Oral Activity of a Methylenecyclopropane Analog, Cyclopropavir, in Animal Models for Cytomegalovirus Infections

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We reported previously that purine 2-(hydroxymethyl)methylenecyclopropane analogs have good activity against cytomegalovirus infection. A second-generation analog, (Z)-9-{{2,2-bis-(hydroxymethyl)cyclopropylidene}methyl}guanine (ZSM-I-62, cyclopropavir [CPV]), has particularly good activity against murine and human cytomegaloviruses (MCMV and HCMV) in vitro. To determine the oral activity of this compound in vivo, BALB/c or severe combined immunodeficient (SCID) mice infected with MCMV and two models using SCID mice implanted with human fetal tissue and subsequently infected with HCMV were used. In MCMV-infected normal mice, CPV at 10 mg/kg of body weight was highly effective in preventing mortality when administered at 24, 48, or 72 h post-viral inoculation and reduced titers of virus in tissues of SCID mice by 2 to 5 log₁₀. In one HCMV model, human fetal retinal tissue was implanted into the anterior chamber of the mouse eye and inoculated with the Toledo strain of HCMV, and in the second, human fetal thymus and liver tissues were implanted under the kidney capsule of mice and then inoculated with HCMV. In general, replication of HCMV in both types of implant tissue increased from 7 through 21 to 28 days and then gradually decreased to undetectable levels by 8 weeks postinfection. Oral treatment with 45 or 15 mg of CPV/kg initiated 24 h after infection was highly effective in reducing replication to undetectable levels in both models and was generally more effective than ganciclovir. These data indicate that the methylenecyclopropane analog, CPV, was highly efficacious in these four animal models and should be evaluated for use in HCMV infections in humans.

Cytomegalovirus (CMV) infections continue to be a major cause of morbidity in patients immunosuppressed as a result of solid organ or stem cell transplants (12, 14, 28). The treatment of choice for these infections has primarily been intravenous ganciclovir (GCV) (16, 47); however, its use for long-term maintenance therapy for persistent CMV infection has been problematic due to the development of toxic side effects, such as neutropenia (16, 47), and the development of resistant isolates (8, 9, 15, 26, 27). Other therapies have included acyclovir (ACV), particularly for long-term prophylaxis (3), or foscarnet (PFA) for GCV-resistant CMV. The advent of orally active valganciclovir and valganciclovir have facilitated the delivery of ACV and GCV (3, 29, 32, 48) but have not reduced the problem of toxicity or emergence of isolates resistant to GCV (8). Although cidofovir and PFA are approved for use in treatment of these infections, both are too toxic for long-term use (24, 38, 41). The ongoing nature of these problems for therapy of CMV infections has continued to underscore the importance of developing additional therapies for these infections, in particular ones that have less toxicity and greater activity against GCV and PFA resistant mutants.

We have previously described the synthesis and antiviral activity of a new class of compounds, the methylenecyclopropane analogs of purine and pyrimidine nucleosides (23, 35, 36, 37, 39, 40). Among the original first-generation compounds,

synadenol and synguanol along with some other analogues had good activity against most of the herpesviruses and were at least as active against CMV as GCV (23, 39, 40). Importantly, these compounds retained their activity against GCV- and PFA-resistant CMV isolates. In addition, some of these compounds were reported to have activity against human CMV (HCMV) and murine CMV (MCMV) in animal models (6, 39). In an attempt to increase the activity of these molecules and enhance their oral activity, a second generation of (Z)- and (E)-2,2-[bis(hydroxymethyl)cyclopropylidene] methylpurines and pyrimidines was synthesized and evaluated for their activity against herpesviruses, hepatitis B virus, and human immunodeficiency virus (49). One of these analogues, (Z)-9-{{2,2-bis-(hydroxymethyl)cyclopropylidene}methyl}guanine (ZSM-I-62, cyclopropavir [CPV]), had particularly good activity against human or murine CMV and was also active against GCV-resistant isolates, including both UL97 and UL54 mutants (22). Its mechanism of action appears similar to that of GCV in that it is an inhibitor of DNA synthesis that involves phosphorylation by UL97, but it may have a substrate specificity different from that of GCV (2, 22).

The purpose of the experiments described in this report was to evaluate the activity of CPV, administered either parenterally or orally, against experimental CMV infection in animal models. The experimental infections used were as follows: (i) MCMV infection of BALB/c mice, utilizing both mortality and inhibition of virus replication in target organs as indications of efficacy; (ii) SCID mice infected with MCMV to determine the influence of treatment on replication in systemic organs in an immunocompromised host; (iii) HCMV infection of human

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FIG. 1. Structure of CPV.

retinal tissue implanted in eyes of SCID mice; and (iv) HCMV infection of thymus and liver tissue implanted under the kidney capsule of SCID mice. The endpoint used to determine efficacy in the two HCMV models was inhibition of HCMV replication in the implanted human tissue.

