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Particular characteristics of the anti-human cytomegalovirus activity of (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) in vitro

Johan Neyts, Robert Snoeck, Jan Balzarini and Erik De Clercq

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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Summary

The acyclic nucleoside phosphonate analogue (*S*)-1-[3-hydroxy-2-phosphonylmethoxypropyl]cytosine (HPMPC) is a potent and selective inhibitor of human cytomegalovirus (HCMV) replication and DNA synthesis. Unlike 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), HPMPC inhibits HCMV replication in cell cultures which have been treated with the compound before infection. Upon short-pulse treatment of HCMV-infected cells with HPMPC, a long-lasting antiviral response is obtained. The antiviral activity of HPMPC, unlike the antiviral activity of other cytosine derivatives (i.e. Ara-C, FIAC), is not readily reversed by 2'-deoxycytidine or cytidine. Neutral and alkaline sucrose gradient analysis of HCMV DNA isolated from HPMPC-treated HCMV-infected cell cultures revealed that HPMPC does not cause (detectable) single- or double-strand breakage of HCMV DNA.

Cytomegalovirus; HPMPC; DNA integrity

Introduction

Human cytomegalovirus (HCMV) infection can lead to serious, debilitating and often life-threatening diseases in immunocompromised individuals, and

Correspondence to: Erik De Clercq, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium.

severe clinical manifestations in the fetus or neonates (Stagno et al., 1982; Verheyden, 1988; Tyms et al., 1989).

The only antiviral drugs that have been reported to be efficacious in the treatment of HCMV infections are ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG)] and foscarnet (phosphonoformic acid, PFA). However, both ganciclovir and foscarnet therapy may give serious side effects (i.e. neutropenia and kidney failure, respectively), and ganciclovir-resistant HCMV strains may emerge in some individuals (Erice et al., 1988). Recurrence of the disease is often observed after treatment is stopped (Collaborative DHPG Treatment Study Group, 1986).

In 1986 we reported on a novel class of acyclic nucleotide phosphonate analogues with broad-spectrum anti-DNA virus [adenovirus, herpes simplex virus (HSV) type 1 and type 2, thymidine-kinase-deficient (TK^-) HSV-1, varicella-zoster virus (VZV), HCMV, Epstein-Barr virus (EBV), vaccinia virus (VV), African swine fever virus] activity (De Clercq et al., 1986, 1987). This class of compounds is characterized by the presence of a stable phosphonylalkyl ether group, and the absence of the usual glycosidic bond. From this class of phosphonylmethoxypropyl derivatives HPMPA [(*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine] and HPMPC [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine] have emerged as highly potent and selective inhibitors of HCMV replication in vitro (Snoeck et al., 1988; Neyts et al., 1990). Moreover, HPMPC has proved to afford a long-lasting inhibition of HCMV replication in vitro (Neyts et al., 1990) and prolonged protection against murine cytomegalovirus (MCMV) infection in vivo (Kern et al., 1989).

In the present report, we have addressed different features of the inhibitory effect of HPMPC on HCMV replication. We found that HPMPC is endowed with a prophylactic and long-lasting antiviral effect, which is not reversed by cytidine or 2′-deoxycytidine. The compound does not cause detectable single- or double-strand breakage of HCMV DNA.

Materials and Methods

Compounds

DHPG (ganciclovir) was kindly provided by Dr. Julian Verheyden (Syntex Inc., Palo Alto, CA, U.S.A.). HPMPC was synthesized by Dr. A. Holý (Czechoslovak Academy of Sciences, Prague, Czechoslovakia). FIAC [1-β-D-(2-fluoro-2-arabinofuranosyl)-5-iodocytosine] was obtained from Dr. J.C. Martin (Bristol Myers, Wallingford, CT, U.S.A.) and Ara-C (Cytarabine) from the Upjohn Company (Puurs, Belgium). The natural nucleosides 2′-deoxycytidine, cytidine and 2′-deoxythymidine were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

[*Methyl*-^{3}H]-2′-deoxythymidine ([*methyl*-^{3}H]dThd; specific radioactivity: 45 Ci/mmol) was purchased from Amersham International Ltd. (U.K.).

