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Antiviral activity of anti-cytomegalovirus agents (HPMPC, HPMPA) assessed by a flow cytometric method and DNA hybridization technique

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Summary

Phosphonylmethoxyalkylpurines and -pyrimidines, particularly (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) and (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) and their cyclic forms (cHPMPC, cHPMPA) and the 3-deaza analogue of HPMPA (HPMPc^{3}A) are selective and potent inhibitors of human cytomegalovirus (CMV) replication in vitro. Their anti-CMV activity has been monitored by flow cytometry and DNA hybridization. The anti-CMV agent ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG)] was included as a reference compound. From the flow cytometric assays, HPMPC and HPMPA were the most active compounds, with an EC_{50} (50% effective concentration) of approximately 0.6 μM. The EC_{50} values obtained by DNA hybridization ranged from 0.05 μM (HPMPC) to 0.74 μM (DHPG). Selectivity indexes, calculated as the ratio of the 50% inhibitory concentration (CC_{50}) for cell growth or [*methyl*-^{3}H]-thymidine incorporation to the EC_{50} for virus replication were highest for HPMPC and its cyclic derivative (cHPMPC). The flow cytometry and DNA hybridization assays thus confirm the results obtained by the classic plaque assay. They allow a quantitative estimation of the anti-CMV potency of the test compounds.

Human cytomegalovirus; DNA hybridization; Flow cytometry

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Introduction

Human cytomegalovirus (CMV) infection can be life-threatening in immunocompromised hosts including transplant recipients (kidney, heart, lung, liver and bone marrow) and patients suffering from the acquired immune deficiency syndrome (AIDS) (Tyms et al., 1989). CMV is the common causative agent of pneumonia and retinitis in such patients, but it can also lead to hepatitis, adrenalitis and gut infection in some cases. The chemotherapy of CMV infection has entered a new era. In recent years, various compounds have been described which achieve a selective inhibition of CMV replication. Two of these compounds, namely 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, ganciclovir) and phosphonoformate (PFA, foscarnet) have been used in the treatment of CMV infection in immunocompromised patients (Klintmalm et al., 1985; Erice et al., 1987; Laskin et al., 1987; Reed et al., 1988). Recently, a new acyclic nucleoside phosphonate derivative, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [HPMPA], has been described as a potent and selective inhibitor of a broad spectrum of DNA viruses, including herpesviruses [herpes simplex (HSV) type 1 and type 2, thymidine kinase-deficient (TK^-) HSV, varicella zoster virus (VZV), CMV, Epstein-Barr virus, poxviruses (vaccinia virus), iridoviruses (African swine fever virus) (De Clercq et al., 1986, 1987) and hepadnaviruses (Yokota et al., 1990a,b). We have also found that (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine [(*S*)-HPMPC], another member of the acyclic nucleoside phosphonate family, achieves a potent and selective inhibition of CMV replication in vitro (Snoeck et al., 1988). Here we describe the anti-CMV activity of HPMPA, HPMPC, their cyclic forms (cHPMPA and cHPMPC), the 3-deaza form of HPMPA ($HPMPc^3A$) and DHPG, as determined by flow cytometric and DNA hybridization assays that were specifically developed for this purpose.

Materials and Methods

Cells

All experiments were carried out in human embryonic lung (HEL) fibroblasts (ATCC CCL-137, American Type Culture Collection, Rockville, MD, USA).

Viruses

The Davis and AD-169 strains (ATCC VR807 and ATCC VR538, respectively) were provided by S. Michelson, Pasteur Institute, Paris, France.

Monoclonal antibodies

The murine monoclonal antibody (mAb) F4a (Amadei et al., 1983), was a

gift from S. Michelson. This mAb (used at a dilution of 1/100) specifically detected viral antigen expression during the late part of the virus cycle.

The fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of rabbit anti-murine immunoglobulin antibody [RaM-IG-$F(ab')_2$-FITC] (Dakopatts, Denmark) were used at a dilution of 1/40.

Compounds

HPMPA, cHPMPA, $HPMPc^3A$, HPMPC and cHPMPC were obtained from Dr. A. Holý (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). DHPG (ganciclovir) was a gift from Syntex Inc. (Palo Alto, CA, USA).

