an Alcott autosampler Model 718 AL HPLC. A C18 Luna column 4.6 mm × 250 mm (Phenomenex, Torrance, CA) was employed. 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 generated rapid and reproducible results. HPLC conditions for these prodrugs have been reported previously (Anand et al., 2004; Katragadda et al., submitted for publication). The limits of quantification were found to be ACV, 25 ng/ml; AACV, 100 ng/ml; SACV, 50 ng/ml; EACV, 50 ng/ml; IACV, 50 ng/ml; and VACV, 50 ng/ml. Intra- and interday precision (measured by coefficient of variation, CV%) was less than 3 and 5%, respectively.

2.11. Statistical analysis

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

All relevant pharmacokinetic parameters were calculated by noncompartmental analyses of aqueous humor concentration–time curves with a pharmacokinetic software package, WinNonlin, v2.1 (Pharsight, CA). Data was fitted to a noncompartmental model, with a constant infusion over a period of time. The fit was examined by observing R^2, correlation or coefficient of variance (CV), weighted residuals and predicted versus observed values. Maximum aqueous humor concentrations (C_{max}) and area under the aqueous humor concentration time curves (AUC_{0-last} and AUC_{0-inf}) were obtained from the aqueous humor concentration–time profiles. The slopes of the terminal phase of aqueous humor profiles were estimated by log-linear regression and the terminal rate constant (λ_z) was calculated from the slope. The terminal aqueous humor half-lives were determined from the equation: $t_{1/2} = 0.693/\lambda_z$. Absorption rate constant (k_a) was obtained with Eq. (2).

3. Results

3.1. Stability studies

3.1.1. Stability in probe perfusate

Stabilities of L-alanine, L-isoleucine, L-serine, γ-glutamate and L-valine ester prodrugs of acyclovir were determined in the probe perfusate (IPBS, pH 7.4) for a period of 96 h (Table 1). The half-lives for AACV, SACV, EACV, VACV and IACV in IPBS, pH 7.4 were 2.2 ± 0.04, 14.6 ± 3.7, 18.3 ± 6.4, 14.2 ± 0.2 and 21 ± 0.05 h, respectively, indicating that chemical hydrolysis may cause regeneration of the parent drug during the course of a microdialysis experiment (Table 1). Comparatively shorter half-life for AACV (2.2 ± 0.04 h) in IPBS, pH 7.4 indicates that it is highly prone to chemical hydrolysis than other prodrugs.

Table 1
First-order hydrolysis rate constants of AACV, SACV, EACV, VACV and IACV in IPBS (pH 7.4), cornea, aqueous humor, iris-ciliary body and lens homogenates

	AACV		SACV		EACV		VACV		IACV	
	Rate ($k \times 10^3$ min^{-1})	Half-life (h)	Rate ($k \times 10^3$ min^{-1})	Half-life (h)	Rate ($k \times 10^3$ min^{-1})	Half-life (h)	Rate ($k \times 10^3$ min^{-1})	Half-life (h)	Rate ($k \times 10^3$ min^{-1})	Half-life (h)
IPBS 7.4	5.34 ± 0.09	2.2 ± 0.04	0.79 ± 0.14	14.6 ± 3.7	0.89 ± 0.19	18.3 ± 6.4	0.81 ± 0.01	14.2 ± 0.2	0.55 ± 0.002	21 ± 0.05
Cornea[a]	80.5 ± 27.6	0.16 ± 0.07	0.84 ± 0.11	13.8 ± 1.8	0.73 ± 0.36	27.1 ± 13.2	20.2 ± 1.7	0.57 ± 0.05	1.67 ± 0.09	6.9 ± 0.04
Aqueous humor[a]	71.2 ± 26.4	0.18 ± 0.06	–	[b]	3.05 ± 1.98	4.9 ± 2.7	1.6 ± 0.3	7.5 ± 1.7	0.34 ± 0.03	34 ± 3.5
Iris-ciliary body[a]	–	[c]	74.6 ± 4.49	0.15 ± 0.009	1.76 ± 0.34	6.7 ± 1.5	–	[c]	31.4 ± 1.7	0.37 ± 0.02
Lens[a]	8.35 ± 0.29	1.38 ± 0.05	1.42 ± 0.05	8.2 ± 0.3	–	[b]	1.75 ± 0.18	6.6 ± 0.7	0.07 ± 0.0007	158 ± 1.6

Values are mean ± S.D. ($n = 3$).
[a] Rate ($k \times 10^3$ min^{-1} mg protein^{-1}).
[b] No measurable degradation during the period of a 96-h experiment.
[c] Complete degradation before 2-min period.