MATERIALS AND METHODS

Antiviral compounds. The methylenecyclopropane analog, CPV, was synthesized by Z. Yan and J. Zemlicka, Wayne State University School of Medicine (Detroit, Mich.), and provided through the Antiviral Research Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, Md.). The synthesis and *in vitro* antiviral activity have been reported previously (23, 49), and the structure is presented in Fig. 1. GCV was purchased from the University of Alabama Hospital Pharmacy and used as a positive control in all experiments. GCV was prepared in sterile saline, and CPV was prepared in 0.4% carboxymethylcellulose (CMC) at various dosages and administered to mice in a 0.1-ml volume intraperitoneally (i.p.) or a 0.2-ml volume orally.

Tissue, cells, and viruses. Human foreskin fibroblast (HFF) cells and murine embryo fibroblast (MEF) cells were prepared as primary cultures and used in assays with HCMV and MCMV. These cells were propagated in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), L-glutamine, and antibiotics as indicated above. Human fetal tissue was obtained from Advanced Biosciences Resources (Alameda, Calif.) and prepared as described below. All viruses were propagated using standard virological methods. The Smith strain of MCMV was obtained originally from June Osborne, University of Wisconsin, and the Toledo strain of HCMV was obtained from Edward Mocarski (Palo Alto, Calif.).

Mice. Female BALB/c mice, 3 weeks old, were obtained from Charles River Laboratories (Raleigh, N.C.). Male SCID mice, 8 to 12 weeks old, were obtained from Charles River Laboratories, National Cancer Institute (Bethesda, Md.). Mice were group housed in microisolator cages and utilized 15 mice per treatment group for statistical analysis. Mice were obtained, housed, utilized, and euthanized according to U.S. Department of Agriculture and Association for Assessment and Accreditation of Laboratory Animal Care regulatory policies. All animal procedures were approved by The University of Alabama at Birmingham, Institutional Animal Care and Use Committee prior to initiation of studies.

Experimental MCMV infections and viral pathogenesis. MCMV infections were initiated by i.p. inoculation of BALB/c or SCID mice, using an approximate 90% lethal dose and observed daily for 21 to 30 days. GCV or CPV was administered i.p. or orally once daily for 5 days. In certain experiments, samples of lung, liver, spleen, kidney, pancreas, and salivary gland were obtained from three BALB/c mice per treatment group or control mice on day 1, 3, 5, 7, 10, 12, or 14 after MCMV infection. With SCID mice the same tissues were harvested on days 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 after infection. Samples were homogenized in medium (10% [wt/vol]) and frozen at -70°C until assayed for virus (19). Tissue samples were thawed and assayed on MEF cells by plaque assay to determine titers of MCMV (19). Briefly, samples of organ homogenates were diluted serially, and a 0.2-ml volume was placed into triplicate wells of 12-well plates containing MEF cell monolayers and incubated for 7 days. Cultures were stained with neutral red (Gibco, Rockland, Md.) for approximately 6 h prior to enumeration of viral plaques.

SCID-hu mouse model for HCMV replication in ocular tissue. Implantation of human fetal retinal tissue and later infection of these implants were performed as described previously (4, 5). In brief, 4- to 8-week-old male SCID mice were anesthetized with an i.p. injection of ketamine (100 mg/kg of body weight) and

xylazine (15 mg/kg), and the topical anesthetic, proparacaine-HCl (0.5%), was instilled in the eyes. A 27 gauge by 1/2-in. winged infusion needle containing mechanically dissociated human fetal retina was then inserted into the medial sclera and into the anterior chambers of both eyes. At the lateral side of the anterior chamber, approximately 5 μl of tissue was injected, and the needle was removed. Using similar procedures, the mice were again anesthetized 6 to 9 weeks after implantation, and 10 μl of 2,000 to 7,500 PFU of HCMV, depending on the experiment, was injected into the anterior chamber containing the implant. Within a given experiment, all animals received the same amount of inoculum. Beginning 24 h after viral inoculation, the mice were treated with drug given i.p. (0.1 ml) or orally (0.2 ml) once daily for 28 days. On days 7, 14, 21, and 28, six animals per group were euthanized, and both eyes were harvested, homogenized, and frozen at -70°C until assayed for HCMV.

