ELSEVIER

Antiviral Research 28 (1995) 343–357

Antiviral Research

Antiviral activity of selected acyclic nucleoside analogues against human herpesvirus 6

D. Reymen [a,*], L. Naesens [a], J. Balzarini [a], A. Holý [b], H. Dvořáková [b], E. De Clercq [a]

[a] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium*

[b] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic*

Received 2 May 1995; revised 12 July 1995; accepted 12 July 1995

Abstract

Human herpesvirus 6 (HHV-6) was examined in vitro for its sensitivity to a broad range of nucleoside analogues, including acyclovir (ACV), ganciclovir (GCV), penciclovir (PCV), buciclovir (BCV), brivudin (BVDU), the N^7-isomer of 6-deoxyganciclovir (S2242), foscarnet (phosphonoformic acid, PFA), and several acyclic nucleoside phosphonate (ANP) analogues such as (*S*)-HPMPA, (*S*)-HPMPC, PMEA and PMEDAP. Antiviral efficacy was monitored microscopically by the inhibitory effect of the compounds on HHV-6-induced cytopathic effect in human T-lymphoblastoid HSB-2 cells. In addition, a newly developed immunofluorescence/flow cytometric assay (FACS) was used to determine HHV-6-specific antigen expression. A close correlation was observed between the antiviral data obtained by the microscopic assay and the flow cytometric assay. Marked antiviral efficacy was noted for S2242, PFA and the ANP analogues (*S*)-HPMPA, (*S*)-HPMPC, (*S*)-cHPMPC, (*S*)-3-deaza-HPMPA, (*S*)-3-deaza-cHPMPA, (*S*)-HPMPG and (*R*)-HPMPG. Also, PMEA and PMEDAP proved highly active against HHV-6 infection, whereas (*S*)-FPMPA and (*R*)-PMPDAP were inactive. ACV was only slightly protective against HHV-6, and no activity was found for GCV, PCV, BCV and BVDU. Overall, the efficacy of the nucleoside analogues against HHV-6 appeared to correlate with their efficacy against human cytomegalovirus (HCMV).

* Corresponding author. Tel.: +32 (16) 332176; Fax: +32 (16) 337340; E-mail: Diane.Reymen@uz.kuleuven.ac.be.

SSDI 0166-3542(95)00058-5

Keywords: Human herpesvirus 6 (HHV-6); Nucleoside analogues; Acyclic nucleoside phosphonate analogues

1. Introduction

Human herpesvirus 6, one of the most recently discovered members of the herpesviridae, was initially isolated from peripheral blood lymphocytes of patients with the acquired immune deficiency syndrome (AIDS) or with various lymphoproliferative diseases (Josephs et al., 1986; Salahuddin et al., 1986). Although HHV-6 is morphologically similar to other human herpesviruses and may share partial homology with human cytomegalovirus (HCMV) (Efstathiou et al., 1988), molecular analysis and immunological data revealed that HHV-6 is clearly distinct from Epstein–Barr virus (EBV), HCMV, herpes simplex virus types 1 and 2, and varicella-zoster virus (Josephs et al., 1986; Salahuddin et al., 1986). Two genetically distinct variants (A and B) of HHV-6 have been described. Interestingly, most strains that have been isolated from immunosuppressed patients (particularly transplant patients) belong to the B subtype (Dewhurst, 1994). These findings suggest that this subtype may be of higher clinical importance than the A subtype, although the relative prevalence of the two subtypes and their virological effects in healthy and in patient populations remain issues that need to be further explored (Yalcin et al., 1994).

HHV-6 has been implicated in the pathogenesis of a number of diseases. It has been causally related to exanthema subitum (roseola infantum) (Yamanishi et al., 1988), heterophile antigen-negative infectious mononucleosis and possibly Kikuchi–Fujimoto's disease (Krueger and Sander, 1989; Salahuddin et al., 1993). Also, reactivation of latently infected cells may contribute to the progression of chronic fatigue syndrome, certain auto-immune diseases (e.g., systemic lupus erythematosus and Sjögren's syndrome) and various lymphoproliferative disorders (e.g., non-Hodgkin's lymphomas) (Krueger and Sander, 1989; Salahuddin et al., 1993).

HHV-6 has also been proposed to be a cofactor in the progression of AIDS, since the cell tropism of HHV-6 is similar to that of human immunodeficiency virus type 1 (HIV-1). Coinfection of HIV-1-infected lymphocytes by HHV-6 has been demonstrated to increase the cytopathic effect of HIV-1 (Agut et al., 1989; Lusso et al., 1989). The stimulating effect of HHV-6 on HIV-1 replication may be based on transactivation of HIV-1 LTR by HHV-6-specific DNA fragments (Ensoli et al., 1989; Horvat et al., 1989; Thompson et al., 1994; Wang et al., 1994). Also, HHV-6 infection of lymphocytic cells has been shown to upregulate CD4 expression, rendering the cells more susceptible to HIV-1 infection (Lusso et al., 1991). However, the clinical relevance of these in vitro data remains to be elucidated. The high levels of HHV-6 that have been detected in AIDS patients, may be related to an activating role of HHV-6 in the progression of AIDS, or may result from an opportunistic HHV-6 infection in the immunosuppressed patients (Spira et al., 1990; Corbellino et al., 1993).

