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Comparative Activity of (*S*)-1-(3-Hydroxy-2-Phosphonylmethoxypropyl)Cytosine and 9-(1,3-Dihydroxy-2-Propoxymethyl)Guanine against Rat Cytomegalovirus Infection In Vitro and In Vivo

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Two antiviral compounds, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [HPMPC] and 9-(1,3-dihydroxy-2-propoxymethyl)guanine [DHPG], were evaluated for their inhibitory effects on human cytomegalovirus (HCMV) replication in human embryonal fibroblasts and on rat cytomegalovirus (RCMV) replication in rat embryonal fibroblasts. The concentrations of HPMPC or DHPG required to inhibit HCMV plaque formation by 50% were 0.1 and 0.6 μg/ml, respectively. For RCMV, these values were 1.1 and 25 μg/ml, respectively. For HCMV, the selectivity indices of HPMPC and DHPG, as determined by the ratio of the 50% inhibitory concentration for cell growth to the 50% inhibitory concentration for virus plaque formation, were 1,250 and 140, respectively, and for RCMV, they were 500 and 76, respectively. HPMPC was far more active than DHPG against RCMV infection in vivo as measured by mortality, histopathological changes, and virus titers in organs of immunocompromised RCMV-infected rats. The minimal effective dosage required to prevent mortality from RCMV infection was a single dose of HPMPC at 2 mg/kg of body weight compared with DHPG therapy twice daily at 20 mg/kg/day for 5 days. Furthermore, HPMPC was more effective than DHPG in reducing virus titers in internal organs ($P < 0.01$) and in RCMV-induced histopathologic lesions. In contrast to DHPG, which did not show activity when administered 1 day before infection, HPMPC was effective even when administered 7 days before RCMV infection.

Human cytomegalovirus (HCMV) has been recognized as one of the most important pathogens in immunocompromised hosts (11, 14), including patients with AIDS and recipients of bone marrow transplants, in whom HCMV is the most frequent cause of death (11, 17). Few nucleoside analogs have been reported to be effective against HCMV in vitro, and most of these have relatively low selectivity indices (18). Only two drugs, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG; ganciclovir) and phosphonoformic acid (foscarnet), have been shown to be therapeutically effective against HCMV disease (16). However, the clinical use of these agents is limited by severe adverse reactions (5). Therefore, the search for more-potent and less-toxic antiviral drugs for chemotherapy in HCMV infections has to be continued. Recently, a new class of acyclic nucleoside phosphonate analogs has been described (7, 8, 20). Of these compounds, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC) seems to be the most potent and most selective inhibitor of CMV replication in vitro (18). In the present study, we compared the inhibitory effect of HPMPC on rat CMV (RCMV) and HCMV replication in cultured cells with that of DHPG. Furthermore, we compared the efficacy of HPMPC with that of DHPG in a rat model (19) for CMV infection in the immunocompromised host and investigated the efficacy of prophylactic treatment with HPMPC on a lethal CMV infection in rats.

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MATERIALS AND METHODS

Viruses. For in vitro tests with HCMV, the AD169 strain (ATCC VR 807) was used. RCMV, isolated by Bruggeman et al. from wild rats (3) and characterized by Meijer et al. (15), was used for studies with the rat model. For in vitro studies, we used RCMV and HCMV obtained from cell culture supernatant (2). For in vivo studies, RCMV was obtained from suspensions of salivary gland tissue of rats after several in vivo passages (2).

Antiviral agents. DHPG was obtained from Syntex Inc., Palo Alto, Calif., and HPMPC was synthesized by I. Rosenberg and A. Holy (Czechoslovak Academy of Sciences, Prague, Czechoslovakia) (12).

Virus plaque assays and virus plaque inhibition assays. Rat embryonal fibroblasts (REF) were obtained as described before (19). REF and human lung embryonal fibroblasts (HEF) (Flow 2002, Amstelstad, Zwanenburg, The Netherlands) were grown in Eagle's minimal essential medium supplemented with 10% inactivated newborn calf serum (NCS; Westburg), 2 mM L-glutamine, and 24 mM sodium bicarbonate (growth medium) in 24-well culture plates (Greiner, Alphen aan de Rijn, The Netherlands).

