Efficacy of (S)-1-(3-Hydroxy-2-Phosphonmethoxypropyl)Cytosine in Various Models of Herpes Simplex Virus Infection in Mice

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The phosphonomethoxyalkyl derivative (S)-1-(3-hydroxy-2-phosphonmethoxypropyl)cytosine (HPMPC) was evaluated for its in vivo efficacy in several model infections for herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) and thymidine kinase-deficient (TK-) HSV-1 in mice. In hairless mice infected intracutaneously with HSV-1 or HSV-2, HPMPC completely suppressed all manifestations of the disease (skin lesions, paralysis of the hind legs, and mortality) if it was administered topically at a concentration of as low as 0.1, 0.3, or 1%. Similarly, HPMPC completely suppressed TK- HSV-1 infection in athymic nude mice if it was administered topically at 0.1 or 0.3% or intraperitoneally at 100 or 250 mg/kg/day. HPMPC was also effective against intraperitoneal HSV infection if it was given orally at a dose of 50 mg/kg/day or higher. In mice inoculatedintracerebrally with HSV-2, intraperitoneal HPMPC treatment achieved a significant and dose-dependent protection at doses ranging from 5 to 400 mg/kg/day. The protective effect of HPMPC (at 200 mg/kg/day) was accompanied by a complete inhibition of virus multiplication in the brain. In all models of infections studied, the efficacy of HPMPC proved to be superior to that of acyclovir. The most remarkable feature of HPMPC was that a single administration of the compound, even as late as 4 days after infection, conferred significant protection against HSV-1 or HSV-2 infection. Topical or systemic HPMPC treatment is efficacious in murine models of HSV-1, HSV-2, and TK- HSV infections.

The phosphonomethoxyalkylpurines and -pyrimidines have been identified as new classes of antiviral agents with broad-spectrum activities against a wide variety of DNA viruses (13, 14). (S)-9-(3-Hydroxy-2-phosphonomethoxypropyl)adenine (HPMPA), the prototype of this class of compounds, has proved to be active against adenoviruses (2), herpesviruses (herpes simplex virus type 1 [HSV-1] and type 2 [HSV-2] [13, 14, 31], varicella-zoster virus [1], cytomegalovirus [29], and Epstein-Barr virus [22]), hepadnaviruses (duck hepatitis B virus [32]), and iridoviruses (African swine fever virus [19]), and poxviruses (vaccinia virus) (13, 14).

(9-(2-Phosphonomethoxethyl)adenine (PMEA), which can be considered as the truncated form of HPMPA in which the hydroxymethyl group has been deleted, is active against herpes-, hepada-, and iridoviruses (13, 14, 19, 33) and is also active against retroviruses (human immunodeficiency virus type 1 [27] and type 2 [5], simian immunodeficiency virus [5], feline immunodeficiency virus [15], Moloney murine sarcoma virus [4], Rauscher murine leukemia virus [8], and murine AIDS [LP-BM5] virus [18]). 9-(2-Phosphonomethoxethyl)-2,6-diaminopurine (PMEDAP) is even more potent as an antiretrovirus agent than PMEA is (25).

HPMPC, PMEA, and PMEDAP have been the subject of a previous study in which the efficacies of these phosphonomethoxyalkyl derivatives were demonstrated in a variety of experimental HSV-1 and HSV-2 infections, including intracutaneous HSV-1 and HSV-2 infections in hairless mice; intracutaneous thymidine kinase-deficient (TK-) HSV-1 infections in athymic nude mice; systemic (intraperitoneal [i.p.]) HSV-1 infections in mice; and intracerebral HSV-1, HSV-2, or TK- HSV-1 infections in mice (12). These animal models for HSV infections have been used to assess the in vivo efficacy of (S)-1-(3-hydroxy-2-phosphonomethoxypropyl)cytosine (HPMPC), another member of the class of phosphonomethoxyalkyl derivatives (14), which, according to previous investigations (7), would be more efficacious than acyclovir [ACV; 9-(2-hydroxyethoxymethyl)guanine] in both the systemic and topical treatment of HSV infections in mice and guinea pigs.

