Intravitreal Toxicology and Duration of Efficacy of a Novel Antiviral Lipid Prodrug of Ganciclovir in Liposome Formulation

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PURPOSE. To evaluate the intraocular safety and antiviral treatment efficacy of the sustained lipid prodrug of ganciclovir, 1-O-hexadecylpropanediol-3-phospho-ganciclovir (HDP-P-GCV), as an intravitreal injectable drug system for viral retinitis.

METHODS. HDP-P-GCV was synthesized by coupling 1-O-hexadecyl-propanediol-3-phosphate to either free hydroxyl of ganciclovir in pyridine with dicyclohexylcarbodiimide as catalyst. The compound was formulated into liposomes. The antiviral activity was assessed by DNA reduction in vitro, and intraocular safety was assessed by ophthalmoscopy, electrophysiology, and histology after intravitreal injections, with resultant intravitreal concentrations of 0.2, 0.632, 1.12, and 2 mM. The treatment efficacy was evaluated by simultaneous intravitreal injection of HDP-P-GCV and herpes simplex virus type 1 (HSV-1) or by intravitreal injection of HDP-P-GCV at various times before HSV-1 intravitreal inoculation. Retinitis was scored with ophthalmoscopy and compared with controls.

RESULTS. In vitro, the IC50 of HDP-P-GCV against HSV-1 and human cytomegalovirus (HCMV)-infected cells was 0.02 and 0.6 μM, respectively. In rabbits in vivo, HDP-P-GCV dispersed evenly and maintained a good vitreous clarity at all doses except 2 mM final intravitreal concentration. Although cataracts were observed in some eyes at the higher doses, they were not observed in eyes with 0.2 mM final intravitreal concentration. No other indications of ocular toxicity were observed. Intravitreal injection of HDP-P-GCV with resultant 0.2 mM intravitreal concentration in the HSV-1 retinitis rabbit model demonstrated a complete protection of the retina with the simultaneous treatment strategy and a 4- (P = 0.03) to 6- (P = 0.058) week significant protection of retina with the pretreatment strategies when compared with ganciclovir or blank liposome controls.

CONCLUSIONS. In the rabbit model of HSV-1 retinitis HDP-P-GCV acts as a long-lasting intravitreal injectable anti-CMV or anti-HSV compound. This self-assembling liposome system could be applicable for many compounds available for intraocular diseases. (Invest Ophthalmol Vis Sci. 2000;41:1523–1532)

Cytomegalovirus (CMV) infection of the retina is the most common opportunistic infection in acquired immune deficiency syndrome (AIDS) patients. Although the incidence of CMV retinitis decreased after the introduction of highly active antiretroviral therapy (HAART),1 CMV retinitis remains a threat to vision and quality of life in AIDS patients who fail HAART or do not qualify for HAART. Ganciclovir (GCV) was the first drug to be approved for CMV infection in AIDS patients. Ganciclovir is effective in treating CMV retinitis by intravenous administration, but the drug does not eliminate the virus from the retina, requiring long-term suppressive maintenance therapy. During maintenance treatment, relapse is almost inevitable in non-immune-reconstituted patients; systemic toxicity such as bone marrow suppression is also a problem. The sustained-release GCV implant is effective treatment for CMV retinitis2 and recurrent CMV retinitis,3 but complications from surgery such as endophthalmitis and retina detachment are sight threatening.3,4

We wished to develop a simple, in-office injectable local therapy that would be effective, minimally toxic, and long-lasting for treatment of CMV retinitis. We have previously reported a self-assembling liposomal foscarnet analog that demonstrated a higher therapeutic index5,6 and longer vitreal half-life than foscarnet, after intravitreal injection.7 In the present study, we evaluated the intravitreal toxicity and experimental treatment efficacy of 1-O-hexadecylpropanediol-3-phosphoganciclovir (HDP-P-GCV; Fig. 1).
monomethoxytrityl-ganciclovir (58% yield). The residue was crystallized in hot toluene to give 5.2 g N-acylated product. Proton nuclear magnetic resonance (1H-NMR) spectra were recorded on a General Electric QE-300 spectrophotometer operating at 300 MHz and reported in units of parts per million (ppm) relative to internal tetramethylsilane at 0.00 ppm. Anal. chromatography was performed using 230 to 400 mesh silica gel (EM Science, Gibbstown, NJ). Thin-layer chromatography was performed using 230 to 400 mesh silica gel (EM Science, Gibbstown, NJ). Combustion analyses were carried out by Oneida Research Services (Whitesboro, NY). Reagents were obtained from Aldrich Chemical (Milwaukee, WI) unless noted otherwise. Ganciclovir, obtained as cytovene oral capsules (Roche Laboratories, Nutley, NJ), was recrystallized from water and dried in vacuo before use.