For plaque assays with organ tissues, 10% homogenates (wt/vol) were prepared in basal medium Eagle (BME) supplemented with 2% NCS, 2 mM L-glutamine, and 24 mM sodium bicarbonate, and after centrifugation, triplicate samples of the supernatant and serial 10-fold dilutions in BME with 2% NCS were incubated for 1 h at 37°C on a 90% confluent monolayer of REF. Then the supernatants were removed and replaced by BME with 2% NCS with agar overlay. After an incubation period of 7 days, the number of plaques was monitored microscopically following formaldehyde fixation and staining with methylene blue.

For plaque inhibition assays, 90% confluent REF and HEF monolayers were infected with about 150 PFU of RCMV or HCMV per well, respectively. The viruses were diluted in BME with 2% NCS and supplements. After 1 h of incubation at 37°C, BME was removed and replaced by medium containing concentrations ranging from 200 to 0.01 μg of the antiviral compound per ml in BME with supplements with and without agarose overlay in triplicate. After 7 and 10 days of incubation for RCMV and HCMV, respectively, the number of plaques was monitored as described above. The MIC of the compound is expressed as the concentration required to inhibit plaque formation by 50% (EC_{50}). The EC_{50} was estimated from the number of plaques on semilogarithmic graphs as a function of the concentration of the antiviral compounds. In time-of-addition experiments, antiviral agents were added to HEF and REF at different days before and after infection (−7, −5, −3, −1, 0, +1, +3, +5, and +7 days) and incubated for 24 h at 37°C. Thereafter, the supernatant containing the antiviral drugs was removed and replaced by fresh BME with 2% NCS. The cell cultures were inoculated as described above, and the plaques were counted at 7 days postinfection (p.i.). The MIC is expressed as the concentration required to inhibit virus plaque formation by 50% (EC_{50}).

Growth inhibition assay. The inhibitory effects of DHPG and HPMPC on HEF and REF growth were determined by seeding 3×10^3 cells per well in 96-well microtiter plates (Greiner) in growth medium. After 24 h, the medium was removed and replaced by growth medium containing various concentrations of the test compound allowing maximal replication. After 3 days of incubation at 37°C, the cell number was determined with a Coulter Counter. The inhibitory concentration of the compound is expressed as the IC_{50}, or concentration required to reduce cell growth by 50%. The selectivity index corresponds to the ratio of IC_{50} for cell growth to the EC_{50} for RCMV replication.

Animal inoculation and treatment. Specific-pathogen-free 8-week-old male Brown Norway rats (140 to 180 g) received total body irradiation (TBI) of 5 Gy and were inoculated intraperitoneally with 1 ml of 10^5 PFU of RCMV as described before (19). DHPG and HPMPC were administered intraperitoneally at different times before and after virus inoculation as described below. The injected dose was adjusted to the actual body weight. Sham-treated animals were injected intraperitoneally with sterile phosphate-buffered saline. Uninfected negative controls received the same dilution of RCMV-negative salivary gland suspension and 5 Gy of TBI. Groups receiving virus or irradiation only were also included in the study.

Survival scores and specimen collection. Rats were examined daily and survival was recorded. The in vivo activity of the drug is expressed as the minimal effective dose, or the daily dose at which at least 50% of the animals survived the lethal infection (ED_{50}).

To study the effect of treatment on virus titers in organs, DHPG- or HPMPC-treated rats and untreated rats were sacrificed 7 days p.i. Virus titers in organs were measured as described earlier (19). In short, spleen, liver, lungs, kidneys, and salivary glands were removed aseptically, homogenized in a tissue grinder, and suspended in BME with 2% NCS, and plaque assays were performed.

For histopathological examination, organs were fixed in paraformol-lysin-periodate, embedded in paraffin, and processed as described before (19).

For detecting RCMV antigen, monoclonal antibodies directed against a 29-kDa polypeptide of RCMV and against 41-, 46-, and 91-kDa polypeptides were used (4). Specificity was analyzed as described previously (19).