The present study was aimed at evaluating the in vitro sensitivity of HHV-6 to a number of antiviral nucleoside analogues, known to be effective in the treatment of infections with other herpesviruses or HIV. We have also evaluated the anti-HHV-6

activity of several acyclic nucleoside phosphonate (ANP) analogues. These are novel nucleotide analogues in which an alkyl side-chain containing a phosphonate group, is linked to a purine or pyrimidine base. This class of broad-spectrum antiviral agents exhibit potent and selective activity not only against herpes- and retroviruses, but also against adeno-, pox-, hepadna- and papillomaviruses (De Clercq et al., 1987; De Clercq, 1991, 1993; Balzarini et al., 1993; Naesens et al., 1994). The antiviral activities found with these compounds were compared with their activities against other human herpesviruses, especially HCMV.

2. Materials and methods

2.1. Cells

HSB-2 cells (American Type Culture Collection No. CCL 120.1), an immature T-lymphoblastoid cell line, established in 1966 from peripheral blood cells (PBL) of a patient with acute lymphoblastic leukemia (Adams, 1968) were propagated in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (2 mM), sodium bicarbonate (0.075%) and gentamycin. Cell cultures were incubated at 37°C in a CO_2-controlled incubator.

2.2. Virus

The prototype GS strain of HHV-6 (Salahuddin et al., 1986) was kindly provided by Dr. D.V.M. Ablashi (National Cancer Institute, National Institute of Health, Bethesda, MD., USA). High-titered virus stocks (10^4 $CCID_{50}$/ml, as calculated according to the method of Reed and Münch) were prepared in HSB-2 cells. Therefore, approximately 15×10^6 cells were pelleted, infected with HHV-6 in a 1-ml volume and incubated for 1–2 h at 37°C. Then, the infected cells were resuspended in medium at a concentration of 0.75×10^6 cells/ml. Cell cultures were subcultivated every three to four days by two- to three-fold dilution with fresh medium. Viral growth was examined microscopically by the appearance of cytopathic effect (CPE) and also monitored for antigen expression by immunofluorescence/flow cytometric analysis (FACS analysis; as described below). When CPE was at its maximum (i.e., at seven to ten days after infection), fetal calf serum was added to a final concentration of 20%. The infected cells were then thoroughly resuspended, divided in aliquots and stored at −70°C.

2.3. Compounds

Phosphonoformic acid (foscarnet, PFA) and (*R*)-9-(3,4-dihydroxybutyl)guanine (buciclovir, BCV) were obtained from Astra Läkemedel (Södertälje, Sweden), 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) was purchased from Wellcome Laboratories (Research Triangle Park, NC, USA) and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir, GCV) was from Syntex (Palo Alto, CA, USA). 9-[4-hydroxy-3-(hydroxy-

methyl)butyl]guanine (penciclovir, PCV) was obtained from Hoechst AG (Frankfurt am Main, Germany). (1*R*-1α, 2β, 3α)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine [(−)BHCG] and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) were obtained from Bristol-Myers Squibb (Wallingford, CT, USA). 2-Amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (S2242) was kindly provided by Dr. G. Jähne (Hoechst AG).

(*E*)-5-(2-Bromovinyl)-1-(β-D-deoxyribofuranos-1-yl)uracil (brivudin, BVDU) and 5-iodo-1-(β-D-2-deoxyribofuranos-1-yl)uracil (idoxuridine, IDU) were synthesized by Dr. P. Herdewijn (Rega Institute for Medical Research, Leuven, Belgium). 1-β-D-Arabinofuranosyl (*E*)-5-(2-bromovinyl)uracil (sorivudine, BVaraU) was from Yamasa Shoyu (Choshi, Japan). The acyclic nucleoside phosphonates evaluated were: 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and its bis(pivaloyloxymethyl)-ester [bis(POM)-PMEA], 9-2(-phosphonylmethoxyethyl)guanine (PMEG), 8-aza-PMEG, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP), (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)HPMPA], (*S*)-3-deaza-HPMPA, (*S*)-cyclic-HPMPA [(*S*)-cHMPA], [(*S*)-3-deaza-cyclic-HPMPA [(*S*)-3-deaza-cHPMPA], (*S*)-7-deaza-HPMPA [(*S*)-7-deaza-HPMPA], (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [cidofovir, (*S*)-HPMPC], (*S*)-cyclic-HPMPC [(*S*)-cHPMPC], (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)guanine [(*S*)-HPMPG], (*R*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)guanine [(*R*)-HPMG], (*S*)-cyclic-HPMPG [(*S*)-cHPMPG], (*R*)-cyclic-HPMPG [(*R*)-cHPMPG], (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-2,6-diaminopurine [(*S*)-HPMPDAP], (*S*)-8-aza-HPMPDAP, (*R*)-9-(2-phosphonylmethoxypropyl)-2,6 diaminopurine [(*R*)-PMPDAP] and (*S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine [(*S*)-FPMPA]. All the acyclic nucleoside phosphonate analogues were synthesized by Dr. A. Holý and Dr. H. Dvořáková (Academy of Sciences of the Czech Republic, Prague, Czech Republic) according to the published procedures (Holý et al., 1989; Holý, 1993; Starrett et al., 1992; Dvořáková et al., 1993; Dvořáková and Holý, 1993; Jindřich et al., 1993; Balzarini et al., 1993), except for PMEA, bis(POM)-PMEA, (*S*)-HPMPC and (*S*)-cHPMPC which were kindly provided by Gilead Sciences (Foster City, CA, USA).