SCID-hu mouse model for HCMV infection in thymus and liver tissue. For the second experimental HCMV infection, 4- to 6-week-old male SCID mice were anesthetized, and fragments of human fetal thymus and liver (thy/liv) were implanted under the capsule of one kidney by using an 18-gauge trocar, using techniques described previously (7, 17). After an implant growth period of 12 to 14 weeks, the grafts were inoculated with 2,000 to 9,000 PFU of HCMV. Beginning 24 h after viral inoculation, the mice were treated once daily for 28 days. On days 14, 21, 28, and 35, 6 to 11 implants were biopsied (approximately 50% of the graft size), homogenized, and frozen at -70°C until assayed for HCMV. Biopsies were also obtained 1 week after treatment ended (day 35) to determine if viral replication rebounded after cessation of treatment.

Viral replication in implant tissue. To monitor HCMV replication in retinal implant tissue, animals were euthanized at various times after infection. The eyes were removed, temporarily stored in sterile irrigating balanced salt solution, and homogenized in MEM containing 10% FBS, 2 mM L-glutamine, 200 U of

TABLE 1. Effect of intraperitoneal treatment with GCV or CPV on mortality of BALB/c mice inoculated with MCMV

Treatment ^a	Mortality ^b		P value	MDD ^c	P value
	No.	(%)			
Placebo					
CMC (0.4%), +24 h	8/15	(53)		5.4	
CMC (0.4%), +48 h	13/15	(87)		5.1	
GCV					
50 mg/kg, +24 h	0/15	(0)	<0.001		
16.7 mg/kg, +24 h	0/15	(0)	<0.001		
5.6 mg/kg, +24 h	1/15	(7)	<0.001	3.0	NS ^d
50 mg/kg, +48 h	1/15	(7)	<0.001	5.0	NS
16.7 mg/kg, +48 h	1/15	(7)	<0.001	10.0	NS
5.6 mg/kg, +48 h	2/15	(13)	<0.001	5.0	NS
CPV					
50 mg/kg, +24 h	5/15	(33)	NS	8.4	NS
16.7 mg/kg, +24 h	0/15	(0)	<0.001		
5.6 mg/kg, +24 h	4/15	(27)	NS	2.8	<0.01
50 mg/kg, +48 h	12/15	(80)	NS	4.6	NS
16.7 mg/kg, +48 h	3/15	(20)	0.001	6.7	NS
5.6 mg/kg, +48 h	0/15	(0)	<0.001		
Toxicity					
GCV					
50 mg/kg, +24 h	0/10	(0)			
CPV					
50 mg/kg, +24 h	2/10	(20)		19.0	

^a Animals were treated once daily for 5 days beginning 24 (+24) or 48 (+48) h post-viral inoculation.

^b No. dead/no. inoculated.

^c MDD, mean day of death.

^d NS, not significant when compared to the placebo control.

TABLE 2. Effect of oral treatment with GCV or CPV on mortality of BALB/c mice inoculated with MCMV

Treatment ^a	Mortality ^b		P value	MDD ^c	P value
	No.	(%)			
Placebo					
CMC (0.4%), +24 h	14/15	(93)		5.1	
CMC (0.4%), +48 h	13/15	(87)		6.0	
GCV					
50 mg/kg, +24 h	3/15	(20)	<0.001	2.7	<0.05
16.7 mg/kg, +24 h	3/14	(21)	<0.001	6.7	NS ^d
5.6 mg/kg, +24 h	6/15	(40)	<0.001	6.0	NS
50 mg/kg, +48 h	1/15	(7)	<0.001	3.0	<0.05
16.7 mg/kg, +48 h	0/15	(0)	<0.001		
5.6 mg/kg, +48 h	7/15	(47)	0.05	6.6	NS
CPV					
50 mg/kg, +24 h	1/15	(7)	<0.001	3.0	NS
16.7 mg/kg, +24 h	0/15	(0)	<0.001		
5.6 mg/kg, +24 h	3/15	(20)	<0.001	4.0	NS
50 mg/kg, +48 h	4/15	(27)	<0.01	4.5	0.01
16.7 mg/kg, +48 h	0/15	(0)	<0.001		
5.6 mg/kg, +48 h	1/15	(7)	<0.001	3.0	<0.05
Toxicity					
GCV					
50 mg/kg, +24 h	0/10	(0)			
CPV					
50 mg/kg, +24 h	0/10	(0)			

^a Animals were treated once daily for 5 days beginning 24 (+24) or 48 (+48) h post-viral inoculation.