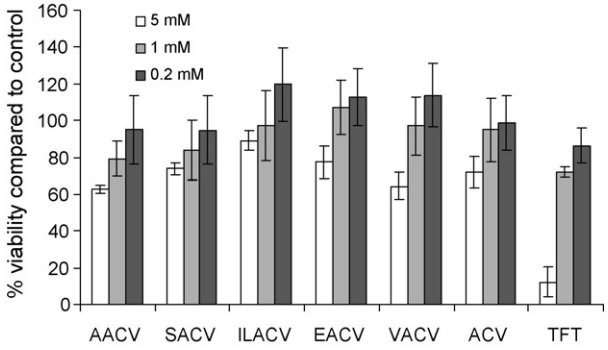

Fig. 4. Cell proliferation assay in the presence of various concentrations of ACV, AACV, SACV, IACV, EACV, VACV and TFT. Data are mean percentage of viable cells ± S.D.

3.1.2. Stability in ocular tissue homogenates

Enzymatic hydrolysis of the prodrugs was assessed in the ocular tissues like iris-ciliary body, cornea, aqueous humor and lens. AACV and VACV were readily converted to ACV exhibiting complete degradation in iris-ciliary body within 2 min (Table 1). On the contrary EACV exhibited excellent stability in ocular tissues (iris-ciliary body, lens and cornea) indicating that it was less prone to enzymatic hydrolysis. The trend was slightly different in aqueous humor, wherein SACV showed excellent stability compared to other prodrugs.

3.2. Cell proliferation assay

TFT, ACV, VACV, EACV, SACV, AACV and IACV all inhibited cell growth in a concentration-dependent manner, but to various degrees. IACV and SACV showed the least inhibition of cell growth at concentrations up to 5 mM. Exposure to 5 mM TFT resulted in almost complete cell death. ACV showed comparable cell survival at the highest concentration tested (5 mM) (Fig. 4).

3.3. In vivo ocular absorption

3.3.1. Total drug (prodrug + parent drug) concentrations

In vivo ocular absorption profiles of all the prodrugs were analyzed by the total (prodrug and regenerated parent drug) drug concentrations (Fig. 5) and by individual profiles as intact prodrug (Fig. 6) and regenerated parent drug, ACV (Fig. 7). In this section the total concentration profiles of the prodrugs and the regenerated parent drug, ACV has been considered. The absorption profiles of the compounds exhibited linear accumulation

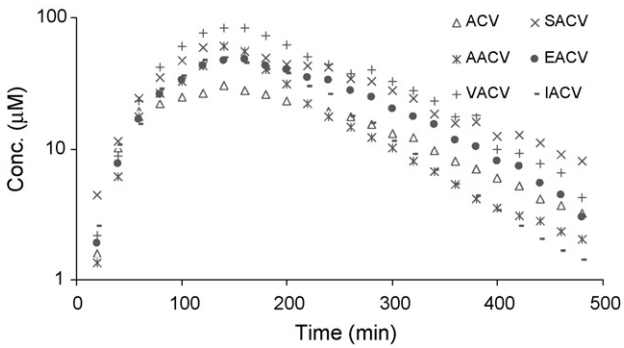

Fig. 5. Aqueous humor concentration–time profile of absorption of total concentration of ACV upon topical administration of (Δ) ACV, (✳) AACV, (×) SACV, (−) IACV, (♦) EACV and (+) VACV.

Table 2
Pharmacokinetic parameters for corneal absorption of amino acid prodrugs of ACV