Antiviral assay

HEL cells were propagated in Eagle's minimum essential medium (MEM; Gibco, Paisley, U.K.) supplemented with 10% inactivated fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 1% L-glutamine (Gibco) and 0.3% sodium bicarbonate (Gibco) in 96-well microtiter plates (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.). All assays were carried out in confluent cells infected with 20 or 100 plaque-forming units (PFU) of virus per well.

The viruses were diluted in MEM supplemented with 2% FCS, 1% L-glutamine and 0.3% sodium bicarbonate. After a 2-h virus adsorption period at 37°C in 5% CO_2 atmosphere, the medium containing residual virus was removed and replaced by MEM containing 2% FCS, 1% L-glutamine, 0.3% sodium bicarbonate and varying concentrations (1200 μM–0.003 μM) of the test compounds. After an incubation period of seven days, the cells were fixed with ethanol and stained with 2.5% Giemsa solution. Virus plaque formation (virus input: 20 PFU) or viral cytopathic effect (virus input: 100 PFU) was monitored microscopically. The antivirally active concentration is expressed as EC_{50}, i.e., the concentration required to reduce virus-induced plaque formation or cytopathogenicity by 50%.

Flow cytometry assay

The method described previously by Schols et al. (1989) was used to evaluate the in vitro antiviral activity of the test compounds. To recount briefly, the CMV (AD-169 strain)-infected cells were trypsinized and fixed for 20 min in acetone/PBS (v/v: 66/34) at 4°C. The cells were resuspended in PBS containing 1% gelatine (PBS/gel; Vel, Haasrode, Belgium) and incubated in the presence of the appropriate mAb for 1 h in a shaking water-bath. The cells were rinsed in PBS/gel and resuspended in PBS/gel containing RaM-IgG-$F(ab')_2$-FITC. After 0.5-h incubation, the cells were rinsed and resuspended in a solution of 0.5% formaldehyde in PBS. The samples were analyzed with a fluorescence-activated cell sorter (Facstar, Becton Dickinson, San Jose, CA, U.S.A.).

DNA hybridization assay

The kit Hybriwix® (Athens, OH, U.S.A.) was used to evaluate the effects of the test compounds on viral DNA production (Gadler, 1983). The assay was carried out in the same conditions as described for the antiviral assay. At seven days after infection with CMV (AD-169 strain), the cells were lysed and after transfer of the lysates onto filters, the latter were incubated at 60°C for 2 h in the presence of the ^{125}I-labeled probes. After washing, the radioactivity remaining on the filters was determined in a gamma counter (Auto-gamma, Packard, Warrenville, IL, USA).

Cytotoxicity assay

Cytotoxicity measurements were based on the inhibition of HEL cell growth, as previously described (Snoeck et al., 1988). To recount briefly, 3×10^3 HEL cells were seeded per well in a 96-well microtiter plate and allowed to proliferate for 24 h in MEM containing 10% FCS. Different concentrations of the test compounds were then added in duplicate, and after three days incubation at 37°C in 5% CO_2 atmosphere, when the cell monolayer was about 70% confluent, cells were detached by trypsinisation and the cell number was determined with a Coulter counter. The minimum cytotoxic dose is expressed as the CC_{50}, i.e., the concentration required to reduce cell growth by 50%.

As a second marker for cytotoxicity, the compounds were evaluated for their inhibitory effects on host cell DNA synthesis. Incorporation of [*methyl*-^{3}H]-thymidine (Amersham, U.K.) was measured under the same experimental conditions as described above for cell growth inhibition. After two days incubation, 0.5 μCi of [*methyl*-^{3}H]-thymidine was added to each well. The amount of [*methyl*-^{3}H]-thymidine incorporated into acid-insoluble material was determined after a 20-h exponential growth period of the cells. The CC_{50} for DNA synthesis is defined as the concentration of compound required to reduce [*methyl*-^{3}H]-thymidine incorporation by 50%.

The selectivity index is defined as the ratio of the CC_{50} for inhibition of cell growth to the EC_{50} for inhibition of CMV plaque formation.

