In Vivo Antiviral Efficacy of a Dipeptide Acyclovir Prodrug, Val-Val-Acyclovir, against HSV-1 Epithelial and Stromal Keratitis in the Rabbit Eye Model

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PURPOSE. A dipeptide prodrug of the antiviral nucleoside acyclovir (ACV), val-val-ACV (VVACV), was evaluated in vivo as a potential drug candidate for improving antiviral efficacy against herpes epithelial and stromal keratitis.

METHODS. The effect of 1% VVACV on epithelial keratitis induced by inoculation of HSV-1 strain McKrae (25 μL of 10^6 plaque-forming units [PFU]) in the scarified rabbit cornea and stromal keratitis induced by intrastromal injection of HSV-1 strain RE (10 μL of 10^5 PFU) was compared with that of 1% trifluorothymidine (TFT) and balanced salt solution as the vehicle control. Both eyes of 10 rabbits were used in each treatment group. Lesions were evaluated by slit lamp examinations over a 2-week period after infection. Aqueous humor samples and corneas were analyzed for drug concentrations at the end of each experiment. Cytotoxicity of VVACV in comparison with valacyclovir (VACV), ACV, and TFT was evaluated in cellular proliferation assays.

RESULTS. The dipeptide prodrug VVACV demonstrated excellent activity against HSV-1 in the rabbit epithelial and stromal keratitis models: 1% VVACV was as effective as 1% TFT. The prodrug was also less cytotoxic than TFT, which is the only effective drug currently licensed and routinely used for topical treatment of ocular herpes infections in the United States.

CONCLUSIONS. The less cytotoxic and highly water-soluble prodrug VVACV, which showed excellent in vivo activity against HSV-1 in rabbit epithelial and stromal keratitis, is a promising drug candidate for treatment of ocular HSV infections. (Invest Ophthalmol Vis Sci. 2003;44:2529–2534) DOI:10.1167/iovs.02-1251

Herpes simplex keratitis is the leading cause of blindness in the United States,1 as well as the most frequent cause of corneal opacities in developed countries.2 Nucleoside analogues developed initially for the treatment of severe herpes simplex virus (HSV) infections (neonatal HSV, HSV encephalitis, and HSV keratitis) include trifluorothymidine (TFT), idoxuridine (IDU), and cytosine arabinoside (Ara-A), all of which were found to be too toxic for systemic use and were, therefore, restricted to topical use for herpetic keratitis.3 However, even long-term treatment with TFT is dose limited because of its cytotoxicity.4

Another nucleoside analogue, acyclovir (ACV), has been shown to be clinically effective against herpes viruses.5 Because of poor aqueous solubility6 and low corneal permeability, however, the drug is not very effective against ocular herpes infections.7 ACV ointment has demonstrated efficacy in treating superficial herpes keratitis,8 although its use has not been approved in the United States because of side effects associated with the ointment therapy. Various lipophilic prodrugs of ACV have also been examined as possible drug candidates against ocular herpes virus infections, but their enhanced lipophilicity renders these compounds poorly water soluble. Because of the complex nature of the cornea, a drug must strike a balance between hydrophilicity and lipophilicity to allow sufficient permeability across the cornea and to be formulated into 1% to 3% eye drops. To date, formulation of lipophilic prodrugs of ACV into eye drops has not been feasible.6,7

In the past, strategies have been used to design prodrugs of various poorly absorbed drugs targeted to receptors and transporters for improved bioavailability.8–15 In this connection, discovery of the oligopeptide transporter on the rabbit cornea14 opened up a new avenue for the development of prodrugs and analogues with improved bioavailability and site specificity due to selective targeting of the transporter.15 Most recently, the peptide transporters PepT1 and -T2 have captured considerable attention in terms of drug delivery through prodrug design. Small peptides, such as di- and tripeptides, are transported by PepT1 and -T2 in intestinal and renal epithelial cells, respectively. The structure, function, mechanism, and substrate specificity of the peptide transporters have been studied extensively.16–25