^b No. dead/no. inoculated.

^c MDD, mean day of death.

^d NS, not significant when compared to the placebo control.

penicillin/ml, 50 µg of gentamicin/ml, and 3 µg of amphotericin B/ml. Each eye was weighed and homogenized individually as a 10% (wt/vol) suspension in medium, using a Kontes tissue grinder (Vineland, N.J.). The homogenate was centrifuged at 1,500 rpm (Eppendorf model 8810 R) for 15 min at 4°C, and the supernatant removed and frozen at -70°C until assayed for HCMV using standard plaque assay techniques on HFF cells. Biopsy samples of thy/liv implants were weighed and homogenized as 10% (wt/vol) suspensions, centrifuged, and assayed as described above. Titers were expressed as log₁₀ PFU/gram of tissue.

Plaque assay for MCMV or HCMV. MEF or HFF cells were seeded into six-well plates and incubated at 37°C. Two days later, tissue homogenates were serially diluted in MEM with 2% FBS. Medium was aspirated from the wells, and 0.2 ml of tissue suspension was added to each well in duplicate. Plates were incubated for 1 h with shaking every 15 min, and 2.0 ml of medium was added to each well. After incubation for 8 days, cells were stained with a 5% neutral red solution in phosphate-buffered saline. The stain was aspirated, cells were washed with phosphate-buffered saline, and plaques were counted by using a stereomicroscope. Under these assay conditions, secondary plaque formation did not alter titers of virus. By comparing drug-treated with untreated wells, 50% effective concentrations were calculated by using MacSynergy II software (33).

Evaluation of efficacy: statistics. In order to determine therapeutic efficacy with these models, animals treated with CPV or GCV were compared to vehicle-treated animals, and differences in mortality rates were evaluated using Fisher's exact test. Difference in mean day of death (MDD) and titers of virus in tissues were determined by a Mann-Whitney U rank sum test. Percentages of implants positive for HCMV infection were calculated and compared using a general linear regression model, and titers of virus (PFU/milliliter ± standard deviation or log₁₀ PFU/gram) were compared using a stratified Wilcoxon rank sum test. The results obtained throughout the entire 28-day treatment period were used to

calculate significance. In general, a *P* value of 0.05 or less was considered significant.

RESULTS

Effect of i.p. treatment with GCV or CPV in MCMV infections of mice. To determine the ability of CPV to influence mortality rates in mice infected with MCMV, compounds were dissolved in deionized water at doses of 50, 16.7, or 5.6 mg/kg and administered i.p. once daily for five consecutive days beginning 24 or 48 h post-viral inoculation. GCV was administered i.p. in similar doses for comparison. Toxicity was associated with the 50-mg/kg dose of CPV with 20% mortality. At the 16.7- or 5.6-mg/kg dose, no toxicity was observed, and both CPV and GCV significantly reduced final mortality rates (*P* ≤

TABLE 3. Effect of oral treatment with GCV or CPV on mortality of BALB/c mice inoculated with MCMV

Treatment ^a	Mortality ^b		P value	MDD ^c	P value
	No.	(%)			
0.4% CMC, +24 h	14/15	(93)		5.4	
GCV, +24 h					
10 mg/kg	4/15	(27)	<0.001	8.0	<0.001
3 mg/kg	4/15	(27)	<0.001	7.0	0.001
1 mg/kg	13/15	(87)	NS ^d	6.4	<0.001
CPV, +24 h					
10 mg/kg	0/15	(0)	<0.001		
3 mg/kg	0/15	(0)	<0.001		
1 mg/kg	2/15	(13)	<0.001	7.5	<0.01
0.4% CMC, +48 h	14/15	(93)		5.5	
GCV, +48 h					
10 mg/kg	3/15	(20)	<0.001	6.7	0.06
3 mg/kg	10/15	(67)	NS	6.5	0.001
1 mg/kg	14/15	(93)	NS	6.1	<0.05
CPV, +48 h					
10 mg/kg	1/15	(7)	<0.001	6.0	NS
3 mg/kg	1/15	(7)	<0.001	6.0	NS
1 mg/kg	7/15	(47)	0.01	5.9	NS
0.4% CMC, +72 h	14/15	(93)		5.6	
GCV, +72 h					
10 mg/kg	14/15	(93)	NS	6.5	<0.01
3 mg/kg	14/15	(93)	NS	5.9	NS
1 mg/kg	15/15	(100)	NS	5.9	NS
CPV, +72 h					
10 mg/kg	4/15	(27)	<0.001	7.5	<0.01
3 mg/kg	8/15	(53)	<0.05	6.0	NS
1 mg/kg	15/15	(100)	NS	6.5	0.001
Toxicity					
CPV, 10 mg/kg	0/10	0			

^a Animals were treated one time daily for 5 days beginning 24 h after viral inoculation (+24 h).

