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PRETREATMENT OF THE HOST CELL WITH 1-(S)-(3-HYDROXY-2-PHOSPHONYLMETHOXYPROPYL)CYTOSINE (HPMPC) IS SUFFICIENT FOR ITS ANTIVIRAL EFFECT

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Summary. – 1-(S)-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) inhibits efficiently human cytomegalovirus (HCMV) replication. The drug exhibited marked antiviral effect *in vitro* not only at single application after virus adsorption, but also if given 48 hr post-infection. The inhibition of HCMV replication by HPMPC occured after mere pretreatment of the host cells with the drug followed by its removal from the medium before virus infection. No free or host cell bound infectious virus was detected in the drug pretreated (1.5 μg/ml) virus infected cells. This effect was drug concentration dependent.

Key words: 1-(S)-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC); antiviral effect; human cytomegalovirus (HCMV)

Introduction

In the past few years it was convincingly demonstrated that 9-(S)-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) acts very efficiently against a broad spectrum of DNA viruses, in particular herpes simplex viruses of the type 1 and 2, cytomegaloviruses, adenoviruses, and vaccinia virus, etc. (DeClercq *et al.*, 1986). Similar effects have been established also for its cytosine congener, 1-(S)-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) (DeClercq *et al.*, 1987). As these acyclic analogues of 5'-mononucleotides already bear the (modified) phosphoric acid functionality, their action is not dependent on the activation by viral thymidine kinase or cellular nucleoside kinases (DeClercq, 1984). Therefore, the drugs are active also against thymidine kinase deficient mutants of HSV-1, HSV-2 or VZV (DeClercq *et al.*, 1987).

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An increased attention is recently being devoted to the therapy of HCMV infections causing serious problems in immunocompromized patients or AIDS patients (Macher *et al.*, 1983). Human cytomegaloviruses do not encode virus specific thymidine kinase; therefore, they are not sensitive to acyclovir (ACV) – a drug which is extremely active against both types of HSV and whose action depends on the phosphorylation by the viral enzyme (DeClercq, 1987). The only drug approved so far for the treatment of CMV infections is an acyclovir analogue Ganciclovir (DHPG, Cytovene) (Laskin *et al.*, 1987). In contrast to ACV, DHPG is phosphorylated by the cellular nucleoside kinases and finally interferes with *de novo* CMV-DNA synthesis (Smee *et al.*, 1985; Mathews and Boehme, 1988). HPMPC has been shown to inhibit efficiently CMV replication (Snoeck *et al.*, 1988; Kern and Vogt, 1989). However, the detailed mechanism of action of this drug is still unknown. Here, we compare the antiviral effect of HPMPC applied on the host cells before and after virus adsorption.

Materials and Methods

Virus and cells. AD 169 strain (Central Institute for Hygiene, Berlin) of human cytomegalovirus (HCMV) was used troughout the whole study. Human embryonic lung cells (HEL cells) were grown in EPL medium (Institute of Sera and Vaccines, Prague). When confluent HEL cells were infected with 3log$TCID_{50}$ infective doses, the adsorption of the virus was achieved after 2 hr at 25 °C. The $TCID_{50}$ values were expressed according to Reed and Munch (1938). The virus suspension was prepared by repeated freezing and thawing of the virus infected cells.

Drugs. HPMPC was synthesized according to the described procedure (Holý *et al.*, 1989) and used as a sodium salt. Ganciclovir was kindly donated by Dr. J. C. Martin, Bristol-Myers Squibb Co. (U.S.A.) and Acyclovir by Prof. E. DeClercq, Rega Institute, Leuven (Belgium).

Antiviral activity assay. The minimum antiviral inhibitory dose was expressed as the concentration required to inhibit completely (MIC_{100}) virus-induced cytopathogenicity. The inhibitors were applied to the culture medium after the virus adsorption period and the cytopathogenicity was evaluated in per cent of destructed cells.

Immunofluorescent staining. Both infected and mock infected cells were examined for the presence of the early and late viral antigens by indirect specific immunofluorescence staining: the early antigen with the aid of chromatographically purified mouse monoclonal antibody (Mo; monoclonal antibody product No. 9220, IgG3, Biotech Research Labs., Du Pont Co., U.S.A.), and/or the late antigen (i.e. on 5th and 6th day p.i.) with human serum – polyclonal antibody (Po; Institute for Hygiene and Epidemiology, Prague) and the corresponding fluorescein isothiocyanate labelled globulin (Institute of Sera and Vaccines, Prague). In both cases the numbers of fluorescein positive cells were counted (in the randomly chosen fields 300 cells were counted), and the results expressed as percentage of the total cells (Adamcová, 1970).