Dipeptide prodrugs of ACV were synthesized with the goal of improving the ocular bioavailability of ACV after topical instillation of an aqueous solution of the drug. It was hypothesized that the dipeptide prodrugs would use the oligopeptide transporter on the cornea for absorption, thereby increasing the permeability of ACV into the cornea. In an earlier report, the ACV dipeptide prodrugs were shown to be substrates for the human peptide transporter hPepT1 on the intestinal cell line Caco-2.26 Also the prodrugs exhibited excellent solution stability in comparison with valacyclovir (VACV), a drug of choice for oral and genital herpes27 because of its improved bioavailability in comparison with ACV.28,29 The dipeptide prodrugs also showed significantly lower cytotoxicity in the Statens Seruminstitut rabbit corneal cell line (American Type Culture Collection, Manassas, VA), in comparison with TFT and ACV itself, and exhibited excellent in vitro antiviral efficacy.
against HSV-1 in comparison with ACV. Finally, the prodrugs were highly soluble and permeable across the cornea in comparison with ACV. The high permeability was probably the result of recognition by the oligopeptide transporter on the cornea, and it is assumed that the increased permeability of the prodrugs will result in higher concentrations in the stromal tissues. The dipeptide ACV prodrugs can be formulated into 1% to 3% eye drops and seem therefore to be promising drug candidates for the treatment of HSV keratitis with stromal involvement.

In this report, we examined the antiviral efficacy of the dipeptide prodrug val-val-acyclovir (VVACV) in an in vivo rabbit HSV epithelial and stromal keratitis model. The antiviral effect of the drug was compared with that of TFF, which is the drug of choice for treatment of HSV keratitis. Also, the cytotoxicity of the prodrug in comparison with TFF, ACV, and VACV was assessed in primary corneal cultures.

**Materials and Methods**

**Materials**

VACV was the generous gift of GlaxoSmithKline (Research Triangle Park, NC). Trifluorothymidine ophthalmic solution (1%) was purchased from Falcon Pharmaceuticals (Fort Worth, TX). The growth medium, modified eagle medium (MEM), was obtained from Life Technologies (Grand Island, NY). MEM nonessential amino acids solution, penicillin, streptomycin, sodium bicarbonate, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). Culture flasks (75-cm² growth area) and 96-well plates were purchased from Costar (Bedford, MA). The solvents were of analytical grade and were obtained from Fisher Scientific (Pittsburgh, PA). ACV and VACV were synthesized in our laboratory. The structures of TFF, ACV, VACV, and VVVACV are shown in Figure 1.

For the in vivo antiviral experiments, New Zealand White (NZW) rabbits were obtained from McNeil Rabbitry (McNeil, MS). Corneas for the primary corneal cultures were obtained from NZW rabbits purchased from Myrtle's Rabbitry (Thompson Station, TN).

**Cellular Proliferation Assay**

Cellular proliferation assays were performed to examine the toxicity of VVVACV in comparison with ACV, TFF, and VACV. The commercial assay used (CellTiter 96 AQueous 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 cultures. Corneas from NZW rabbits were excised and washed thoroughly with Dulbecco's phosphate-buffered saline (DPBS). The corneas were blotted dry and transferred to sterile culture dishes containing 0.5 mL trypsin (0.25%) or 1.2 U/mL protease (Dispase II, Sigma), after which the corneas were placed with the epithelial surface touching the protease and incubated at 37°C for 30 minutes. The epithelial cells were stripped off with gentle scraping from the periphery (1–1.5 mm from the limbus) to the center. Care was taken to peel only the epithelial layer and not the underlying stromal layer, as that would contaminate the epithelial cells with keratinocytes. The cells were washed with MEM and placed in culture flasks. After 12 hours, the medium was removed and fresh medium supplemented with insulin (5 μg/mL), transferin (5 μg/mL), sodium selenite (5 ng/mL), amphotericin B (0.25 μg/mL), polymyxin B sulfate (0.5 μg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), human recombinant epidermal growth factor (10 ng/mL), 0.4% bovine pituitary extract, and 10% fetal bovine serum was added. The medium was changed twice a week, and the cells were subcultured every 7 to 10 days (subculture ratio 1:5).

The cells were seeded onto 96-well plates for the cell proliferation assay. Solutions of TFF, ACV, VACV, and VVVACV at different concentrations (0.05–5 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% CO₂ atmosphere for a period of 24 or 48 hours to evaluate the time-dependent 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 495 nm (reference at 590 nm) using a 96-well microtiter plate reader (SpectraFluor Plus; Tecxan, Manndorf, Switzerland).

