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Antiviral Effect in Human Cytomegalovirus-Infected Cells, Pharmacokinetics, and Intravitreal Toxicology in Rabbits of Acyclovir Diphosphate Dimyristoylglycerol

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Acyclovir diphosphate dimyristoylglycerol (ACVDP-DG) is a lipid prodrug which is active against ACV-resistant strains of herpes simplex virus because of its intracellular metabolism to ACV monophosphate. In human cytomegalovirus (HCMV)-infected MRC-5 cells, ACVDP-DG was ninefold more active than ACV. When liposomal [8-^{3}H]ACVDP-DG was injected intravitreally at the maximum nontoxic dose of 1 μmol in rabbits, the drug remained above its estimated 90% HCMV-inhibitory concentration for 18 days. Intravitreal ganciclovir persists above its 90% inhibitory concentration for only 1 to 2 days. ACVDP-DG may be useful as a local treatment for HCMV retinitis.

Human cytomegalovirus (HCMV) retinitis occurs in 15 to 42% of patients with AIDS (9, 10, 12, 14, 17). Two antiviral drugs currently available for the treatment of HCMV retinitis are ganciclovir (GCV) and foscarnet, both of which are associated with significant toxicities (5) and the emergence of resistance (4, 16). Intravitreal injections with GCV have been used in patients not tolerating systemic therapy (11, 23). The main disadvantage of this local therapy is the need for four to six injections per eye per month (1, 8, 13, 21).

Acyclovir diphosphate dimyristoylglycerol (ACVDP-DG) is an analog of CDP diacylglycerol and is very active in herpes simplex virus type 1- and type 2-infected cells in vitro (15). ACVDP-DG is metabolized to ACV monophosphate intracellularly and retains substantial in vitro antiviral activity in ACV-resistant thymidine kinase-deficient mutants of herpes simplex virus type 1 and type 2 because of its unique metabolism (15). In this paper, we report the antiviral activity of ACVDP-DG in HCMV-infected MCR-5 cells and the ocular toxicity and pharmacokinetics after intravitreal injections of liposomal ACVDP-DG in rabbit eyes.

Human embryonic lung fibroblast cells (MRC-5) and HCMV (strain AD169) were obtained from the American Type Culture Collection (Rockville, Md.). Virus stocks were prepared by infecting subconfluent MRC-5 cells; the titers of HCMV in the cell-free supernatant were determined, and aliquots were stored in liquid nitrogen. Subconfluent MRC-5 cells in 24-well culture dishes were pretreated for 24 h with drugs in minimal essential medium containing 2% fetal bovine serum and antibiotics. The medium was removed, and virus was added, absorbed for 1 h at 37°C, aspirated, and replaced with the drug dilutions. After 5 days of incubation, HCMV DNA was quantified in triplicate by nucleic acid hybridization with an HCMV antiviral susceptibility test kit from Diagnostic Hybrids, Inc. (Athens, Ohio). Results are expressed as percentages of the amount of HCMV-specific DNA in untreated HCMV-infected control cells.

The concentration of ACVDP-DG which inhibited the replication of the AD169 strain of HCMV in MRC-5 cells by 50% (IC_{50}) was at 2.4 μM, compared with IC_{50}s of 22 and 1.1 μM for ACV and GCV, respectively (Fig. 1). The concentration of GCV required to reduce HCMV DNA by 90% was 28 μM, compared with 36 μM for ACVDP-DG and 316 μM for ACV. The 50% toxic dose of ACVDP-DG in subconfluent human lung fibroblasts is >1,000 μM (15).

Liposomes containing dioleoylphosphatidylcholine–dioleoylphosphatidylglycerol–cholesterol–ACVDP-DG in a molar ratio of 40:10:30:20 were prepared by drying 50 μmol of the lipid mixture under nitrogen in a 1.5-ml vial to which was added 1.0 ml of 250 mM sorbitol–20 mM sodium acetate (pH 5.5). The vial was sealed and sonicated at 56°C in a cup horn sonicator (Heat Systems Ultrasonics, Plainview, N.Y.) at maximum output for 30 min. The resulting total lipid concentration was 100 mM, and the concentration of ACVDP-DG was 20 mM. Control liposomes consisting of dioleoylphosphatidylcholine-dioleoylphosphatidylglycerol-cholesterol (60:10:30 molar ratio) were prepared similarly at a final lipid concentration of 100 mM. ACVDP-DG was synthesized and purified as described previously (15).