Results

In order to compare the antiviral effects of HPMPC with that of ACV and DHPG, the drugs (0.1 $\mu g \cdot ml^{-1}$ – 200 $\mu g \cdot ml^{-1}$) were added to the cultivation medium immediately after virus adsorption and the cytopathic effect of CMV on host HEL (human embryonic lung) cells was evaluated after 11 days of incu-

Table 1. Activity of HPMPC and of the reference compounds against CMV in HEL cells

Compound	[a]MIC_{100} ($\mu g.ml^{-1}$)
HPMPC	0.4
DHPG	3.0
ACV	>200

[a]Minimal concentration required for complete inibition of virus-induced cytopathogenicity.

Table 2. Inhibitory activity of HPMPC and DHPG applied at various times after infection against CMV in HEL cells

[a]Time (hr)	[b]MIC_{100} ($\mu g.ml^{-1}$)	
	HPMPC	DHPG
2	0.4	6
24	0.4	3
48	0.8	15
72	c	c

[a]At indicated intervals after infection the inhibitors were added to the medium at various concentrations and than MIC_{100} was determined.
[b]Minimal concentration required to inhibit completely virus-induced cytopathogenicity.
[c]At 12.5 $\mu g.ml^{-1}$ the inhibition of virus-induced cytopathogenicity was not complete.

bation. The results presented in Table 1 demonstrate markedly higher activity of HPMPC ($MIC_{100} = 0.4\ \mu g \cdot ml^{-1}$) compared to DHPG ($MIC_{100} = 3.0\ \mu g \cdot ml^{-1}$); as expected, ACV was quite inactive under these conditions. In another experiment, the efficiency of HPMPC and DHPG was compared to determine the phase of CMV replication during which the addition of drug still causes significant antiviral effect. The data of Table 2 unequivocally witness that the both compounds cause total suppression of the CMV cytopathic effect in HEL cells when administered 48 hr p.i. at the latest. The addition of HPMPC to the cultivation media 72 hr p.i. causes a partial inhibition of the virus cytopathogenicity at the non-cytotoxic concentrations only. The MIC_{100} value for HPMPC added 48 hr p.i. ($0.8\ \mu g \cdot ml^{-1}$) is 2x higher than that for compound added 24 hr p.i. A similar effect occurs with DHPG (Table 2).

HCMV replicating in the presence of HPMPC at the concentration which merely causes a partial suppression of the virus cytopathogenicity (e.g., 0.15 $\mu g \cdot ml^{-1}$) loses completely its infectivity (Table 3). ACV which was used for comparison (as the negative control) in this experiment is quite inactive in this

respect. To verify the loss of CMV infectivity to the intact HEL cells caused by HPMPC, the CMV infected HEL cells treated after virus adsorption with HPMPC (for conditions, see Table 3) were co-cultivated with intact HEL cells. Although the presence of CMV early antigen in the HPMPC treated infected cells was proven by the fluorescence method, it was found that the CMV in the HPMPC treated cells is unable to infect additional (uninfected) cells. In the control experiment performed under the indentical conditions without HPMPC treatment or, with ACV replacing HPMPC, the virus cytopathic effect reached completion.

The presence of CMV early and late antigens in the cells treated with HPMPC or DHPG was studied separately; it was found that in the both cases

Table 3. Infectivity of CMV in HEL cells after HPMPC treatment[a]

Compound	($\mu g.ml^{-1}$)	Virus yield			
		1. passage		2. passage	
		CP[b]	$log_{10}TCID_{50}$	CP[b]	$log_{10}TCID_{50}$
HPMPC	0.15	5	n	0	n
ACV	0.15	100	3.5	100	3.5
untreated cells		100	3.5	100	3.5

[a]Compounds were in both passages added immediately after infection at the concentration which cause only a partial inhibition of virus-induced cytopathogenicity.
[b]The cytopathogenicity (CP) was evaluated in per cent of destructed cells.
[n]No cytopathogenicity.