**In Vivo Antiviral Efficacy**

**Epithelial Keratitis.** HSV-1 strain McKrae was propagated on primary rabbit kidney (PRK) cell monolayers and titered by plaque assay on African green monkey kidney cell (CV-1) monolayers. The mildly scarified corneas of NZW rabbits (1.5–2.5 kg) were inoculated with 25 μL of a suspension of HSV-1 strain McKrae (1 × 10⁸ PFU). The eyelids were closed and the viral suspension rubbed gently on the corneal surface for 20 to 40 seconds. Care was taken to avoid leakage of the suspension. Animal care and treatment in this investigation was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Three groups of 10 rabbits each were used, one group each for treatment with TFF, VVVACV, and a vehicle control (BSS; Cytosol Ophthalmics, Lenoir, NC). Both eyes of all rabbits were used. Each compound (50 μL at concentration of 1%) was applied topically five times per day every 2 hours starting at 8 AM and ending at 4 PM. Treatment was begun on postinfection (PI) day 3 and continued for five consecutive days. Slit lamp examination (SLE) was performed on a masked basis once a day from PI days 3 though 10 and then again on

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**TABLE 1. SLE Scoring System for Epithelial Keratitis Caused by HSV-1 McKrae in Rabbit Eyes**

<table>
<thead>
<tr>
<th>SLE Score</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0–0.5</td>
<td>Normal to nonspecific, random superficial lesion</td>
</tr>
<tr>
<td>0.6–0.9</td>
<td>Punctate ulcerations</td>
</tr>
<tr>
<td>1.0–1.9</td>
<td>One or more dendritic ulcerations</td>
</tr>
<tr>
<td>2.0–2.9</td>
<td>Geographic ulceration or trophic erosion (less than 50% of cornea involved)</td>
</tr>
<tr>
<td>3.0–3.9</td>
<td>Geographic ulceration or trophic erosion (more than 50% of cornea involved)</td>
</tr>
</tbody>
</table>
Table 2. SLE Scoring System for Stromal Keratitis Caused by HSV-1 Strain RE in Rabbit Eyes

<table>
<thead>
<tr>
<th>SLE Score</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>Normal</td>
</tr>
<tr>
<td>0.5–0.9</td>
<td>Mild edema; not diffuse; no haze</td>
</tr>
<tr>
<td>1.0–1.9</td>
<td>Significant edema; slight haze; iris clearly visible</td>
</tr>
<tr>
<td>2.0–2.9</td>
<td>Gross edema; stromal swelling; cloudy, diffuse; can see anterior chamber; visible</td>
</tr>
<tr>
<td>3.0</td>
<td>Severe stromal edema; very cloudy, cannot see anterior chamber; pupillary border no longer distinct</td>
</tr>
<tr>
<td>4.0</td>
<td>Opaque cornea; anterior chamber structure not visible</td>
</tr>
</tbody>
</table>

PI days 12 and 14. The ocular lesions were characterized as deep punctate lesions, dendritic lesions, or geographical epithelial defects. The HSV-1 scoring system used in the rabbit eyes is summarized in Table 1. At the conclusion of the examination period, 100 μL of aqueous humor was collected from each experimental eye and analyzed for VVACV or TFT.

**Stromal Keratitis.** Corneas of anesthetized NZW rabbits were injected intrastromically with 10 μL of RE strain HSV-1 (105 PFU). Three groups of 10 rabbits each were used, one group each for treatment with TFT, VVACV, and a vehicle control (BSS; Cytosol Ophthalmics). Aliquots of each compound (50 μL at a concentration of 1%) were applied topically every 2 hours starting at 6 AM and ending at 4 PM. Treatment was begun on PI day 3 and continued for five consecutive days. SLE was performed on a masked basis once a day on PI days 3 through 14. Corneas were evaluated for stromal edema and visibility into the aqueous chamber with simultaneous visualization of the pupil and iris. The scoring system used to evaluate stromal disease is summarized in Table 2. At the end of the examination period, 100 μL of the aqueous humor from each experimental eye was aspirated and analyzed for VVACV or TFT. Also, corneas were excised and analyzed for VVACV or TFT.

**Analytical Procedures**

**HPLC Conditions.** The aqueous humor and corneas were assayed using high pressure liquid chromatography (HPLC). The system consisted of a pump (Rainin model SD-200; Dynamax, Woburn, MA), a UV detector (Rainin model UV-C; Dynamax) at 254 nm, a fluorescence detector (model 1100; Hewlett Packard, Palo Alto, CA) at excitation of λ 285 nm and emission of λ 370 nm, and an autosampler (model 718 AL HPLC; Alcott, Norcross, GA, with a C18 Luna column, 4.6 × 250 mm; Phenomenex, Torrence, CA). The mobile phase consisted of a mixture of buffer and an organic modifier. The percentage of the organic phase was varied to elute compounds of interest. HPLC conditions for the various compounds are summarized in Table 3. At the end of the examination period, 100 μL of the aqueous humor from each experimental eye was aspirated and analyzed for VVACV or TFT. Also, corneas were excised and analyzed for VVACV or TFT.