Ganciclovir (4.4 g, 17.2 mmol) was suspended in dry pyridine (100 ml) and cooled to 0°C. Chlorotrimethylsilane (11.2 g, 103 mmol) was added drop-wise over 5 minutes. The mixture was allowed to warm to room temperature. Monomethoxymethyl chloride (6.18 g, 20 mmol) and dimethylaminopyridine (0.25 g, 2 mmol) were added, and the mixture was stirred overnight. The reaction mix was then cooled to 5°C with ice/H2O, water (20 ml) was added, and the mixture was stirred for 15 minutes. Concentrated NH4OH (25 ml) was then added, and the mixture was stirred an additional 15 minutes. The mixture was filtered, the solvent evaporated in vacuo, and the residue was chromatographed (gradient: CH2Cl2 → 15% EtOH/CH2Cl2) to give the coupled product as a white solid (36 g, 61% yield). This solid was suspended in 80% aqueous acetic acid and heated to 55°C for 5 hours to remove the monomethoxymethyl protecting group. The solution was concentrated in vacuo and the residue chromatographed (gradient: CH2Cl2 → 80:20:1:1 CH2Cl2:MeOH: NH4OH:H2O) to give HDP-P-GCV as a white powder that was recrystallized from 2:1 (1,4-dioxane/water) to give analytically pure HDP-P-GCV (9 g, 36% yield). The material was characterized by 1H-NMR as follows: (CDCl3 + DMSO-d6) d 0.852 (t, 3H), 1.249 (br s, 28H), 1.52 (m, 2H), 1.94 (t, 2H), 3.17 (m, 2H), 3.56 (t, 2H), 3.76 (d, 2H), 3.76 (s, 3H), 3.95 (m, 1H), 4.23 (m, 2H) 4.89 (m, 2H), 6.79 (d, 2H), 7.19 to 7.50 (m, 16H), 7.60 (s, 1H), 10.57 (s, 1H) [s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad singlet].

**Preparation of Liposomes Containing HDP-P-GCV for Antiviral Assays**

Chloroform solutions of the phospholipids, cholesterol, and drugs were mixed to provide dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), cholesterol (CH), and HDP-P-GCV at a molar ratio of 50/10/30/10. The chloroform was removed under a stream of nitrogen, and the thin lipid film was hydrated by the addition of 360 μl of 250 mM sorbitol/20 mM sodium acetate, pH 5.5. The small multiple dose vial was sealed under nitrogen with a Teflon-lined cap and sonicated for 1 hour at 42°C. The clear preparation of sonicated liposomes, representing a nominal HDP-P-GCV concentration of 5 mM, was diluted sequentially with Dulbecco’s modified Eagle’s medium containing 4% fetal bovine serum and antibiotics. The medium was removed, HCMV (AD-169 strain) was added at a dilution that resulted in 3 to 4 cytopathic effect in the control wells at 5 days, and the wells were incubated at 37°C for 1 hour. The supernatant was removed, replaced with drug dilutions at the indicated concentrations, and incubated for 5 days. HCMV DNA was quantified by nucleic acid hybridization using probes supplied by Diagnostic Hybrids (Athens, OH). The herpes simplex virus type 1 (HSV-1), strain F, assays were performed similarly but without drug pretreatment. HSV-1-infected cells were incubated for 24 hours with drug dilutions at the indicated concentrations. After incubation, the medium was removed, the cells were lysed, and the HSV-1 DNA present was quantified by nucleic acid hybridization using HSV-1 DNA probes obtained from Diagnostic Hybrids. The results are presented as a percentage of the no drug control HCMV or HSV-1 DNA levels (mean ± SD).
Liposome Preparation for Intravitreal Injection

HDP-P-GCV (5 mg, dry powder) was placed in a vial with DOPC, DOPG, and cholesterol at a molar ratio of 12:48:10:30 (HDP-P-GCV/DOPC/DOPG/CH). Solvent from the carrier lipids was removed by evaporation under nitrogen. Trace solvent was eliminated by lyophilization overnight. 5% dextrose was added to make a final drug concentration of 28 mM, and the mixture was sonicated for 1 h at 45°C, producing a opalescent solution of small unilamellar liposomes (diameter < 220 nanometers). 10 This solution was used directly or diluted with sterile 5% dextrose for injection at final intravitreal concentrations of approximately 2.0 mM, 1.12 mM, 0.632 mM, and 0.2 mM. For the treatment study, liposomes were made at a drug concentration of 8.85 mM and diluted to 2.8 mM to give a final intravitreal concentration of approximately 0.2 mM.