Parameters	ACV[a]	SACV	AACV	EACV	IACV	VACV
$AUC_{(0-t)(TC)}$ (min μM)	7109 ± 1680	13,859 ± 1632*	10,167 ± 3531	10,529 ± 2570	8921 ± 2324	13,784 ± 4233*
$AUC_{inf(TC)}$ (min μM)	7558 ± 1851	14,875 ± 1997*	8764 ± 5002	10,819 ± 2616	9067 ± 2387	14,217 ± 4312*
$C_{last(TC)}$ (μM)	3.2 ± 1.2	8 ± 2.6*	2.2 ± 0.6	3 ± 0.9	1.4 ± 0.6	4.3 ± 2.7
$C_{max(TC)}$ (μM)	31 ± 4	85 ± 18*	66 ± 11*	54 ± 15*	52 ± 10*	86 ± 36*
$T_{max(TC)}$ (min)	152 ± 7	140 ± 8	135 ± 4	160 ± 11	140 ± 6	147 ± 6
$k_{a(TC)} \times 10^5$ (min^{-1})	2.8 ± 0.5	6.3 ± 1.1*	4.7 ± 1.9	4.6 ± 1.6	4.9 ± 0.9	5.7 ± 1.7*
$\lambda_{z(TC)}$ (min^{-1})	0.009 ± 0.001	0.015 ± 0.005	0.011 ± 0.0007	0.01 ± 0.0007	0.015 ± 0.003	0.01 ± 0.002
$MRT_{(TC)}$ (min)	128 ± 9	148 ± 2	103 ± 7	149 ± 11	113 ± 6	140 ± 13
$t_{1/2(TC)}$ (min)	82 ± 11	47 ± 13	72 ± 12	82 ± 6	64 ± 14	55 ± 10

Values presented are for total (prodrug + parent drug) drug concentrations. Values are mean ± S.E. (n = 4–6). TC, total concentration in terms of ACV; MRT, mean residence time, AUC, area under curve; C_{max}, maximum concentration; C_{last}, concentration achieved at the last time point; T_{max}, time to reach maximum concentration; λ_z, terminal elimination rate constant; k_a, absorption rate constant; $t_{1/2}$, half-life.

[a] Control.

* $p < 0.05$; compared to control.

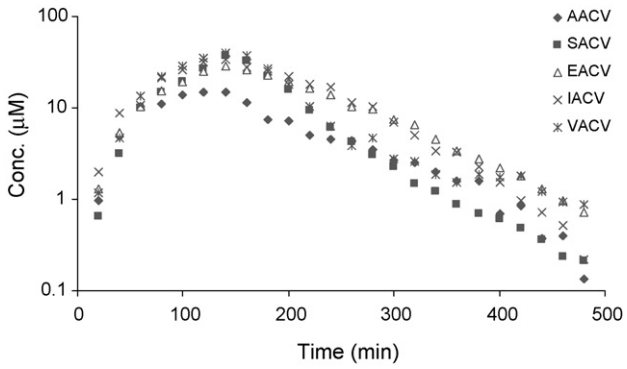

Fig. 6. Aqueous humor concentration–time profile of intact prodrug upon topical administration of (♦) AACV, (■) SACV, (×) IACV, (Δ) EACV and (✳) VACV.

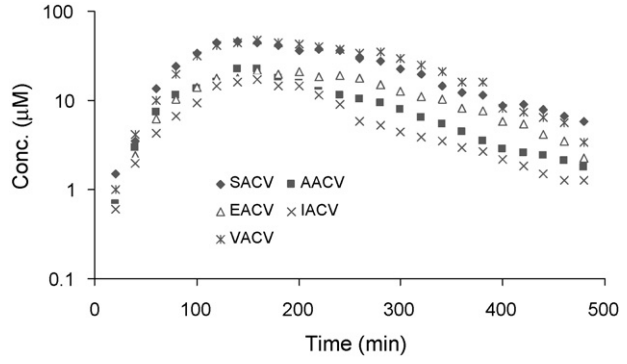

Fig. 7. Aqueous humor concentration–time profile of regenerated ACV upon topical administration of (■) AACV, (♦) SACV, (×) IACV, (Δ) EACV and (✳) VACV.

until the removal of the well (Figs. 5–8). The post-infusion phase showed first-order decline in total drug concentrations in aqueous humor (Figs. 5–8). Pharmacokinetic profiles for the absorption phases are shown in Fig. 5, whereas pharmacokinetic parameters have been summarized in Table 2. Area under curve (AUC_{inf}) of the total ACV concentrations plot after administration of ACV, SACV, AACV, EACV, IACV and VACV were calculated as 7558 ± 1851, 14,875 ± 1997, 8764 ± 5002, 10,819 ± 2616, 9067 ± 2387, and 14,217 ± 4312 min μM, respectively. SACV and VACV administration exhibited approximately twofold increase in AUC_{inf} relative to an ACV administration (Fig. 8). C_{last} of SACV was observed to be 8 ± 2.6 μM in aqueous humor which is two and three times higher than VACV and ACV, respectively. C_{max} values for total concentration of ACV also exhibited twofold increment for

