Effect of Maternal Treatment with Cyclic HPMPC in the Guinea Pig Model of Congenital Cytomegalovirus Infection

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Background. Cytomegalovirus (CMV) infection of the fetus is the leading cause of congenital infection. Using the guinea pig model of congenital CMV infection, we sought to determine whether antiviral treatment of a CMV-infected dam could improve the outcome of offspring.

Methods. Pregnant Hartley guinea pigs were inoculated with guinea pig CMV (GPCMV) during the late second/early third trimester of gestation. Guinea pigs received either 1 dose of cyclic 1-[(S)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine dihydrate (cHPMPC; 35 mg/kg) or placebo 24 h after GPCMV infection. Guinea pigs were monitored until delivery or were killed 10 days after infection, for the evaluation of the effect of cHPMPC on viral replication by polymerase chain reaction analysis in various dam and pup tissues.

Results. cHPMPC treatment of infected dams improved pup survival from 28.2% (11/39) to 83.2% (36/43) (P<.001) and extended the duration of pregnancy. cHPMPC treatment did not prevent infection of the placenta or disseminated infection of the dam and pup but significantly decreased the amount of GPCMV in tissues. GPCMV DNA levels in the placenta were reduced from 3.54 to 2.12 log_{10} genome copies/μg of DNA (P<.0001).

Conclusions. Treatment of the GPCMV-infected pregnant dam with 1 dose of cHPMPC improves the outcome of congenital infection and decreases viral replication in a guinea pig model.
and teratogenicity of available anti-CMV drugs limits the use of antiviral therapy during pregnancy at present.

CMVs are highly species specific. Guinea pig CMV (GPCMV) is the only small-animal CMV that crosses the placenta; therefore, this model has been used by us and others to study the pathogenesis and treatment of congenital CMV infection [16–19]. In the studies reported here, we evaluated the effect of antiviral therapy of the GPCMV-infected dam on the outcome of congenital CMV infection. Cyclic 1-[(s)-2-hydroxy-2-oxo-1,4,2- dioxaphosphorinan-5-y]methyl]cytosine dihydrate (cHPMPC) was selected for these studies because of its relatively long half-life [20–23], which allows treatment with a single intraperitoneal (ip) injection, and its efficacy against GPCMV [24, 25]. Although the pharmacokinetics of cHPMPC in guinea pigs is not known, a number of species—including mice, rats, rabbits, and humans—have been evaluated for the bioavailability and antiviral activity of cHPMPC and its parent compound, HPMPC, against herpesviruses [20–23]. To our knowledge, this is the first controlled evaluation of maternal antiviral therapy for congenital CMV.

MATERIALS AND METHODS

**Virus.** GPCMV strain 22122 (American Type Culture Collection) stock was prepared by sequential in vivo passage in strain 2 male guinea pigs (Cincinnati Children’s Hospital Research Foundation), as described elsewhere [26]. A salivary-gland virus stock (passage 11) was used for all experiments. Titers of virus stocks were determined by plaque assay using guinea pig lung fibroblast monolayers (cell line CCL-158; American Type Culture Collection).

**Guinea pigs.** Pregnant strain 2 guinea pigs (Cincinnati Children’s Hospital Research Foundation) were used for an initial safety study. Hartley pregnant guinea pigs were obtained from Harlan at ~35–45 days gestation (of a 65–70-day gestation) and were used for antiviral evaluations. Guinea pigs were housed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all procedures were approved by the Institutional Animal Care and Use Committee.

**Antiviral drug.** cHPMPC, the cyclic congener of HPMPC (cidofovir), was supplied by Gilead Sciences. cHPMPC was prepared and administered ip to guinea pigs at a dose of 35 mg/kg, as described elsewhere [25]. Sterile saline solution was used as a control treatment.