Viruses

The human cytomegalovirus (HCMV) strains AD-169 (ATCC VR-538) and Davis (ATCC VR-807) were kindly provided by Dr. S. Michelson (Institut Pasteur, Paris, France).

Cells

Human embryonic lung (HEL) fibroblast (ATCC CCL 137) cells (at low passage) were grown in minimum essential medium (MEM; Gibco, Paisley, U.K.), containing 10% inactivated fetal calf serum (FCS; PEAK, Zaandam, The Netherlands), 1% L-glutamine (Gibco) and 0.02% sodium bicarbonate (Gibco). Murine leukemia L1210 cells were grown in RPMI medium (Gibco) containing 10% FCS, 1% L-glutamine and 0.02% sodium bicarbonate.

Inhibition of the cytopathic effect of HCMV by HPMPC and DHPG

Confluent HEL cells in 96-well microtiter plates (Falcon, Becton Dickinson, NJ, U.S.A.) were infected with 100 plaque-forming units (PFU) per well. Virus was diluted in MEM supplemented with 2% FCS, 1% L-glutamine, and 0.02% sodium bicarbonate. After a 2-h adsorption period, unadsorbed virus was removed and the cells were incubated for seven days in the presence of varying concentrations of the test compounds. In some experiments, the compounds were added to the cells for short time-periods before or after virus infection. After the limited exposure times, the compounds were removed by aspirating the supernatants, and the cells were washed twice with MEM and further incubated in compound-free medium. After seven days, cell cultures were fixed, stained and prepared for visual inspection as described before (Snoeck et al., 1988). The antiviral effective concentration is expressed as EC_{50}, or concentration of compound required to inhibit virus-induced cytopathogenicity by 50%.

Inhibitory effect of the test compounds on HEL and L1210 cell proliferation

The inhibitory effects of the test compounds on the growth of murine leukemia L1210 and human embryonic lung fibroblast cells was determined essentially as described by Balzarini and De Clercq (1983) and Snoeck et al. (1988), respectively.

Inhibitory effect of HPMPC on HCMV DNA synthesis

HEL cells were seeded in 60-mm petri dishes (Falcon, Becton Dickinson, NJ, U.S.A.) at 8×10^5 cells per petri dish containing 4 ml MEM supplemented with 10% FCS. Confluent HEL cells were infected with HCMV (strain Davis)

at a multiplicity of infection (MOI) of approximately 1. Unadsorbed virus was removed after 2 h, and the virus-infected cell monolayers were incubated in the presence of HPMPC at 0.1 or 0.3 μg/ml in MEM containing 0.5% FCS, 1% L-glutamine and 0.02% sodium bicarbonate. After six days incubation, 17 μCi of [*methyl*-^{3}H]dThd were added, and the cells were harvested 24 h later. The cell monolayers were washed twice with phosphate-buffered saline (PBS) and the cells were lysed with 200 μl of a solution containing 0.2% sodium dodecyl sulfate (SDS), 0.5% N-laurylsarcosylate, 1 mM sodium EDTA, 100 mM NaCl and 10 mM Tris HCl, pH 7.4. Samples were frozen and stored at −70°C until use. Each sample (200 μl) was layered on top of 8 ml of a CsCl solution (density: 1.7067 g/ml) and centrifuged to equilibrium at 30 000 rpm in a Beckman L7-55 ultracentrifuge for 65 h, at 20°C, using a TFT 65-13 rotor (Kontron, Beun de Ronde, Amsterdam, The Netherlands). Seven-drop fractions were collected from the bottom of the tubes, and the refractive index of every fifth fraction was determined. The acid-insoluble material of the fractions was precipitated on Whatman GF/C filters (Whatman International, Maidstone, U.K.) with 5% ice-cold trichloroacetic acid. Filters were dried with ethanol, and the radioactivity was determined in a soluene-based scintillant.

