Published in final edited form as:

Invest Ophthalmol Vis Sci. 2004 November ; 45(11): 4138–4144. doi:10.1167/iovs.04-0064.

Characterization of a novel intraocular drug delivery system using crystalline lipid antiviral prodrugs of ganciclovir and cyclic cidofovir

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Abstract

Purpose—We have previously reported a novel intraocular drug delivery system using hexadecyloxypropyl-phospho-ganciclovir (HDP-P-GCV) as a prototype. We hypothesized that many biologically effective compounds could be modified to crystalline lipid prodrugs and be delivered directly into the vitreous as a long-lasting, slow release system. This study is to further characterize this new drug delivery system using small particles of HDP-P-GCV and hexadecyloxypropyl-cyclic cidofovir (HDP-cCDV).

Methods—HDP-P-GCV was microfluidized into 4.4 μm (median) particles, injected into rabbit vitreous, then the vitreous drug level was measured at different time points. Crystalline HDP-cCDV was synthesized, suspended in 5% dextrose and injected into the rabbit's vitreous with 10, 55, 100, 550, or 1000 μg in 50 μl vehicle per eye to determine the highest non-toxic dose. This non-toxic dose, 100 μg , was injected into 24 rabbit eyes to evaluate pharmacokinetics, into 14 rabbit eyes with established HSV retinitis to evaluate its efficacy, and into 58 rabbit eyes prior to HSV infection to evaluate its intraocular antiviral duration.

Results—Microfluidized particles of HDP-P-GCV showed an increased drug release rate compared to the large particle drug formulation, with area under concentration-time curve (AUC) of 219.8 ± 114.1 (n=3) versus 108.3 ± 47.2 (n=3) for unmodified HDP-P-GCV during the 12 week period after a 2.8 μmole intravitreal injection. There was a 103% increase of the drug released from the microfluidized formulation of HDP-P-GCV versus the unmodified formulation. Intravitreal injections of HDP-cCDV at doses of 100 $\mu\text{g/eye}$ or lower revealed no toxicity. After the 100 $\mu\text{g/eye}$ intravitreal injections, HPLC analysis showed a vitreous HDP-cCDV level of 0.05 μM at week 5, falling to 0.002 μM at week 8. The concentration at week 8 (0.002 μM) remained above the IC_{50} for CMV (0.0003 μM). The pretreatment study demonstrated an antiviral effect that lasted 100 days after a single intravitreal injection.

Conclusion—This crystalline lipid prodrug intravitreal delivery system is an effective approach to achieving sustained, therapeutic drug levels in the eye. Small microfluidized particles of HDP-P-GCV provide more rapid dissolution and higher vitreous drug levels.

Keywords

Intravitreal drug delivery; lipid prodrug; intraocular toxicity; ocular pharmacokinetics; HSV-1 retinitis

Introduction

Drug delivery to the vitreous, retina, and choroid is a challenging task due to the formidable obstacles posed by the blood-retinal barrier and tight junctions of the retinal pigment epithelium. Only small fractions of drug administered orally, intramuscularly, or intravenously, ¹ reach the target. Therefore, large and potentially toxic doses of drug are required. Another challenge to retinal drug delivery is the fact that drug levels need to be sustained for prolonged periods at the target site. It is difficult to use intravitreal injections if the half-life of the injected drug is short, since frequent injections would be necessary. To facilitate localized delivery to the posterior segment via an injectable, sustained-release system, we have developed and reported an intravitreal drug-delivery system that uses a crystalline lipid prodrug of ganciclovir (GCV), HDP-P-GCV.² In our previous study, we demonstrated that a single intravitreal injection of crystalline HDP-P-GCV provides 20-weeks of protection from HSV infection in a rabbit retinitis model. Furthermore, we hypothesized that changing the drug particle size might alter the ganciclovir release kinetics, and that this delivery system could be applied to many low molecular weight compounds that are known to have antiviral or antiproliferative effects.² In the current study, we further characterize this long-lasting intraocular drug delivery system by studying the relationship between HDP-P-GCV particle size and GCV release profile, and by synthesizing and testing a new crystalline compound, hexadecyloxypropyl-cyclic cidofovir (HDP-cCDV), using the same technology we previously reported.² Although we studied two individual compounds, they belong to the same family of lipid conjugated crystalline compounds and they share the property of crystalline formulation, water insolubility, and long-lasting slow release after intravitreal delivery.

