Oral Activity of Ether Lipid Ester Prodrugs of Cidofovir against Experimental Human Cytomegalovirus Infection

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Infection with human cytomegalovirus (HCMV) can cause serious complications in bone-marrow and solid-organ transplant recipients, and current therapies are not optimal. We evaluated 2 orally active ether lipid ester analogues of cidofovir (CDV)—hexadecyloxypropyl-CDV (HDP-CDV) and octadecyloxyethyl-CDV (ODE-CDV)—in severe combined immunodeficient mice in which either human fetal retinal tissue or human fetal thymus and liver tissue had been implanted and was later infected with HCMV. Our results indicate that orally administered treatment with either HDP-CDV or ODE-CDV is 4–8-fold more active, on a molar basis, than is intraperitoneally administered CDV. These data suggest that HDP-CDV and ODE-CDV should be further evaluated as potential antiviral agents for treatment of HCMV infection.

Human cytomegalovirus (HCMV) infection continues to be a major cause of morbidity in bone-marrow and solid-organ transplant recipients [1] and is reported to be the most-frequent congenital infection worldwide, occurring in 0.2%–2.5% of all live births [2]. At present, there are 5 therapeutics approved for treatment of HCMV, but, in many cases, long-term therapy is ineffective and may result in the development of resistance or of serious adverse effects that require termination of treatment [3]. These problems clearly indicate the need for better antiviral agents against HCMV.

One of the more-active drugs for treatment of HCMV infection is cidofovir (CDV); however, its lack of oral activity requires administration via intermittent intravenous infusion, and this treatment often results in significant nephrotoxicity. To produce orally active prodrugs of CDV, 2 ether lipid ester analogues, hexadecyloxypropyl-CDV (HDP-CDV) and octadecyloxyethyl-CDV (ODE-CDV) were synthesized [4, 5]. In vitro evaluation of these compounds indicate that both are 100–10,000 times more active against various laboratory, clinical, and drug-resistant isolates of HCMV than are CDV and ganciclovir [5]. This increased activity has been attributed, at least for HDP-CDV, to enhanced cellular uptake, as well as to increased intracellular levels of CDV diphosphate, compared with those of CDV [6]. Pharmacokinetic evaluation of the 2 analogues of CDV in mice indicate that the oral bioavailability of HDP-CDV and of ODE-CDV is 93% and 88%, respectively, both of which are much higher than that of CDV (<5%) [7]. These excellent oral bioavailabilities, in combination with the observation that accumulation of these analogues in the kidney is markedly reduced, suggest that HDP-CDV and/or ODE-CDV may be more conveniently administered and be less nephrotoxic than is CDV.

In the present experiments, we used 2 animal models of HCMV infection in humans to evaluate the oral efficacy of both HDP-CDV and ODE-CDV. In these models, either human fetal retinal tissue or human fetal thymus and liver (thy/liv) tissue was implanted into
severe combined immunodeficient (SCID) mice and was later infected with HCMV. The mice were treated with either vehicle (saline), HDP-CDV, or ODE-CDV, and, at various times after infection, implanted tissue was removed and titers of HCMV were quantified, by plaque assay.

**MATERIALS AND METHODS**

**Antiviral drugs.** CDV (Vistide) was provided by Gilead Pharmaceuticals, and the ether lipid esters HDP-CDV and ODE-CDV were synthesized as described elsewhere [4, 5]. To provide the desired dose levels, compounds were diluted in either 0.1 mL of sterile saline (for intraperitoneal [ip] administration) or 0.2 mL of sterile saline (for oral administration).

**Preparation of viral pools.** The Toledo strain of HCMV was obtained from E. Mocarski (Stanford University School of Medicine, Stanford, CA) and was propagated in human foreskin fibroblast cells that had been derived from primary cultures [8, 9].

**Implantation and infection of retinal tissue.** The implantation and later infection of human fetal retinal tissue was performed as described elsewhere [8, 10]. In brief, 4–8-week-old male SCID mice (Charles River Laboratories) were anesthetized via ip injections of 100 mg of ketamine/kg of body weight and of 15 mg of xylazine/kg of body weight; proparacaine-HCl (0.5%) was instilled in the eyes. A winged infusion needle (27G × one-half inch) containing mechanically dissociated human fetal retinal tissue in Optisol (Chiron Ophthalmics) was then inserted into the anterior chamber of the mouse eye, where ~5 µL of tissue was injected. By a similar procedure, the mice were again anesthetized 8–12 weeks later, and 10 µL of virus (4000–5000 pfu) was injected near the implants. Beginning 24 h after infection, once-daily treatment of the mice, by different treatment methods, was initiated and was continued for 28 days.