^b No. dead/no. inoculated.

^c MDD, mean day of death.

^d NS, not significant when compared to the placebo control.

FIG. 2. Effect of oral treatment with CPV or GCV on replication of MCMV in BALB/c mice. Groups of mice were infected with MCMV and treated with either vehicle, 10 mg of GCV/kg, or 10 mg of CPV/kg. Three mice per group were euthanized on various days after infection, and lung, liver, spleen, kidney, pancreas, and salivary glands were removed and homogenized (10% [wt/vol]) for quantitation of virus. Titers of virus are expressed as $\log_{10}$ PFU/gram of tissue.

0.001) when treatment was initiated at 24 to 48 h postinfection (Table 1).

Effect of oral treatment with GCV or CPV on MCMV infections of mice. We next performed a study similar to the one above to determine if CPV had antiviral activity when delivered orally. GCV or CPV was given at 50, 16.7, or 5.6 mg/kg once daily for 5 days beginning 24 or 48 h post-viral inoculation. Oral treatment with GCV or CPV provided significant protection from mortality when initiation of treatment was delayed until 24 to 48 h at concentrations as low as 5.6 mg/kg (Table 2). Since we did not achieve an endpoint in these studies, an additional experiment was performed using doses of 10, 3, and 1 mg/kg beginning 24 to 72 h postinfection. The results presented in Table 3 indicate that doses of CPV as low as 1 mg/kg still significantly reduced mortality rates due to MCMV infection when treatment was delayed until 48 h. The results also indicated that CPV significantly reduced mortality rates at 10 and 3 mg/kg even when treatment was delayed until 72 h after infection, a time when some animals begin to exhibit signs of infection (Table 3).

Effect of oral GCV or CPV treatment on replication of MCMV in mice. To compare the effect of treatment with oral GCV or CPV on the replication of MCMV in target organs of mice, animals were inoculated with MCMV and treated with 10 mg of GCV or CPV/kg once daily for 5 days beginning 24 h after infection. On various days postinfection, animals were

euthanized and tissues were removed and assayed for MCMV. Both treatment regimens resulted in a significant reduction in mortality ($P < 0.001$) (data not presented), and treatment with CPV resulted in a 3 to 5 $\log_{10}$ decrease in titers of virus in liver, spleen, and kidney ($P < 0.01$) but only a 1 $\log_{10}$ reduction in lung and pancreas and no reduction in salivary gland. For animals treated with GCV, only a small reduction in titer of virus was observed in the liver and kidney (Fig. 2).

Since CMV infections are a problem primarily in the immunocompromised host, we have utilized SCID mice which are deficient in both T cells and B cells and are very sensitive to infection with MCMV as a model for evaluating drug efficacy in an immunocompromised host (19). Groups of animals were infected with 100 PFU of MCMV and treated orally with 10 mg of GCV or CPV/kg given once daily for 28 days beginning 24 h after infection. On various days postinfection, animals from control and treated groups were euthanized, and tissues were removed and assayed for MCMV. The vehicle-treated animals had an MDD of 20 days, and those treated with GCV had an MDD of 23 days. In contrast, the animals treated with CPV remained alive as long as the animals received drug, but all died after treatment was discontinued on day 28 with an MDD of 32 (data not presented). In mice treated with GCV, there was some inhibition of virus replication in the liver; however, treatment with CPV decreased virus titers in liver, lung, spleen, and kidney by 2 to 5 $\log_{10}$ ($P < 0.01$). CPV but not GCV

FIG. 3. Effect of oral treatment with CPV or GCV on replication of MCMV in SCID mice. Groups of mice were infected with MCMV and treated with either vehicle, 10 mg of GCV/kg, or 10 mg of CPV/kg. Three mice per group were euthanized on various days after infection, and lung, liver, spleen, kidney, pancreas, and salivary glands were removed and homogenized (10% [wt/vol]) for quantitation of virus. Titers of virus are expressed as $\log_{10}$ PFU/gram of tissue.

significantly altered virus replication in salivary gland ($P < 0.05$) (Fig. 3).