Materials and methods

Synthesis of compounds

1. HDP-P-GCV: HDP-P-GCV was synthesized as previously reported.² To prepare a small particle formulation and eliminate the population of large particles, HDP-P-GCV was suspended in distilled water and the slurry was subjected to five passes through a microfluidizer (Microfluidics, Newton, MA). The slurry was then flash frozen in a 1 liter round bottom flask and lyophilized overnight to remove the water. Unmodified HDP-P-GCV and microfluidized HDP-P-GCV were subjected to laser light scattering particle size analysis at Cirrus Pharmaceuticals, Inc., Durham, NC. Measurements were performed using a HELOS laser diffraction instrument (Sympatec, Lawrenceville, NJ), equipped with a R3 lens (0.5 to 175 microns). For each measurement, approximately 100 mg of dry sample was dispersed at a feed rate of 75% using the VIBRI (controlled feeder) and RODOS (dry dispersing) attachments set at a main pressure of 4.0 bar and a venturi pressure of 100 mbar. The triggering conditions were set to start measurement when channel 25 reached 1% total signal and to stop when channel 25 dropped below 0.5%. Data were analyzed using the Fraunhofer method by means of the WINDOX analytical software (Sympatec, version 3.2, release 4).
2. HDP-cCDV: cCDV was prepared from CDV as described previously³ except that the compound was isolated as the dicyclohexyl-morpholinocarboxamidinium salt. The scheme of the synthesis is illustrated in Figure 1. The purity (greater than 98%) of the

compounds used in this study was confirmed by analytical thin layer chromatography, nuclear magnetic resonance spectroscopy, and mass spectroscopy as reported previously.^{4,5}

Animal studies—All procedures were adherent to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research

1. Intravitreal pharmacokinetics of small particle formulation of HDP-P-GCV: Three rabbits received 2.8 μ moles of the drug in 50 μ l of 5% dextrose in their left eyes. 2.8 μ moles were previously determined to be non-toxic.² Vitreous sampling was performed at post-injection week 1, week 2, week 3, week 5, week 8, and week 12. Rabbit eyes were well dilated before anesthesia by topical application of a combination of tropicamide 1% and phenylephrine hydrochloride 2.5%. Anesthesia was performed as previously described.⁶ Under anesthesia, the rabbit eye was proptosed through a hole made on a piece of Latex rubber. A cornea ring (depth = 2.5 mm) made from a 5 ml plastic syringe barrel, was placed on the cornea. Methylcellulose was applied to the cornea within the inner area of the ring, followed by a glass cover slip. Under the direct view of a surgical microscope, fifty to one hundred microliters of vitreous fluid was aspirated through the pars plana with a 23-gauge needle attached to a 0.5 ml syringe. Vitreous fluid was obtained from a location deliberately chosen to be away from the drug depot.² Subsequent vitreous aspirations were performed with 28.5 gauge needle. Vitreous samples were placed in a pre-weighed vial for HPLC analysis of HDP-P-GCV levels as described before.²
2. Intravitreal toxicity and pharmacokinetics of HDP-cCDV: For toxicity studies, five doses (10 μ g or 0.018 μ mole, 55 μ g or 0.1 μ mole, 100 μ g or 0.18 μ mole, 550 μ g or 1.01 μ mole, 1000 μ g or 1.84 μ mole in 50 μ l of 5% dextrose) were tested in 8 rabbits, 16 eyes for 8 weeks. One eye of each animal was injected with drug in 50 μ l of 5% dextrose and the fellow eye was injected with 50 μ l of 5% dextrose as the control. Before drug injection, baseline IOP and fundus examination were documented. Drug or 5% dextrose in a volume of 50 μ l was intravitreally injected into vitreous cavity as previously described.² After injection, eyes were monitored with a hand held tonometer (Tonopen, Medtronic, Jacksonville, FL), slit-lamp, and indirect ophthalmoscope at day 3, week 1, week 2, week 3, week 5, and week 8. Any change from the baseline was documented. All animals were sacrificed at 8 weeks following the intravitreal drug injection. Before sacrifice, full field scotopic ERGs were obtained from all animals as previously described.² Following enucleation, globes were processed for paraffin sections and light microscopic examination as described previously.⁶

