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Effect of (S)-1-[(3-hydroxy-2-phosphonyl methoxy) propyl] cytosine on the replication and morphogenesis of herpes simplex virus type 1

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Summary

Treatment of African green monkey kidney cells with 1 μg/ml of (S)-1-[(3-hydroxy-2-phosphonyl methoxy) propyl] cytosine (HPMPC) inhibited the release of infectious herpes simplex virus type 1 (HSV-1) by more than 90%. Electron microscopic observations of HPMPC-treated monkey kidney cells demonstrated few intracellular or extracellular viral particles. The viral particles seen were without dense cores. In addition, HPMPC blocked cell fusion induced by HSV-1 in monkey kidney cells. Immunoblot analysis showed that HPMPC significantly blocked the expression of HSV-1-specific proteins. Furthermore, HPMPC inhibited the synthesis of viral DNA as determined by in situ hybridization. These results indicate that HPMPC inhibits the replication of HSV by blocking one of the events involved in DNA synthesis.

Herpes simplex Type 1; HPMPC, mechanism of action; Viral replication, inhibition of

Introduction

Infections caused by human herpesviruses are exceedingly prevalent and result in a broad spectrum of disease, including life-threatening illnesses. The seven human herpesviruses include, cytomegalovirus (CMV), Epstein-Barr

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virus (EBV), varicella-zoster virus (VZV), herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2, respectively), human herpesvirus 6 (HHV-6) and the newly recognized human herpesvirus 7 (HHV-7; Frenkel et al., 1990). Of these seven herpesviruses, HSV-1 and HSV-2 have been studied extensively for their unique biological and molecular properties. These properties include, among others, (1) an ability to cause a broad spectrum of clinical disease, (2) gene expression and protein transport, and (3) a unique propensity to establish latency and recur even in the presence of humoral and cell-mediated immunity (Stevens and Cook, 1971; Stevens et al., 1972; Bastian et al., 1972; Warren et al., 1977; Fraser et al., 1981). Because these viruses are important causes of human disease, a number of therapeutics have been developed to treat HSV infections. Acyclovir (ACV) has been shown to modify the course of herpes simplex encephalitis, neonatal herpes, HSV infections in immune compromised hosts and primary and recurrent genital herpes (Baker, 1990). However, the development of HSV isolates which are resistant to ACV and the recognition that outcome following therapy still needs to be improved has prompted the continued development of new antiviral therapies for HSV.

Recently, it was reported that the phosphonate nucleoside analogue (S)-1-[(3-hydroxy-2-phosphonyl methoxy) propyl] cytosine (HPMPC) has broad spectrum activity against members of the herpesvirus group (De Clercq et al., 1987; Snoeck et al., 1988; Bronson et al., 1989). The 50% effective concentration (EC_{50}) against a number of HSV-1 isolates is approximately 1 μg/ml and the 50% inhibitory concentration (IC_{50}) for toxicity is greater than 100 μg/ml (Kern and Vogt, 1990). The mode of action of HPMPC, however, on the replication of HSV-1 is unknown. The purpose of our studies was to determine the effect of HPMPC on the replication and morphogenesis of HSV-1 and to investigate the mechanism of action of this compound. In these studies, we demonstrated that HPMPC significantly inhibited the replication of HSV-1 in African green monkey kidney cells. The block in replication appeared to be early in the replication cycle since viral DNA and protein synthesis were significantly inhibited in HPMPC-treated cells.

Materials and Methods

Cell cultures and virus

African green monkey kidney (BS-C-1) cells were obtained from the American Type Culture Collection, Rockville, MD, and were grown in medium 199 containing 10% heat-inactivated fetal bovine serum and gentamicin (50 μg/ml). The 'F' strain of HSV-1 was obtained from B. Roizman, The University of Chicago, Chicago, IL.

Reagents and radioisotope

(S)-1-[(3-hydroxy-2-phosphonyl methoxy) propyl] cytosine was provided by Bristol-Myers Company, Wallingford, CT, through the Antiviral Substances

Program, NIAID, NIH. Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. Na ^{125}I (15.5 Mci/μg of iodine) was purchased from Amersham Corporation, Arlington Heights, IL.