Linear sucrose gradient analysis

HCMV (strain Davis) DNA was separated from cellular DNA by CsCl gradient analysis as described above, collected and dialysed for 4 h against 4 l of neutral sucrose buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA·2 Na, 1 M NaCl) to remove the CsCl. Then, 180 μl of the DNA solutions were placed on top of a 4.8-ml linear neutral or alkaline sucrose gradient (from 8% to 30%, w/w) containing ultrapure sucrose (Schwarz/Mann, Becton Dickinson). The neutral sucrose buffer contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA·2 Na, 1 M NaCl. The alkaline sucrose buffer contained 50 mM Tris-HCl, 1 mM EDTA·2 Na, 1 M NaCl, 0.3 M NaOH. The sucrose gradients were centrifuged at 25 000 rpm for 2.5 h in an SW 56-1 titanium rotor (MSE, Scientific instruments) at room temperature. Then 140-μl fractions were collected from the top of the gradient. The density of each peak was determined with a Zeiss refractometer. The DNA in each fraction was then precipitated on Whatman GF/C filters with 5% trichloroacetic acid, and the filters were dried with ethanol (70%). Radioactivity was determined in a soluene-based scintillant.

Virus yield reduction assay

HEL cells were grown to confluency in 24-well titer plates. Then the cells were infected with HCMV (strain Davis) at a MOI of 1, and treated with HPMPC at 20 μg/ml, either immediately after virus infection or at four days after infection. At different times after infection (see footnote to Table 3), the supernatants of the cell cultures were collected and the virus titer was

determined by titration on confluent HEL cell cultures in 96-well microtiter plates.

Results

Treatment of HEL cell cultures with HPMPC and DHPG for different time-periods before and after HCMV infection

When HCMV-infected cultures were incubated for the first 6 h post-infection in the presence of the different test compounds, and then further incubated in drug-free medium, a significant difference in the antiviral activity of the test compounds was observed (Table 1). DHPG and FIAC had virtually lost all antiviral potency, whereas HPMPC was about as active as FIAC or DHPG that had been in continuous contact (seven days) with the HCMV-infected cells.

In a second set of experiments, HPMPC and DHPG were administered at day 3, 2 or 1 before HCMV infection. After a 12- or 24-h incubation period, the compounds were removed, and the cell cultures were rinsed twice with fresh medium and further incubated in drug-free medium until infection. After virus adsorption, the HCMV-infected cells were incubated for seven days in drug-free medium. Under these experimental conditions, preincubation of the cell cultures with HPMPC caused a significant inhibition of HCMV replication (Table 2). For example, treatment of the cells with HPMPC from three to two days pre-infection, resulted in a marked inhibition of HCMV-induced cytopathogenicity, as measured seven days post-infection (EC_{50}: 2.4 μg/ml). In contrast, DHPG had no or very weak antiviral activity under these conditions, even if exposed to the cells for 24 h immediately before HCMV infection.

TABLE 1

Inhibitory effects of HPMPC, FIAC and DHPG on HCMV (strain Davis)-induced cytopathogenicity in HEL cells following short-pulse treatment of the cells with the compounds immediately after virus infection

Compound	EC_{50}[a] (μg/ml)	
	Continuous incubation with the cells (0–7 days)	Following a 6-h treatment period post-infection
HPMPC[b]	0.25 ($\pm$ 0.1)	1.5 ($\pm$ 0.6)
DHPG[b]	0.9 ($\pm$ 0.4)	49 ($\pm$ 8.4)
FIAC	5.9 ($\pm$ 3.0)	$\geqslant$ 260

[a] Concentration required to effect a 50% inhibition of HCMV-induced cytopathogenicity (recorded at seven days post-infection). Data are mean values for three to six independent experiments. The standard deviations are given between parentheses.
[b] Data for HPMPC and DHPG are in agreement with the data obtained by Snoeck et al. (1988).