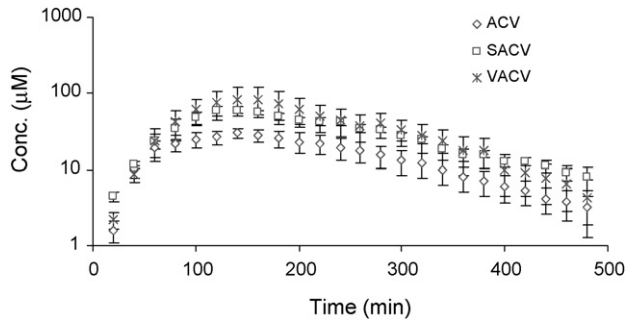

Fig. 8. Aqueous humor concentration–time profile of absorption of total concentration of ACV upon topical administration of (◊) ACV, (□) SACV and (✳) VACV.

SACV and VACV compared to ACV. AACV, EACV and IACV exhibited comparable AUC_{inf} even though C_{max} values were higher than ACV. Time to reach maximum concentration (T_{max}) for all the prodrugs did not vary significantly ($p < 0.05$) and was found to vary from 130 to 170 min. The absorption rate constants (k_a) also exhibited twofold increment for SACV and VACV compared to ACV (Table 2). The absorption rate constants (k_a) of all the compounds obtained from the linear portion of the cumulative concentration profile were found to be lower than the elimination rate constants (λ_z) obtained from the terminal portion of the profile. The elimination rate constants for all the prodrugs were similar. The mean residence time values (MRT) for all the prodrugs did not indicate any significant changes (Table 2).

3.3.2. Prodrug concentration

Pharmacokinetic parameters of intact SACV, AACV, EACV, IACV and VACV upon topical administration of SACV, AACV, EACV, IACV and VACV (Fig. 6) have been listed in Table 3A . The AUC_{inf} and C_{max} values of the intact prodrugs were similar for all the prodrugs. The $C_{last(PD)}$ value of the intact prodrug SACV was significantly high when compared to other prodrugs.

3.3.3. Parent drug concentration

Pharmacokinetic parameters of regenerated ACV upon topical administration of SACV, AACV, EACV, IACV and VACV (Fig. 7) have been listed in Table 3B. In contrast to intact prodrug, AUC_{inf} and C_{max} values of the regenerated ACV from SACV and VACV were significantly higher compared to other prodrugs. The C_{last} value of the regenerated ACV from SACV and VACV was also significantly high when compared to other prodrugs. Except SACV, C_{max} values for regenerated ACV ($C_{max(ACV)}$) were almost similar to C_{max} values for intact prodrugs ($C_{max(PD)}$).

Table 3
Pharmacokinetic parameters for corneal absorption of amino acid prodrugs of ACV

Parameters	SACV	AACV	EACV	IACV	VACV
(A) Values presented are for intact prodrug					
$AUC_{(0-t)(PD)}$ (min μM)	3157 ± 290	4277 ± 1934	5081 ± 1621	5784 ± 1719	3377 ± 433
$AUC_{inf(PD)}$ (min μM)	3437 ± 290	4300 ± 2369	5134 ± 1623	5793 ± 1724	3471 ± 443
$C_{last(PD)}$ (μM)	2.1 ± 0.3	0.2 ± 0.04	0.7 ± 0.1	0.2 ± 0.1	0.9 ± 0.4
$C_{max(PD)}$ (μM)	27 ± 3	28 ± 15	32 ± 11	33 ± 7	42 ± 18
(B) Values presented are for regenerated ACV from prodrug					
$AUC_{(0-t)(ACV)}$ (min μM)	10,702 ± 1453	4263 ± 2024	5448 ± 1726	3747 ± 601	10,407 ± 3233
$AUC_{inf(ACV)}$ (min μM)	11,495 ± 1795	4502 ± 2213	5725 ± 1817	3913 ± 643	10,745 ± 3625
$C_{last(ACV)}$ (μM)	5.8 ± 2.4	1.8 ± 1.4	2.3 ± 0.8	1.2 ± 0.5	3.3 ± 2.5
$C_{max(ACV)}$ (μM)	49 ± 4	23 ± 8	25 ± 6	22 ± 4	47 ± 18

Values are mean ± S.E. (n = 4–6). PD, concentration of prodrug; AUC, area under curve; C_{max}, maximum concentration; ACV, concentration of generated parent drug.