Antisera

Monoclonal antibodies to glycoproteins B, D, and E were prepared in this laboratory (Koga et al., 1986; Chatterjee et al., 1989). Polyclonal rabbit antiserum to HSV-1 was obtained from Lee Biomolecular Research Laboratories, San Diego, CA.

Electron microscopy

Virus-infected, HPMPC-treated and virus-infected untreated cells grown in 60 mm dishes were processed for electron microscopy as described previously (Chatterjee et al., 1982). In brief, cells were washed with phosphate-buffered saline (PBS) and fixed with 1% glutaraldehyde. Cells were post-fixed with 1% osmium tetroxide and embedded in an epoxy resin mixture. Thin sections were stained with uranyl acetate and lead citrate and were examined under a Philips EM 301 electron microscope.

Polyacrylamide gel electrophoresis and immunoblotting

HPMPC-treated and untreated monkey kidney cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and, subsequently, processed for immunoblotting, as described previously (Chatterjee et al., 1985). Nitrocellulose strips were incubated for an additional 30 min at room temperature with rabbit anti-mouse IgG (Organon Teknika Corporation, West Chester, PA) if mouse monoclonal antibodies were used. The strips were finally reacted with ^{125}I-protein A for 1 h at room temperature followed by autoradiography.

In situ DNA hybridization

African green monkey kidney cells, grown in 4-chamber glass slides were infected and treated with HPMPC for 18 h as described previously. Cells were fixed in acetone for 10 min and processed for in situ DNA hybridization using ENZO Color Gene DNA hybridization test kit (ENZO Diagnostics, New York, NY). In brief, biotinylated HSV DNA probe was added to the cells, covered with a glass coverslip and placed on a 92°C heating block for 2 min. Slides were then removed from the heating block and incubated for 10 min at room temperature. The samples were next treated with post-hybridization buffer and incubated further at room temperature for 10 min. The slides were rewashed and incubated with avidin-biotinylated horseradish peroxidase and subsequently with 3-amino-9-ethylcarbazole mixed with hydrogen peroxide for 15 min at room temperature. Finally, the samples were washed and counter-stained with Fast Green in acetic acid for 30 s, washed and observed under a Nikon TMS microscope.

Viral DNA-containing cells exhibit red nuclear staining, whereas, the uninfected cells displayed no nuclear staining.

Results

Effect of HPMPC on the replication of HSV-1 in African green monkey cells

To determine the effect of HPMPC on the release of infectious HSV-1 particles, BS-C-1 cells were infected with F strain of HSV-1 (multiplicity of infection = 5). After a 1-h adsorption, cells were washed and treated with 1, 2, 5 or 10 μg/ml of HPMPC for 18 h. One set of cells served as untreated control. Supernatant fluids from treated and untreated cells were collected and tested for the ability to form plaques. This experiment demonstrated that 1 μg/ml of HPMPC blocked the release of infectious HSV-1 by greater than 90% (Table 1, expt. 1). Treatment of cells with 5 μg/ml of HPMPC inhibited the production of infectious HSV-1 by greater than 99% (Table 1, expt. 2). Similar results were obtained when human fibroblast cells were used instead of BS-C-1 cells (data not shown).

In addition, HPMPC also blocked the cell fusion induced by HSV-1 in BS-C-1 cells (Fig. 1). In brief, monkey cells were infected with HSV-1 and then treated with different concentrations of HPMPC for 18 h. Cells were stained with May-Grunwald-Giemsa, as described previously (Chatterjee and Hunter, 1979). The block in replication and in cell-to-cell fusion was not due to toxic effects of HPMPC. No changes in uninfected cells were observed after HPMPC treatment either in cell number or cell morphology (data not shown). Additionally, it has been reported that concentrations of HPMPC as high as 100 μg/ml were not cytotoxic to human fibroblast cells (Kern, 1991).

TABLE 1

Effect of HPMPC on the replication of HSV-1 in Monkey Kidney Cells[a]

Experiment	Concentrations (μg/ml)	Plaque-forming units/ml	% Inhibition
	0	5×10^5	0
1	1	3×10^4	94.0
	2	1×10^4	98.0
	0	1×10^5	0
2	1	1×10^4	90.0
	5	7×10^2	99.3
	10	5×10^1	99.9

[a]African green monkey kidney cells were infected with HSV-1 and then treated with different concentrations of HPMPC as mentioned above. One set of cells served as untreated control. Supernatant fluids from treated and untreated cells were collected and tested for their ability to form plaques on monkey kidney cells.