**DNA extraction, primer design, and conventional polymerase chain reaction (PCR).** DNA was extracted from 200 µL of a 10% tissue homogenate or blood, using the Qiagen QIAamp DNA mini kit DNA extraction system (Qiagen), in accordance with the manufacturer’s instructions. For conventional PCR assays, 2.5% of the final eluted DNA was tested using a PCR master mix (Promega) and a Bio-Rad block thermal cycler. The amplification program included 40 cycles of 30 s at 94°C, followed by 30 s at 57°C and 1 min at 72°C. GPCMV DNA isolated from infected guinea pig salivary glands and uninfected guinea pig spleen DNA was used as positive and negative controls, respectively. Amplification products were examined by 2% agarose gel electrophoresis (Invitrogen). Primers that would yield 100- and 300-bp DNA products were designed for PCR assays using the GPCMV UL97 sequence (GenBank accession number U26058) [27]. Nine primer pairs were synthesized (Sigma), and 2 primer sets were selected on the basis of specific binding and the expected fragment size. The first primer pair was designated as UL97F1 (5’-GATCGCTTCTGTC-AACAGC-3’) and UL97FR1 (5’-CGCAACTGATCGAATATCAC-TG-3’). A second primer pair, flanking the Fl/R1 sequence, was designated as UL97F3 (5’-GAAGCTGCTTCTCCTGCT-3’) and UL97R3 (5’-GTCAATGGTACCTCGGAGC-3’). These primers amplified regions of the GPCMV UL97 gene of 100 and 306 bp, respectively. Positive and negative control reactions were included for all PCR assays. For both first-round PCR and nested PCRs, negative controls were found to be negative and positive control reactions yielded the expected size fragment.

**Real-time PCR.** To quantify viral loads, the UL97F1/R1 primers were used to evaluate samples, using LightCycler Real-Time PCR SYBR Green I fluorescent dye (Roche). A 306-bp fragment of the UL97 gene was amplified by PCR using the UL97F3/R3 primers, cloned into the pGEM-T Easy Vector System (Promega), and designated as plasmid pH97. The UL97 fragment was verified by sequence analysis using M13 universal sequencing primers (Promega) and UL97F3/R3 primers. A standard curve was generated using a serial 10-fold dilution of pH97 DNA (1 × 10^5–1 × 10^6 plasmid copies) in 50 µg of uninfected guinea pig DNA from spleen. The conditions of amplification were optimized by testing the following parameters: number of cycles, annealing and elongation time, temperature, primer concentration, and reduction of primer dimer formation. Controls included uninfected guinea pig DNA and a template with no DNA. FastStart DNA MasterPLUS SYBR Green I reaction mix (Roche) that contained primers (1 µmol/L) and nuclelease-free water was used for all reactions. Samples (5 µL) that contained either 50 or 100 ng of eluted DNA were added for a total reaction volume of 20 µL. The following cycling program was used: 10 min at 95°C followed by 50 cycles of 10 s at 95°C, 5 s at 59°C, and 5 s at 72°C. For negative reactions, a 1:10 dilution of the samples was tested to determine the possibility of PCR inhibition. The limit of detection of the assay was between 1 and 10 copies, with a faint band detectable at 1 copy in some experiments. For statistical comparisons, neg-
samples were obtained from dams at 5 and 10 days after infection. Placenta, spleen, and liver from dams and liver and spleen from pups were evaluated for GPCMV DNA by PCR. To evaluate viral loads by PCR, blood infected and treated as in the experiments described above and samples were saved for DNA extraction and PCR analysis. Control dams were used in 2 experiments. Guinea pigs were treated 24 h later with cHPMPC or saline solution. Dams were observed daily for delivery. Specimens of infected with guinea pig cytomegalovirus and treated with cyclic 1-[(s)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine dihydrate (cHPMPC). A, Pup survival. In the cHPMPC-treated group, 83.7% (39/47) pups survived, compared with 28.2% (11/39) in the control group (P<.001). B, Gestation period. Gestation was extended in cHPMPC-treated dams: the mean no. of days to delivery was 24.4, compared with 13.6 in the control group (P=.01).

Figure 1. Pup survival and duration of gestation in pregnant guinea pigs treated with cyclic 1-[(s)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine dihydrate (cHPMPC). A, Pup survival. In the cHPMPC-treated group, 83.7% (39/47) pups survived, compared with 28.2% (11/39) in the control group (P<.001). B, Gestation period. Gestation was extended in cHPMPC-treated dams: the mean no. of days to delivery was 24.4, compared with 13.6 in the control group (P=.01).

Table 1. Viral detection in offspring of pregnant guinea pigs infected with guinea pig cytomegalovirus and treated with cyclic 1-[(s)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine dihydrate (cHPMPC).

<table>
<thead>
<tr>
<th>Group, organ</th>
<th>Positive cultures (live pups) Positive nested PCR (live and dead pups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cHPMPC treated</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0/27 (0)</td>
</tr>
<tr>
<td>Liver</td>
<td>7/27 (25.9)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>2/8 (22.2)</td>
</tr>
<tr>
<td>Liver</td>
<td>2/8 (22.2)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. positive/total no. (%) PCR, polymerase chain reaction.