2.4. Anti-HHV-6 assay

The inhibitory effect of the compounds on HHV-6 replication in HSB-2 cells was monitored by the inhibition of virus-induced cytopathogenicity (CPE), which was based on the appearance of large refractile cells in HSB-2 cells. Therefore, HSB-2 cells were infected with HHV-6 at a multiplicity of infection (MOI) of 0.001. Immediately after infection, 100 μl of the infected cell suspension was transferred to the wells of a flat-bottomed microtiter plate containing 100 μl of various concentrations of the test compounds. At day 3 and day 7, cell cultures were subcultivated by two-fold dilution with medium containing fresh compound. Microscopical evaluation of viral CPE was performed on day 10. Antiviral activity of the test compounds was expressed as EC_{50}, i.e., the concentration required to inhibit HHV-6-induced cytopathogenicity (CPE) in HSB-2 cells by 50%. The selectivity index was defined as the ratio of the cytostatic (CC_{50}) to the antivirally effective (EC_{50}) concentration (FACS results).

2.5. Flow cytometric assay

After microscopical evaluation, cell cultures were further analyzed by an indirect immunofluorescence/flow cytometric assay (FACS analysis). The cells were centrifuged, washed twice with phosphate-buffered saline, and a high-titered anti-HHV-6 polyclonal antiserum (kind gift from Dr. G.R.F. Krueger, University of Cologne, Germany) was added. After 50 min incubation at 37°C, the cells were washed twice with PBS. Then, the cells were incubated with fluorescein isothiocyanate conjugated $F(ab')_2$-fragments of rabbit anti-human immunoglobulin antibody [RaH-Ig-$F(ab')_2$-FITC] (Prosan, Ghent, Belgium) for 50 min at 37°C, washed once with PBS and fixed in 0.37% paraformaldehyde in PBS. The samples were then analyzed with a fluorescence-activated cell sorter (FACStar, Becton Dickinson, Mountain View, CA, USA) equipped with an argon-ion laser (Spectraphysics Model 164) at 488 nm and 250 mW light output. The green fluorescence was filtered through a 530/30 band pass adsorption filter. For each sample, 5000 to 10,000 cells were analyzed by a CONSORT 30 system (Becton Dickinson). The threshold of positivity for the green fluorescence was arbitrarily set, based on the uninfected control samples. All the data were expressed in a log fluorescence histogram form; a three-dimensional display of the histograms was obtained by the LYSYS software program (Becton Dickinson). The EC_{50}, that is the concentration required to suppress HHV-6-specified antigen expression by 50%, was calculated from the concentration-dependent inhibition curves.

2.6. Cytostatic assay

HSB-2 cells were transferred to the wells of a flat-bottomed microtiter plate and cultured in the presence of different concentrations of the test compounds. The cultures were subcultivated as mentioned above. On day 7, the cells were counted with a Coulter Counter. Cytostatic effects of the compounds were expressed as CC_{50}, or concentration required to inhibit cell growth by 50%.

3. Results

The anti-HHV-6 activity of different nucleoside analogues was investigated in HHV-6-infected HSB-2 T-lymphoblastoid cells. As shown in Fig. 1, infection of the cells resulted in a clear HHV-6-induced cytopathic effect (CPE), i.e., refractile ballooning cells (panel B), as compared to mock-infected cells (panel A). Suppression of viral CPE by (*S*)-HPMPC at a concentration of 4 μg/ml (14 μM), is visualised in panel C. Potential toxicity of the compounds was also scored microscopically [panel D; (*S*)-HPMPC at a concentration of 100 μg/ml (360 μM)]. This toxicity could be easily distinguished from the cytopathic effect of the virus (compare panel B with D). This enabled accurate determination of the antiviral efficiency of the test compounds, relative to their cytostatic effect.

In addition, the efficacy of the test compounds against HHV-6 was determined from their inhibitory effect on HHV-6-induced antigen expression in HHV-6-infected HSB-2

cells, as measured by a newly developed, highly sensitive indirect immunofluorescence/flow cytofluorographic method (FACS analysis). This technique specifically distinguishes between the uninfected and the infected cell population and allows a reliable determination of the percentage of HHV-6-infected cells in the whole cell population. With this method, concentration-dependent inhibition curves were obtained, as exemplified for PFA, PMEDAP, (*S*)-HPMPC and acyclovir (ACV) in Fig. 2.