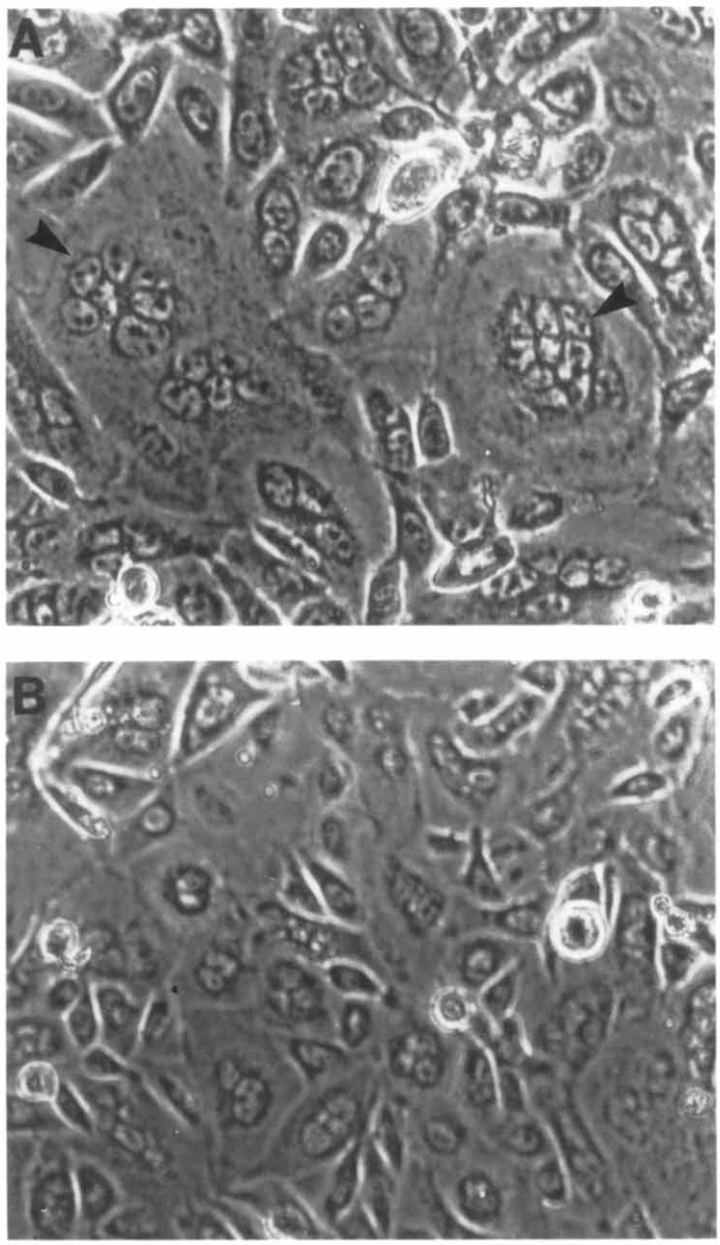

Fig. 1. Effect of HPMPC on the cell fusion induced by HSV-1 in monkey kidney cells. (A) HSV-1-infected cells without any HPMPC treatment showing several multinucleate cells (arrows). (B) HSV-1-infected, HPMPC-treated (2 μg/ml) cells.

Effect of HPMPC on the release of total extracellular viral particles

Since the release of infectious virus particles was significantly blocked by

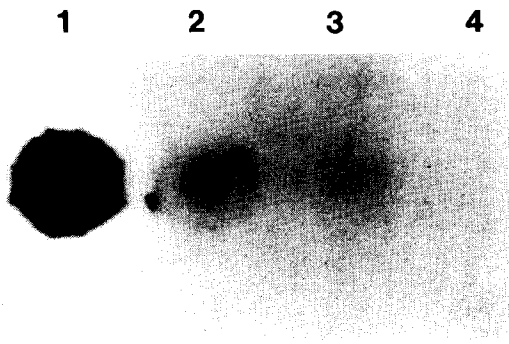

Fig. 2. Release of total extracellular viral particles from HPMPC-treated and untreated monkey kidney cells. The detailed procedure is described in the text. Lane 1, no HPMPC; lane 2, 1 μg/ml HPMPC; lane 3, 2 μg/ml HPMPC; lane 4, 5 μg/ml HPMPC.