**Sample Preparation.** Aqueous humor (100 μL) was mixed with 100 μL of a 4:5 chilled mixture of acetonitrile and methanol to precipitate the proteins. The sample was then centrifuged, and the supernatant was analyzed for VVACV and TFT. The standards were prepared in a similar fashion to account for losses in recovery of the produrg and the metabolites. Excised corneas were homogenized in 1 mL of a chilled 50:50 mixture of isotonic PBS (pH 7.4) and a chilled 4:5 mixture of acetonitrile and methanol for approximately 4 minutes in a tissue homogenizer (model 985-370 Tissue Tearer; Biospec Products, Inc., Bartlesville, OK). Subsequently the corneal homogenates were centrifuged at 12,500 rpm for 25 minutes at 4°C to remove cellular debris, and the supernatant was used for hydrolysis studies.

**Statistical Analysis**

For slit lamp scores, nonparametric one-way analysis of variance (ANOVA: Kruskal-Wallis test) was used. Wilcoxon scores were used for comparison among groups in this analysis. Analyses were performed on computer (SAS/Stat Guide for Personal Computers, Ver. 8.D1; SAS Institute, Cary, NC). Cellular proliferation assays were conducted in triplicates with three sets each and the results expressed as means ± standard deviation. ANOVA was performed to detect statistical significance with $P < 0.05$ considered to be statistically significant.

**Results**

**Cellular Proliferation Assay**

TFT, ACV, VACV, and VVACV all inhibited cell growth in a concentration-dependent manner (Table 4), but to various degrees. VACV and VVACV showed the least inhibition of cell growth at concentrations up to 5 mM at both 24 and 48 hours.

The effect of the drugs on cellular proliferation was also found to be time dependent (Table 4). Exposure to 5 mM TFT for 48 hours resulted in almost complete cell death. Only VVACV showed no significant different in cell survival from 24 to 48 hours at the highest concentration tested (5 mM).

**In Vivo Efficacy Studies**

**Epithelial Keratitis.** The effects of 1% TFT and 1% VVACV were comparable at 14 days (Fig. 2). However, the dipeptide produrg VVACV produced the same therapeutic effect at half the molar concentration (1% TFT = 35.3 mM; 1% VVACV = 18.6 mM). At the end of the epithelial keratitis experiments, the concentration of VVACV in the aqueous humor was 25.2 ± 16.8 μM ($n = 10$). TFT was not detected in the aqueous humor samples.

**Stromal Keratitis.** Treatment for HSV keratitis with stromal involvement requires absorption of topically applied drug through the deeper layers of the corneal epithelium. Our results show that 1% VVACV was as effective as 1% TFT, although the molar concentration of VVACV was only approximately half that of TFT; SLE scores were significantly lower in VVACV-treated eyes than in control eyes ($P < 0.05$; Fig. 3). Thus, the val-val produrg can be applied effectively at a lower concentration in stromal keratitis without the complications of cytotoxicity. It should be noted that both epithelial and stromal disease were self-limited in this model, so that SLE scores of the

<table>
<thead>
<tr>
<th>Drug</th>
<th>Composition of Aqueous Phase (pH 2.5)</th>
<th>Composition of Organic Phase</th>
<th>Mobile Phase (Aq:Org)</th>
<th>Parent Drug</th>
<th>Amino Acid Prodrug</th>
<th>Dipeptide Prodrug</th>
<th>Retention Times (Min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclovir (ACV)</td>
<td>25 mM NH₄H₂PO₄</td>
<td>Acetonitrile</td>
<td>98:2</td>
<td>8.8</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ValACV (VACV)</td>
<td>25 mM NH₄H₂PO₄</td>
<td>Acetonitrile</td>
<td>95:5</td>
<td>5.2</td>
<td>8.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ValValACV (VVACV)</td>
<td>25 mM NH₄H₂PO₄</td>
<td>Acetonitrile</td>
<td>94:6</td>
<td>3.9</td>
<td>5.5</td>
<td>32.1</td>
<td>—</td>
</tr>
<tr>
<td>TFT</td>
<td>10 mM CH₃COONa†</td>
<td>Methanol</td>
<td>87.5:12.5</td>
<td>5.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* UV detection at $\lambda_{max}$ 254 nm.
† pH 3.88.
eyes treated with the vehicle control (BSS) also returned to normal as the days progressed (Figs. 2, 3).