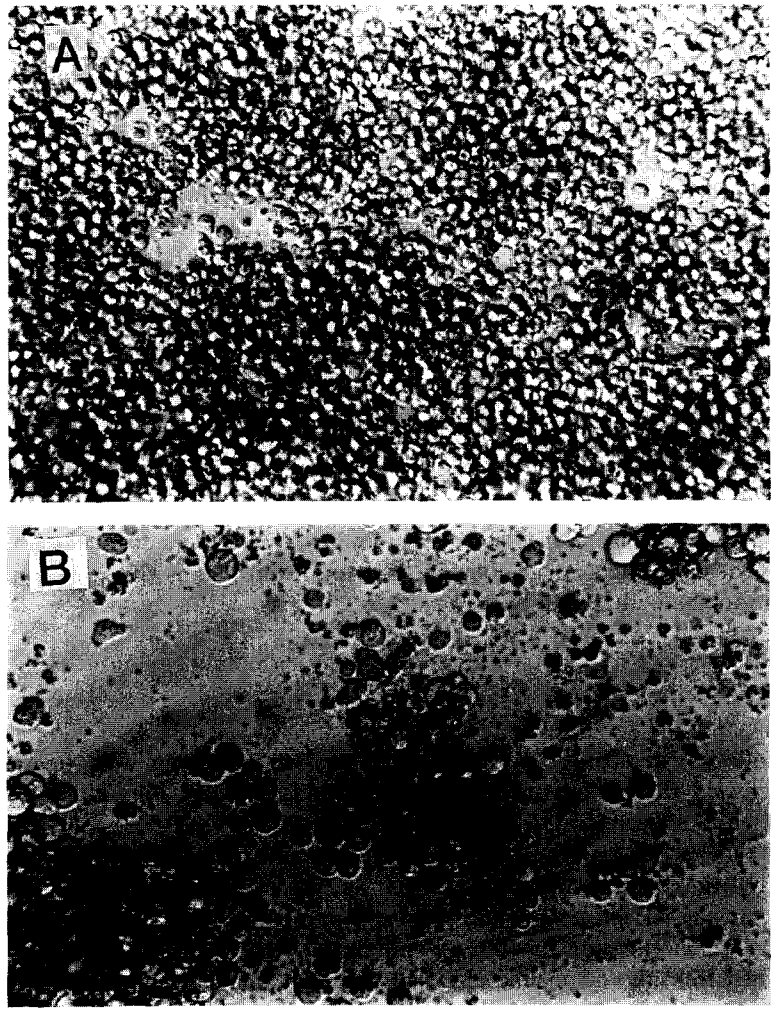

Fig. 1. Inhibitory effect of (*S*)-HPMPC against HHV-6-induced cytopathic effect (CPE) in HHV-6-infected human T-lymphoblastoid HSB-2 cells. Panel A: mock-infected cells; Panel B: HHV-6-infected HSB-2 cells at day 7 post infection; Panel C: HHV-6-infected cells treated with (*S*)-HPMPC at a concentration of 4 μg/ml; Panel D: mock-infected cells treated with (*S*)-HPMPC at a concentration of 100 μg/ml.

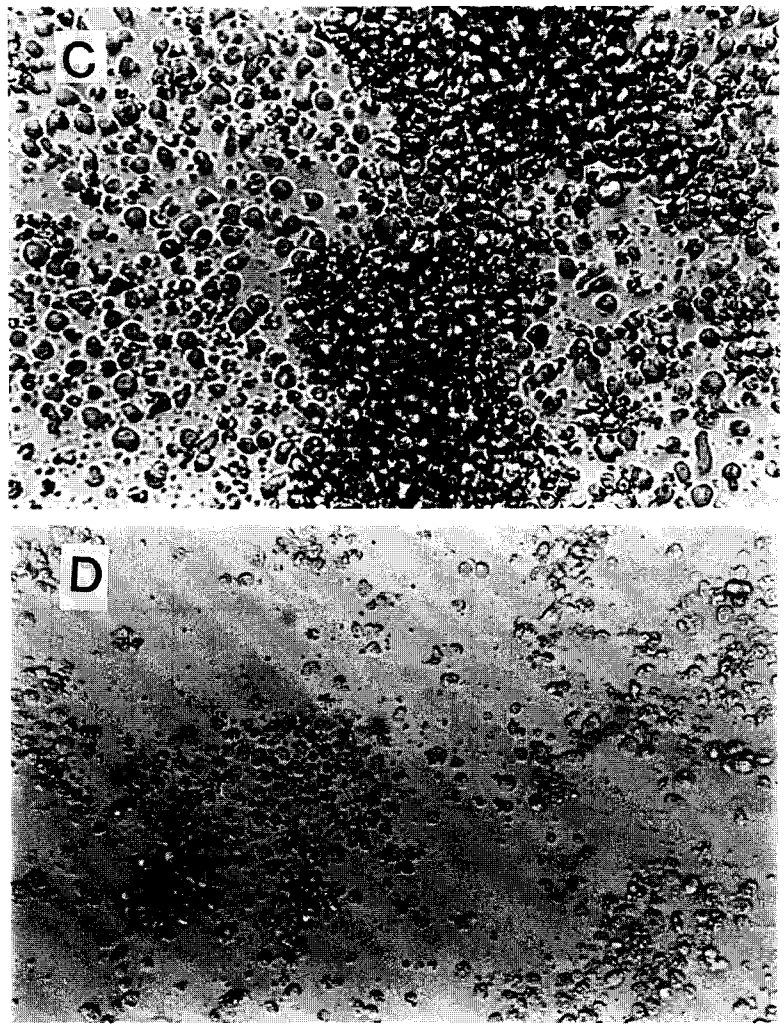

Fig. 1 (continued).