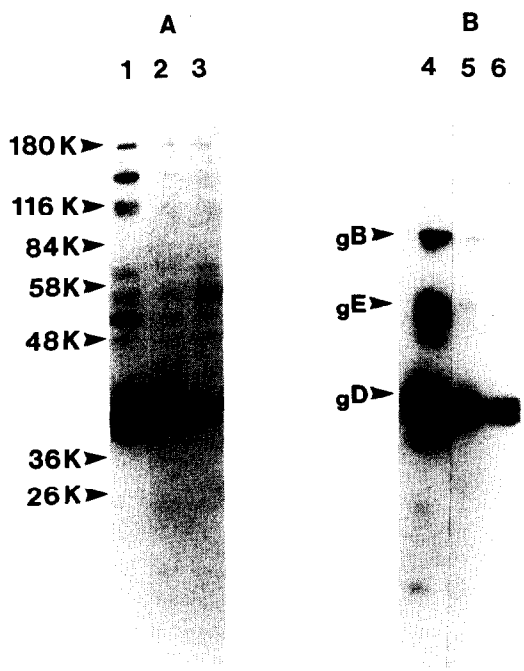

Fig. 3. Effect of HPMPC on the expression of HSV-1 proteins in treated and untreated monkey kidney cells. A: the blot was incubated with rabbit antiserum to HSV-1. B: the blot was incubated with monoclonal antibodies to gB, gD and gE. Lanes 1 and 4, no HPMPC; lanes 2 and 5, 1 μg/ml HPMPC; lanes 3 and 6, 2 μg/ml HPMPC.

HPMPC, it was important to determine whether any particles including non-infectious were released from HPMPC-treated cells. Monkey kidney cells were infected with HSV-1 and treated with 1, 2 or 5 μg/ml of HPMPC for 18 h as before. Supernatant fluids were collected, clarified by low-speed centrifugation