At the end of the stromal keratitis experiments, the concentration of VVACV was 25.1 ± 10.4 µM (n = 10) in the aqueous humor; no drug was detected in the cornea. TFT was not detected in the either aqueous humor or the cornea.

**DISCUSSION**

Therapy for HSV keratitis in the United States and elsewhere may involve the topical application of TFT drops and/or IDU, Ara-A, or ACV ointments, all of which are associated with poor ocular absorption and dose-limiting cytotoxicity that restricts long-term use. Chemotherapeutic regimens can vary, with the initiation of antivirals beginning as early as PI day 2 and as late as day 7.

IDU and TFT are nucleoside analogues that are incorporated into the viral DNA, causing disruption of viral DNA synthesis. At present, topical application of TFT is the gold standard in the treatment of HSV keratitis, although cytotoxicity limits its use. One of the mechanisms for the antiviral activity of Ara-A may involve inhibition of DNA polymerase and terminating the viral DNA chain. ACV is specifically activated by viral thymidine kinase and then phosphorylated by cellular enzymes to ACV triphosphate, which binds preferentially to HSV-1 DNA polymerase and blocks viral replication. 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. 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. In addition, ACV ointment is not effective against stromal keratitis or when deeper ocular tissues are involved. VACV, a drug of choice for genital herpes, increases the bioavailability of ACV by due to its recognition by hPEPT1 after oral absorption. However, because of its limited stability in solution, formulation of higher concentrations of VACV into stable aqueous drops is not feasible, and, therefore, this drug cannot be used against HSV epithelial and stromal keratitis. Thus, the development of a safe, long-acting, effective, nontoxic, and stable topical antiviral drops that require less frequent doses for fewer days or lower concentrations would represent a significant improvement over the currently available antiviral drugs.

In an earlier report, VVACV showed extremely low cytotoxicity in comparison with TFT and ACV, making VVACV a safe and promising drug candidate for the treatment of HSV epithelial and stromal keratitis. In the present study, similar

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**Table 4.** Cellular Proliferation Assay in the Presence of Various Concentrations of Drugs as a Function of Time

<table>
<thead>
<tr>
<th>Drug Conc. (mM)</th>
<th>TFT</th>
<th>ACV</th>
<th>VACV</th>
<th>VVACV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 2.1</td>
<td>100 ± 2.1</td>
<td>100 ± 2.1</td>
<td>100 ± 2.1</td>
</tr>
<tr>
<td>0.05</td>
<td>96.8 ± 8.6</td>
<td>70.1 ± 2.7</td>
<td>96.2 ± 2.5</td>
<td>102.1 ± 2.7</td>
</tr>
<tr>
<td>0.1</td>
<td>89.1 ± 4.9</td>
<td>65.6 ± 5.1</td>
<td>94.4 ± 6.2</td>
<td>105.2 ± 2.9</td>
</tr>
<tr>
<td>0.5</td>
<td>61.7 ± 5.6</td>
<td>42.7 ± 4.3</td>
<td>83.6 ± 4.3</td>
<td>77.5 ± 3.8</td>
</tr>
<tr>
<td>1.0</td>
<td>55.1 ± 5.1</td>
<td>53.3 ± 4.4</td>
<td>77.6 ± 11.3</td>
<td>66.2 ± 5.6</td>
</tr>
<tr>
<td>5.0</td>
<td>11.1 ± 3.2</td>
<td>1.11 ± 1.7</td>
<td>60.1 ± 2.7</td>
<td>37.8 ± 2.8</td>
</tr>
</tbody>
</table>

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**FIGURE 2.** SLE scores for VVACV and TFT in a rabbit model of HSV-1 epithelial keratitis. *Significant difference (P < 0.05) for VVACV compared with BSS. †Significant difference (P < 0.05) for TFT compared with vehicle control.