MATERIALS AND METHODS

Compounds. HPMPC was prepared as described previously (21). ACV was obtained from the Wellcome Research Laboratories, Research Triangle Park, N.C. Stock solutions of the compounds were prepared in phosphate-buffered saline, if the compounds were administered i.p. or subcutaneously (s.c.), or in drinking water, if the compounds were given perorally (p.o.), whereupon the compounds were administered twice daily in 0.2-ml volumes. If intended for topical use, the compounds were dissolved in dimethyl sulfoxide (DMSO) at the indicated concentrations and were applied topically four times a day.

Mice. The animals used throughout the experiments were (i) 25-day-old NMRI (Naval Medical Research Institute) mice (weight, 11 to 13 g), (ii) 25- to 30-day-old hairless (hrl/hr) mice (weight, 15 to 20 g), and (iii) 25- to 30-day-old athymic nude (nu/nu) mice (weight, 15 to 20 g). The NMRI mice were randomly bred. The hrl/hr mice were bred by backcrossing and intercrossing of the homozygous parents. The nu/nu mice were bred by breeding scheme IV of Giovanella and Stehlin (20). All the mice were obtained from the Animal Production Center (Proefdierencentrum) of the Katholieke Universiteit Leuven. The mice were housed under conventional conditions in groups of 5 (hrl/hr and nu/nu mice) or 10 (NMRI mice) and were given food and drinking water ad libitum. Throughout all experiments, male and female mice were used at random.

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**Viruses.** The origins of the virus strains have been described previously: for HSV-1 (strain KOS) and HSV-2 (strain 196), see reference 11; for TK- HSV-1 (VMW-1837), see references 10, 13, and 29. The latter variant was isolated from an immunosuppressed patient with a chronic HSV-1 infection that had become resistant to ACV treatment (29); this HSV-1 variant originally consisted of 92% TK- and TK+, as demonstrated by plaque autoradiography. It was plaque purified. All HSV stocks were prepared in primary rabbit kidney cells. Titers of the virus stocks were as follows: HSV-1 (KOS), 10^6.7 PFU/ml; HSV-2 (196), 10^6.0 PFU/ml or 10^5.5 PFU/ml or 10^5.0 PFU/ml or 10^5.0 PFU/ml or 50% cell culture infective doses (CCID50s/ml) per ml; and TK- HSV-1 (VMW-1837), 10^6.3 CCID50s/ml. PFU were determined in Vero cell cultures; titers based on the CCID50 were determined in primary rabbit kidney cells.

**Intracutaneous HSV-1 and HSV-2 infections in hairless mice.** Hairless mice were inoculated intracutaneously (at the lumbosacral area by scratching the skin with a scarificator) with either HSV-1 (KOS) at 10^5.7 PFU/0.05 ml per mouse or HSV-2 (196) at 10^5.7 PFU/0.05 ml per mouse. As a rule, the mice were treated for 5 days, starting 1 h after virus infection, with the test compounds, which were applied topically at the indicated concentrations in DMSO (four times daily) in a volume of 0.05 ml over an area of 1.5 cm^2. In some experiments, the test compounds were applied only once. The mice were monitored daily for the development of herpetic skin lesions, paralysis of the hind legs, and cumulative mortality up to day 20 postinfection.

**Intracutaneous TK- HSV-1 infection in athymic nude mice.** Athymic nude mice were inoculated intracutaneously in the lumbosacral area with TK- HSV-1 (VMW-1837) at 10^5 CCID50s/0.05 ml per mouse. The mice were treated for 5 days, starting 1 h after virus infection, with the test compounds, which were either applied topically at the indicated concentrations in DMSO (four times daily) or administered i.p. (twice daily). The mice were monitored daily for the development of herpetic skin lesions, paralysis of the hind legs, and mortality through 60 days.

**i.p. HSV-1 infection of NMRI mice.** NMRI mice were inoculated i.p. with HSV-1 (KOS) at 10^3 PFU/0.2 ml per mouse. The mice were treated for 5 days, starting 1 h after virus infection, with the test compounds, which were administered p.o. (twice daily) by gavage at the indicated doses. In some experiments, the test compounds were administered s.c. at the indicated times and doses. Mortality was recorded daily through day 20 postinfection.