Effect of cHPMPC treatment on the outcome of pregnancy. To determine the safety of cHPMPC in pregnant guinea pigs, 3 pregnant, uninfected, strain 2 guinea pigs were treated with a single dose (35 mg/kg) of cHPMPC administered by ip injection. Guinea pigs were monitored for signs of toxicity and pregnancy outcome. None of the dams developed evidence of toxicity (e.g., decreased activity or weight loss); although 1 dam delivered 3 stillborn pups 24 h after treatment, the remaining pups appeared healthy at birth and remained healthy during the week of observation.

The aim of the first set of experiments was to evaluate the effect of maternal cHPMPC treatment on pregnancy outcome after GPCMV infection. As is seen in figure 1A, antiviral treatment with cHPMPC significantly increased the percentage of live-born pups, from 28.2% (11/39) in the control group to 83.7% (36/43) in the cHPMPC-treated group (P<.01). Treatment also increased the mean number of days from infection until delivery, from 13.6±9.5 in control dams to 24.4±6.6 in cHPMPC-treated dams (P<.01; figure 1B). To determine whether antiviral treatment had an effect on the transmission of virus to the fetus and infection of the pups, live pups were killed within 3–7 days of delivery, and their livers and spleens were evaluated by culture. As is seen in table 1, virus was recovered infrequently from live-born pups, and there was little effect of treatment on virus recovery. As further documentation of the limited quantities of virus present in pup tissues, first-round PCR was unable to detect viral DNA (data not shown). However, when a second round of PCR amplification (nested) with primers UL97F1/R1 was performed, 70%–80% of spleen

Means were compared by Student’s t test. Mortality was compared by Fisher’s exact test. All comparisons were 2-tailed.

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and liver samples from both the cHPMPC-treated and control groups were positive (table 1). This suggests that only low levels of virus were present in pups and that treatment did not prevent infection.

**Effect of antiviral treatment on maternal and fetal viral load by conventional PCR analysis.** To further examine the effects of cHPMPC treatment on viral replication in dams and pups, the same experimental design was used, except that dams were killed at 10 days after infection (this was expected to precede delivery in control dams). In the first of 2 experiments, blood was obtained from dams at 5 and 10 days after virus infection and was then examined by PCR. No viral DNA was detected in day-5 samples by first-round PCR when primers UL97F3/R3 were used (figure 2A). We therefore examined the samples by nested PCR and found that, after a second amplification using primers UL97F1/R1, all of the dams (5/5) in the control group at day 5 had detectable DNA loads in the blood. However, no viral DNA was detected in the cHPMPC-treated group (0/4; $P<.01$). By 10 days after infection, both groups had detectable viral DNA (figure 2B), and no significant difference was found (2/3 cHPMPC-treated dams vs. 5/5 untreated dams; 1/4 cHPMPC-treated dams died before day 10). This data suggests that cHPMPC treatment delayed but did not prevent viremia.

The impact of cHPMPC treatment on viral replication in livers, spleens, and placentas from these dams was also examined by first-round PCR using primers UL97F1/R1. Viral DNA was detected in 100% (5/5) of livers and spleens from control dams, whereas viral DNA was detected in only 50% (2/4) of livers and spleens from cHPMPC-treated dams. Although these differences were not significant ($P = .17$), the signal intensity of the UL97 PCR products in control dams appeared to be greater than that in cHPMPC-treated dams, which suggests a quantitative difference in viral loads. Similarly, only 63% of placentas from cHPMPC-treated dams were positive for GPCMV DNA (5/8), whereas 100% of pla-
centas from control dams (figure 3) were positive (15/15; \( P = .03 \)). The results from this initial experiment were confirmed, along with those of a second experiment, using a real-time PCR assay as described below.

To determine the effects of treatment on viral loads in fetal tissues at 10 days after infection, fetal liver and spleen were examined by first-round PCR. The results of these assays were negative for the detection of GPCMV DNA. However, when nested PCR was used (table 2), GPCMV DNA was detected in the livers of fetuses from cHPMPC-treated dams (71.4% [15/21]) and control dams (83.3% [20/24]), as well as in 76.2% (16/21) of spleens from fetuses of cHPMPC-treated and control dams (all values nonsignificant). The intensity of the bands of the \( UL97 \) PCR product in cHPMPC-treated tissues, however, appeared to be less than that in the control group, which suggests a lower viral load in cHPMPC-treated fetuses. These results were again confirmed by real-time PCR, as described below.