For the pharmacokinetic studies, the highest non toxic dose from the results of the toxicity studies, 100 μ g/eye, was intravitreally injected into 24 eyes of 16 rabbits, and the remaining 8 eyes were injected with the same volume (50 μ l) of 5% dextrose as control. Two animals, 3 eyes with drug and 1 eye with 5% dextrose, were used at each time point. The time points used were post-injections on day 1, day 3, week 1, week 2, week 3, week 5, week 8, and week 10. After animal sacrifice, globes were enucleated and kept on ice before freezing and dissecting. The globes were then submerged in -40 °C 2-methylbutane in a beaker sitting in a dry ice-ethanol bath for 30 seconds. The frozen globe was cut into two halves through the optic nerve, then the lens and anterior chamber blocks were removed immediately. The globe halves were then placed under the surgical microscope at room temperature for 40 to 70 seconds to allow the interface between retina and vitreous to thaw before the vitreous block was removed with a forceps. The retina was gently scraped off the bed of retinal pigment epithelium (RPE)/choroid layer using a fine spatula. The remaining RPE/

choroid layer was forcefully scraped off from underneath the sclera. The dissection was completed under direct view of a surgical microscope within 2 to 3 minutes to avoid cross-contamination. These different tissues from the same eyes were stored separately in the pre-weighed and pre-labeled glass vials. The vials were kept at -70 °C until HPLC analysis.

3. HSV-1 rabbit retinitis treatment study of HDP-cCDV: For the retinitis intervention study, 72 rabbits were used, including 14 rabbits for the treatment study and 58 for the pretreatment study. Only the right eye of each rabbit was used. Ophthalmoscopic retinitis grading was performed using a previously reported method with a standardized grading scheme.⁷ A very experienced observer graded retinitis throughout the experiments in an unmasked manner. For the treatment study, the right eyes of 14 rabbits were intravitreally injected with 0.06 mL of a 5×10^{-5} dilution of $10^{-7.6}$ mean tissue culture infective dose (TCID₅₀)/mL HSV-1. When retinitis developed and reached grade 1 (earliest detected retinitis grade: 1 or 2), 5 infected eyes received 100 micrograms of HDP-cCDV in 50 μ L of 5% dextrose, 4 infected eyes received an equivalent dose of free CDV in 50 μ L of 5% dextrose, and the other 5 infected eyes received 50 μ L of 5% dextrose. For the pretreatment study, 100 μ g/eye was tested. 58 rabbits were divided into four groups: the 21 day, 47 day, 68 day, and 100 day groups. 15 rabbits were used for each time point of the pretreatment study (except 13 rabbits were used for the 100 day time point). At each time point 5 rabbits received 100 micrograms (in 50 μ L 5% dextrose) of HDP-cCDV, 5 rabbits received an equivalent dose of CDV in 50 μ L of 5% dextrose, and 5 rabbits received 50 μ L of 5% dextrose (for the 100 day pre-treatment only 3 rabbits were used for HDP-cCDV). HSV-1 virus was inoculated as scheduled at 21 days, 47 days, 68 days, or 100 days after the intraocular drug injections. The HSV-1 virus dose, injection method, and clinical retinitis grading were done as previously described.^{2,7} Rabbits were killed 2 weeks after development of retinitis. Retinitis was graded on days 3, 6, 9, 11, and 14. Rabbits without retinitis 3 or 4 weeks after HSV-1 inoculation were sacrificed.
4. Histological evaluation of retinal damage and choroidal inflammation for the rabbit eyes in the treatment group. After death, globes were enucleated and processed for light microscopy. After H&E staining, slides next to the vertical meridian were selected from each eye. The section that was used to measure retinal thickness for each eye included the entire anterior and posterior retina in cross-section. The thickness of the retina was measured at 5 locations in the inferior retina and 3 locations in the superior retina as illustrated by individual bars in Figure 2. Thickness was measured with a reticule installed in the eyepiece of the microscope. All measurements and grading were performed under 100 X magnification. Each scale unit of the reticule is equivalent to 27.8 micrometers. The thickness of each retina was expressed as a mean derived from all eight measurement locations.
5. Statistical methods. The continuous variables such as the total area under the concentration-time curve, intraocular pressure, and thickness of the retina were compared between or among groups using T-test or Tukey test. The ordinal variables such as retinitis scores were analyzed across the groups using Kruskal-Wallis test and followed by a nonparametric Tukey-type test to locate the differences if a null hypothesis was rejected by the Kruskal-Wallis test. Differences of $p < 0.05$ were considered to be significant. All statistical simulations were performed with two-tailed comparison unless otherwise indicated.