**Intracerebral HSV-2 infection of NMRI mice.** NMRI mice were inoculated intracerebrally with HSV-2 (196) at 0.2 CCID50s/0.02 ml per mouse. As a rule, the mice were treated for 5 days, starting 1 h after virus infection, with the test compounds, which were administered i.p. (twice daily) at the indicated doses. In some experiments, the test compounds were administered s.c. at the indicated times and doses. Mortality was recorded daily through day 20 postinfection.

The brains of NMRI mice that were inoculated intracerebrally with HSV-2 (196) and treated i.p. twice daily for 5 days, starting 1 h after virus infection, with the test compounds (phosphate-buffered saline, HPMPC at 200 mg/kg of body weight per day, or ACV at 200 mg/kg/day) and were examined for virus content at 3, 5, 7, and 9 days postinfection. The mice were sacrificed, the brains were harvested and homogenized, and the brain homogenates of three mice per group were pooled and assayed for virus plaque forma-

**RESULTS**

**Intracutaneous infection.** In hairless mice inoculated intracutaneously with HSV-1, HPMPC brought about a reduction in the mortality rate from 100 to 0% if it was applied topically at a concentration of as low as 0.1% (Table 1). At concentrations of 0.3 and 1%, HPMPC completely suppressed all manifestations of the disease (herpetic skin lesions, paralysis of the hind legs, and mortality). Similarly, in hairless mice inoculated intracutaneously with HSV-2, HPMPC achieved a significant reduction in mortality (at 0.03% [P < 0.02]) if it was applied topically at a concentration of 0.1% or higher. ACV did not offer significant protection against intracutaneous HSV-2 infection at a concentration of 1% (Table 1). One daily application for 5 days, or even a single application (at day 0), protected the mice against all manifestations of the disease, including mortality. The complete protection achieved by a single topical application of HPMPC at 5% (1 h postinfection) contrasted with the lack of efficacy shown by ACV under the same experimental conditions (data not shown).

Topical treatment with HPMPC at 0.3% completely suppressed the development of herpetic skin lesions and paralysis of the hind legs in athymic nude mice inoculated intracutaneously with TK- HSV-1; also, the mortality rate of these mice (at 60 days postinfection) was reduced from 100 to 0% (Fig. 1). Even at a concentration of 0.1%, HPMPC suppressed the development of lesions, and the resulting mortality, in all but one mouse. When evaluated under the same conditions, topical ACV, even at a concentration of 3%, did not prevent the development of lesions or alter mortality (Fig. 1).

When HPMPC was given systemically (i.p.) to athymic nude mice that were inoculated intracutaneously with TK- HSV-1, it completely suppressed all manifestations of the disease, including skin lesion development, paralysis of the

<table>
<thead>
<tr>
<th>Compound concn (% [w/vol])</th>
<th>HSV-1 (KOS)</th>
<th>HSV-2 (196)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPMPC</td>
<td>ACV</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.01</td>
<td>80</td>
<td>ND</td>
</tr>
<tr>
<td>0.03</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a The compounds were applied topically four times a day for 5 days, starting 1 h after virus infection.

b With DMSO as the vehicle.

c At day 20 postinfection. There were 10 mice per group (20 mice for the control group). Reduction in the mortality rate by 40% was significant at P < 0.02; reductions in the mortality rate by ≥60% were significant at P < 0.001.

d ND, Not determined.

Statistical analysis. Statistical significance of the differences in the mortality rate by day 20 postinfection between the treatment groups and the control group was assessed by the χ² test (with the Yates correction).
Inhibitory effects of topical HPMPC or ACV administration on the development of lesions (skin lesions and/or paralysis of the hind legs) and mortality of athymic nude mice inoculated intracutaneously with TK-HSV-1 (VMW-1837). The compounds were applied topically four times a day for 5 days, starting 1 h after virus infection. Compound concentrations (weight/volume) were as follows: 0% (control) ( ), 0.03% HPMPC ( ), 0.1% HPMPC ( ), 0.3% HPMPC ( ), 3% ACV ( ). DMSO served as the vehicle. There were 10 mice per group. The reductions in the number of mice that developed lesions (A) or succumbed to the infection (by day 60 postinfection) (B) following treatment with 0.1 or 0.3% HPMPC were highly significant (P < 0.001).