Table 4. Effect of HPMPC and DHPG on the presence of early and late antigens in CMV infected HEL cells

[a]Time (hr)	[b]Antibody	Per cent of positive cells		
		Control	HPMPC	DHPG
24	Mo	81.7	29.5	39.8
48	Mo	79.6	27.3	38.7
120	P	100	44.7	73.8

[a] Both compounds were added to the medium immediately after infection and at indicated times cells were fixed for fluorescent staining.
[b] Mo .. monoclonal antibody, P .. polyclonal antibody.

Table 5. Cytopathogenicity of CMV after pretreatment of HEL cells with HPMPC

HPMPC ($\mu g \cdot ml^{-1}$)	[a]Intervals of pretreatment (hr)					
	0–24	12–18	12–36	24–30	24–48	24–168
	[b]Cytopathogenicity					
No addition	100	60	50	50	50	50
0.15	15	5	5	3	0	0
1.50	0	0	0	0	0	0

[a] HEL cells were treated by (S)-HPMPC during the indicated time intervals after seeding. The drug was then removed by successive washing with fresh medium and cells were infected by CMV at $50 \log_{10} TCID_{50}$.
[b] The cytopathogenicity was evaluated in per cent of destructed cells (after the lethal-vital staining the cells were counted).

the relative amount of the positive (infected) HEL cells is significantly lessened by the drug treatment (Table 4). This experiment demonstrates an evident interference of HPMPC with an early stage of the virus replication which, however, persists to the late stages of CMV development (Table 4).

Finally, we have investigated whether cellular metabolic pathways of the host cells are directly involved in the antiviral effect of HPMPC. The drug was added to the cultivation media at diverse stages of HEL cells proliferation (12 or 24 hr after the seeding, respectively) and the cells were cultivated for 6 or 24 hr, respectively. Afterwards, the cell culture was extensively washed with phosphate buffered saline (PBS) followed with the fresh medium, and cultivated for 3 hr in the fresh medium before CMV infection. After virus adsorption, the cells were incubated and the cytopathic effect evaluated after 11 days of incubation. The data summarized in Table 5 show that virus cytopathogenicity was completely or extensively suppressed by pretreatment of the host cells with HPMPC. The effect depends on the drug concentration, duration of the treatment and the stage of cell proliferation. The infectivity of virus isolates obtained from thus pretreated cells was studied separately with the use of intact HEL cells. On day 12 after infection of pretreated cells (which did not show any cytopathic effect) they were tested on the presence of the persisting intracellular virus. One portion of the experimental material was used for co-cultivation with the fresh confluent HEL cells, the other one was collected and desintegrated by repeated freezing-thawing and finally centrifuged; additional portions of the fresh confluent HEL cells were infected by the supernatant and sediment, respectively. In all three cases the cytopathic effect was evaluated after eleven days of incubation (data not shown). The results of this experiment confirmed the non-infectivity of the free as well as of the cell-bound virus particles (data not shown).

Discussion

Our data unequivocally demonstrate that HPMPC inhibits replication of HCMV at lower concentrations than DHPG; it is considerably efficient even when applied 48 hr after the infection of the host cells. At concentrations which do not cause complete inhibition of virus-induced cytopathogenicity, HPMPC results in the formation of virus particles with reduced infectivity (Table 3); these particles preserve a positive though diminished level of the "early" and "late" antigens (Table 4). The suppression of CMV multiplication can also be achieved by a single pretreatment of the host cells with HPMPC *prior* to infection (Table 5). Most probably, the drug does not only affect the virus-encoded enzymes, but also a part of cellular metabolic pathways which participate in virus replication. Recently, Bronson *et al.* (1990) have observed that in addition to the phosphorylation leading to the mono- and diphosphate (analogues of nucleoside 5'-di- and triphosphate) which proceeds under the participation of cellular enzymes (Bronson *et al.*, 1990; Merta *et al.*, 1990), HPMPC is *in vivo* transformed to an additional metabolite which was tentatively assigned the structure of CDP-choline analogue (HPMPC-choline). We presume that the antiviral effect of HPMPC might be based on this metabolite which would most probably interfere with the *de novo* synthesis of the lipoid component of the virion envelope (Ben-Porat and Kaplan, 1971). This aspect is a subject of our present investigation.

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