Effect of treatment with GCV or CPV on HCMV replication in SCID-hu retinal tissue implants. In addition to MCMV for evaluating efficacy of potential new antiviral agents for HCMV, we have utilized two models of HCMV in SCID mice. In the first experiment, we determined the efficacy in the SCID-hu mouse ocular model. SCID mice with retinal implants infected with the Toledo strain of HCMV were treated i.p. with 45 mg of GCV/kg or orally with 45 or 15 mg of CPV/kg once daily for 28 days starting 1 day after infection. On 7, 14, 21, and 28 days after infection, eyes were removed from vehicle-, GCV-, or CPV-treated animals and homogenized, and HCMV replication was quantified by plaque assay. The results are shown in Table 4. In comparison with vehicle-treated animals, treatment with GCV significantly reduced mean titers of virus in implants on day 28 from $5,050 \pm 3,996$ PFU/ml in control implants to 167 ± 444 PFU/ml. In implants from mice treated with CPV at 45 or 15 mg/kg, there was a complete reduction in mean titers of virus. These data suggested that CPV was highly effective against HCMV replication at 15 mg/kg and indicated the need to determine the efficacy of CPV at lower concentrations. A second study was performed, using CPV concentrations of 10 and 3 mg/kg, and the results are summarized in Table 4, experiment 2. Although not significant at the $P < 0.05$ level marked, inhibition of HCMV replication was observed at the 10-mg/kg dose, but little inhibition was observed with 3 mg/kg.

Effect of treatment with GCV or CPV on HCMV replication in SCID-hu thy/liv tissue implants. Since infection of the eye with HCMV may present a strong blood-eye barrier to the systemic delivery of an antiviral compound, we next used SCID mice implanted with human fetal thymus and liver tissue under the kidney capsule to determine the efficacy of the methylene-cyclopropane analog against HCMV replication in a visceral organ. In this model, the implanted thymus and liver tissue have been shown to grow and become vascularized. It might be expected that antiviral drugs that do not penetrate well into the eye may be more efficacious in this model and the effect of CPV compared with that of GCV was examined with the thy/liv tissue implant model. In the first experiment, thy/liv implants were infected with 6,700 PFU of the Toledo strain of HCMV. Starting 24 h after infection, mice were treated with vehicle, 45 mg of GCV/kg, or 45 or 15 mg of CPV/kg once daily for 28 days. At 14, 21, 28, and 35 days after infection, implants were biopsied and HCMV titers were quantified by plaque assay. The results summarized in Table 5 indicated that both GCV and CPV were also efficacious in this model. At 28 days after infection, during the peak of HCMV replication, GCV inhibited HCMV replication by 1 to 2 $\log_{10}$ PFU/g, whereas both doses of CPV inhibited viral replication by greater than 4 $\log_{10}$ PFU/g. Since an endpoint was not obtained using these concentrations of CPV, a second experiment was performed using doses of 10 and 3 mg/kg. Treatment was administered as described above. The results are also presented in Table 5 as

TABLE 4. Effect of treatment with GCV or CPV on replication of HCMV in SCID-hu retinal tissue implants

Treatment ^c	Parameter	Value on days postinfection:				P value
		7	14	21	28	
Expt 1						
Vehicle (saline) ^a	No. of PFU/g ^c	292 ± 498	2,000 ± 1,719	4,408 ± 4,041	5,050 ± 3,996	0.002
	% Positive (<i>n</i> ^d)	33 (4/12)	75 (9/12)	83 (10/12)	80 (8/10)	
GCV (45) ^b	No. of PFU/g	0 ± 0	0 ± 0	0 ± 0	167 ± 444	
	% Positive (<i>n</i>)	0 (0/12)	0 (0/12)	0 (0/10)	17 (2/12)	
CPV (45) ^a	No. of PFU/g	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.001
	% Positive (<i>n</i>)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/10)	
CPV (15) ^a	No. of PFU/g	0 ± 0	0 ± 0	200 ± 632	0 ± 0	0.002
	% Positive (<i>n</i>)	0 (0/12)	0 (0/12)	10 (1/10)	0 (0/10)	
Expt 2						
Vehicle ^a	No. of PFU/g	0	608 ± 1,497	3,575 ± 6,216	17,500 ± 26,592	<0.01
	% Positive (<i>n</i>)	0 (0/12)	25 (3/12)	58 (7/12)	63 (5/8)	
GCV (30) ^b	No. of PFU/g	0	50 ± 173	50 ± 122	0	
	% Positive (<i>n</i>)	0 (0/12)	8 (1/12)	16 (1/6)	0 (0/4)	
CPV (10) ^a	No. of PFU/g	0	150 ± 239	200 ± 411	2,863 ± 7,736	0.08
	% Positive (<i>n</i>)	0 (0/12)	33 (4/12)	25 (3/12)	38 (3/8)	
CPV (3) ^a	No. of PFU/g	0	275 ± 717	4,500 ± 11,993	8,442 ± 20,226	NS ^f
	% Positive (<i>n</i>)	0 (0/12)	17 (2/12)	58 (7/12)	50 (5/10)	