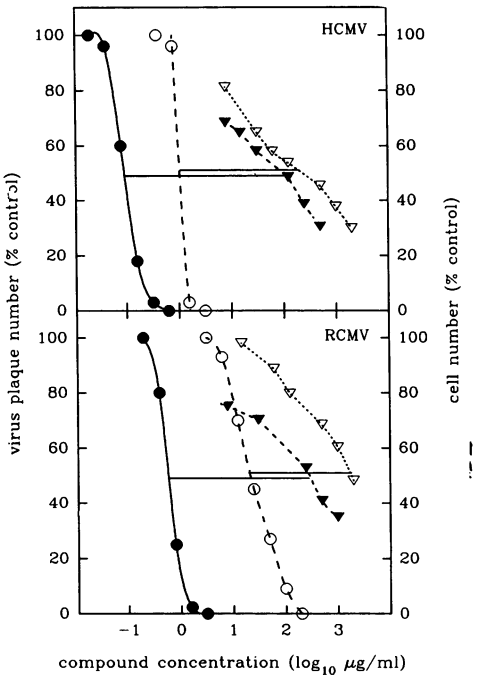

FIG. 1. Dose-response curves for antiviral activity of DHPG and HPMPC in vitro. Virus plaque inhibition by HPMPC (●) and DHPG (○) and cytotoxicity of HPMPC (▼) and DHPG (▽) are shown. Points represent the mean values of two separate experiments performed in triplicate. Standard deviation is $x \times 1.5$.

Statistical analysis. For statistics on in vitro results, the Mann-Whitney test and Fisher's exact test were applied. For comparison survival curves, the generalized Wilcoxon test by Gehan was used (9). P values of <0.05 were considered statistically significant.

RESULTS

Inhibition of virus plaque formation and cytotoxicity of HPMPC and DHPG in vitro. Dose-response curves for the antiviral activity and cytotoxicity of HPMPC and DHPG are presented in Fig. 1. HPMPC inhibited HCMV plaque formation at an EC_{50} of 0.10 (0.07 to 0.16) μg/ml and RCMV plaque formation at an EC_{50} of 0.6 (0.4 to 0.9) μg/ml. The EC_{50} of DHPG was 1.1 (0.6 to 1.8) μg/ml for HCMV and 25 (14 to 40) μg/ml for RCMV. The IC_{50}s of DHPG and HPMPC in uninfected HEF were 150 (100 to 220) and 125 (80 to 185) μg/ml, respectively, whereas the corresponding values in REF were 1,900 (1,260 to 2,800) and 300 (230 to 390) μg/ml, respectively (Fig. 1). The selectivity indices (ratio of IC_{50} to EC_{50} for CMV plaque formation) of DHPG for HCMV and RCMV were 140 and 76, respectively. The selectivity indices of HPMPC for HCMV and RCMV were 1,250 and 500, respectively. In HEF, HPMPC has a ninefold-higher selectivity than DHPG, whereas in REF, the selectivity index of HPMPC is seven times higher than that of DHPG.

Time-of-addition experiments. When drugs were added at 6 h p.i. and removed 24 h later, the EC_{50}s of HPMPC and DHPG in HCMV-infected HEF cells were 0.8 and 35, respectively (Fig. 2). However, when drugs were added 1 day p.i. and removed 24 h later, the EC_{50}s of HPMPC and

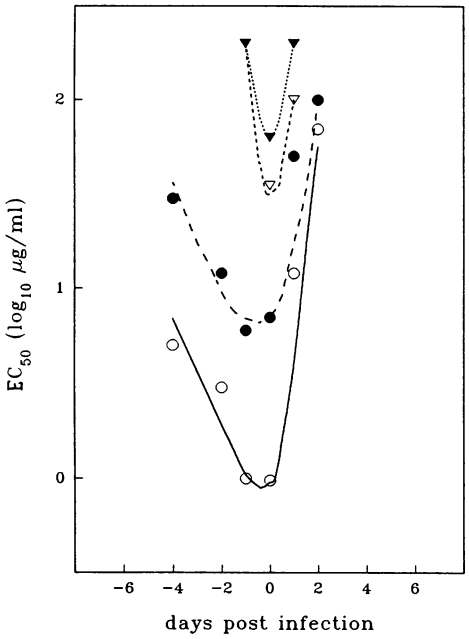

FIG. 2. Time-of-addition curves for antiviral activity in vitro (EC_{50}). Symbols: ○, EC_{50} of HPMPC for HCMV; ●, EC_{50} of HPMPC for RCMV; ▽, EC_{50} of DHPG for HCMV; ▼, EC_{50} of DHPG for RCMV. Points represent the mean values of two experiments. Standard deviation is $y \times 1.5$.