PFA was a potent inhibitor of HHV-6 replication with an EC_{50} of 5.8 μM, as measured by the indirect immunofluorescence/flow cytometric assay (Table 1; FACS). The selectivity index of PFA, or ratio of cytostatic over antivirally effective concentration, was 141. These data are in agreement with those published elsewhere (Agut et al., 1989a, 1991; Streicher et al., 1988). Therefore, PFA was used as a reference compound throughout the subsequent antiviral activity and cytotoxicity experiments.

Acyclovir (ACV) had a rather poor protective effect with an EC_{50} of 270 μM and a selectivity index of only 3. However, compounds that are structurally related to ACV, namely, ganciclovir (GCV), buciclovir (BCV) and penciclovir (PCV) displayed no

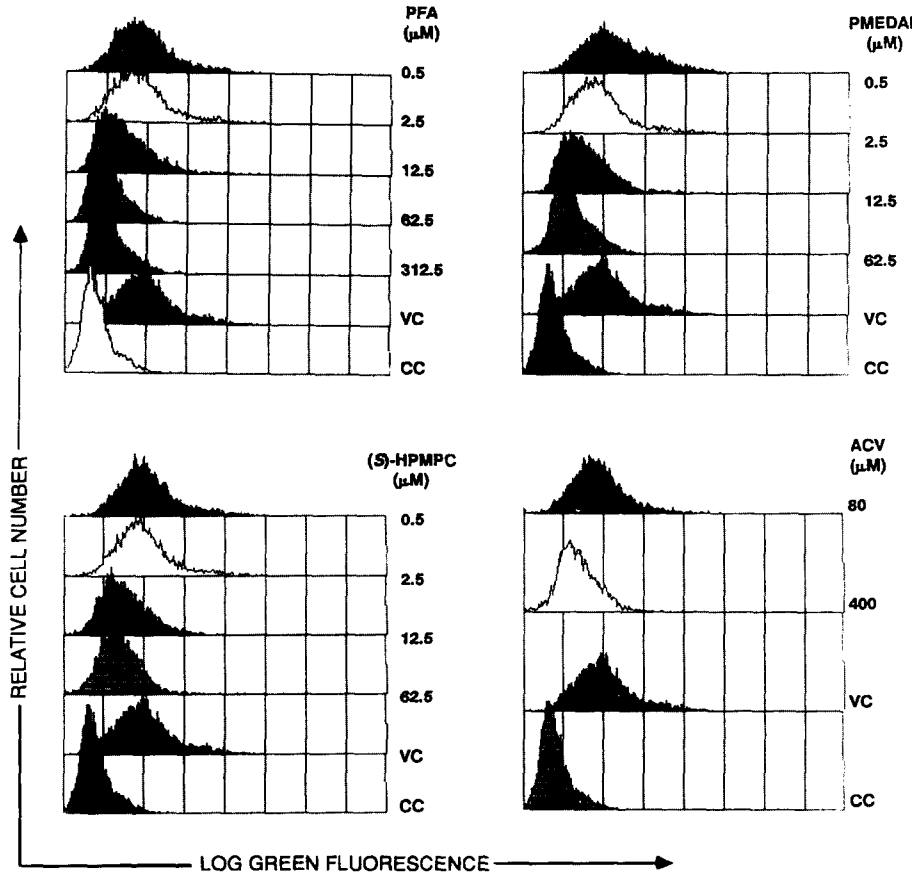

Fig. 2. Inhibitory effect of PFA, PMEDAP, (*S*)-HPMPC and acyclovir (ACV) against HHV-6-specific antigen expression in HHV-6-infected HSB-2 cells, as determined by indirect immunofluorescence/flow cytometric analysis (FACS). CC = mock-infected cell control; VC = virus-infected cell control.

anti-HHV-6 activity at subtoxic concentrations (Table 1). Nor did FIAC, BVDU and its congeners BVaraU and IDU. In contrast (−)BHCG displayed moderate anti-HHV-6 activity with and EC_{50} of 8.4 μM and a selectivity index of 4. Marked antiviral activity was also noted for the N^7-isomer of 6-deoxy-ganciclovir S2242 (EC_{50}: 0.02 μM, selectivity index: 42), which is in agreement with the data reported by Neyts et al. (1994).

Several acyclic nucleoside phosphonates displayed a marked inhibitory effect on HHV-6 replication. The highest antiviral activity was noted with the HPMP derivatives, their EC_{50} values being (in increasing order): 1.2 μM [(*S*)-3-deaza-HPMPA], 3.7 μM [(*S*)-3-deaza-cHPMPA], 5.6 μM [(*S*)-cHPMPC], 5.6 μM [(*S*)-HPMPG], 6.2 μM [(*R*)-HPMPG], 10 μM [(*S*)-cHPMPG] and 11 μM [(*S*)-HPMPC]. For these compounds, the selectivity index was 10–30. A strong anti-HHV-6 activity was also observed for the PME derivatives PMEA and its bis(POM) ester prodrug bis(POM)-PMEA, PMEG and

Table 1
Antiviral efficacy of selected nucleoside analogues in HHV-6 infected HSB-2 cells