Results

When the compounds were evaluated for their inhibitory effects on CMV (strain AD-169 or Davis) plaque formation, the five phosphonyl derivatives showed an EC_{50} ranging from 0.2 to 0.7 μM (Table 1). Under the same conditions, DHPG showed EC_{50}s of 2.7 and 1.95 μM for AD-169 and Davis, respectively.

The results obtained in the flow cytometric assay are depicted in Table 2 and Figs. 1 and 2. In agreement with the results obtained in the plaque assay, the EC_{50} values of the phosphonyl derivatives were 3–10-fold lower than the EC_{50}

TABLE 1

Antiviral activity and cytotoxicity of anti-CMV agents in human embryonic lung (HEL) cells

Compound	Antiviral activity (EC_{50}[a], μM)		Cytotoxicity (CC_{50}, μM)		Selectivity index[d]	
	AD-169 strain	Davis strain	Cell growth[b]	[*Methyl*-^{3}H]-thymidine incorporation[c]	AD-169 strain	Davis strain
HPMPC	0.25	0.36	360	72	1500	1000
cHPMPC	0.72	0.72	720	108	1000	1000
HPMPA	0.28	0.57	57	6	200	100
cHPMPA	0.23	0.43	115	20	500	260
HPMPc^{3}A	0.18	0.12	90	6	500	750
DHPG	2.73	1.95	390	97	150	100

[a] Concentration required to reduce virus plaque formation by 50%.
[b] Concentration required to reduce cell growth by 50%.
[c] Concentration required to reduce [*methyl*-^{3}H]-thymidine incorporation by 50%.
[d] Ratio of CC_{50} (for cell growth) to EC_{50}.

TABLE 2

Comparison of antiviral activity of anti-CMV agents (EC_{50}, μM; AD-169 strain) as monitored by plaque formation, flow cytometry and DNA hybridization

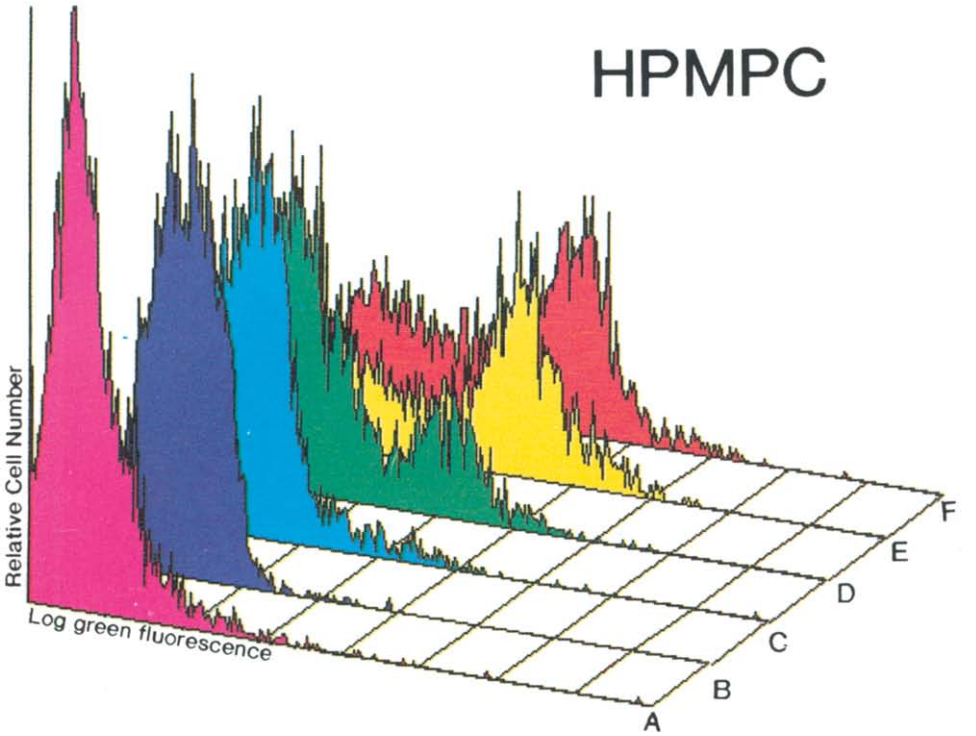
Compound	Plaque formation[a]	Flow cytometry[b]	DNA hybridization[c]
HPMPC	0.18	0.72	0.05
cHPMPC	3.6	1.29	0.10
HPMPA	0.21	0.37	0.05
cHPMPA	0.23	1.78	0.16
HPMPc^{3}A	0.18	1.14	0.18
DHPG	1.9	4.7	0.74