DHPG increased significantly to 12 and >200 μg/ml, respectively. When HPMPC was added at 48 h p.i. and removed 24 h later, it was less inhibitory to HCMV plaque formation (70 μg/ml), and when treatment was delayed until 3 days p.i. or later, no differences between treated and untreated cells were recorded (EC_{50} > 100 μg/ml).

Even when administered 4 days before infection and removed 24 h later, HPMPC inhibited HCMV replication (Fig. 2). In contrast, DHPG at 100 μg/ml added 1 day before infection did not affect HCMV replication. For RCMV infection, the EC_{50}s of HPMPC were 10 times higher than those for HCMV, but otherwise, the kinetics were similar (Fig. 2). DHPG had almost no effect on HCMV or RCMV infection if added for a short time (24 h).

Effects of HPMPC and DHPG treatment on survival of RCMV-infected rats. As outlined in Table 1, DHPG therapy started 6 h p.i. and continued for 5 days with a dose interval of 12 h prevented mortality from RCMV infection in irradiated rats, but DHPG treatment for 3 days was not effective. In contrast, HPMPC administered for 3 days or as a single dose prevented death from RCMV infection.

The ED_{50}s were determined with these treatment schedules (Table 2). The ED_{50} for DHPG (twice daily for 5 days) was 20 mg/kg/day. The ED_{50} for HPMPC (administered in a single dose at 6 h p.i.) was 2 mg/kg.

In the control groups, rats which had received either TBI or RCMV only survived, as did the rats which had received CMV-negative salivary gland homogenate. Untreated irradiated RCMV-infected animals did not survive infection.

Time-response relation between HPMPC and DHPG treatment and survival. A single dose of HPMPC (20 mg/kg) was effective if given on day 0, 1, or 2 p.i. but not on day 3, 5, or 7 p.i. (Table 2). DHPG was not effective if given as a single dose (6 h p.i.). DHPG treatment was effective only if treatment was started at 6 h p.i. and doses were given twice daily for 5 days. Neither HPMPC nor DHPG was effective if therapy started at 3 days (or later) after infection.

The effect of preventive treatment with HPMPC or DHPG on survival was determined by giving a single dose (10 mg/kg) of HPMPC on day −7, −5, −3, or −1 before TBI and RCMV inoculation. All HPMPC-treated rats survived. In contrast, all rats which received one dose (100 mg/kg) of DHPG at 12 h before virus inoculation and all untreated rats

TABLE 1. Effects of different treatment schedules on survival of RCMV-infected immunocompromised rats[a]

Treatment schedule (day[s] of treatment)[b]	No. of survivors/ total no. of rats
DHPG, 20 mg/kg/day	
0–7	10/10
0–5	10/10
0–3	1/10
0[c]	0/5
HPMPC, 20 mg/kg	
0–5	5/5
0–3	10/10
0[d]	10/10

[a] All experiments were performed with groups of five rats each. Important data were run in duplicate experiments.
[b] DHPG was administered intraperitoneally twice daily, and HPMPC was administered once daily.
[c] One dose of DHPG (100 mg/kg).
[d] One dose of HPMPC (20 mg/kg) at 6 h p.i.