FIG. 1. Inhibitory effects of topical HPMPC or ACV administration on the development of lesions (skin lesions and/or paralysis of the hind legs) and mortality of athymic nude mice inoculated intracutaneously with TK- HSV-1 (VMW-1837). The compounds were applied topically four times a day for 5 days, starting 1 h after virus infection. Compound concentrations (weight/volume) were as follows: 0% (control) ( ), 0.03% HPMPC ( ), 0.1% HPMPC ( ), 0.3% HPMPC ( ), 3% ACV ( ). DMSO served as the vehicle. There were 10 mice per group. The reductions in the number of mice that developed lesions (A) or succumbed to the infection (by day 60 postinfection) (B) following treatment with 0.1 or 0.3% HPMPC were highly significant (P < 0.001).

Inhibitory effects of topical HPMPC or ACV administration on the development of lesions (skin lesions and/or paralysis of the hind legs) and mortality of athymic nude mice inoculated intracutaneously with TK- HSV-1 (VMW-1837). The compounds were applied topically four times a day for 5 days, starting 1 h after virus infection. Compound concentrations (weight/volume) were as follows: 0% (control) ( ), 0.03% HPMPC ( ), 0.1% HPMPC ( ), 0.3% HPMPC ( ), 3% ACV ( ). DMSO served as the vehicle. There were 10 mice per group. The reductions in the number of mice that developed lesions (A) or succumbed to the infection (by day 60 postinfection) (B) following treatment with 0.1 or 0.3% HPMPC were highly significant (P < 0.001).
TABLE 2. Inhibitory effects of infrequent and delayed subcutaneous administration of HPMPC or ACV on the mortality rate of NMRI mice inoculated i.p. with HSV-1 (KOS) or intracerebrally with HSV-2 (196)

<table>
<thead>
<tr>
<th>Compound dose (mg/kg/day)</th>
<th>Time and frequency of administration</th>
<th>HPMPC</th>
<th>ACV</th>
<th>HPMPC</th>
<th>ACV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td></td>
<td>100</td>
<td>90</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td>20</td>
<td>Days 0, 1, 2, 3, 4 (once a day)</td>
<td>50</td>
<td>50</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>Day 0 (once)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>Day 0 (once)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>Day 0 (once)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>Day 2 (once)</td>
<td>30</td>
<td>100</td>
<td>60</td>
<td>100</td>
</tr>
</tbody>
</table>

* At day 20 postinfection. There were 10 mice per group (20 mice for the HSV-1 [KOS] control group and 30 mice for the HSV-2 [196] control group). Reductions in mortality by 40 and 50% were significant at P < 0.02 and P < 0.005, respectively; reductions in the mortality rate by ≥60% were significant at P < 0.001.

clear time-response effect, with maximal protection (100% reduction in mortality) occurring if treatment was started at day 0 (1 h postinfection) and no protection whatsoever if treatment was initiated at day 5 postinfection (data not shown). The last day of starting treatment which remained effective was 3 days postinfection, for which the mortality rate was 50% (P < 0.05).

Virus titers in the brains of control mice (infected intracerebrally with HSV-2) rose to 10^6 PFU/g at 3 days postinfection and to ≥10^7 PFU/g at 5, 7, and 9 days postinfection (Fig. 3). A reduction in virus titer was noted following ACV treatment, although ACV treatment did not prevent virus titers to exceed the 10^6 PFU/g threshold at 7 and 9 days postinfection. Following HPMPC treatment, virus remained undetectable in the brains during the whole observation period (3 to 9 days postinfection) (Fig. 3).