TABLE 2

Effects of HPMPC and DHPG on HCMV (strain AD-169)-induced cytopathogenicity in HEL cells following treatment of the cells for different time-periods before infection or following continuous treatment of the cells post-infection

Treatment period before infection	EC_{50}[a] (μg/ml)	
	HPMPC	DHPG
From day -3 until day -2 (24 h)	2.4 (± 1.4)	> 200
From day -3 until day -2.5 (12 h)	10.8 (± 4.5)	ND
From day -2 until day -1 (24 h)	1.8 (± 0.8)	⩾46
From day -2 until day -1.5 (12 h)	7.5 (± 4.3)	ND
From day -1 until day 0 (24 h)	0.9 (± 0.3)	40 (± 4.8)
Continuous incubation post-infection (0-7 days)	0.16 (± 0.13)	0.6 (± 0.24)

[a] Concentration required to effect a 50% inhibition of HCMV-induced cytopathogenicity (recorded at seven days post-infection). Data are mean values for four independent experiments. Standard deviations in parentheses.
ND: not determined.

Inhibition of virus yield following late addition of HPMPC to HCMV-infected HEL cells

HPMPC was examined on its inhibitory effect on HCMV yield following its addition at four days post-infection. From day 5 post-infection, virus titers in the cell supernatants were as high as 4×10^8 PFU/ml. HPMPC effected a 97% reduction in extracellular virus yield following a 24-h treatment period (from day 4 to day 5 post-infection). When the cell cultures were incubated with HPMPC for another 24 or 48 h (from day 4 to day 6–7 post-infection), virus yield was reduced by 4 $\log_{10}$ (Table 3).

Effect of natural nucleosides on the anti-HCMV activity of HPMPC, FIAC and Ara-C

To gain some insight in the mechanism of action of HPMPC, the effects of dCyd and Cyd on its antiviral action were examined. While dCyd (at 500 μg/ml) completely reversed the antiviral effects of FIAC (EC_{50} in the absence or presence of dCyd: 9 and > 200 μg/ml, respectively) and ara-C (EC_{50} in the absence or presence of dCyd: 0.3 and 99 μg/ml, respectively), it did not markedly affect the antiviral activity of HPMPC (EC_{50} in the absence or presence of dCyd: 0.49 and 0.8 μg/ml, respectively). These observations are in agreement with those reported by Snoeck et al. (1988). Similarly, addition of Cyd had no influence on the antiviral activity of HPMPC, but resulted in a 100-fold increase of the EC_{50} value for ara-C. At the concentration tested (500 μg/ml), neither dCyd nor Cyd by themselves had any effect on HCMV replication.

TABLE 3

Inhibitory effect of HPMPC on HCMV yield at different time-periods after infection following treatment with the compound for different time-periods

Treatment period after infection	Virus yield (log_{10} PFU/ml)
Mock treatment	
From day 4 until day 5	4.0×10^8
From day 6 until day 7	3.8×10^8
From day 7 until day 8	3.3×10^8
HPMPC treatment (20 μg/ml)	
From day 4 until day 5	8.3×10^6
From day 4 until day 6	6.3×10^4
From day 4 until day 7	1.2×10^4
From day 4 until day 8	2.2×10^4
From day 0 until day 8	$< 10^2$

HEL cell cultures were infected with HCMV (strain Davis) at a MOI of 1, and incubated either in the presence of HPMPC (20 μg/ml) or normal culture medium for the indicated time-periods. Culture supernatants were harvested after five, six, seven or eight days. Cultures treated with the test compound or normal MEM had a daily change of medium after four days.