TABLE 2. Survival after RCMV infection[a] with different antiviral treatment schedules and dosage regimens

Antiviral agent[b]	Dosage (mg/kg/day)	Schedule (day)	No. of survivors[c]/ total no. of rats
PBS	0	0–5	0/20
DHPG	5	0–5	0/10
	10	0–5	0/10
	20	0–5	15/20[d]
	40	0–5	9/10[d]
	40	2–7	0/10
	40	5–11	0/5
	40	0	0/5
	100	1	0/5
HPMPC	0.5	0	0/10
	1	0	3/10
	2	0	10/10[d]
	5	0	10/10[d]
	10	0	10/10[d]
	10	1	10/10[d]
	10	2	8/10[d]
	10	3	0/10
	10	6	0/5
	10	−7	10/10[d]
	5	−7	6/10
	2	−7	0/10
	1	−7	0/5

[a] To establish lethal RCMV infection, rats received 5 Gy of TBI and 10^5 PFU of RCMV. Experiments were carried out with groups of five rats each, and cumulative data of separate experiments are presented.
[b] Treatment, started at day 0, was administered from 6 h p.i. PBS, phosphate-buffered saline.
[c] Measured at 21 days p.i.
[d] $P < 0.05$, as calculated with the generalized Wilcoxon test by Gehan (9).

TABLE 3. Effects of HPMPC and DHPG treatment on virus titers on organs of RCMV-infected irradiated rats[a]

Treatment	Virus titer (log_{10} PFU/g of tissue [mean ± SD]) in[b]:			
	Salivary gland	Spleen	Liver	Lungs
DHPG, 20 mg/kg[c]	1.89 ± 0.22[d]	3.99 ± 1.54[d]	2.94 ± 1.21[d]	2.87 ± 1.84[d]
HPMPC[e]				
5 mg/kg	2.27 ± 0.62[d]	3.05 ± 1.26[d]	1.79 ± 0.54[d]	2.96 ± 0.96[d]
20 mg/kg	<1.39[f]	<1.39[f]	<1.39[f]	<1.39[f]
None	3.51 ± 0.27	6.17 ± 0.52	5.49 ± 0.26	5.12 ± 0.30

[a] Groups of five rats each received 5 Gy of TBI and 10^5 PFU of RCMV.
[b] Detection limit was 1.39.
[c] Treatment was started at 6 h p.i. and continued twice daily for 5 days.
[d] $P < 0.05$.
[e] Administered as a single dose at 6 h p.i.
[f] $P < 0.01$.

died from RCMV infection ($P < 0.01$) and had high virus titers in internal organs (data not shown).

A dose-response relationship was established with RCMV-infected rats which received a single HPMPC dose (different dosages) at 7 days before virus inoculation. The ED_{50} for HPMPC under these conditions was 10 mg/kg (Table 2).

Effects on virus titers in organs. Table 3 shows the effects of these treatment regimens on the virus titers in several organs at 7 days p.i. In the DHPG (20 mg/kg/day for 5 days) treatment group, virus titers in spleen, liver, and lungs were reduced by 3 log_{10} values compared with titers in untreated controls ($P < 0.01$). However, DHPG given at 10 mg/kg/day for 5 days did not markedly reduce virus titers (data not shown). In contrast, a single dose of HPMPC at 5 mg/kg caused a significant reduction in virus titers in liver and lung compared with those in untreated controls ($P < 0.05$); furthermore, a single dose of HPMPC at 20 mg/kg reduced virus titers in all organs to below detection level ($P < 0.01$). A single dose of HPMPC at 1 mg/kg did not affect virus titers in internal organs (data not shown).

Histopathological changes in RCMV-infected rats treated with HPMPC. Histopathological abnormalities in untreated lethally RCMV-infected rats have been described previously (19). In short, massive infection of the spleen is accompanied by an almost complete disappearance of the periarteriolar lymphocyte sheaths and massive congestion with RCMV-containing macrophages. The liver parenchyma shows patchy necroses, many mononuclear cells, and numerous virus-infected hepatocytes with characteristic inclusion bodies. In the lungs, the interstitium shows congestion, with hemorrhages and infiltrating macrophages. Bone marrow smears show intensely immunoreactive mononuclear cells, whereas the kidneys show only discrete abnormalities (19).