HSV-1 for In Vivo Study

HSV-1 PH strain, 11 provided courtesy of Jang Oh (Proctor Foundation, University of California, San Francisco), was cultured as described previously. 5 Immediately before intraocular viral inoculations, an aliquot of the virus was thawed and further diluted 10,000-fold.

Animal Studies

For the safety and toxicity study, 10 New Zealand white rabbits (weighing 1.7–3.6 kg) were used in accordance with the guidelines of the University of California, San Diego, Office of Campus Veterinary Services. Only the right eye of each rabbit was used for the study. The rabbits were divided into five groups: simultaneous treatment (22 rabbits), 1-week pretreatment (14 rabbits), 2-week pretreatment (15 rabbits), 4-week pretreatment (15 rabbits), and 6-week pretreatment (8 rabbits). For the simultaneous treatment study, 7 rabbits received intravitreal injections of 0.1 ml saline and 0.06 ml of a 10−6 TCID50/ml titered HSV-1; 5 rabbits received intravitreal injections of 7.6 TCID50/ml titered HSV-1; 5 rabbits received intravitreal injections of 71.4 μg GCV in 0.1 ml (equivalent vitreous concentration of 0.2 mM, i.e., 0.1 ml of 2.8 mM HDP-P-GCV) and 0.06 ml of a 10−4 dilution of 10−7.6 TCID50/ml titered HSV-1; and four rabbits were intravitreally injected with 0.1 ml of 2.8 mM HDP-P-GCV and 0.06 ml of a 10−4 dilution of 10−7.6 TCID50/ml titered HSV-1. For the 1-week pretreatment study, 6 rabbits were injected with 0.1 ml of 2.8 mM HDP-P-GCV, whereas the other 7 rabbits were injected with 700 μg GCV in 0.1 ml as control. One week later those 13 eyes were inoculated with 0.06 ml of a 10−4 dilution of 10−7.6 TCID50/ml titered HSV-1. For the 2-week pretreatment study, 8 rabbits were intravitreally injected with HDP-P-GCV, and 4 rabbits were injected with 700 μg GCV as control. Two weeks later those 12 eyes were inoculated with 0.06 ml of a 10−4

Electroretinography (ERG) was performed on all drug-injected rabbits and control rabbits postoperatively before they were killed as described previously. 5

At 2 or 8 weeks after injection, the rabbits were euthanized. Enucleated globes were fixed, dissected, and processed as previously described 7 for light and electron microscopic examination.

For the treatment study, a total of 72 pigmented rabbits were used in accordance with the guidelines of the University of California, San Diego, Office of Campus Veterinary Services. Only the right eye of each rabbit was used for the study. The rabbits were divided into five groups: simultaneous treatment (22 rabbits), 1-week pretreatment (14 rabbits), 2-week pretreatment (15 rabbits), 4-week pretreatment (15 rabbits), and 6-week pretreatment (8 rabbits). For the simultaneous treatment study, 7 rabbits received intravitreal injections of 0.1 ml saline and 0.06 ml of a 10−6 TCID50/ml titered HSV-1; 5 rabbits received intravitreal injections of 7.6 TCID50/ml titered HSV-1; 5 rabbits received intravitreal injections of 71.4 μg GCV in 0.1 ml (equivalent vitreous concentration of 0.2 mM, i.e., 0.1 ml of 2.8 mM HDP-P-GCV) and 0.06 ml of a 10−4 dilution of 10−7.6 TCID50/ml titered HSV-1; and four rabbits were intravitreally injected with 0.1 ml of 2.8 mM HDP-P-GCV and 0.06 ml of a 10−4 dilution of 10−7.6 TCID50/ml titered HSV-1. For the 1-week pretreatment study, 6 rabbits were injected with 0.1 ml of 2.8 mM HDP-P-GCV, whereas the other 7 rabbits were injected with 700 μg GCV in 0.1 ml as control. One week later those 13 eyes were inoculated with 0.06 ml of a 10−4 dilution of 10−7.6 TCID50/ml titered HSV-1. For the 2-week pretreatment study, 8 rabbits were intravitreally injected with HDP-P-GCV, and 4 rabbits were injected with 700 μg GCV as control. Two weeks later those 12 eyes were inoculated with 0.06 ml of a 10−4