To monitor the replication of HCMV in the retinal-tissue implants, ~6 mice from each treatment group were killed at 7, 14, 21, and 28 days after infection. The eyes were removed and were homogenized in 1.0 mL of MEM containing 10% fetal bovine serum, 2 mmol/L l-glutamine, 200 U/mL penicillin, 50 µg/mL gentamicin, and 3 µg/mL fungizone. Homogenates were centrifuged, and supernatants were removed and were frozen, at −70°C, until being assayed for HCMV. In most assays, the threshold of detection was 10^3 pfu/g of tissue.

**Implantation and infection of thy/liv tissue.** The implantation and later infection of human fetal thy/liv tissue in SCID mice was performed as described elsewhere [9]. In these experiments, 4–8-week-old male SCID mice were anesthetized, and fragments of human fetal thy/liv tissue were implanted, by use of an 18-gauge trocar, under the kidney capsule. After recovery, for 2 weeks the mice were given, in drinking water, 0.8 mg of sulfamethoxazole/mL of water and 0.16 mg of trimethoprim/mL of water.

The implants were allowed to develop for 12–16 weeks before being inoculated with 6000–7000 pfu of HCMV. Treatment was initiated 24 h after infection and was continued once daily for 28 days. On days 14, 21, 28, and 35, ~10 implants from each group were biopsied (~50% of implant), were weighed, and were homogenized in MEM. Homogenates were centrifuged, and supernatants were removed and were frozen, at −70°C, until being assayed for HCMV.

**Statistical evaluation.** Because the data were not normally distributed and because there were a number of zero values in several of the data sets, a stratified, nonparametric Wilcoxon rank sum test was used to evaluate the differences between virus replication in the mice receiving vehicle and that in the mice receiving drug treatment. Days were used as strata, and data collected during the entire 28 days of treatment were used.

**RESULTS**

Initially, several experiments were performed to determine the maximal tolerated dose and the minimal effective dose of each of the analogues. In the first experiment, SCID-human (SCID-hu) retinal-tissue implants were used, and the results indicated that daily treatment with 20 mg of CDV/kg of body weight, with 10 mg of HDP-CDV/kg of body weight, and with 5 mg of HDP-CDV/kg of body weight all effectively reduced the replication of HCMV, compared with the mice that were treated with vehicle (data not shown). In the second experiment, SCID-hu thy/liv-tissue implants were used, and the results indicated that, compared with the mice that were treated with vehicle, treatment with 10 mg of CDV/kg of body weight or with either 10, 5, or 2.5 mg of HDP-CDV/kg of body weight were effective in inhibiting the replication of HCMV (data not shown). To determine the in vivo activity of ODE-CDV, another series of SCID-hu thy/liv-tissue implants were infected and were treated either orally with vehicle; orally with either 5, 2.5, or 1.25 mg of ODE-CDV/kg of body weight; or ip with 20 mg of CDV/kg of body weight. These experiments indicated that treatment with either CDV or ODE-CDV was effective in inhibiting the replication of HCMV in a dose-dependent manner (data not shown).

In the experiments presented here, a direct comparison between ip administered CDV, orally administered HDP-CDV, and orally administered ODE-CDV was made, in both the SCID-hu retinal-tissue implant model and the SCID-hu thy/liv-tissue implant model. In the first of these models, SCID-hu retinal-tissue implants were infected with 4700 pfu of HCMV/eye. Beginning 24 h after infection, the mice were either treated ip once daily with 20 mg of CDV/kg of body weight or were treated orally once daily with vehicle, with either 10 or 5 mg of HDP-CDV/kg of body weight, or with either 5 or 2.5 mg of ODE-CDV/kg of body weight. Treatment was continued for 28 days; on days 7, 14, 21, and 28, ~6 mice from each treatment group were killed, and their eyes were removed, were homogenized, and were as-
Efficacy of Oral Cidofovir Prodrugs against HCMV

Figure 1. Effect that treatment with intraperitoneally administered cidofovir (CDV), with orally administered hexadecyloxypropyl-CDV (HDP-CDV), and with orally administered octadecyloxyethyl-CDV (ODE-CDV) have on the replication of human cytomegalovirus, in human fetal retinal-tissue implants in SCID mice. The nos. in parentheses after each drug in the legend indicate the no. of milligrams per kilogram of body weight the mice received each day.