In the livers of the DHPG-treated group (20 mg/kg/day for 5 days), parenchymal necrosis was almost absent, although mononuclear cells scattered throughout the parenchyma and relatively few RCMV-containing hepatocytes showed nuclear inclusions. In the spleen, periarteriolar lymphocyte sheaths remained at almost normal size, but congestion of sinuses with macrophages was noted. In the lungs, hemorrhages were prevented, but RCMV-containing macrophages persisted, and in bone marrow, few immunoreactive mononuclear cells persisted.

No virus-associated or drug-associated pathological changes could be detected in any of the internal organs of the RCMV-infected rats treated with HPMPC at 20 mg/kg at 6 h p.i. In hematoxylin-and-eosin-stained sections, organs showed no histopathological changes which indicated hepatotoxicity, nephrotoxicity, or bone marrow toxicity at this dosage regimen.

DISCUSSION

HPMPC is a very potent and selective inhibitor for HCMV in vitro (18). The compound inhibits the replication of HCMV in HEF at a 10-fold-lower concentration than DHPG does. Our in vitro studies confirm that HPMPC is a potent and selective inhibitor of HCMV in HEF and indicate that the drug is also effective against RCMV in REF (Fig. 1). Compared with DHPG, HPMPC is nine- and sevenfold more selective against HCMV and RCMV, respectively. Snoeck et al. (18) also reported that HPMPC added immediately p.i. for 24 h is very effective against CMV infection in vitro. We confirmed that in contrast to DHPG, HPMPC is active against HCMV following a short (24-h) incubation. In time-of-addition experiments, we found that HPMPC was effective in vitro if added early (within 24 h) after virus infection. Also, HPMPC was effective against HCMV and RCMV when cell monolayers were exposed to the drug for 24 h as early as 4 days prior to infection. Under similar conditions, DHPG was not effective against CMV infection. Thus, the in vitro data suggest that HPMPC has three advantages over DHPG: a higher selectivity index, effectiveness after a short incubation, and prolonged duration of activity after removal from cell culture medium.

HPMPC was also more potent than DHPG in the treatment of RCMV infection in vivo. Thus, the total amount of drug required to protect rats against RCMV infection was 50 times less for HPMPC than for DHPG. These studies also found evidence of a prolonged residual effect in that rats given one dose of HPMPC 7 days before RCMV infection (10 mg/kg) or at 6 h after infection (2 mg/kg) were completely protected against the lethal outcome of disease. It has been reported that HPMPC and its active metabolites have a relatively long intracellular half-life (24 to 48 h) (10). This long half-life also may occur in vivo and account for the prolonged prophylactic and therapeutic responses noted after a single dosing of the compound.

Treatment with a single dose of HPMPC at 5 mg/kg at 6 h p.i. led to a substantial reduction in virus titer comparable to the virus titer reduction achieved with DHPG at 20 mg/kg/day for 5 days. Moreover, one dose of HPMPC (20 mg/kg) at 6 h p.i. completely inhibited RCMV replication in the internal organs. These findings are in contrast to the findings of Li et al. (13), who reported that virus titers in organs of guinea pigs infected with guinea pig CMV were not influenced by HPMPC treatment at 2.5 mg/kg/day for 5 days. In

guinea pigs treated with HPMPC at 5 or 10 mg/kg/day for 5 days, Li et al. (13) observed death and histopathological changes in kidneys and bone marrow due to toxicity of the compound. However, in rats receiving HPMPC at 20 mg/kg/day for 5 days, no deaths were noted, nor were there any toxic histopathological changes. Moreover, toxicity studies in mice revealed that the toxic dose for HPMPC for mice is well above 200 mg/kg/day (1). Also, De Clercq (6) noted that even if HPMPC is administered at 400 mg/kg/day for 5 days, it is not toxic to mice but instead completely protects the mice against a lethal intracerebral herpes simplex infection.

In summary, the present study has demonstrated that HPMPC is a selective and potent inhibitor of HCMV and RCMV replication in vitro. The compound is also active against RCMV infection in immunocompromised (irradiated) rats at doses that are nontoxic to these animals. The compound effectively reduces virus titers in internal organs and prevents RCMV-induced pathological changes. Furthermore, HPMPC is active prophylactically if given as a single dose 7 days before infection. HPMPC in the treatment of CMV infection in the immunocompromised host warrants further evaluation.

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