Results

1. **Effect of Microfluidization of HDP-P-GCV on particle size:** We subjected an aqueous slurry of HDP-P-GCV to five consecutive cycles of microfluidization. After lyophilization and recovery of the powder, both the unmodified and the microfluidized HDP-P-GCV formulations were subjected to laser light scattering particle size analysis (Figure 3). Unmodified HDP-P-GCV (Panel A) showed a bimodal distribution with a volume median diameter (X50) centered around 8.0 microns. After microfluidization (Panel B), a more monodispersed population of smaller particles was noted, having an X50 of 4.4 microns and a 99th percentile diameter (X99) of 20 microns and a 90th percentile diameter (X90) of 10 microns. No large particles remained after microfluidization treatment. Finally, we also analyzed an untreated formulation of HDP-cCDV powder (Panel C). This compound showed a population of particles having an X50 of 8.9 microns.
2. **Intravitreal pharmacokinetics of the unmodified and microfluidized small particle formulations of HDP-P-GCV:** Intravitreal injection of the microfluidized HDP-P-GCV demonstrated a similar intravitreal drug depot to that of the unmodified HDP-P-GCV. The total area under the concentration-time curve (AUC) of the microfluidized HDP-P-GCV at week 12 was 219.8 ± 114.1 (n=3) versus 108.3 ± 47.2 (n=3) for the unmodified HDP-P-GCV following an intravitreal dose of 2.8 μ moles. After logarithmic transformation of the data, t-test revealed a p value of 0.15. The mean concentration-time curves are shown in Figure 4. The result indicates that microfluidized HDP-P-GCV may provide a faster release rate and higher free drug concentration in the vitreous fluid. This is likely due to the larger surface area of the microfluidized particles, leading to a more rapid rate of dissolution.
3. **Intravitreal toxicity and pharmacokinetics of HDP-cCDV:** The toxicity study showed that the highest non-toxic dose is 100 μ g/eye. Doses of 550 μ g/eye and 1000 μ g/eye showed local cataract with mild iritis and/or local retinal toxicity (Figure 5) in two eyes, one with the 550 μ g dose and one with the 1000 μ g dose. However, the other eye with the 550 μ g dose showed no toxicity until the end of the study while the drug depot was floating in the vitreous cavity without contacting any intraocular tissue. The drug depot was still visible at the end of the study (week 8) in the eyes with 100 μ g or higher doses (Figure 6). Intraocular pressure (IOP) was measured at baseline and post-injection at day 3, week 1, week 2, week 3, week 5, and week 8 from each eye. At week 8 after drug injection, the eyes with the 100 μ g dose or lower showed an average IOP of 8.7 ± 1.0 mmHg on the right eyes and 9.8 ± 1.1 mmHg on the left eyes (p=0.175, paired t-Test); the eyes with the 550 and the 1000 μ g doses showed an average IOP of 8.7 ± 2.1 mmHg on the right eyes and 11.3 ± 2.3 mmHg in the left eyes (p=0.01, paired t-Test). At all other time points, IOP was not significantly different between the treated and the control eyes. ERG examination revealed that all eyes had normal ERGs, including the eye with the 1000 μ g injections. The pathology study confirmed the normal retina, vitreous, and choroids in the eyes with 100 μ g intravitreal injections.

The pharmacokinetic study revealed that the whole vitreous samples showed an average concentration of 0.54 μ M HDP-cCDV at post-injection day 1 and an average concentration of 0.002 μ M at post-injection week 8, with an estimated vitreous half-life of 6.3 days. At the end of this experiment (post-injection week 10), HDP-cCDV was still detectable in whole vitreous samples (0.0006 μ M) (Figure 7). However, the HDP-cCDV was below the limit of detection in the retina.
4. **HSV-1 retinitis treatment studies:**