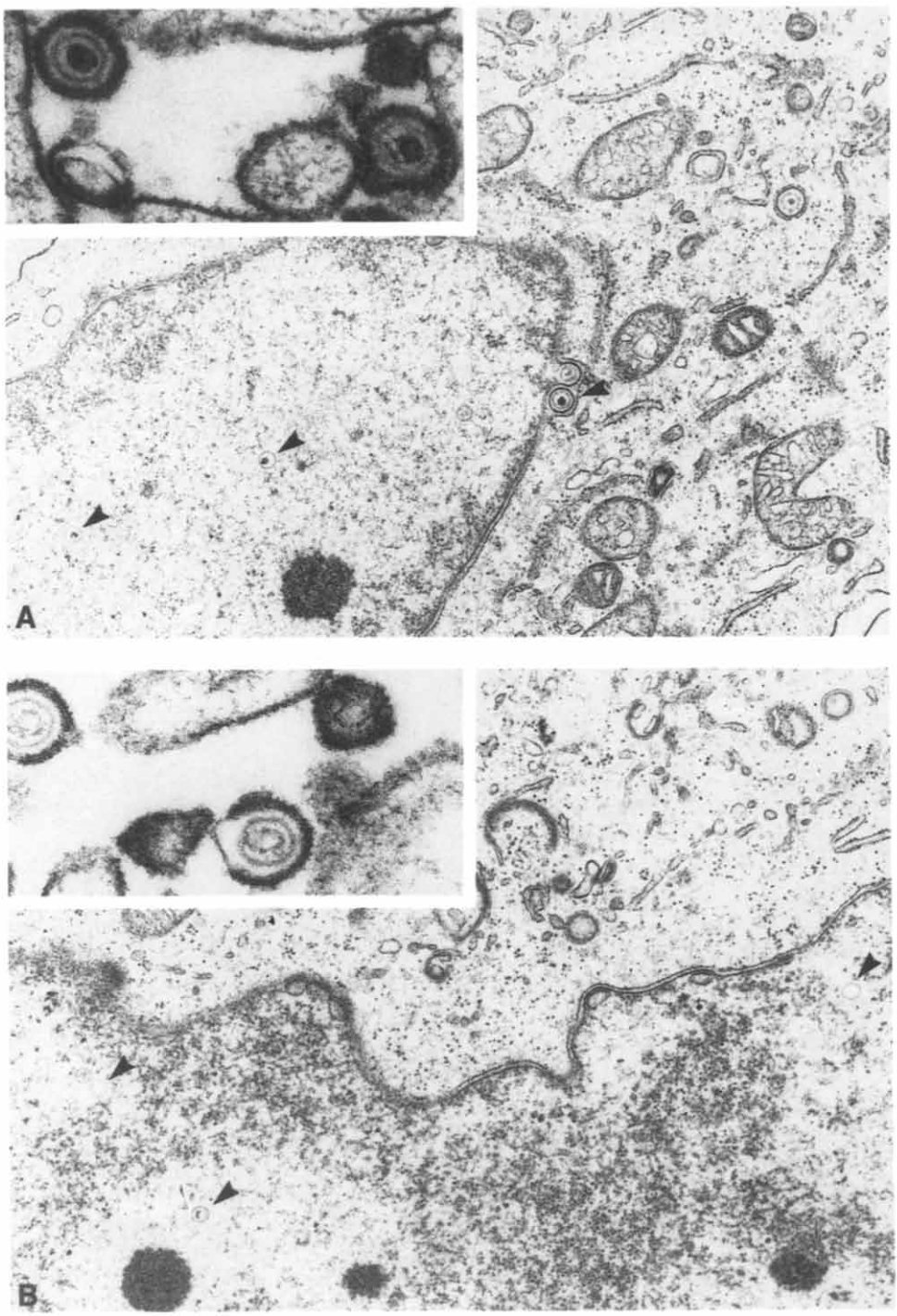

Fig. 4. Electron microscopic observations of HSV-1-infected, HPMPC-treated and untreated monkey kidney cells. A: no HPMPC; note the distinct nucleocapsids and budding particles (arrowheads). Inset shows complete particles with dense cores. B: HPMPC-treated (2 μg/ml) cell; few intranuclear capsids are observed (arrowheads). Inset shows particles without dense cores. A, B, × 9,750. Insets, × 31,980.

in a Damon/IEC centrifuge for 15 min at 1000 × *g*. The virus was pelleted by ultracentrifugation at 35000 rpm in a Sorvall TH 641 rotor for 1 h. The virus pellets were lysed and spotted onto nitrocellulose filter paper. The filter paper was washed with PBS, blocked in Bovine Lacto Transfer Technique Optimizer (5% non-fat milk in PBS with sodium azide, BLOTTO; Johnson et al., 1984) and incubated with rabbit antiserum to HSV-1 for 16 h at 4°C. Finally, bound antibodies were detected by reacting the blot with ^{125}I-protein A for 1 h at room temperature followed by autoradiography. After autoradiography, the blot was counted in a gamma counter for quantitation. The result of this experiment (Fig. 2) showed a significant reduction (87%, 97%, and 99% as determined by gamma counts) in the release of total extracellular viral proteins from treated (1, 2, or 5 μg/ml respectively) cells.

Expression of viral polypeptides and assembly of nucleocapsids in HPMPC-treated cells

To determine whether the block in replication correlated with a reduction in the expression of virus-specific proteins, the following experiment was performed. African green monkey kidney cells were infected with HSV-1 and treated with 1 or 2 μg/ml of HPMPC for 18 h as described. Cell lysates were collected and processed for polyacrylamide gel electrophoresis and immunoblotting as described in Materials and Methods. The nitrocellulose blot was reacted with rabbit anti-HSV-1 antiserum. A separate but identical blot was incubated with monoclonal antibodies to gB, gD, and gE. The result of this experiment demonstrated that the expression of capsid proteins as well as gB and gE was significantly inhibited in HPMPC-treated cells (Fig. 3A,B). However, the expression of gD, although reduced was not completely inhibited. Similar results were obtained when human fibroblast cells were used instead of BS-C-1 cells (data not shown).