Effect of natural nucleosides on the cytotoxicity of HPMPC and Ara-C

The concentration at which HPMPC inhibits HEL cell growth by 50% (IC_{50}) is 100 μg/ml. Upon addition of dCyd (500 μg/ml) or Cyd (500 μg/ml), the IC_{50} of HPMPC for exponentially growing HEL or Vero cells was not markedly altered (data not shown). The cytotoxicity of HPMPC was also determined in murine leukemia L1210 cells. Only at a concentration of 500 μg/ml did HPMPC afford approximately 50% inhibition of cell growth, and addition of dCyd at 1000 μg/ml did not result in a complete reversal but only partially restored cell growth (increase in cell growth from 50% to 80%). These observations are in agreement with previously published data (Snoeck et al., 1988). In contrast, the IC_{50} of ara-C for L1210 cell growth was 0.007 μg/ml, and addition of dCyd at 100 μg/ml completely restored cell growth, even in the presence of higher ara-C concentrations (i.e., 0.1 μg/ml) that inhibited cell growth by 90%.

Effect of HPMPC on HCMV DNA synthesis in HEL cells

The effect of HPMPC on HCMV DNA synthesis was evaluated in HEL cells infected with HCMV (strain Davis). Fig. 1 shows the data for a representative experiment. Although the cells were infected at a high multiplicity of infection (MOI: 1), giving rise to 100% cytopathicity within 24–48 h, HPMPC achieved marked reduction of viral DNA synthesis at 0.1–0.3 μg/ml, as recorded at seven days post-infection. At a concentration of 0.3 μg/ml, HPMPC reduced HCMV DNA synthesis by 86% (Fig. 1, panel C); at 0.1 μg/ml, it effected 50% reduction of viral DNA synthesis (Fig. 1, panel B).

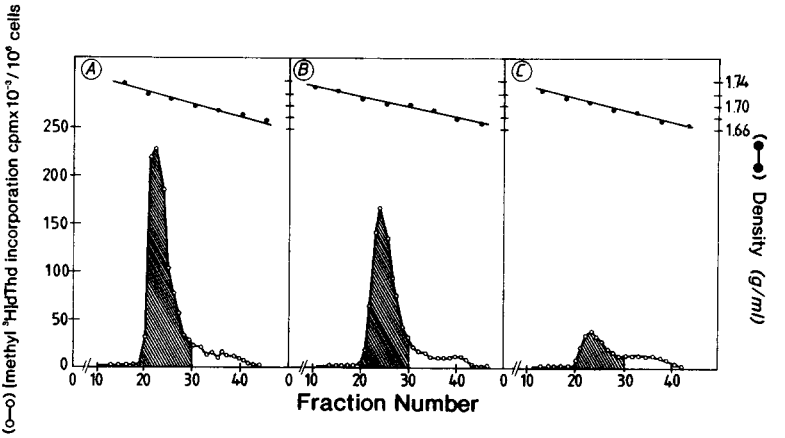

Fig. 1. CsCl equilibrium gradient analysis of DNA from HCMV (strain Davis)-infected HEL cells, which had not been treated (panel A), treated with 0.1 μg/ml HPMPC (panel B), or treated with 0.3 μg/ml HPMPC (panel C). The HCMV-infected cells were exposed to the drug for seven days, at which time the cells were harvested and analyzed for their HCMV DNA content. [*Methyl*-^{3}H]dThd (17 μCi) was added 24 h before the cells were harvested. Shadowed areas represent HCMV DNA

Effect of HPMPC on the integrity of HCMV DNA, as determined by neutral and alkaline sucrose gradient analysis

Under alkaline conditions DNA is denaturated, and centrifugation on alkaline sucrose gradients allows the determination of whether single-strand DNA breaks may have occurred. Similarly, sucrose gradient analysis of non-denaturated DNA under neutral conditions allows the detection of double-strand DNA breaks (Zubroff and Sarma, 1976). As is evident from Fig. 2, the viral DNA which was synthesized in the presence of HPMPC at 0.3 μg/ml (panels C1 and C2) or 0.1 μg/ml (panels B1 and B2) gave a peak in the sucrose gradient with the same density as the viral DNA synthesized in the absence of HPMPC (panels A1 and B1). Similar results were obtained under neutral (panels A1, B1, C1) and alkaline (panels A2, B2, C2) conditions, indicating that no (significant) double- or single-strand breaks of HCMV DNA had occurred following exposure of HCMV-infected cells to HPMPC.