A. Treatment Study

Out of 14 eyes of 14 rabbits that received 0.04 ml of 1×10^{-4} dilution of 10-7.6 TCID₅₀ HSV-1, 12 eyes developed retinitis at day 4 after virus inoculation. Rabbits were randomly divided into three groups. Retinitis scores were not significantly different among three groups before the intravitreal drug or dextrose injections ($p=0.77$, Kruskal-Wallis Test). The four eyes that received the 5% dextrose solution after development of retinitis (median score of 1) progressed to complete retinitis with retinal detachment and severe vitreous cloudiness. The four eyes that received intravitreal HDP-cCDV and the 4 eyes that received cidofovir following induction of retinitis (median score of 1.5 and 2), showed similar clinical scores of retinitis to the 5% dextrose controls at all check time points ($P>0.05$, Kruskal-Wallis Test). However, vitreous cloudiness was noticeably less severe than the control eyes. The measurement of thickness of retina from histological evaluation revealed that the thickness of the retina in the eyes with intravitreal injection of CDV and HDP-cCDV was $95 \pm 40 \mu\text{m}$ and $80 \pm 10 \mu\text{m}$ while the retinal thickness of the 5% dextrose injected eyes was $46 \pm 8 \mu\text{m}$. There was no significant difference between the CDV and HDP-cCDV treated groups or between HDP-cCDV and 5% dextrose treated groups; however, a significant difference was detected between CDV and 5% dextrose treated groups ($P<0.05$, Tukey HSD test).

B. Pretreatment study

For the 3-week pretreatment study, all five rabbits that received intravitreal injection of 5% dextrose developed typical retinitis. In contrast, none of the rabbits that received the intravitreal injection of HDP-cCDV or CDV developed retinitis. At each time point, retinitis scores were analyzed across three groups using Kruskal-Wallis test and followed by a nonparametric Tukey-type tests to locate the difference(s) if the null hypothesis was rejected by the Kruskal-Wallis test (Table 1). For the 47-day pretreatment, at day 6 after virus inoculation all control eyes developed retinitis with a median grade of 4. The rabbits that received CDV pretreatment all developed retinitis with a median grade of 3. In contrast, out of 5 rabbits that received HDP-cCDV pretreatment, only 2 developed grade 2 retinitis. There is a significant difference in retinitis scores during the 14 day grading period between HDP-cCDV pretreated eyes and CDV pretreated eyes or control eyes (Table 1). For the 68-day HDP-cCDV pretreatment study, at day 14 following virus inoculation, only two rabbits developed grade 3 and grade 4 retinitis, the other three rabbits had complete protection from HSV-1 infection. The other two groups (10 eyes) with dextrose or CDV pretreatment all had grade 4 retinitis (Table 1). For the 100-day pretreatment study, one of four rabbits in the HDP-cCDV pretreated group died before virus inoculation. Among the other three rabbits, one did not develop retinitis, and the other two had grade 3 retinitis at day 6. The HDP-cCDV pretreated group had lower retinitis scores than the retinitis scores of the dextrose pretreated group (Table 1).

Discussion

The goal of this study is to further characterize the novel crystalline lipid prodrug intraocular drug delivery system that we reported earlier with HDP-P-GCV.² We found that the unmodified crystalline lipid prodrug of GCV possesses a slow release property after intravitreal

injection. In our previous report, HDP-GCV could prevent HSV-1 viral infection of the retina for 20 weeks following a single intravitreal injection, while a single intravitreal injection of GCV provided less than one-week protection.² In the current study, we demonstrated that microfluidized small particle HDP-P-GCV might have released a greater amount of free drug into the vitreous fluid than the unmodified large particle formulation of HDP-P-GCV. AUCs from animals in the large particle group ranged from 60.7 to 155 while AUCs in the small particle group ranged from 140 to 351. The drug release was twice as high from the small particle group; there was a trend which was not statistically significant probably because of the sample size of the data. We believe that small particles have a larger surface area that increases the contact surface with the dissolution medium, resulting in a higher dissolution rate than large particles. It is possible that very small amounts of crystalline prodrug may diffuse to distant part of vitreous and were sampled into the vitreous tap, which may be responsible for the relatively large variation of the vitreous fluid drug concentrations between individual animal eyes. We have used three animals per time point to get a mean value from which the data curve showed a clear trend and valid information. In this study, vitreous fluid was sampled by multiple vitreous taps at different time points. Although we sampled the vitreous fluid through pars plana and we did not observe vitreous or anterior chamber fibrin formation, it is still possible that multiple vitreous taps might cause intraocular environmental change and influence the subsequent vitreous drug level measurement. Data are not available from this experiment to delineate this concern.