Compound [a]	EC_{50} [b] (μM) based on		CC_{50} [c] (μM)	Selectivity index [d]
	CPE	FACS		
PFA	8.4 ± 4.8	5.8 ± 2.3	818 ± 316	141
S2242	0.03 ± 0.02	0.02 ± 0.003	0.85 ± 0.22	42
PMEDAP	11 ± 4.1	9.1 ± 6.1	164 ± 23	18
PMEA	30 ± 14.3	28 ± 22.1	175 ± 38	6
bis-(POM)-PMEA	0.56 ± 0.08	0.53 ± 0.1	1.9 ± 1.4	4
PMEG	1.7 ± 0.4	1.5 ± 0.9	1.6 ± 0.2	1
8-aza-PMEG	> 25	> 25	45 ± 1.6	–
(*S*)-HPMPA	10 ± 3.6	8.0 ± 3.0	46 ± 9	6
(*S*)-cHPMPA	42 ± 1.3	32 ± 14.8	146 ± 47	5
(*S*)-3-deaza-HPMPA	1.4 ± 0.25	1.2 ± 0.3	36 ± 0.5	30
(*S*)-3-deaza-cHPMPA	3.6 ± 1.3	3.7 ± 2.7	45 ± 30	12
(*S*)-7-deaza-HPMPA	> 10	> 10	12 ± 2	–
(*S*)-HPMPG	4.9 ± 0.9	5.6 ± 0.5	122	22
(R)-HPMPG	5.6 ± 0.01	6.2 ± 0.2	87	14
(*S*)-cHPMPG	6.9 ± 1.3	10 ± 4.1	153 ± 32	15
(*R*)-c-HPMPG	> 25	> 25	57 ± 40	–
(*S*)-HPMPDAP	26 ± 3.1	27 ± 8.5	404 ± 147	15
(*S*)-8-aza-HPMPDAP	> 300	> 300	> 300	–
(*S*)-HPMPC	14 ± 6.6	11 ± 10.6	141 ± 16	13
(*S*)-c-HPMPC	6.3 ± 3.0	5.6 ± 5.5	109 ± 86	20
(*S*)-FPMPA	> 300	> 300	> 300	–
(*R*)-PMPDAP	> 300	> 300	> 300	–
ACV	179 ± 52	270 ± 70	861 ± 73	3
GCV	> 25	> 25	54 ± 2.0	–
BCV	> 200	> 200	277 ± 53	–
PCV	> 400	> 400	ND	–
(−)BHCG	7.4 ± 0.0	8.4 ± 0.4	37 ± 8.0	4
FIAC	> 1	> 1	2.4 ± 2.7	–
BVDU	> 1	> 1	1.2 ± 0.8	–
IDU	> 1500	> 1500	> 1500	–
BVaraU	> 75	> 75	> 75	–

ND = not determined.
[a] For abbreviations: see section 2.3.
[b] Antiviral activity was expressed as EC_{50}, or compound concentration required to exert a 50% inhibition of HHV-6-induced cytopathic effect (CPE), or a 50% inhibition of HHV-6-specific antigen expression, as determined by immunofluorescence/flow cytometric analysis (FACS).
[c] Cytostatic effect was expressed as CC_{50}, or compound concentration that reduced cell growth by 50%.
[d] Selectivity index was defined as the ratio of CC_{50} over EC_{50} (as determined by FACS analysis).

PMEDAP, the latter having an EC_{50} of 9.1 μM and a selectivity index of 18. In contrast, no anti-HHV-6 activity was observed for the PMP and FPMP derivatives.

In parallel with FACS analysis, microscopy was performed to determine the inhibitory effect of the test compounds on HHV-6-induced cytopathogenicity. The microscopical data showed a close correlation with the results obtained by the indirect immunofluorescence/flow cytometric method (Table 1). A correlation coefficient of

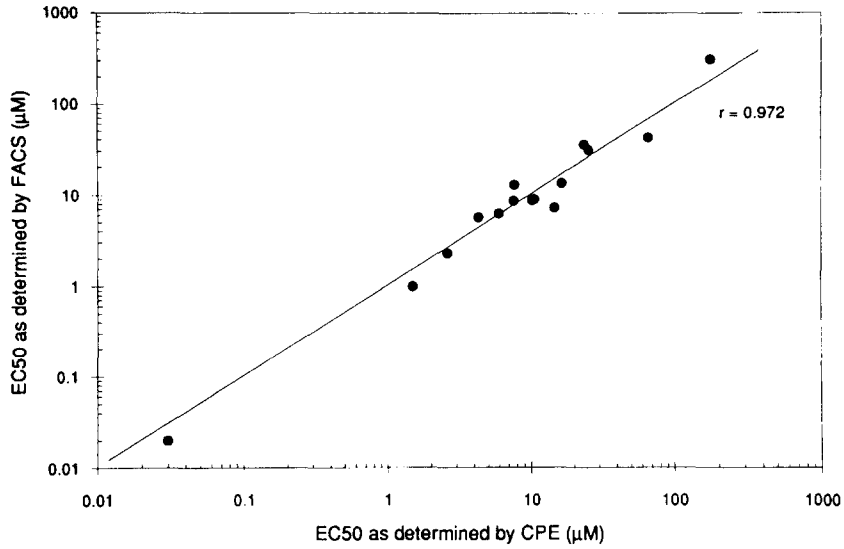

Fig. 3. Comparison of the antiviral data based on HHV-6-specific antigen detection, as determined by immunofluorescence/flow cytometric analysis (FACS), and on microscopical evaluation of HHV-6-induced cytopathic effect (CPE). Data shown are the EC_{50} values extracted from Table 1, and for: PFA, compound S2242, ACV, PMEDAP, PMEA, (*S*)-HPMPA, (*S*)-HPMPC, (*S*)-HPMPDAP, (*S*)-3-deaza-HPMPA, (*S*)-3-deaza-cHPMPA, (*S*)-cHPMPA, (*S*)-HPMPG, (*R*)-HPMPG, (*S*)-cHPMPG and (*S*)-cHPMPC.