New Zealand White rabbits were used in accordance with the guidelines of the University of California, San Diego, Office of Veterinary Affairs. For retinal toxicology, electroretinography was performed before intravitreal injections and before euthanasia at either 3 or 10 weeks, as described previously (3). Intravitreal injections of 0.05 to 0.2 ml were given at ACVDP-DG doses of 0.25, 0.5, 1.0, 2.0, or 4.0 μmol. Empty liposomes and normal saline were used as controls. Indirect ophthalmoscopic examination of the eyes was performed prior to injection, immediately after injection, at 1 to 2 weeks postinjection, and before euthanasia. For retinal toxicity evaluation,

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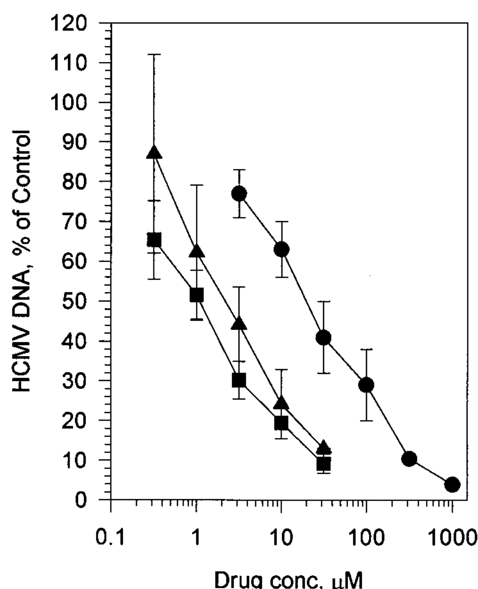

FIG. 1. Inhibition of HCMV replication by ACV (●), GCV (■), and ACVDP-DG (▲). Results are means ± standard deviations (bars) of three separate experiments. Data are expressed as percentages of the value for a drug-free control. HCMV-specific DNA assays were done in triplicate as described in the text.

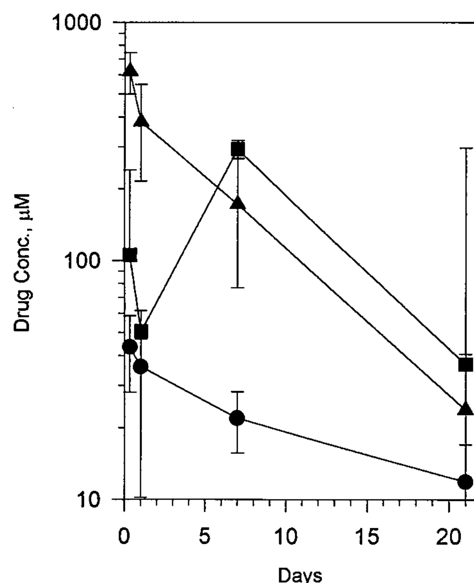

FIG. 2. [^{3}H]ACVDP-DMG levels in rabbit vitreous following injection. Results are expressed as the mean concentrations of [^{3}H]ACVDP-DG ± standard deviations (bars) (n = 4). Symbols: ▲, vitreous; ■, retina; ●, sclera.

at the conclusion of 3 or 10 weeks the rabbits were euthanized and their retinas were perfusion fixed and processed for light and electron microscopy as previously described (3). Tissues from the areas above and below the medullary ray were taken for electron microscopy. A vertical section containing part of the optic nerve was studied by light microscopy.