Injection of lower drug levels could eliminate the local retinal toxicity resulting from contact of drug depot with retina due to gravitational effects and positioning.^{2,8} The current studies were conducted within unvitrectomized eyes. The drug aggregation and release profile could be quite different if injected into a vitrectomized eye. Further studies are warranted in vitrectomized eyes. Based on our findings, we hypothesize that controlled release could be achieved by using mixtures of different sizes of crystalline drug in an intravitreal administration. These mixtures could be designed to have release profiles tailored to treat different kinds of vitreoretinal diseases.

In the previous report, we first described this novel intraocular drug delivery system using HDP-P-GCV as a prototype.² We reasoned that the hexadecyloxypropanol moiety could be coupled to many nucleoside phosphates or phosphonates to form solid hydrophobic crystals that slowly dissolve in water. The dissolved molecules enter the cells and are cleaved intracellularly by phospholipases C into hexadecyloxypropanol and the parent drug.⁹ In the current study, we used the same concept and technique to synthesize an ether lipid ester of cyclic cidofovir, HDP-cCDV. Intravitreal injection of 100 µg or lower doses of HDP-cCDV crystals demonstrated an ideal drug depot that floated in the inferior vitreous cavity without disturbing the visual axis. The vitreous elsewhere was clear and no toxicity was found in the eyes with 100 µg or lower doses. Local toxicity seen with higher doses was caused by the contact of drug depot with retina or lens, which was similar to the local retinal toxicity caused by intravitreal high dose HDP-P-GCV.² The higher dose forms a larger drug depot in vitreous, which tends to contact intraocular tissues to cause toxicity. An intraocular pressure drop associated with intravitreal injection of CDV was not observed in the eyes that received 100 µg or lower doses in the current study. The eyes with higher doses showed a mild IOP drop at the last time point (8.7 ± 2.1 versus 11.3 ± 2.3 mmHg, $P=0.01$). However, no hypotony was found. Hypotony, intraocular pressure of 5 mmHg or lower with associated retinal edema, has been well known as a complication following local or systemic cidofovir administration.¹⁰ The absence of hypotony may be due to the fact that cyclic CDV and HDP-cCDV are not picked up avidly by organic anion transporters in the ciliary body. Intravitreal pharmacokinetics showed that HDP-cCDV was still detectable at week 10 after a single intravitreal injection of 100 µg per eye. The estimated vitreous half-life for HDP-cCDV was 6.3 days, which favorably compares to 20 hours for CDV or 10 hours for cCDV.¹² The detected

concentration at week 8 was 0.002 μM , which is above the IC_{50} for CMV. In this study we did not detect HDP-cCDV in the retina, which could be due to low sensitivity of HPLC and fast conversion of HDP-cCDV into cCDV then into CDV by cellular phosphohydrolases. Using HDP-P-GCV, we have shown that the prodrug may be metabolized by participation of vitreous cells. There is little parent drug detectable when prodrug was incubated with heat inactivated vitreous sample but conversion can be detected readily by native vitreous that contains cells.⁹ It has been known that CDV is phosphorylated to cidofovir diphosphate, the active form of cidofovir that has a long intracellular half-life.¹³ Indeed, the pretreatment studies indicated an at least 100 days pharmacologic effect against HSV-1 infection of the rabbit retina. In the current studies, retinitis was graded in an unmasked manner. It could lead to bias if retinitis severity was similar as in the treatment study. Therefore, we performed further objective analysis of retinal thickness from the pathologic examination for the treatment groups to confirm the findings. In the pretreatment studies, the severity of retinitis among the three groups was so obvious that it is unlikely that unmasked grading influenced the results.

In summary, this novel intraocular drug delivery system has promise with challenging refractory chronic vitreoretinal diseases that require prolonged drug treatment. Small crystals of HDP-P-GCV have been found to release more drug over time than large unmodified particles and the delivery system has been extended to crystalline HDP-cCDV. The concept and technique delineated here can be applied to many compounds including antiproliferative drugs such as phosphonmethoxyethylguanine (PMEG), arabinofuranosylguanine (Ara-G), 5-fluoro-2'-deoxyuridine (5-FUdR), and other antiproliferatives that are under our investigation.