[a] Concentration required to reduce viral plaque formation by 50%.
[b] Concentration required to reduce CMV late antigen expression by 50%; mean of two independent experiments.
[c] Concentration required to reduce viral DNA synthesis by 50%; mean of three independent experiments.

of DHPG. HPMPC and HPMPA may be marginally more active than their derivatives in the flow cytometric assay with an EC_{50} of 0.3–0.7 μM (Table 2). Figs. 1 and 2 show that, as the concentrations of the test compounds (HPMPC, DHPG) increased, the percentage of cells expressing late viral antigens clearly decreased.

In the DNA hybridization assay (Table 2), again HPMPC (EC_{50}: 0.05 μM) and HPMPA (EC_{50}: 0.05 μM) proved more active than DHPG (EC_{50}: 0.74 μM). Among the phosphonyl derivatives, HPMPc^{3}A, cHPMPA and cHPMPC were only slightly less active (EC_{50}: 0.10–0.18 μM) than the parent compounds (HPMPA, HPMPC). Yet, the phosphonyl derivatives were 3–10 times more active in inhibiting viral DNA synthesis than DHPG (Table 2).

Of the test compounds, HPMPC, cHPMPC and DHPG were less toxic than


HPMPC
Relative Cell Number
Log green fluorescence
A
B
C
D
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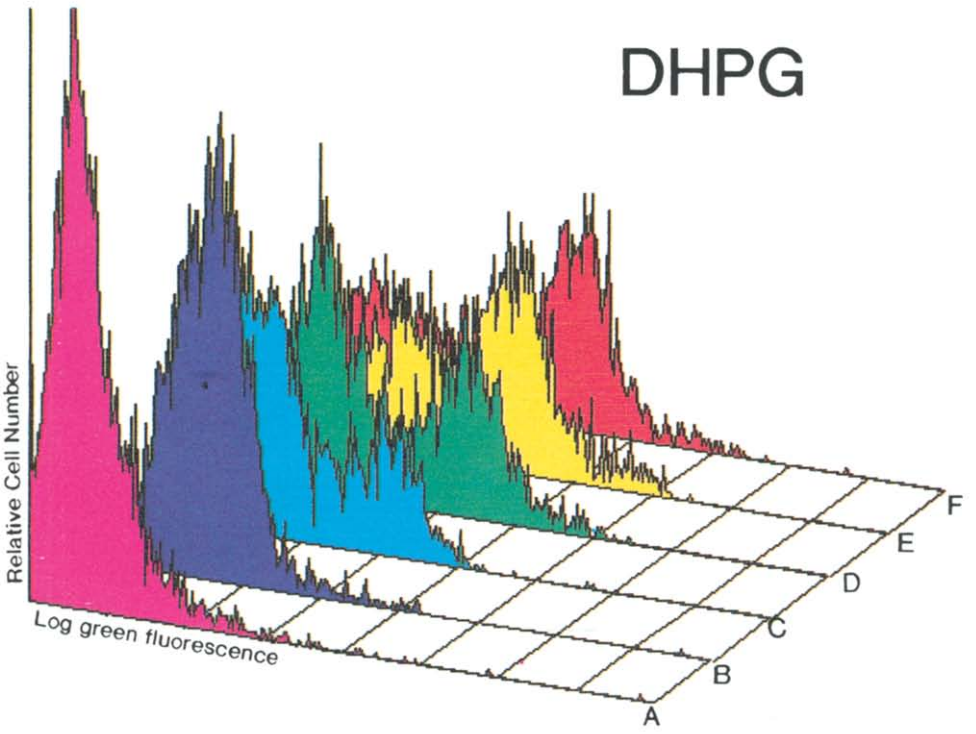
DHPG
Relative Cell Number
Log green fluorescence
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the adenine derivatives HPMPA, cHPMPA and HPMPc^{3}A, whether toxicity measurements were based on inhibition of cell growth or thymidine incorporation. Thus, the greatest selectivity index was achieved by HPMPC and cHPMPC, irrespective of the method used to assess their anti-CMV activity (plaque assay, flow cytometry or DNA hybridization) or cytotoxicity (cell growth or thymidine incorporation). If the selectivity index was defined as the ratio of CC_{50} for inhibition of cell growth to EC_{50} for inhibition of viral plaque formation (Table 1), the selectivity index of HPMPC and cHPMPC was about 1000–1500.