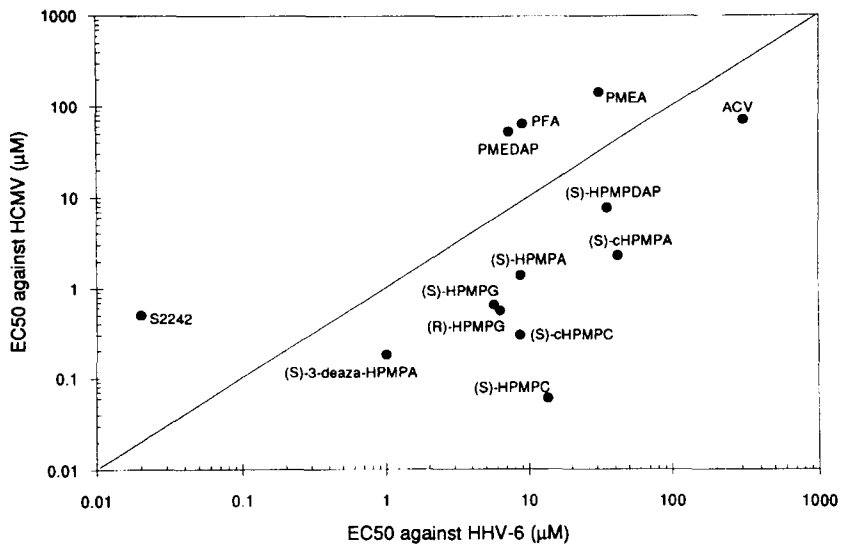

Fig. 4. Comparison of the anti-HHV-6 activity (FACS results) of several nucleoside analogues and their efficacy against HCMV [data taken from Snoeck et al. (1988), Andrei et al. (1991), Balzarini et al. (1993) and Neyts et al. (1994)].

0.972 was calculated upon correlation analysis of the EC_{50} values obtained by the flow cytometric method and those obtained by the microscopic assay (Fig. 3).

4. Discussion

Among the different compounds examined here, PFA emerged as the most selective anti-HHV-6 agent, with an EC_{50} of 5.8 μM (as measured by FACS analysis) and a selectivity index of 141. This result is in agreement with the data obtained by several other groups (Streicher et al., 1988; Agut et al., 1989a, 1991). Overall, PFA and its closely related pyrophosphate analogue phosphonoacetic acid appear as strong inhibitors of HHV-6 replication. Therefore, PFA can be regarded as a reference compound for HHV-6 drug sensitivity determinations. In contrast, literature data (Streicher et al., 1988; Russler et al., 1989; Agut et al., 1989a, 1991; Burns and Sandford, 1990; Di Luca et al., 1990) on the anti-HHV-6 activity of ACV and related acyclic nucleoside analogues show less consistency. It should be noted that these reported results were obtained under different experimental conditions, i.e., with different HHV-6 strains, in different cell types and using various procedures for viral infection.

These differences in sensitivity appear not to be related to the HHV-6 strain used since Agut et al. (1991) have demonstrated homogeneous susceptibility of different HHV-6 isolates from HHV-6 subtype A or B to a number of antiviral compounds in vitro. However, the cell type used may be a more important factor in the discrepancies seen in the antiviral results. Data on efficacy of ACV and GCV against HHV-6 have in all previous cases been obtained in fresh human lymphocytes (Streicher et al., 1988; Russler et al., 1989; Agut et al., 1989a, 1991; Burns and Sandford, 1990), the antiviral concentrations ranging from 2 to 20 μM for ACV and from 1 to 25 μM for GCV. In HSB-2 cells, we and others (Streicher et al., 1988) observed only moderate anti-HHV-6 activity for ACV and little if any activity for GCV.

Several authors (Streicher et al., 1988; Burns and Sandford., 1990; Di Luca et al., 1990; Gompels et al., 1995) have suggested that HHV-6 lacks its own phosphorylating enzymes [such as viral thymidine kinase (TK)]. Thus it appears that, in order to exert their antiviral effect on HHV-6, ACV and GCV merely depend on the phosphorylating capacity of the cells, which may differ considerably from one cell type to another. The inactivity of BVDU and related analogues (i.e., BVaraU) against HHV-6, may be explained by the absence of a virus-encoded TK in the HHV-6-infected HSB-2 cells. In contrast to nucleoside analogues which are substrate analogues of the DNA polymerase, PFA interacts with the active site of the enzyme as a product analogue of pyrophosphate. In this context, the consistency in the antiviral data for PFA is most probably related to the fact that no phosphorylation is required for PFA to be active against the viral DNA polymerase.