Table 1. Effect that treatment with cidofovir (CDV), with hexadecyloxypropyl-CDV (HDP-CDV), and with octadecyloxyethyl-CDV (ODE-CDV) have on the replication of human cytomegalovirus (HCMV), in human fetal retinal-tissue implants in SCID mice.

<table>
<thead>
<tr>
<th>Treatment, parameter</th>
<th>Time after infection, days</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Vehicle (saline)a</td>
<td></td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; pfu/g</td>
<td>2.98 ± 3.35</td>
</tr>
<tr>
<td>Positive for HCMV, % (proportion)</td>
<td>25 (3/12)</td>
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<tr>
<td>CDV (20 mg/kg)b</td>
<td></td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; pfu/g</td>
<td>1.40 ± 1.94</td>
</tr>
<tr>
<td>Positive for HCMV, % (proportion)</td>
<td>8 (1/12)</td>
</tr>
<tr>
<td>HDP-CDV (10 mg/kg)a</td>
<td></td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; pfu/g</td>
<td>1.40 ± 1.94</td>
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<tr>
<td>Positive for HCMV, % (proportion)</td>
<td>8 (1/12)</td>
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<tr>
<td>HDP-CDV (5 mg/kg)a</td>
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<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; pfu/g</td>
<td>1.40 ± 1.94</td>
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<tr>
<td>Positive for HCMV, % (proportion)</td>
<td>8 (1/12)</td>
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<tr>
<td>ODE-CDV (5 mg/kg)a</td>
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<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; pfu/g</td>
<td>1.40 ± 1.94</td>
</tr>
<tr>
<td>Positive for HCMV, % (proportion)</td>
<td>8 (1/12)</td>
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<tr>
<td>ODE-CDV (2.5 mg/kg)a</td>
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<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; pfu/g</td>
<td>1.40 ± 1.94</td>
</tr>
<tr>
<td>Positive for HCMV, % (proportion)</td>
<td>8 (1/12)</td>
</tr>
</tbody>
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NOTE. Data are mean ± SD, except where noted.

a Treatment was initiated 24 h after infection and was administered orally once daily for 28 days.
b Treatment was initiated 24 h after infection and was administered intraperitoneally once daily for 28 days.

said for HCMV. The results indicate that, compared with treatment with vehicle, treatment with either CDV, HDP-CDV, or ODE-CDV significantly reduce (P < .0001) replication of HCMV in implanted tissue (figure 1 and table 1). By day 21 after infection, the mean titer of HCMV in the retinal-tissue implants for the group of mice treated with vehicle was 4.25 log<sub>10</sub> pfu/g of biopsy sample; in comparison, by day 21 after infection, the mean titer of HCMV in the retinal-tissue implants for the group of mice treated with CDV was 1.88 log<sub>10</sub> pfu/g of biopsy sample, and, in the groups of mice treated with either 5 mg of HDP-CDV/kg of body weight or 2.5 mg of ODE-CDV/kg of body weight, the mean titers of HCMV in the retinal-tissue implants were 1.88 and 2.44 log<sub>10</sub> pfu/g of biopsy sample, respectively. Importantly, in the retinal-tissue implants of the groups of mice treated with either 10 mg of HDP-CDV/kg of body weight or 5 mg of ODE-CDV/kg of body weight, no significant amount of virus was detected.

In a second comparison between the 3 compounds, SCID-hu thy/liv-tissue implants were infected with 7000 pfu of HCMV. Beginning 24 h later, the mice were treated, and tissue was harvested according to a regimen similar to that described above for the retinal-tissue implant experiment. The results, presented in figure 2 and in table 2, indicate that, compared with treatment with vehicle, treatment with either CDV, HDP-CDV, or ODE-CDV is effective (P < .001) in inhibiting HCMV replication. By day 28 after infection, the mean titer of HCMV in the thy/liv-tissue implants for the group of mice treated with vehicle was 5.70 log<sub>10</sub> pfu/g of biopsy sample; in comparison, by day 28 after infection, the mean titers of HCMV in the thy/liv-implant tissue for the groups of mice treated with either 20 mg of CDV/kg of body weight or 2.5 mg of ODE-CDV/kg of body weight were 3.30 and 2.61 log<sub>10</sub> pfu/g of biopsy sample, respectively. In the groups of mice treated with either 10 or 5
mg of HDP-CDV/kg of body weight or with 5 mg of ODE-CDV/kg of body weight, no HCMV was detectable by day 28 after infection, a result similar to that of the retinal-tissue implant experiment.