Discussion

The acyclic nucleoside phosphonate analogue HPMPC is a highly potent and selective anti-HCMV agent (Snoeck et al., 1988), which possesses a long-lasting antiviral activity that is not observed for DHPG (Neyts et al., 1990).

We have now established (Table 2) that exposure of HPMPC to the cells before infection with HCMV also results in a strong antiviral activity. This implies that the active metabolite(s) of HPMPC must remain present in the cells

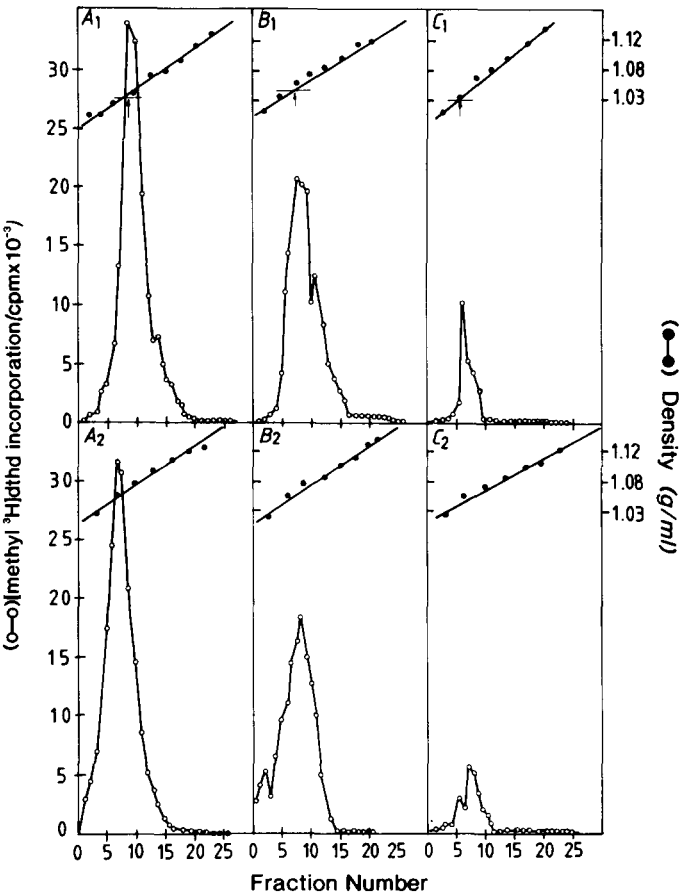

Fig. 2. Sucrose gradient analysis of the HCMV DNA recovered from the shadowed areas of Fig. 1 (panels A, B and C). Panels A1, B1 and C1 represent HCMV DNA analyzed under neutral conditions, whereas panels A2, B2 and C2 represent HCMV DNA analyzed under alkaline (denaturating) conditions. Panels A1 and A2 represent virus control (HCMV DNA recovered from shadowed area of Fig. 1, panel A); panels B1 and B2 represent HCMV-infected cultures treated with HPMPC at 0.1 μg/ml (HCMV DNA recovered from shadowed area of Fig. 1, panel B); and panels C1 and C2 represent HCMV-infected cultures treated with HPMPC at 0.3 μg/ml (HCMV DNA recovered from shadowed area of Fig. 1, panel C).

for several days so as to inhibit HCMV DNA synthesis. In fact, it has been reported that the phosphorylated metabolites of HPMPC are highly stable within the host cells (Hitchcock et al., 1989).