Table 1. Clinical Safety and Toxicity with Intravitreal Injection of HDP-P-GCV

<table>
<thead>
<tr>
<th>Intravitreal Concentrations</th>
<th>No. of Eyes</th>
<th>2 Weeks after Injection</th>
<th>8 Weeks after Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VCS(+)</td>
<td>Ant Seg</td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>0, 0, 0, 0</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>HDP-P-GCV</td>
<td>0.2 mM</td>
<td>4</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td></td>
<td>0.632 mM</td>
<td>4</td>
<td>0, 1, 0, 1</td>
</tr>
<tr>
<td></td>
<td>1.12 mM</td>
<td>4</td>
<td>2, 2, 1, 2</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>4</td>
<td>4, 4, 4, 4</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.002</td>
<td>0.016</td>
</tr>
</tbody>
</table>

VCS, vitreous clarity scale, see text.

Ant Seg, anterior segment: 0 for normal; 1 for mild iris and conjunctive congestion; 2 for moderate iris and conjunctive congestion; and 3 for severe iris and conjunctive congestion.

Cataract: 0 for clear lens; 1 for local or mild lens opacity with permission of clear view of fundus; 2 for moderate lens opacity with obscuration of fundus detail; and 3 for complete lens opacification without view of fundus.

NL, normal; NV, no view.

P comparison of scores across all doses using Kruskal–Wallis.

* Graded by grossing.
dilution of $10^{-7.6}$ TCID$_{50}$/ml titered HSV-1. For the 4-week pretreatment study, 7 rabbits were intravitreally injected with 0.1 ml of 2.8 mM HDP-P-GCV, and 7 rabbits were injected with 700 µg GCV in 0.1 ml as control. Four weeks later those 14 rabbits were inoculated with 0.06 ml of a $1 \times 10^{-7}$ dilution of $10^{-7.6}$ TCID$_{50}$/ml titered HSV-1. For the 6-week pretreatment, 5 eyes were injected with 0.1 ml of 2.8 mM HDP-P-GCV, and 3 eyes were injected with saline as control to confirm the virus viability. In addition, 1 rabbit each was used to confirm the virus viability with intravitreal virus inoculation in the 1-, 2-, and 4-week pretreatment groups.

The HSV-1 was intravitreally inoculated under direct observation using a surgical microscope as described before by us. After the intravitreal HSV-1 inoculation, both eyes were examined on days 2, 4, 6, 9, 11, and 14. After careful fundus examination with an indirect ophthalmoscope, diagrams were made and the retinitis was graded according to the previously published score criteria. In selected cases the fundus was photographed. Most rabbits were killed 2 weeks after receiving HSV-1; however, a few of them were killed before the 2-week point because HSV-1 encephalitis developed or the fellow eye became infected. After eye enucleation, the globes were fixed in 10% formalin for 2 to 5 days before processing for routine histopathologic examination.

**Immunohistochemical Assay for HSV-1 Antigen**

Paraffin sections (5-µm-thick) were deparaffinized, then placed in 1% hydrogen peroxide in absolute methanol solution to neutralize endogenous peroxidase activity. Tissue sections were hydrated in a series of graded alcohols to water and then to a Tris buffer (0.05 M Tris, 1.5% NaCl, 2 mM CaCl$_2$, pH 7.6) and placed in proteinase K in Tris buffer (+4 µg/ml) at 37°C for 20 minutes. Sections were then transferred into 2% glycine in phosphate-buffered saline (PBS) for 5 minutes and subsequently placed in PBS (pH 7.5) for 5 minutes. The sections were then covered with a 1:40 dilution of antibody (horseradish peroxidase-conjugated rabbit anti-human HSV-1 antibody, No. P0175; DAKO, Carpinteria, CA) in a fish gelatin block, coverslipped, and incubated at 4°C overnight in a humidity chamber. The next day the coverslips were removed in a beaker of dH$_2$O. The slides were washed with PBS, followed by 0.1 M acetate buffer (pH 5.2), and then incubated with aminothiophenol and 3,3′-diaminobenzidine at 37°C in a humidity chamber for 20 to 30 minutes. The reaction was killed in dH$_2$O, and the sections were counterstained with Mayer’s hematoxylin.

**Statistical Analysis**

Statistical analysis was done using the Kruskal-Wallis test and JMP software, version 3.1 (SAS, Cary, NC). The scores from evaluation of vitreous clarity, anterior segment inflammation, cataract, and vitritis were computerized across the doses to reveal the meaningful differences. Differences in retinitis scores in HDP-P-GCV-treated versus control groups were also compared with the Kruskal-Wallis test.