**DISCUSSION**

The results of our experiments indicate that HPMPC is highly effective in the treatment of various experimental HSV model infections, including encephalitis, and cutaneous and generalized HSV infections. In particular, HPMPC proved effective in the treatment of TK− HSV infection in athymic nude mice, a model that could be considered representative of TK− HSV infections in immunocompromised patients (9, 16, 17, 28, 30, 32). TK− HSV infection in nude mice is refractory to ACV treatment (Fig. 1 and 2) but is fully responsive to topical or parenteral HPMPC treatment.

HPMPC proved to be more efficacious than ACV in both the topical and systemic treatment of HSV-1 and HSV-2 infections in mice and guinea pigs (7, 23), and our present findings indicate that HPMPC is effective against HSV encephalitis under conditions in which ACV is totally ineffective (Table 2). When compared at the same dose schedules, HPMPC was found to arrest virus replication in the brain, whereas ACV did not achieve more than a modest reduction in virus titers (Fig. 3).

HPMPC also offers potential for topical treatment of HSV infections, since a concentration of 0.1% would suffice to completely block the infection and the symptoms associated with it (Table 1 and Fig. 1). No compounds other than the phosphonylmethoxyalkyl derivatives (HPMPA, PMEA, PMEDAP [13], and HPMPC [this report]) have ever been reported to suppress cutaneous HSV infection, whether it is caused by HSV-1, HSV-2, or TK− HSV strains, at such a low concentration.

HPMPA is approximately 20 times more active (against vaccinia virus infection) when administered by the parenteral (i.p. or s.c.) route than it is when administered by the p.o. route (12). Also, HPMPC proved to be effective against HSV infection at a dose of 5 mg/kg/day given i.p., whereas following p.o. administration, the dosage had to be increased to 50 mg/kg/day to significantly reduce the mortality rate (data not shown). These findings point to the limited bioavailability of the phosphonylmethoxyalkyl derivatives following oral administration.

No acute toxicity (as based on lethality) was observed with HPMPC in mice, if given i.p. or p.o. at doses of up to 200 mg/kg/day for 5 days (6). In the present study, HPMPC was given i.p. at doses of up to 400 mg/kg/day, for 5 days, without causing lethality. In fact, this dosage regimen appeared to be completely protective against a lethal intracerebral HSV-2 infection. On the other hand, significant protection has been obtained with HPMPC against systemic HSV-1 or HSV-2 infection if given i.p. at a dose of as low as 0.1 mg/kg/day (7, 23). This means that the in vivo selectivity index (or therapeutic ratio) of HPMPC is 4,000.

A remarkable feature is that HPMPC, administered as a single dose up to day 4 after infection, causes significant reductions in mortality (Table 2). The marked antiviral activity exhibited following single or infrequent dosing appears to be a unique feature of the phosphonylmethoxyalkyl derivatives. In another study, HPMPC was found to be equally effective in the treatment of HSV-1 keratitis if instilled (as eyedrops) nine times daily, three times daily, or only once daily (24). Also, HPMPC has been found to be found effective against murine cytomegalovirus and simian vari
cella-zoster virus when administered systemically at infrequent doses to mice and monkeys, respectively (21a, 29a). Furthermore, PMEA has proved to be more efficient against Moloney murine sarcoma virus-induced tumor formation in newborn mice when administered as a single dose (on day 0) than when this dose was spread over 2, 4, or 7 administrations (on days 0 and 3; days 0, 2, 4, and 6; or days 0, 1, 2, 3, 4, 5, and 6 postinfection, respectively) (3).

The marked activity shown by HPNPC following single or infrequent dosing points to the long-lasting nature of its antiviral effect. In fact, a short-pulse treatment with HPNPC (for only 6 h postinfection) suffices to suppress virus (i.e., cytomegalovirus) replication for several days in cell culture (26). This prolonged antiviral effect may be related to the fact that the metabolites of HPNPC, i.e., the diprophosphate and choline derivatives, persist for a long time within the cells that have been exposed to HPNPC (7a, 20a).

In conclusion, HPNPC is a potent and selective broad-spectrum anti-herpesvirus agent which holds promise for the therapy of herpesvirus infections. The findings of this study document the efficacy of HPNPC in the topical and systemic treatment of HSV-1, HSV-2, and TK- HSV infections in murine models.

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