**FIGURE 3.** SLE scores for VVACV and TFT in rabbit model of HSV-1 stromal keratitis. *Significant difference (P < 0.05) VVACV vs. vehicle; †significant difference (P < 0.05) TFT vs. vehicle.
results were obtained from cytotoxicity studies on primary corneal cultures (Table 4), wherein VVACV was found to be significantly less cytotoxic than TFT or ACV (P < 0.05). The reduced cytotoxicity of VACV and VVVACV in comparison with ACV could be explained by the requirement for a free hydroxyl group essential for the antiviral activity of ACV. In VVACV, ACV could be explained by the requirement for a free hydroxyl or aqueous humor. There are several possible explanations for VVVACV and ACV. VVACV was detected in the aqueous humor, in inhibit viral cytopathogenicity by 50%. Corneal tissues were experiments were higher than the concentration necessary to 10% of the rabbits during the experiments. The extremely low cytotoxicity of the prodrugs allows for long-term treatment, even at higher doses, without the concern for major side effects.

HSV-1 keratitis often manifests as dendritic lesions, progressing to contiguous spread of viral lesions laterally beneath the apical epithelium. Reduction of the viral load at the level of the stroma reduces the severity and frequency of recurrent disease. The anti-HSV-1 activity of VACV was not compromised at the esterification of ACV necessary to form the dipeptide prodrug. The concentrations of drugs required to reduce HSV-induced disease scores to 0.3 in the rabbit epithelial keratitis model and 0.2 in the stromal keratitis rabbit model by day 14 were 33.5 mM for TFT and 18.6 mM for VACV. The results show that VVVACV is as effective as TFT at approximately half the molar concentration and is therefore more potent against HSV epithelial and stromal keratitis than TFT. We suggest that treatment of herpetic keratitis with dipeptide prodrugs of ACV may reduce the frequency and severity of recurrent keratitis and reduce stromal damage, thereby reducing the incidence of scarring.

The molar concentrations of drug remaining in the aqueous humor at the end of the epithelial and stromal keratitis experiments with VVVACV were 25.2 ± 16.8 and 25.1 ± 10.4 μM, respectively. In an earlier study, we showed that VVVACV had an EC50 of 6.14 μM against HSV-1. Therefore, the concentrations achieved in the aqueous humor at the end of the in vivo experiments were higher than the concentration necessary to inhibit viral cytopathogenicity by 50%. Corneal tissues were analyzed for TFT, VACV, and the metabolites of VVVACV (VACV and ACV). VVVACV was detected in the aqueous humor, but not in the corneas. TFT was not detected in either cornea or aqueous humor. There are several possible explanations for these findings. Because the preparation of the corneal samples involved several extraction procedures, it could be that the amount of drug remaining in the tissue was below the detectable limit. It is also possible that the drugs were degraded in the corneal tissues, or that VVVACV is in fact concentrated in the aqueous humor. Both the appearance of VVVACV in the aqueous humor and the reduction in the SLE scores in the epithelial and stromal keratitis models provide evidence that the topically applied drug traversed the tissues of the cornea. Because our study assessed tissue drug concentrations at only one time point, it is difficult to draw conclusions from these data. A better understanding of this point would require a detailed evaluation of the aqueous humor kinetics of this drug, but this type of investigation was beyond the scope of the present study.

Topical trifluridine is very effective for treatment of HSV-1 ocular disease. Consequently, a new topical ocular antiviral agent should exhibit similar or greater therapeutic effects than TFT as the current drug of choice, as well as lesser toxicity. Because of the recognition of VVVACV by the oligopeptide transporter on the cornea and its ability to be formulated into stable aqueous eye drops of 1% to 3% concentration, higher concentrations of ACV can be achieved in the deeper tissues of the cornea, and diseases such as HSV epithelial and stromal keratitis can be cured. In the future, a broader series of dipeptide prodrugs of ACV should also be examined for in vivo antiviral efficacy to develop the safest and most effective antiviral agents against ocular HSV infection.

Conclusions

VVACV, the dipeptide prodrug of ACV, is highly soluble and stable in water, allowing formulation into 1% to 3% eye drops. VVVACV is also significantly less cytotoxic than ACV and the currently recommended agent, TFT. In vivo, VVVACV exhibits excellent antiviral activity against epithelial and stromal keratitis and is as effective as TFT in the rabbit eye model. The stability, solubility, and in vivo antiviral efficacy of VVVACV makes formulation into stable aqueous solutions at higher concentrations a feasible goal. The VVVACV solution is effective in treating the infections of the underlying deeper stromal tissues. Therefore, the dipeptide prodrugs of ACV may constitute a significant therapeutic advantage in the treatment of ocular HSV infections.

References