**RESULTS**

**In Vitro Anti–HSV-1 and Anti-HCMV Activity of HDP-P-GCV**

The antiviral activities of GCV and HDP-P-GCV were determined in MRC-5 human lung fibroblasts infected with HSV-1. The concentration that lowered viral DNA by 50% for GCV was 0.03 µM and 0.02 µM for HDP-P-GCV ($P > 0.5$). In HCMV-infected cells, the 50% effective concentrations were 1.6 µM for GCV compared with 0.6 µM for HDP-P-GCV ($P < 0.006$).

**Clinical Evaluation of Safety and Toxicity**

After injection, HDP-P-GCV dispersed evenly in the vitreous in all eyes except the eyes that received the highest dose (0.1 ml of 28 mM). The vitreous was totally clear at 0.2 mM final intravitreal concentration. Vitreous clarity decreased with increasing dose. Both 0.632 and 1.12 mM final intravitreal concentrations demonstrated a vitreous clarity that permitted a clear view of small vessels on the medullary ray. The 2 mM final intravitreal dose resulted in a cloudy vitreous with the obscuration of the optic nerve head and medullary ray (Table 1). Variable cataract and anterior segment injection were observed in these doses, except for 0.2 mM final intravitreal concentration (Table 1). The frequency and severity of cataract and anterior segment injection were associated with drug concentration (Table 1). Indirect ophthalmoscopy of fundus did not reveal any abnormality in any eye. All eyes at all concentrations of HDP-P-GCV exhibited normal ERGs when compared with the saline controls (amplitude and latency; Fig. 2).

**Pathologic Evaluation of Toxicity**

Histologic examination demonstrated normal retinal morphology in all eyes at the second and eighth week (Fig. 3) except for one eye that received the highest dose. This eye demonstrated a poor preservation of inner and outer segments of photoreceptors. Mild vitritis or iritis and ciliary body inflammation were present in some eyes. Vitritis was more frequent in eyes with high doses; this was statistically significant ($P = 0.0028$, Kruskal–Wallis; Table 2). The inflammatory cells were predominantly chronic (lymphocytes and macrophages, some of which contained lipids). Electron microscopic examination of retina and ciliary body from eyes with 1.12 mM and lower final calculated intravitreal concentrations of HDP-P-GCV confirmed normal ultrastructure when compared with saline control eyes (Fig. 4).

**Clinical Evaluation of the HDP-P-GCV Treatment**

In the simultaneous treatment group, 4 rabbits that received HDP-P-GCV did not develop retinitis after up to 2 weeks of observation, whereas the 7 rabbits that received saline and the 6 rabbits that received blank liposomes all developed typical HSV-1 retinitis. This retinitis started at day 4 after HSV-1 intravitreal inoculation, with initial hyperemia and swelling (grade 0.5) and flame hemorrhaging (grade 1) of the optic nerve head. On day 6, the retinitis progressed with a few scattered retinal lesions on the inferior retina, with mild vitreous haze (grade 2) or confluent retinal lesions on the inferior retina and more vitreous opacification (grade 3; Fig. 5). On day 9, retinitis typically spread throughout the retina and caused severe vitreous opacification and retinal detachment (grade 4). In the simultaneous treatment group, of 5 rabbits receiving 71.4 µg GCV (a dose equivalent to 0.1 ml of 2.8 mM HDP-P-GCV intravitreal injection), none developed HSV-1 retinitis except for 1 rabbit who developed retinitis after day 6 and demonstrated a grade 4 retinitis on day 11 after virus inoculation. Comparison of the retinitis scores among eyes receiving HDP-P-GCV, saline, blank liposomes, or GCV revealed significant
differences between saline or blank liposomes and HDP-P-GCV or GCV; there was no difference between HDP-P-GCV– and GCV-injected eyes (Table 3).

In the 1-week pretreatment group, of 6 eyes with HDP-P-GCV, 3 did not develop retinitis and 3 developed a mild and delayed retinitis. In contrast, of 7 eyes injected with 700 mg GCV, 6 developed retinitis. The retinitis scores between the two groups were significantly different (Table 3).

In the 2-week pretreatment study, of 8 rabbits with HDP-P-GCV, 2 did not develop retinitis and 6 developed delayed retinitis. All 4 rabbits treated with 700 µg GCV developed typical retinitis, as did the saline controls. There were significant differences between HDP-P-GCV– and the GCV-treated eyes (Table 3).