4. Discussion

Enzymatic and chemical processes are known to play an important role in the hydrolysis of prodrugs and it is well known that enzymatic processes frequently play a dominant role than the chemical process (Anand et al., 2003). The rank order of esterase activity in ocular tissues can be arranged in the following order; iris-ciliary body > cornea > aqueous humor ≫ lens (Lee et al., 1982, 1985). These enzymes are predominantly responsible for the bioreversion of amino acid prodrugs of ACV. The rank order of the stability of prodrugs in ocular tissue homogenates are as follows: EACV ≫ SACV > IACV > VACV > AACV. Results indicate that except EACV, tissue half-lives of the prodrugs were significantly low, which suggests that prodrugs are quite labile in the enzymatic environment of the ocular tissues especially, iris-ciliary body (Table 1). The lower enzymatic lability of EACV can be explained by the lack of amino terminus near the ester bond (hydrolysis site) which is formed between γ-carboxy terminus of glutamic acid and hydroxyl group of ACV. Further studies are needed to confirm the importance of amino terminus in facilitating the enzymatic hydrolysis of the ester bond. The higher stability of SACV in aqueous humor (the microdialysis sampling site) can also explain for its highest intact prodrug concentration at the last time point ($C_{last(PD)}$) amongst all prodrugs.

Results obtained from cytotoxicity studies on primary corneal cultures (Fig. 4) indicate ACV and its prodrugs to possess significantly lower cytotoxicity than TFT ($p < 0.05$) at 5 mM making them a safe and promising drug candidates for the treatment of HSV epithelial and stromal keratitis. The cytotoxic effects of all the compounds were found to be concentration dependent, with TFT being the most cytotoxic. The reduced cytotoxicity of ACV and its prodrugs in comparison with TFT could be explained by their mechanism of action (Elion, 1993). The mechanism for the antiviral activity of TFT is primarily due to inhibition of DNA polymerase and termination of viral DNA chain (Lee and Pavan-Langston, 1994). Further, ACV is specifically activated by viral thymidine kinase and then phosphorylated by cellular kinases to ACV triphosphate, which binds preferentially to herpes simplex virus (HSV) DNA polymerase and blocks viral replication (Elion, 1993). In superficial herpes keratitis, the clinical efficacy of 3% ACV ophthalmic ointment applied five times a day for up to 14 days has been reported (Richards et al., 1983). However, ACV ointment has not been approved by the U.S. Food and Drug Administration for clinical use against HSV keratitis in the United States (Richards et al., 1983; Crowe and Mills, 1988). In addition, ACV ointment is not effective against stromal keratitis or when deeper ocular tissues are involved. Thus, the development of a safe, long-acting, effective, nontoxic, and stable topical antiviral drops that require less frequent dosing over a few days would represent a significant improvement over the currently available therapy. The extremely low cytotoxicity of the prodrugs allows for long-term treatment, even at higher doses, without the concern for major side effects.

The rates of elimination for all the compounds were found to be much higher than the rates of absorption (Table 2). Previously, Eller et al. studied the absorption of carbonic anhydrase inhibitors using a well model and reported that the elimination rate constants were two to three orders of magnitude higher than the absorption rate constants (Eller et al., 1985). The elimination rate of the ACV and its prodrugs did not appear to be dependent on physicochemical properties. EACV, a hydrophilic compound was found to have a half-life of 82 min and IACV, a relatively lipophilic compound was found to have a half-life of 64 min. However, ACV having a lipophilicity that is an intermediate between EACV and IACV was found to have a half-life of 82 min.

Aqueous humor is presumed to be the primary route of elimination of the drug from the eye. In the rabbit eye, the turnover rate of aqueous humor equals a bulk flow of ~1.5% (4.2 μl/min) of the volume of the anterior chamber per minute. Hence the half-life a drug, whose elimination is exclusively by the aqueous humor elimination pathway, should be near about 30 min (McMaster and Macri, 1967). Half-lives of the prodrugs were in the range of 47–82 min, which is longer than the aqueous humor turnover rate, suggesting that tissue binding may have an influence on the elimination of these compounds. Systemic uptake through the highly vascular anterior uvea has been proposed as a route of elimination for compounds having very short half-lives. Therefore, this route may not be the predominant route of elimination for the compounds used in this study.