Discussion

The current drugs of choice for the treatment of CMV infections in immunocompromised patients are ganciclovir (DHPG) and foscarnet. They have been used particularly in the treatment of pneumonia in bone-marrow transplant recipients (Klintmalm et al., 1985; Erice et al., 1987). Recently, the association of immunoglobulins with DHPG has proved to contribute to the survival of such patients (Reed et al., 1988). Nevertheless, there is an urgent need for selective and potent anti-CMV agents, and the recently described phosphonylmethoxyalkylpurines and -pyrimidines (HPMPA and HPMPC) show great promise in this regard (De Clercq et al., 1987; Snoeck et al., 1988).

In the present study, HPMPA, HPMPC and some of their derivatives (cHPMPA, cHPMPC and HPMPc^{3}A) were compared for their inhibitory effects on both late CMV antigen expression (measured by flow cytometry) and CMV DNA synthesis (measured by DNA hybridization). With both methods, the parent compounds HPMPA and HPMPC were the most potent anti-CMV agents, and the order of anti-CMV activity obtained with these methods was similar to the order of activity obtained with the plaque assay.

From a comparative study of the three methods to monitor CMV infection in vitro using the same multiplicity of infection, the DNA hybridization assay proved more sensitive than the plaque assay which, in turn, proved more sensitive than the flow cytometric assay, the difference between the least and most sensitive assay methods being roughly 10-fold. Despite this difference in sensitivity, the relative order of activity of the individual compounds remained unchanged (Table 2).

Fig. 1. (opposite page, top). Fluorescence histogram of CMV (AD-169 strain)-infected fibroblasts treated with different concentrations of HPMPC and stained at day 7 with monoclonal antibody F4a. The first row (A) corresponds to the non-specific fluorescence of uninfected cells. The other rows represent infected cells treated with different concentrations of HPMPC: 0 μM (row F), 0.14 μM (row E), 1.4 μM (row D), 14 μM (row C) and 144 μM (row B).

Fig. 2. (opposite page, bottom). Fluorescence histogram of CMV (AD-169 strain)-infected fibroblasts treated with different concentrations of DHPG and stained at day 7 with monoclonal antibody F4a. The first row (A) corresponds to the non-specific fluorescence of uninfected cells. The other rows represent infected cells treated with different concentrations of DHPG: 0 μM (row F), 0.15 μM (row E), 1.5 μM (row D), 15 μM (row C) and 156 μM (row B).

In all assays, HPMPC proved 10-fold more active against CMV than DHPG; these results are in accord with the findings of Neyts et al. (1990), who monitored the effects of HPMPC and DHPG on CMV DNA synthesis (analyzed by CsCl gradient ultracentrifugation). When DHPG and HPMPC were compared for their selectivity as anti-CMV agents, HPMPC also showed a 5–10-fold higher selectivity index than DHPG (Table 1).

From the toxicity data, it appears that the adenine derivatives HPMPA, cHPMPA and HPMPc^{3}A are 5–10-fold more toxic to the host cells than HPMPC, cHPMPC and DHPG. A similar differential toxicity has been observed when these compounds were evaluated for their inhibitory effect on bone marrow cell proliferation (granulocyte-macrophage colony formation; Snoeck et al., 1990).

Flow cytometry and DNA hybridization cannot be recommended as the most appropriate test systems for the large-scale evaluation of new antiviral compounds because of the high costs (i.e. monoclonal antibodies, DNA probes) involved. Yet, these techniques may be quite useful for confirmatory purposes. They also allow quantitative assessment of anti-CMV activity. Whereas the DNA hybridization assay allows a direct quantitation of the inhibitory effects on viral DNA synthesis, flow cytometry may provide additional information on the site of action of the compounds, depending on the choice of the monoclonal antibody (i.e. whether directed against early or late antigens) used in the flow cytometric assay.

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