Consistent with the above observation, electron microscopy indicated few capsids inside the nucleus of HPMPC-treated cells (Fig. 4A). These capsids were empty and without any dense cores. In contrast, the nucleus of untreated cells displayed budding nucleocapsids with dense cores at the nuclear membrane (Fig. 4B). Furthermore, the extracellular viral particles (Fig. 4A, inset) released from untreated cells had dense cores as compared to the few extracellular particles released from treated cells (Fig. 4B, inset).

Synthesis of viral DNA in HPMPC-treated cells

The reduction in virus-specific proteins in HPMPC-treated cells was correlated with the status of viral DNA as determined by in situ DNA hybridization of HPMPC-treated and untreated cells. This experiment (Fig. 5) indicated that even 1 μg/ml of HPMPC significantly inhibited the DNA synthesis in monkey kidney cells. Treatment of monkey cells with 5 μg/ml of HPMPC almost completely inhibited the synthesis of HSV-1 DNA. Similar results were obtained when human fibroblast cells were used instead of BS-C-1 cells (data not shown).

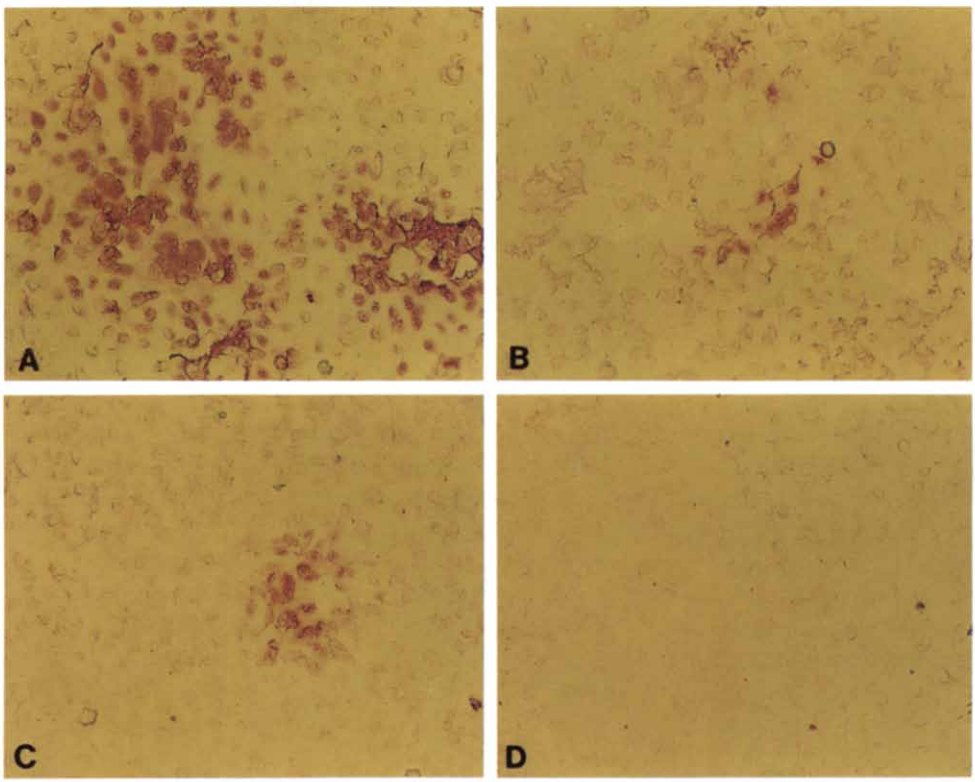

Fig. 5. Effect of HPMPC on the synthesis of viral DNA in monkey kidney cells as determined by in situ hybridization using biotinylated HSV DNA probe. A: no HPMPC; B: 1 μg/ml HPMPC; C: 2 μg/ml HPMPC; D, 5 μg/ml HPMPC.

Discussion

Recently, significant effort has been devoted to the treatment of herpes virus infections by using a variety of antiviral drugs. However, concerns for development of resistance and need to improve efficacy have prompted a re-examination of current therapeutic efforts. One of the recently described phosphonate nucleoside analogues, HPMPC, has excellent activity against HSV-1 and HSV-2 in tissue culture cells and is at least as effective as ACV in murine models of herpes encephalitis and neonatal herpes (Kern and Vogt, 1990). A recent report also indicated that HPMPC is active against thymidine kinase-deficient strains of HSV-1 which are resistant to ACV (Bronson et al., 1989). The results described in our studies demonstrated that HPMPC significantly blocked the release of total extracellular HSV-1 particles from treated cells. This inhibition could be due to a block at an early or late stage in viral morphogenesis. We, thus, examined the effect of HPMPC on the different stages of virus replication cycle which include the expression of viral proteins