^a Treatment was initiated 24 h after infection and administered orally once daily for 28 days.^b Treatment was initiated 24 h after infection and administered intraperitoneally once daily for 28 days.^c Values are expressed as means ± standard deviations.^d *n*, no. positive/no. tested.^e Parenthetical number is daily dosage, given in milligrams per kilogram.^f NS, not significant.

experiment 2. A significant reduction was observed in HCMV replication in the implant tissue in mice that received 10 mg of CPV/kg but not in those given 3 mg/kg. These results were similar to those presented above for the SCID-hu retinal implant tissue. In HCMV infections of BALB/c or SCID mice and in both HCMV infections in the SCID-hu models, CPV was highly active when given orally and was superior to GCV given i.p. or orally.

DISCUSSION

Long-term treatment of chronic CMV infection in transplant patients continues to be a major problem in the management of these infections. Although the development of oral prodrugs for both ACV and GCV has helped alleviate some of the problems of long-term therapy, there still remain the issues of GCV-induced toxicity and the selection of drug-resistant

TABLE 5. Effect of treatment with GCV or CPV on replication of HCMV in SCID-hu thy/liv tissue implants

Treatment ^c	Parameter	Value on day postinfection:				P value
		14	21	28	35	
Expt 1						
Vehicle (saline) ^a	Log ₁₀ PFU/g ^c	4.8 ± 4.9	5.0 ± 5.1	5.2 ± 5.5	4.9 ± 5.3	0.002
	% Positive (<i>n</i> ^d)	73 (8/11)	56 (5/9)	50 (5/10)	56 (5/9)	
GCV (45) ^b	Log ₁₀ PFU/g	0 ± 0	0 ± 0	2.2 ± 2.5	3.2 ± 3.6	<0.0001
	% Positive (<i>n</i>)	0 (0/9)	0 (0/9)	29 (2/7)	17 (1/6)	
CPV (45) ^a	Log ₁₀ PFU/g	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.0003
	% Positive (<i>n</i>)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	
CPV (15) ^a	Log ₁₀ PFU/g	2.3 ± 2.8	0 ± 0	0 ± 0	0 ± 0	0.0003
	% Positive (<i>n</i>)	20 (2/10)	0 (0/8)	0 (0/10)	0 (0/10)	
Expt 2						
Vehicle (CMC) ^a	Log ₁₀ PFU/g	4.2 ± 4.6	4.0 ± 4.1	3.7 ± 3.9	3.8 ± 4.1	0.008
	% Positive (<i>n</i>)	73 (8/11)	63 (5/8)	36 (4/11)	33 (3/9)	
GCV (25) ^b	Log ₁₀ PFU/g	2.3 ± 2.8	0 ± 0	1.4 ± 1.9	2.6 ± 2.9	0.006
	% Positive (<i>n</i>)	20 (2/10)	0 (0/9)	9 (1/11)	38 (3/8)	
CPV (10) ^a	Log ₁₀ PFU/g	2.4 ± 2.8	1.2 ± 1.7	1.9 ± 2.3	3.4 ± 3.9	NS ^f
	% Positive (<i>n</i>)	27 (3/11)	9 (1/11)	9 (1/11)	18 (2/11)	
CPV (3) ^a	Log ₁₀ PFU/g	3.4 ± 3.5	3.6 ± 3.8	4.1 ± 4.3	3.8 ± 4.0	NS ^f
	% Positive (<i>n</i>)	40 (6/10)	70 (7/10)	50 (4/8)	29 (2/7)	

^a Treatment was initiated 24 h after infection and administered orally once daily for 28 days.^b Treatment was initiated 24 h after infection and administered intraperitoneally once daily for 28 days.^c Values are expressed as means ± standard deviations.^d *n*, no. positive/no. tested.^e Parenthetical number is daily dosage, given in milligrams per kilogram.^f NS, not significant.

mutants. While cidofovir (CDV) is the most active of the current drugs against CMV in tissue culture and in animal models (20, 43), its lack of oral bioavailability and the severe nephrotoxicity associated with its use severely limit its usefulness for prophylaxis or chronic therapy. While there are orally active CDV prodrugs that are very active in animal model infections and have altered distribution which spares the kidney (11, 34), these compounds have not yet been evaluated for humans. In addition to the CDV prodrugs, there are other orally active compounds under development including the benzimidazole ribonucleoside, maribavir, previously known as 1263W94, which has been reported to have a satisfactory safety profile and to reduce CMV titers in semen of human immunodeficiency virus-infected men (10, 25, 44). Additionally, the compound has activity against GCV-resistant mutants (46).