Acknowledgements

Supported by NIH EY 07366 (WRF) and NIH EY 11832 (KYH, WRF) Commercial relationship policy: NS Scientific Section Code: RE

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Reagents: a) N,N-dicyclohexyl-morpholinocarboxamidine, 1,3-dicyclohexylcarbodiimide, pyridine, 100 °C; b) 1-bromo-3-hexadecyloxypropane, N,N-dimethylformamide, 80 °C

Figure 1.
Synthesis of hexadecyloxypropyl-cyclic cidofovir (HDP-cCDV).

Figure 2. Retinal section sketch for measuring retinal thickness. There are eight locations to measure retinal thickness.

Figure 3.

Laser Light Scattering Particle Size Analysis of HDP-P-GCV and HDP-cCDV Formulations: Panel A, Unmodified HDP-P-GCV; Panel B, Microfluidized HDP-P-GCV; Panel C, HDP-cCDV

Small particle HDP-P-GCV versus large particle in PK study (2.8 μ mole dose) **Figure 4.**

Micromolar concentration of HDP-P-GCV in vitreous aspirates at different time points following intravitreal injection of 2.8 μ mole dose of small particle size formulation (S2.8 μ mole) and unmodified particle size formulation (2.8 μ mole). Data are presented as mean $\pm$ standard deviation (n=3).

Figure 5. Microphotograph from an eye with 550 μ g HDP-cCDV intravitreal injection, showing localized retinal toxicity (between the two arrows). Disorganized outer nuclear layer and marked retinal pigment epithelium proliferation is observed

Chart: HPLC Analysis of Whole Vitreous for HDP-cCDV in Rabbit Eyes

Figure 6.

Fundus photograph taken at week 5 following an intravitreal injection of 100 μg of crystalline HDP-cCDV into rabbit vitreous, showing a drug depot floating in the vitreous with an estimated size of 1×1.5 disc diameters.

Table 1
Time course and Median Retinitis Scores from pretreatment

Group	Time Point	# of animals	day 6	day 9	day 14
HDP-cCDV	21 day	5	0	0	0
5% dextrose		5	2	4	4
Free CDV		5	0	0	0
P-value (Kruskal-Wallis test)					
#P-value (Nonparametric Tukey test)			0.0069	0.0062	0.0062
			HDP vs Dex, <0.01; HDP vs CDV, NS; CDV vs Dex, <0.01	HDP vs Dex, <0.01; HDP vs CDV, NS; CDV vs Dex, <0.01	HDP vs Dex, <0.01; HDP vs CDV, NS; CDV vs Dex, <0.01
HDP-cCDV	47 days	5	0	2	2
5% dextrose		5	4	4	4
Free CDV		5	3	4	4
P-value (Kruskal-Wallis test)					
#P-value (Nonparametric Tukey test)			0.0033	0.007	0.0314
			HDP vs Dex, <0.01; HDP vs CDV, <0.01; CDV vs Dex, NS	HDP vs Dex, <0.01; HDP vs CDV, <0.01; CDV vs Dex, NS	HDP vs Dex, <0.01; HDP vs CDV, <0.01; CDV vs Dex, NS
HDP-cCDV	68 days	5	0	0	0
5% dextrose		5	4	4	4
Free CDV		5	4	4	4
P-value (Kruskal-Wallis test)					
#P-value (Nonparametric Tukey test)			0.0042	0.0012	0.0067
			HDP vs Dex, <0.01; HDP vs CDV, <0.01; CDV vs Dex, NS	HDP vs Dex, <0.01; HDP vs CDV, <0.01; CDV vs Dex, NS	HDP vs Dex, <0.01; HDP vs CDV, <0.01; CDV vs Dex, NS
HDP-cCDV	100 days	3	3	3	3
5% dextrose		5	4	4	4
Free CDV		5	3	4	4
P-value (Kruskal-Wallis test)					
			0.0367	0.0357	0.0541

Group	Time Point	# of animals	day 6	day 9	day 14
*P-value (Nonparametric Tukey test)			HDP vs Dex, <0.05; HDP vs CDV, NS; CDV vs Dex, NS	HDP vs Dex, <0.05; HDP vs CDV, NS; CDV vs Dex, NS	HDP vs Dex, <0.05; HDP vs CDV, NS; CDV vs Dex, NS
#	HDP=HDP-cCDV; CDV=cidofovir; Dex=dextrose; NS: not significant				
*	indicating nonparametric Tukey-type test with equal sample sizes (Nemenyi test)				
*	indicating nonparametric Tukey-type test with unequal sample sizes (Dunn test)				