In the 4-week pretreatment study, the retinitis scores between HDP-P-GCV– and the maximum (700 µg) GCV–treated eyes on day 4 and day 6 still demonstrated significant differences (P = 0.0044 and P = 0.03). The eyes treated with HDP-P-GCV showed a delay in time of retinitis onset and less severity in clinical appearance.

All 3 control eyes used for confirmation of virus viability in the 1-, 2-, and 4-week pretreatment groups developed typical HSV-1 retinitis.

With the 6-week pretreatment, of 5 eyes pretreated with HDP-P-GCV, 3 developed similar retinitis to the saline control eyes, and 2 eyes were completely protected from infection (P = 0.058).

Figure 2. Electrograms taken from eyes with saline or the highest dose (2 mM final intravitreal concentration) at the second week (A) and at the eighth week (B) after HDP-P-GCV intravitreal injections. Compared with the tracing from saline controls (interrupted lines marked with black dots), the tracings of treated eyes (2 mM final intravitreal concentration) showed normal amplitude and latency (solid lines marked with clear circles).

Figure 3. Light microscopic photograph showing normal retinal architecture from an eye with 0.2 mM final intravitreal concentration of HDP-P-GCV at the second week. Magnification, ×82.5.
Pathologic Evaluation of the Retinitis

Pathologic examination of eyes with retinitis showed destruction of whole layers of retina with retinal cell necrosis accompanied by severe choroiditis, optic nerve inflammation, vitritis, and retinal detachment (Fig. 6). In less severe cases, a sharp border between infected retina and normal retina was observed, reflecting a leading edge of spreading of viral retinitis (Fig. 7). HSV-1 infection of retina was confirmed by immunohistochemical assay using anti–HSV-1 antibody. The treated eyes with normal clinical appearance of the fundus also showed normal retinal architecture histologically.

DISCUSSION

Our studies suggest that HDP-P-GCV is a potent and selective antiviral agent against HSV-1 and HCMV. HDP-P-GCV formulates readily into liposomes and provides long-lasting protection against HSV-1 retinitis of at least 4 weeks. HDP-P-GCV liposomes provided good vitreous clarity and demonstrated minimal retinal toxicity except at the highest doses. However, at the 0.2 mM intravitreal concentration used in the treatment study, cataract formation was not observed and the vitreous was clear; both light and electron microscopy confirmed normal retina and ciliary body morphology. If we assume 0.2 mM is the highest nontoxic dose and divide by the 50% effective antiviral concentrations for HSV (0.023 μM) and HCMV (0.6 μM), we can estimate the selectivity indexes of HDP-P-GCV at 8600 for HSV-1 and 330 for HCMV, suggesting a high likelihood of therapeutic usefulness in vivo.

To evaluate the in vivo duration of efficacy, we conducted a treatment study in our HSV-1 retinitis animal model. In this

<table>
<thead>
<tr>
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<th>No. of Eyes</th>
<th>2 Weeks after Injection</th>
<th>8 Weeks after Injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cornea</td>
<td>Iris and Ciliary Body</td>
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<tr>
<td>Saline</td>
<td>2</td>
<td>NL, NL</td>
<td>NL, NL</td>
</tr>
<tr>
<td>HDP-P-GCV, mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>2</td>
<td>NL, NL</td>
<td>NL, NL</td>
</tr>
<tr>
<td>0.632</td>
<td>2</td>
<td>NL, NL</td>
<td>NL, MI</td>
</tr>
<tr>
<td>1.12</td>
<td>2</td>
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<td>2.0</td>
<td>2</td>
<td>NL, NL</td>
<td>MI, NL</td>
</tr>
</tbody>
</table>

* Comparison of vitritis scores between 0.2, 0.632 mM, and 1.12, 2.0 mM, using Kruskal–Wallis in all HDP-P-GCV eyes.

Vitritis: 0, no inflammatory cells seen; 1+, a few inflammatory cells at low magnification; 2+, sparse inflammatory cells at low magnification; 2+1, sparse inflammatory cells at low magnification; 4+, dense inflammatory cells at low magnification; and 3+, between 2+ and 4+.

NL, normal.

ABNL, abnormal with outer segments of photoreceptor cells shortened or not well preserved.