In a series of nucleoside analogs containing a *Z*- or *E*-methylenecyclopropane moiety, a number of compounds have been synthesized and evaluated in vitro and in animal models for activity against HCMV or MCMV (23, 39, 40). A number of these, including the prototypes, synadenol and synguanol, had activity that was comparable to that of GCV. In addition, the compounds were active when given orally to mice and were active against GCV-resistant mutants (39, 40). At least one of these analogs was also active in two models of HCMV infection in SCID mice that was comparable to GCV (6). In order to increase the potency of these compounds and increase their activity against other viruses, a second generation of the 2,2-bis(hydroxymethyl)methylenecyclopropane analogs was synthesized and evaluated for antiviral activity. One of these, CPV, had particularly good activity against HCMV and MCMV, with 50% effective concentrations of 0.5 to 0.8 μ M, compared with that of GCV, which was 3 to 5 μ M (22).

The purpose of the experiments reported here was to compare the activity of CPV with that of GCV, using a number of animal models for CMV. Inoculation of immunocompetent mice with MCMV provides a model that has many of the characteristics seen in HCMV infection. After inoculation with a large inoculum of MCMV, animals progress rapidly through acute disease and die by 5 to 7 days, or if the inoculum is reduced 10-fold to about 10^6 PFU, all mice survive. In either case, high titers of virus are present in essentially all visceral and glandular tissues for weeks to months (18, 19). In this model, although GCV treatment significantly reduced mortality rates and virus replication in target organs, it was considerably less active than CDV (19). In the experiments reported here, treatment with CPV was more active than that with GCV in that lower concentrations could be used and significantly reduce mortality rates, and when evaluated at comparable dosages, CPV was much more effective in reducing viral replication in target organs, particularly in lung, liver, and spleen.

Members of our group and others have used SCID mice inoculated with MCMV as a model for CMV infection in the murine compromised host (13, 19, 30, 42, 45). Since SCID mice are profoundly immunocompromised, approximately 1 to 3 PFU will result in a lethal infection. However, animals can be kept alive for weeks while on effective therapy, such as GCV or CDV, and most tissues have high levels of virus, as in a chronic infection in humans (19). Thus, the model can be used to evaluate the effect of antiviral therapy in a host that is unable to clear the virus and succumbs without effective therapy. In

this model, CDV again was more effective than GCV in reducing virus replication. In the present experiments, treatment with CPV was also superior to that with GCV in reducing viral replication. In comparison with historical results, CPV appeared to be less efficacious than CDV (19) or the ether lipid ester prodrugs of CDV in reducing viral replication in target organs of mice (21) or in human fetal tissue implants (7).

Although a number of mouse models for HCMV replication have been used (1, 31, 45), we believe that SCID mice implanted with fetal human tissue that can subsequently be infected with HCMV are the models of choice. We have utilized both retinal tissue implanted into the eye or thy/liv tissue implanted under the kidney capsule. While the retinal model represents a model of HCMV infection of an ocular structure that requires systemic therapy to cross the blood-eye barrier, the thy/liv system may mimic a visceral organ in which drug is delivered directly to the site of infection through the blood. We have reported previously that while GCV can effectively reduce viral replication in both types of implants (20), CDV and the oral CDV prodrugs are much more effective than GCV (7). In the results reported here, orally administered CPV was compared with GCV using both retinal and thy/liv implants in SCID mice. While GCV was effective in significantly reducing viral replication in the implanted tissue at levels of 30 to 45 mg/kg, CPV was equally effective at concentrations of 10 to 15 mg/kg.

In these studies, we have compared the activity of orally administered CPV with parenteral or oral GCV in MCMV infections in both immunocompetent and immunocompromised mice and in two models in which fetal retinal or thy/liv tissue was implanted into SCID mice and infected with HCMV. In all four models, CPV was highly effective in reducing mortality rates and/or virus replication in visceral organs or implanted tissues at levels of about 3 to 10 mg/kg. In each model, CPV was more active than GCV.

These results, along with those obtained in vitro, indicate that CPV is very active against CMV infections, and the compound should be evaluated for its pharmacologic and toxicologic properties in order to determine its potential for use in treatment of CMV infection in humans.

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