and glycoproteins, assembly and budding of nucleocapsids, and viral DNA synthesis. In contrast to prior studies with IFN-treated cells (Chatterjee et al., 1985; Chatterjee and Whitley, 1989; Chatterjee and Burns, 1990), a significant reduction in the expression of HSV-1-specific capsid proteins was observed in HPMPC-treated cells. We also analyzed the expression of HSV-1 glycoproteins in treated cells since these molecules are necessary for important biological functions, including envelopment of nucleocapsids and cell-to-cell fusion. The expression of gB and gE was significantly inhibited in infected monkey kidney cells after HPMPC treatment (Fig. 3B). However, although reduced, the expression of gD was not completely blocked in HPMPC-treated cells (Fig. 3B). Similar observation was previously reported using inhibitors of DNA replication which allowed some gD synthesis although at a reduced level (Peake et al., 1982; Gibson and Spear, 1983). Thus, gD has been designated $\beta\gamma$ (early–late) polypeptide. In general, the expression of HSV-1 proteins was significantly reduced in HPMPC-treated cells. As a result of this block in protein expression, cell-to-cell fusion induced by HSV-1 was also affected significantly (Fig. 1B), since this important biological property appears to be regulated by several HSV-1 gene products (Chatterjee et al., 1989). Thus, HPMPC prevented the spread of virus as it has been reported that cell fusion or syncytium formation leads to cell-to-cell spread of viral infection (Hoggan and Roizman, 1959; Lodmell and Notkins, 1974).

The block in replication exhibited by HPMPC occurred prior to the formation of infectious virus in treated cells. This conclusion is supported by the fact that HSV-1 DNA synthesis was reduced significantly and in the electron microscopy few intracellular or extracellular virus particles were found in HPMPC-treated cells. These data suggested that HPMPC blocks HSV-1 morphogenesis at an early stage in virus replication. Although the exact mechanism of action of HPMPC has not been elucidated, it is anticipated that HPMPC may be an inhibitor of DNA polymerase or other enzymes involved in DNA synthesis. In fact, recently an inhibitory effect of HPMPC on HSV-1 and human α DNA polymerase has been observed (Hitchcock, 1991). In both cases, HPMPC was a competitive inhibitor with respect to d CTP, as expected; however, the binding constant or K_i is much higher (K_i = 51 μM) with the human enzyme than with viral polymerase (K_i = 0.87 μM). Thus, a selective inhibition of viral enzyme is achievable. The fact that HPMPC showed greater in vivo potency than ACV against HSV-1 and HSV-2 (Bronson et al., 1989; Kern and Vogt, 1990) and also has excellent activity against CMV and VZV (De Clercq et al., 1987; Snoeck et al., 1988; Kern, 1991) has prompted continued efforts to evaluate the efficacy of HPMPC both in vivo and in vitro.

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References

Baker, D.A. (Ed) (1990) Acyclovir Therapy for Herpesvirus Infections. Marcel Dekker, New York.

Bastian, F.O., Rabson, A.S., Yee, C.L. and Tralka, T.S. (1972) Herpesvirus hominis: isolation from human trigeminal ganglion. Science 178, 306–307.

Bronson, J.J., Ghazzouli, I., Hitchcock, M.J.M., Webb, R.R., Kern, E.R. and Martin, J.C. (1989) Synthesis and antiviral activity of nucleotide analogues bearing the (S)-(3-hydroxy-2-phosphonylmethoxy) propyl moiety attached to adenine, guanine, and cytosine. In: J.C. Martin (Ed), Nucleotide Analogues as Antiviral Agents. pp. 88–102, ACS Symposium Series, American Chemical Society, Washington.

Chatterjee, S., Bradac, J. and Hunter, E. (1982) Effect of monensin on Mason–Pfizer monkey virus glycoprotein synthesis. J. Virol. 44, 1003–1012.

Chatterjee, S. and Burns, P. (1990) Expression of herpes simplex virus type 1 glycoproteins in interferon-treated human neuroblastoma cells. J. Virol. 64, 5209–5213.