In eyes receiving 1 μmol of ACVDP-DG (5 μmol of total lipid), vitreous opacification improved within 3 to 5 days and the vitreous became clear (data not shown). When higher doses were injected, the vitreous remained opacified, with only modest improvement in 3 or 10 weeks. The haziness was seen as diffuse white particles throughout the vitreous. Injection of 2.0 μmol and higher doses of ACVDP-DG and 10 μmol and higher doses of total lipid also caused posterior subcapsular cataract after 3 weeks. The electroretinograms were normal for eyes receiving 0.25-, 0.50-, 1.0-, and 2.0-μmol intravitreal injections of ACVDP-DG at both 3 and 10 weeks. The eyes receiving the empty liposomes also had normal electroretinogram waveforms, amplitude, and latency. With 4.0-μmol intravitreal doses of ACVDP-DG, there were severe reductions in amplitude at both 3 and 10 weeks. Light microscopy displayed normal retinal histology at 3 and 10 weeks with 0.25, 0.50, 1.0 and 2.0 μmol of ACVDP-DG. Electron microscopy of eyes receiving 1.0 μmol of ACVDP-DG and empty liposome injections revealed normal histology at 10 weeks. For the 4.0-μmol intravitreal injection of ACVDP-DG, light microscopy sections revealed toxic changes to the retina, from local retinal pigment epithelium hypertrophy to diffuse loss of height of the photoreceptor outer segments at 3 weeks (data not shown). From the foregoing information, we conclude that 1 μmol of ACVDP-DG is the maximal tolerated dose.

To assess vitreous pharmacokinetics, liposomes containing 1 μmol of [8-^{3}H]ACVDP-DG (specific activity, 0.5 mCi/mmol) were injected into rabbit vitreous and the levels of ^{3}H-drug in vitreous, retina, and sclera were determined at 0.25, 1, 7, and 21 days following injection. For high-performance liquid chromatography analysis, the vitreous was homogenized at 4°C and an aliquot was removed for liquid scintillation counting. One hundred microliters was extracted with chloroform-methanol (2:1, vol/vol) by the method of Folch et al. (7). An aliquot was injected into a Jones APEX II DIOL column (4.6 by 250 mm) and eluted with a linear gradient from 0.5 mM NaH_2PO_4 in 95% tetrahydrofuran to 5.5 mM NaH_2PO_4 in 45% tetrahydrofuran from 5 to 25 min at a flow rate of 0.5 ml/min. A peak which corresponded to the [^{3}H]ACVDP-DP drug standard could be detected (retention time, 16.6 min) as late as 21 days after the initial administration. No other radioactive peaks were detected. The intravitreal concentrations of [^{3}H]ACVDP-DG were subjected to regression analysis with RSTRIP II statistical software (MicroMath Scientific Software, Salt Lake City, Utah). The maximum concentration of ACVDP-DG in the vitreous was calculated to be 437 μM at time zero. The drug level in the vitreous humor was 385 ± 83 μM (mean ± standard error of the mean) at day 1 and declined by monoexponential decay to 24 ± 7 μM at day 21 (Fig. 2). The vitreal half-life of ACVDP-DG was 5.2 days, and its mean residence time was 6.1 days. Retinal levels of [8-^{3}H]ACVDP-DG and its ^{3}H-labeled tissue metabolites were low at day 1 (50 ± 13) but rose by day 7 to 293 ± 13 μM and declined to 37 ± 12 μM by day 21. Drug levels in sclera were much lower.

In summary, our data indicate that ACVDP-DG is nine times more active than ACV in HCMV-infected cells in vitro. Injections of 1.0 μmol of liposomal ACVDP-DG (0.916 mg) administered to rabbits' eyes resulted in an initial estimated intravitreal concentration of 437 μM, 82 times the IC_{50} for HCMV-specific DNA synthesis. The ocular therapeutic index (calculated as maximum tolerated nontoxic dose/IC_{50}) of ACVDP-DMG is 182, which compares favorably with approximately 135 for GCV and 17 for foscarnet (2, 6, 20, 22). ACVDP-DG remains above its IC_{90} for 18 days, compared with only 1 to 2 days for intravitreal GCV (19) and 24 to 54 h for transcleral foscarnet administered by iontophoresis or by injection (2, 18, 22). Prolonged retention of ACVDP-DG in the vitreous may make it especially suitable for local treatment of HCMV retinitis, and substantial uptake of the drug by the

retina may provide for prolonged antiviral action. Intravitreal administration of liposomal ACVDP-DG or other liposomal antiviral liponucleotide prodrugs may be useful for local intravitreal treatment of HCMV.

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