**Quantification of viral load in dams and fetuses by real-time PCR analysis.** To quantify the viral load in tissues, we developed a real-time PCR assay for GPCMV. The assay was first established by optimizing a number of conditions, such as the number of cycles, temperatures and times for annealing and extension of the primers, primer concentration, and specificity of amplification product. The fluorescence data were analyzed for product specificity according to the amount of fluorescence obtained at a specific temperature. The correct size of the 100-bp \( UL97 \) product was confirmed by gel electrophoresis.

Using the real-time PCR assay, dam liver, spleen, and placenta and fetal tissues were examined from 7 cHPMPC-treated dams and 9 control dams at 10 days after infection, as described above (table 3). Results revealed that there was no significant difference between the number of positive liver samples from cHPMPC-treated dams (7/7 [100%]) and that in samples from control dams (9/9 [100%]). However, there was a significant difference (\( P < .0001 \)) between the mean ± SE viral loads in livers from cHPMPC-treated dams (2.73 ± 0.4 \( \log_{10} \) genome copies/\( \mu \)g of DNA) and that in livers from control dams (4.80 ± 0.8 \( \log_{10} \) genome copies/\( \mu \)g of DNA). For spleen samples from dams, the number that were positive for GPCMV DNA by real-time PCR was also not significantly different between the cHPMPC-treated (6/7 [85.7%]) and control (9/9 [100%]) groups, but, again, cHPMPC treatment decreased the viral load from 4.13 ± 0.7 \( \log_{10} \) genome copies/\( \mu \)g of DNA in control dams to 2.61 ± 0.9 \( \log_{10} \) genome copies/\( \mu \)g of DNA (\( P < .01 \)) in cHPMPC-treated dams. Similarly, there were no significant differences between the percentage of placentas found to be positive for viral DNA obtained from cHPMPC-treated dams (15/16 [93.8%]) and that in control dams (22/22 [100%]). However, the viral load in cHPMPC-treated dams (2.12 ± 0.6 \( \log_{10} \) genome copies/\( \mu \)g of DNA) was significantly lower than that in control dams (3.54 ± 0.9 \( \log_{10} \) genome copies/\( \mu \)g of DNA) (\( P < .0001 \)).

We also quantified viral loads in fetal tissues from the cHPMPC-treated and control groups (table 3). There were

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**Table 3. Detection by real-time polymerase chain reaction of guinea pig cytomegalovirus in dam and pup organs 10 days after infection.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no.</th>
<th>Liver</th>
<th>Spleen</th>
<th>Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Dams</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cHPMPC treated</td>
<td>21</td>
<td>15/21 (71.4)</td>
<td>16/21 (76.2)</td>
<td>15/16 (93.8)</td>
</tr>
<tr>
<td>Control</td>
<td>27(^a)</td>
<td>20/24 (83.3)</td>
<td>18/23 (78.3)</td>
<td>22/22 (100)</td>
</tr>
<tr>
<td>Pups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cHPMPC treated</td>
<td>21</td>
<td>15/21 (71.4)</td>
<td>16/21 (76.2)</td>
<td>15/16 (93.8)</td>
</tr>
<tr>
<td>Control</td>
<td>27(^a)</td>
<td>20/24 (83.3)</td>
<td>18/23 (78.3)</td>
<td>22/22 (100)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. positive/total no. (%) or mean ± SE. cHPMPC, cyclic 1-[1(s)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl]methylcytosinedihydrate.

\(^a\) Total of 2 separate experiments.

\(^b\) \( P < .0001 \) vs. control.

\(^c\) \( P < .01 \) vs. control.

\(^d\) Unable to recover some tissues because of the size or condition of fetuses.

\(^e\) \( P < .05 \).

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Dams

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Table 2. Detection by nested polymerase chain reaction of guinea pig cytomegalovirus in pup organs harvested 10 days after infection.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total no.</th>
<th>Liver</th>
<th>Spleen</th>
<th>Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15/21</td>
<td>16/21</td>
<td>15/16</td>
</tr>
<tr>
<td>cHPMPC treated</td>
<td>21</td>
<td>15/21</td>
<td>16/21</td>
<td>15/16</td>
</tr>
<tr>
<td>Control</td>
<td>27(^a)</td>
<td>20/24</td>
<td>18/23</td>
<td>22/22</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. positive/total no. (%) or mean ± SE. cHPMPC, cyclic 1-[1(s)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl]methylcytosinedihydrate.