MI, mild iritis.
model, virus is inoculated onto the retina, drug efficacy can be assessed, and the duration of action of anti-HSV drugs can be determined.6,14 A direct measurement of the intravitreal concentration of HDP-P-GCV would be preferable. However, based on our previous studies,6,7 we have found that with the IC50 or IC90 obtained by the in vitro study and the drug level in living tissues determined by pharmacokinetics, the duration of protection provided by the antiviral compound against the virus cannot be precisely assessed. The pretreatment strategy used by us provides valuable information and allows comparison of relative duration of antiviral action in the eye of various compounds and formulations of antiviral drugs. We use this model because a natural rodent model of HCMV retinitis does not exist.15 Recently, attempts have been made to develop models of HCMV infection in which human retina was implanted in the anterior chambers of laboratory animals to support human cytomegalovirus infection.16,17 These models allow HCMV infection of human retina to be established in an experimental setting, but these are not natural retinitis models. It is difficult to clinically evaluate the antiviral effects of anti-CMV compounds in these settings. The murine CMV retinitis model is available,18 but there are essential differences between murine CMV and HCMV. For instance, acyclovir is highly effective against murine CMV but relatively ineffective against HCMV.19

**TABLE 3. Time-Course and Average Retinitis Scores in HDP-P-GCV–Treated and Untreated Control Rabbits**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 14</th>
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<tr>
<td>HDP-P-GCV TX simultaneous</td>
<td>4</td>
<td>0 (0/4)</td>
<td>0 (0/4)</td>
<td>0 (0/4)</td>
<td>0 (0/4)</td>
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<tr>
<td>Saline control simultaneous</td>
<td>7</td>
<td>0.95 (7/7)</td>
<td>3.43 (7/7)</td>
<td>4 (7/7)</td>
<td>4 (7/7)</td>
</tr>
<tr>
<td>Blank liposome simultaneous</td>
<td>6</td>
<td>2 (6/6)</td>
<td>3.8 (6/6)</td>
<td>4 (6/6)</td>
<td>4 (6/6)</td>
</tr>
<tr>
<td>71.4 μg GCV simultaneous</td>
<td>5</td>
<td>0 (0/5)</td>
<td>0.6 (1/5)</td>
<td>0.8 (1/5)</td>
<td></td>
</tr>
<tr>
<td>P vs saline</td>
<td></td>
<td>0.0058</td>
<td>0.0051</td>
<td>0.0016</td>
<td>0.0016</td>
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<tr>
<td>P vs liposome</td>
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<td>0.0077</td>
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<td>0.0027</td>
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<tr>
<td>P vs GCV</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDP-P-GCV 1 wk pre-TX</td>
<td>6</td>
<td>0 (0/6)</td>
<td>0.25 (2/6)</td>
<td>0.83 (5/6)</td>
<td>1.5 (5/6)</td>
</tr>
<tr>
<td>700 μg GCV 1 wk pre-TX</td>
<td>7</td>
<td>0.5 (5/7)</td>
<td>1.8 (6/7)</td>
<td>3.67 (6/7)</td>
<td>3.67 (6/7)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.021</td>
<td>0.029</td>
<td>0.015</td>
<td>NS</td>
</tr>
<tr>
<td>HDP-P-GCV 2 wk pre-TX</td>
<td>8</td>
<td>0.07 (1/8)</td>
<td>0.94 (5/8)</td>
<td>1.57 (5/8)</td>
<td>2 (6/8)</td>
</tr>
<tr>
<td>700 μg GCV 2 wk pre-TX</td>
<td>4</td>
<td>2 (4/4)</td>
<td>3.5 (4/4)</td>
<td>4 (4/4)</td>
<td>4 (4/4)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.0038</td>
<td>0.0057</td>
<td>0.0058</td>
<td>0.017</td>
</tr>
<tr>
<td>HDP-P-GCV 4 wk pre-TX</td>
<td>7</td>
<td>0 (0/7)</td>
<td>0.64 (6/7)</td>
<td>2.83 (6/7)</td>
<td>3.17 (6/7)</td>
</tr>
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<td>700 μg GCV 4 wk pre-TX</td>
<td>7</td>
<td>0.71 (6/7)</td>
<td>2.14 (6/7)</td>
<td>2.86 (6/7)</td>
<td>3.14 (6/7)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.0044</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDP-P-GCV 6 wk pre-TX</td>
<td>5</td>
<td>0.3 (3/5)</td>
<td>2 (3/5)</td>
<td>2.4 (3/5)</td>
<td>2.4 (3/5)</td>
</tr>
<tr>
<td>Saline control</td>
<td>3 + 3*</td>
<td>1.1 (6/6)</td>
<td>3.0 (6/6)</td>
<td>3.7 (6/6)</td>
<td>3.7 (6/6)</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.058</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

P Comparison of retinitis scores across groups using Kruskal-Wallis.
NS, nonsignificant; infection %, number of infected eye by total eye; TX, treatment.
* Control rabbits used for virus viability confirmation in 1-, 2-, and 4-week pretreatment.
In our model, drug treatment at the time of virus inoculation (simultaneous treatment) can be used to assess drug efficacy in treating retinitis. Pretreatment strategies with this model (drug injections days or weeks before virus inoculation) can assess duration of the antiviral effect of different intravitreally injected compounds. In the present study, the HSV-1 retinitis model produces a focal, nonlethal, and expanding infection that is highly consistent and reproducible as seen in the control eyes and a previous report.