During the course of several of these experiments, the mice were weighed on a thrice-weekly basis. In general, the mice lost ~1%–2% of their original body weight by day 4 after infection but subsequently gained that weight back and then maintained it for the remainder of the treatment period. On the last day of treatment, blood-chemistry analyses were performed, and most of the values obtained for the mice treated with HDP-CDV or CDV were similar to those for the mice treated with vehicle (data not shown). These results indicate that there was a lack of both liver and kidney toxicity.

**DISCUSSION**

In the present study, we have demonstrated that orally administered HDP-CDV and ODE-CDV are highly effective against HCMV infection in vivo. In each experiment, both compounds, administered orally, were found to be 4–8 fold more efficacious, on a molar basis, than was ip administered CDV. In the SCID-hu thy/liv-tissue implant model, the activity of both HDP-CDV and ODE-CDV indicate that oral administration of these compounds results in drug levels that are sufficient to inhibit replication of HCMV in target organs. In the SCID-hu retinal-tissue implant model, the efficacy of these compounds suggests that, in addition to producing systemic activity, both HDP-CDV and ODE-CDV can cross the blood-ocular barrier and inhibit HCMV infection of ocular tissue.

These data further support the usefulness of both the SCID-hu retinal-tissue implant model and the SCID-hu thy/liv-tissue implant model for the evaluation of various antiviral therapies against both ocular and systemic HCMV infection in vivo. Stud-
ies validating the use of these models for the evaluation of antiviral therapies have shown that, although the models do not precisely mimic HCMV infection in humans, the results of treatment with either ganciclovir or CDV [8, 9, 11] appear to correlate with clinical outcome [3].

In addition to their activity against HCMV, HDP-CDV and ODE-CDV also are highly efficacious against other herpesviruses in vitro [12]. The activity of these compounds typically has been shown to be 2–3 logs more efficacious against herpes simplex virus types 1 and 2, against Epstein-Barr virus, and against human herpesviruses 6 and 8 than that of CDV [12]. We also have reported the enhanced in vitro activity of these compounds against both vaccinia and cowpox virus, compared with that of CDV [4]. Further in vivo evaluation of these compounds against orthopoxvirus infection has confirmed that, in mice inoculated intranasally with these viruses, both HDP-CDV and ODE-CDV were as effective as CDV in the inhibition of viral replication and in the prevention of mortality [13].

It is interesting to note that, whereas both HDP-CDV and ODE-CDV are significantly more efficacious than is CDV against vaccinia and cowpox viruses in vitro, the in vivo efficacy of these orally administered compounds do not appear to be significantly different from that of parenterally administered CDV [13]. In vitro, the HDP and ODE analogues of CDV exhibit a 2.5–4.0-log increase in activity against various isolates of HCMV [5]. In contrast, the present study indicates that the oral activity of HDP-CDV and of ODE-CDV is only 4–8-fold more active than is parenterally administered CDV in inhibiting HCMV replication. In the SCID-hu thy/liv-tissue implant model (where tissue implantation and infection occur under the kidney capsule), these results can be partially explained by pharmacokinetic data that indicate that the levels (as measured by the area under the curve from 0 to 72 h) of HDP-CDV and of ODE-CDV in the kidney are 20%–25% of those for CDV [7]. Thus, such parameters as in vitro efficacy, bioavailability, and tissue levels of drugs all must be considered in parallel when the potential of these compounds against various viral infections are interpreted.

HDP-CDV and ODE-CDV are orally bioavailable compounds that have enhanced activity against HCMV, both in vitro [5] and in vivo. After oral administration of these analogues, levels of CDV in plasma and tissue are higher and persist longer in all tissues examined, with the exception of the level in kidney tissue versus that after treatment with parenterally administered CDV [7]. These observations suggest that treatment with orally administered HDP-CDV and/or ODE-CDV may result in less nephrotoxicity than that in treatment with CDV, by minimizing the accumulation of drug in the kidney. The data presented in this study strongly suggest that both HDP-CDV and ODE-CDV are excellent candidates for treatment of HCMV infection—as well as of other DNA-virus infections—and should be further evaluated.

Acknowledgments

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References