\(^a\) Total of 2 separate experiments.
no significant differences in the number of infected tissues between the cHPMPC-treated and control pups. A significant difference (P < .05) was, however, found between the mean ± SE viral load in livers from cHPMPC-treated pups (2.52 ± 1.14 log$_{10}$ genome copies/µg DNA) and that in livers from control pups (3.20 ± 0.58 log$_{10}$ genome copies/µg DNA). However, no significant difference was detected in spleens between groups.

Last, we examined the relationship of the placental viral load to infection of the fetus in the control group. We found that the mean ± SE viral load in the placenta of pups that were not infected (negative PCR results) were significantly lower (2.7 ± 0.3 log$_{10}$ genome copies/µg of DNA) than that in the placenta of congenitally infected pups (3.7 ± 0.7 log$_{10}$ genome copies/µg of DNA; P = .04).

In summary, these experiments demonstrated that the treatment of GPCMV-infected pregnant guinea pigs with a single dose (35 mg/kg) of cHPMPC improves the outcome of congenital infection by allowing the continuation of pregnancy and increasing the survival of pups. cHPMPC treatment also significantly decreased viral load detected in the tissues of treated guinea pigs. However, at the dose regimen that we used, cHPMPC did not prevent the infection of fetuses.

**DISCUSSION**

Although the importance of congenital CMV infection as a significant public health issue is well recognized and the development of a successful vaccine is a priority, this strategy may be years from becoming a viable alternative. Therefore, the development and evaluation of safe and effective anti-CMV drugs for this and other diseases caused by CMV is a priority. However, as with all therapies for pregnant women, the risk of teratogenicity and toxicity must be weighed against the benefits of treatment. Thus, the known teratogenic and toxic effects of the currently available therapies severely limit the use of these drugs, and the present experience with treatment during pregnancy is limited to isolated instances of pregnancy occurring in transplant recipients receiving ganciclovir [29–31] and a report of the unsuccessful treatment of an infected fetus [14]. Recently, the successful use of CMV hyperimmune globulin for the treatment of pregnant women with a primary CMV infection was reported [32].

In the studies reported here, we evaluated the effect of antiviral treatment in pregnant guinea pigs infected with GPCMV during mid-to-late gestation. cHPMPC is an effective anti-CMV antiviral, and, because of its relatively long half-life, treatment can be given as a single dose (35 mg/kg) by ip injection [20–25]. Furthermore, as has been demonstrated here, this dose is well tolerated in the pregnant guinea pig. A single dose of cHPMPC administered 24 h after GPCMV infection decreased the maternal viral load at 5 days after infection but did not prevent viremia: by 10 days after infection, most dams had detectable virus in their blood. Treatment also decreased the viral load detected in the dam liver, spleen, and, more importantly, the placenta, but it did not prevent infection of these tissues. Interestingly, by day 10 days after infection, 100% of the control placentas were infected, which suggests an efficient infection rate of the placenta in the model. Most importantly, our results indicate that treatment significantly reduced the mortality of pups and extended the duration of the pregnancy to normal. Treatment, however, did not prevent infection of the pups but did reduce the viral load in the liver. Further studies are needed to determine whether alternative dosing regimens of cHPMPC or a more effective drug could prevent or delay infection of the placenta and/or fetuses in this model and further improve survival of the pups.

Similar to what has been found in human and mouse CMV studies [33–35], the use of real-time PCR allowed us to show quantitative differences in viral loads that would otherwise not have been subject to evaluation. Also similar to what has been found in human studies, nested PCR improved our ability to detect CMV in fetuses [35]. CMV infection of the placenta without infection of the fetus is thought to be common [36, 37] and may result in spontaneous abortion [36–39]. In the guinea pig, infection of the placenta is thought to occur before infection of the fetus [40], so our inability to detect infection in some fetuses or newborns may have been related to the relatively early timing of specimen collection in relation to maternal infection.

Although most CMV infections of pregnant women go unrecognized, recent advances in the diagnosis of infection in pregnancy have been reported [10–15, 32]. These diagnostic tests are performed in pregnant women during the first trimester in many European countries, although treatment options are limited. Future development of antiviral compounds with less toxicity and teratogenicity, coupled with improved diagnostic testing, may make maternal antiviral treatment a viable therapeutic choice after the diagnosis of congenital CMV infection. As has been shown in the studies presented here, the treatment of maternal infections should have a beneficial effect on the developing fetus and would be expected to be more effective than treatment of the newborn.

**References**