Chatterjee, S. and Hunter, E. (1979) The characterization of Mason-Pfizer monkey virus-induced cell fusion. Virology 95, 421–433.

Chatterjee, S., Hunter, E. and Whitley, R.J. (1985) Effect of cloned human interferons on protein synthesis and morphogenesis of herpes simplex virus. J. Virol. 56, 419–425.

Chatterjee, S., Koga, J. and Whitley, R.J. (1989) A role for herpes simplex virus type 1 glycoprotein E in induction of cell fusion. J. Gen. Virol. 70, 2157–2162.

Chatterjee, S. and Whitley, R.J. (1989) Effect of recombinant hybrid human interferon on replication and morphogenesis of HSV-1 in monkey cells. Virus Res. 12, 33–42.

De Clercq, E., Sakuma, T., Baba, M., Pauwels, R., Balzarini, J., Rosenberg, I. and Holy, A. (1987) Antiviral activity of phosphonyl-methoxyalkyl derivatives of purine and pyrimidines. Antiviral Res. 8, 261–272.

Fraser, N.W., Lawrence, W.C., Wrobleska, Z., Gilden, D.H. and Koprowski, H. (1981) Herpes simplex type 1 DNA in human brain tissue. Proc. Natl. Acad. Sci. USA 78, 6461–6465.

Frenkel, N., Schirmer, E.C., Wyatt, L.S., Katsafanas, G., Roffman, E., Danovich, R.M. and June, C.H. (1990) Isolation of a new herpesvirus from human $CD4^+$ T-cells. Proc. Natl. Acad. Sci. USA 87, 748–752.

Gibson, M.G. and Spear, P.G. (1983) Insertion mutants of herpes simplex virus have a duplication of the glycoprotein D gene and express two different forms of glycoprotein D. J. Virol. 48, 396–404.

Hitchcock, M., Bristol-Myers Squibb Company, Wallingford, Connecticut (1991) Personal communication.

Hoggan, M.D. and Roizman, B. (1959) The isolation and properties of a variant of herpes simplex virus producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. 70, 28–39.

Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H. (1984) Improved technique utilizing non-fat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Technol. 1, 3–8.

Kern, E.R. (1991) Value of animal models to evaluate agents with potential activity against human cytomegalovirus. Transplantation Proc. 23, 152–155.

Kern, E.R. and Vogt, P.E. (1990) Effect of treatment with HPMPC on mortality and pathogenesis of experimental herpes simplex virus infections. Antiviral Res. (Suppl. 1) abst. 34, p. 110.

Koga, J., Chatterjee, S. and Whitley, R.J. (1986) Studies on herpes simplex virus type 1 glycoproteins using monoclonal antibodies. Virology 151, 385–389.

Lodmell, D.L. and Notkins, A.L. (1974) Cellular immunity to herpes simplex virus mediated by interferon. J. Exp. Med. 140, 764–778.

Mertz, G.J. (1990) Herpes simplex virus. In: G.J. Galasso, R.J. Whitley and T.C. Merigan (Eds), Antiviral Agents and Viral Diseases of Man, pp. 265–300. Raven Press, New York.

Peake, M.L., Nystrom, P. and Pizer, L.I. (1982) Herpesvirus glycoprotein synthesis and insertion into plasma membranes. J. Virol. 42, 678–690.

Snoeck, R., Sakuma, T., De Clercq, E., Rosenberg, I. and Holy, A. (1988) (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine, a potent and selective inhibitor of human cytomegalovirus replication. Antimicrob. Agents Chemother. 32, 1839–1844.

Stevens, J.G. and Cook, M.L. (1971) Latent herpes simplex virus in spinal ganglia of mice. Science 173, 843–845.

Stevens, J.G., Nesburn, A.B. and Cook, M.L. (1972) Latent herpes simplex virus from trigeminal ganglia of rabbits with recurrent eye infection. Nature 235, 216–217.

Warren, K.G., Gilden, D.H., Brown, S.M., Devlin, M., Wroblewska, Z., Subak-Sharpe, J. and Koprowski, H. (1977) Isolation of herpes simplex virus from human trigeminal ganglia, including ganglia from one patient with multiple sclerosis. Lancet ii, 637–639.