As shown in Table 3, HPMPC is also able to significantly reduce HCMV yield, when administered several days after virus infection. A similar observation was made previously for DHPG (Tyms et al., 1987). The de novo synthesis of viral DNA is essential to maintain the production of infectious virus (Tyms et al., 1987), and thus remains vulnerable to inhibition by HPMPC, even late after the initial infection. Indeed, when administered from day 4 until day 6–8 after infection, HPMPC at 20 μg/ml afforded a 5000

to 10 000-fold decrease in virus yield (Table 3). These observations again point to the pronounced antiviral potency of HPMPC.

To gain some insight in the mechanism of action of HPMPC, the natural nucleosides dCyd and Cyd were evaluated for their potential to reverse the antiviral and cytotoxic properties of HPMPC. In marked contrast with FIAC and ara-C, the antiviral and cytotoxic effects of HPMPC were not readily reversed by dCyd or Cyd. The dCyd analogues such as ara-C (and also 2′,3′-dideoxycytidine) are phosphorylated by dCyd kinase (Balzarini and De Clercq, 1983; Balzarini et al., 1987), which explains why dCyd can reverse their antiviral and cytotoxic activities. The fact that dCyd does not reverse the antiviral activity and cytotoxicity of HPMPC may be interpreted to mean that HPMPC does not depend on dCyd kinase for its phosphorylation.

To assess the integrity of HCMV DNA synthesized in the presence of HPMPC, neutral and alkaline sucrose gradient centrifugation analyses were carried out. Analysis of DNA in neutral sucrose gradients allows the detection of double-strand DNA breaks, whereas analysis in alkaline sucrose gradients reveals single-strand DNA breaks. No evidence for either double-strand or single-strand DNA breaks was found when HCMV DNA, recovered from HCMV-infected cells treated with HPMPC, was analyzed by neutral or alkaline sucrose gradients (Fig. 2). Similar approaches have been used by others to study the integrity of HSV or VZV DNA that had incorporated different antiviral compounds. Double- and single-strand DNA breaks were noted for HSV DNA containing AIU (5-iodo-5′-amino-2′,5′-dideoxyuridine), while this was not the case for HSV DNA containing IDU (5-iodo-2′-deoxyuridine) (Fisher et al., 1980). Recently, Balzarini et al. (1990) reported a much greater heterogeneity for HSV DNA that had incorporated [^{125}I]IVDU [(*E*)-5-(2-iodovinyl)-2′-deoxyuridine] than for HSV DNA that had incorporated its carbocyclic analogue [^{125}I]C-IVDU. Also, Mancini et al. (1983) reported that HSV DNA containing BVDU [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine] is more liable to single-strand DNA breaks. Similar findings were reported by Shigeta et al. (1985) for VZV DNA that had incorporated BVDU.

Although we demonstrated that HPMPC does not result in (detectable) single- or double-strand DNA breaks, this does not exclude the possibility that HPMPC is incorporated into viral DNA. Votruba et al. (1987) could not unequivocally show the incorporation of [^{14}C]HPMPA into the viral DNA of HSV-infected HEL or Vero cells, probably because the compound inhibited viral DNA synthesis at the concentrations required for establishing its incorporation, and/or because of the relatively low radiospecificity of the compound used in this study. Holý et al. (1990) have recently demonstrated that HPMPA (in its diphosphate form) can actually serve as an alternative substrate of dATP and be incorporated into DNA, even inside the chain via an internucleotide linkage.

In conclusion, HPMPC is a potent inhibitor of HCMV replication and HCMV DNA synthesis. It does not lead to detectable breakage of the viral DNA, and its antiviral activity is not reversed by dCyd. The compound

possesses a long-lasting antiviral effect, and remains active even after it has been exposed for short periods to the cells before virus infection. Based on these in vitro findings, one may expect HPMPC to offer great potential for the treatment of HCMV infections. The long-lasting nature of its antiviral response may allow infrequent and/or prophylactic dosing, which are attractive features for a compound that has to be used in the therapy and prevention of a persistent and readily recurring virus infection, as is the case with CMV.

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