Both HDP-P-GCV and equivalent GCV in the simultaneous treatment study protected retinas from viral infection, except for one eye with GCV. In contrast, all saline and blank liposome control eyes developed typical HSV-1 retinitis. We wished to compare the potency of HDP-P-GCV and GCV with injected equivalent doses of GCV (yielding 0.2 mM final intravitreal concentration). The results did not show a difference between HDP-P-GCV and GCV with the dose tested (0.2 mM of final intravitreal concentration), although there was one eye in the GCV group that developed delayed retinitis. It is possible that doses lower than 0.2 mM final intravitreal concentration might reveal a difference between the two drugs if tested.

In the pretreatment study, we used 0.2 mM final intravitreal concentration of HDP-P-GCV, whereas the maximum tolerated dose of GCV was used. Based on a recent report, 2000 mg of GCV can safely be intravitreally injected in human eyes. Assuming 1.4 ml of rabbit vitreous volume, 700 mg was

**Figure 6.** Light microscopic photograph of HSV-1 retinitis, showing whole layers of retinal necrosis, severe choroiditis, and vitritis. Magnification, ×82.5.

**Figure 7.** Immunohistochemical micrograph of HSV-1 retinitis from a 6-week HDP-P-GCV-pretreated eye. Retinitis involved whole retinal layers accompanied with vitritis. There is a fairly sharp border between infected and relatively normal retinas, which reflect a leading edge of spreading of viral retinitis. HSV-1 antigen was identified as dark red staining by immunohistochemical assay. Magnification, ×82.5.
equivalent to 2000 μg for the human eye. We used 700 μg GCV intravitreal injection in all GCV pretreated eyes. The pretreatment was designed to measure duration of antiviral effect after a single intravitreal injection. The pretreatment studies revealed that the antiviral effect of HDP-P-GCV lasted more than 4 weeks after a single intravitreal injection, whereas the maximum tolerated dose of GCV did not show antiviral effect at 1 week after intravitreal injection when compared with simultaneous saline control (P = 0.1343).

GCV can be intravitreally injected to treat viral retinitis with no systemic toxicity. However, this local treatment often requires frequent intravitreal injections because of the short vitreous half-life of GCV. Frequent injection may cause endophthalmitis or retinal detachment and also decrease quality of life. Currently the only nonfrequent intravitreal injectable antiviral drug for local therapy is cidofovir (HPMPC). Cidofovir could provide 5- to 6-week intervals between injections, but, unfortunately, it causes iritis and even irreversible visually significant hypotony. Decreasing the dose does decrease the incidence of iritis and hypotony, but the treatment effect decreases.

HDP-P-GCV provided a 4- to 6-week period of antiviral effect in our retinitis model in this study. It should be noticed that retinitis in this model is much more severe and progressive than CMV retinitis or other viral retinitis seen in humans. So, it may provide even longer antiviral duration if used to treat human viral retinitis. Compared with liposomal formulations of a lipid prodrug of foscarnet (1-O-octadecyl-sn-glycerol-3-phosphonoformate, ODG-PFA) and lipid prodrug of acyclovir (acyclovir diphosphate dimyristoylglycerol), HDP-P-GCV also demonstrated longer antiviral duration after a single injection in the same retinitis rabbit model (Table 4).

In the toxicity study, we found variable cataract formation with the higher intravitreal doses of HDP-P-GCV. The pathogenesis of cataract is not clear at the present, and it is under investigation. Reformulation might allow injection of a higher dose (higher than 0.2 mM final intravitreal concentration), or newer analogs of GCV designed for optimal liposomal formulation may be developed. This new antiviral agent, HDP-P-GCV, may be very useful as a local therapy for treating CMV retinitis, HSV retinitis, and other intraocular viral infections in both immunocompromised and immunocompetent individuals. This type of self-assemblying liposomal prodrug provides a prototype for intraocular drug delivery and may be applied to the delivery of many currently available drugs for chorioretinal or vitreoretinal diseases.

### References