C_{max} and AUC_{inf} values of total (prodrug and regenerated parent drug) drug concentrations associated with SACV and VACV were about twofold higher than ACV (Fig. 8). The reason for this observation can be attributed to the *in vivo* corneal absorption rate constant (k_a) (Table 2). Incremental rise in k_a values appears to cause an increase in the extent of absorption leading to higher AUC_{inf} and C_{max} values, since elimination rate constants essentially remained the same. The individual profiles of intact prodrug and regenerated ACV (Fig. 6, 7) indicate that rise in $AUC_{inf(TC)}$ was mainly due to the regenerated ACV ($AUC_{inf(ACV)}$) as intact prodrug ($AUC_{inf(PD)}$) values were similar for all the prodrugs (Table 3A and B). C_{max} values of the individual profiles also seem to follow similar trend.

The time to reach maximum concentration (T_{max}) for all the prodrugs was observed to be in the range of 130–160 min, which exceeds the time of removal (120 min) of the drug from the topical well. Therefore, we can assume that cornea is acting as a reservoir and releasing the drug/prodrug for about 10–40 min even after the cessation of the infusion. Detectable levels of SACV ranging from 5.4 to 10.6 μM ($C_{last(TC)}$) were observed in aqueous humor at 480 min as the last time point of detection. This result may be attributed to the longest half-life of SACV in aqueous humor (sampling site) relative to other prodrugs.

The concentrations of ACV at the conclusion of the experiment, $C_{last(TC)}$ following topical administration of ACV, SACV, AACV, EACV, IACV and VACV were 3.2 ± 1.2, 8 ± 2.6, 2.2 ± 0.6, 3 ± 0.9, 1.4 ± 0.6, and 4.3 ± 2.7 μM, respectively. Amongst the prodrugs, $C_{last(TC)}$ for SACV exceeded the EC_{50} for HSV-1 (7.1 μM for ACV) (Anand et al., 2004) isolates whereas, $C_{last(TC)}$ for other prodrugs was below the EC_{50} for HSV-1. Therefore, at the end of an experiment the concentration of ACV in the aqueous humor following administration of SACV was higher than the concentration necessary to inhibit viral cytopathogenicity by 50%. Such superior $C_{last(TC)}$ value of SACV seems to have been generated from the intact prodrug ($C_{last(PD)}$) and regenerated ACV ($C_{last(ACV)}$). The reason for lower $C_{last(TC)}$ value of VACV despite showing comparable improvement in AUC, C_{max}, and k_a to SACV, may be probably due to rapid degradation of the prodrug (Table 1), leading to regeneration of ACV and rapid elimination.

Amino acid prodrugs could be readily absorbed across corneal epithelium there by penetrating into the deeper layers of the cornea, i.e., stroma and simultaneously can undergo hydrolysis to yield ACV. This strategy would be helpful in treating HSV infections of the deeper tissues without having to administer the drug frequently. Moreover, the current drug of choice TFT is not indicated for the treatment of keratitis with deep stromal invasion, and recurrent epithelial keratitis. Also in addition to its cytotoxicity, trifluoridine has a very short half-life (18–20 min) and must be administered every 2 h. The half-life of the cumulative ACV from the amino acid prodrugs were in the range of 47–82 min (Table 2), which would allow the dosing frequency to be reduced resulting in improved patient compliance and safety.

This study indicates that topical administration of amino acid prodrugs of ACV resulted in appreciable therapeutic concentrations of ACV in the aqueous humor. SACV along with other prodrugs shows significantly less cytotoxicity than the currently recommended agent, TFT. This study demonstrates that the ocular bioavailability of ACV can be elevated by approximately twofold upon topical administration of the amino acid prodrug, SACV and VACV in comparison to ACV. Less enzymatic stability of VACV compared to SACV seem to limit its C_{last} value. High C_{last}, AUC_{inf}, C_{max} and k_a values of SACV constitute a significant therapeutic advantage, not only over the current drug of choice trifluorothymidine but also on VACV in the treatment of ocular herpes infections. In conclusion, a combination of the corneal well infusion model and microdialysis can be a valuable tool to determine *in vivo* ocular pharmacokinetics of topically applied ophthalmic drugs.

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