This is the first report on the anti-HHV-6 activity of the acyclic nucleoside phosphonate (ANP) analogues. Interestingly, several compounds belonging to the HPMP series emerged as potent and selective inhibitors of HHV-6: (*S*)-HPMPC, (*S*)-cHPMPC, (*S*)-HPMPA, (*S*)-3-deaza-HPMPA, (*S*)-3-deaza-cHPMPA, (*S*)-HPMPG, (*S*)-cHPMPG and (*R*)-HPMPG, their EC_{50} values ranging from 1 to 11 μM and their selectivity index

ranging from 6 to 30. Thus, the broad-spectrum anti-herpesvirus activity of the HPMP analogues extends to HHV-6.

Marked anti-HHV-6 effect was also noted with the PME compounds PMEA and PMEDAP (EC_{50}: 28 μM and 9.1 μM, respectively; selectivity index: 6 and 18, respectively), which have previously been shown to inhibit retroviruses as well as herpesviruses (Balzarini et al., 1989, 1991; Naesens et al., 1989, 1994). In contrast, (*S*)-FPMPA and (*R*)-PMPDAP, which are known to be active against retroviruses, but not herpesviruses (Balzarini et al., 1991a, 1993), likewise failed to show efficacy against HHV-6.

The activity of the ANP analogues against HHV-6 was compared with their activity against HCMV (Fig. 4). To plot this figure, data for HHV-6 were taken from Table 1 (FACS analysis), whereas the HCMV data were taken from the literature (Snoeck et al., 1988; Andrei et al., 1991; Balzarini et al., 1993; Neyts et al., 1994). It should be taken into account that the activity against HHV-6 and HCMV was determined at different occasions, in different cells and by different methods. A better correlation may have been found if experiments with both viruses could have been done in the same cells. Nevertheless, it appears that the DNA polymerases of HCMV and HHV-6, which have been shown to be structurally related, also display a similar sensitivity to polymerase inhibitors (Bapat et al., 1989).

HHV-6 seems to be sensitive to those nucleoside analogues, such as (*S*)-HPMPC and other ANP analogues, that for their antiviral activity do not depend on activation by the viral TK (Neyts et al., 1990). It is generally hypothesized that the anti-HCMV activity of (*S*)-HPMPC is based on inhibition of HCMV DNA polymerase by the active metabolite (*S*)-HPMPCpp, which is formed from (*S*)-HPMPC by cellular enzymes (Ho et al., 1991; Sullivan et al., 1993). Further studies are needed to reveal whether (*S*)HPMPCpp, and the ANP analogues in general, act against HHV-6 according to the same mechanism as for HCMV.

Several authors have suggested, based on in vitro data, that HHV-6 may enhance HIV replication and play a crucial role in the progression of AIDS (Agut et al., 1989; Ensoli et al., 1989; Horvat et al., 1989; Lusso et al., 1989, 1991; Thompson et al., 1994; Wang et al., 1994). The clinical implications of these in vitro findings remain to be clarified (Spira et al., 1990). Also, the clinical role of HHV-6 as an opportunistic pathogen in HIV-infected individuals remains unclear. Nevertheless, compounds that inhibit HHV-6 replication may be beneficial in the treatment of AIDS patients, either directly by suppressing an opportunistic HHV-6 infection, or indirectly by inhibiting the stimulation of HIV replication by HHV-6. In this context, the dual antiviral activity of PMEA and PMEDAP against both HIV and HHV-6 makes these compounds of potentially great value in the treatment of HIV-infected individuals.

In conclusion, from the present studies, several acyclic nucleoside phosphonate analogues proved to be efficient against HHV-6. The highest potency and selectivity was exhibited by the HPMP congeners [i.e., (*S*)-HPMPC], and these agents should be further explored in their own right for the therapy of HHV-6 infections. Also, clinical studies should be performed to assess the potential of PMEA and PMEDAP in the treatment of AIDS patients suffering from concurrent infections by herpesviruses such as HHV-6. Biochemical studies are underway to delineate which enzymes are involved in the

activation and antiviral action of the ANP analogues. Furthermore, we are planning to evaluate in vitro the efficacy of the ANP analogues against dual infections by HIV and HHV-6.

Acknowledgements

We thank Christiane Callebaut for fine editorial help. Diane Reymen is a Research Assistant from the Belgian "Nationaal Fonds voor Wetenschappelijk Onderzoek" (NFWO). This work was supported by grants from the Biomedical Research Programme of the European Community, the Belgian Fonds for Geneeskundig Wetenschappelijk Onderzoek (Krediet No. 3.0180.95), the Belgian Geconcerteerde Onderzoeksacties (Krediet No. 95/5), and the Grant Agency of the Czech Academy of Sciences (No. 455407).

References

Adams, R.A. (1968) Direct implantation and serial transplantation of human acute lymphoblastic leukemia in hamsters, SB-2. Cancer Res. 23, 1121–1125.

Agut, H., Kérouédan, D., M'Pelé, P., Collandre, H., Samba-Lefèvre, C., Rosenheim, M., Ingrand, D., Itoua-Ngaporo, A., Gentilini, M., Montagnier, L. and Huraux, J.M. (1989) Co-cultivation of human herpesvirus 6 and HIV-1 from blood lymphocytes. Res. Virol. 140, 23–26.

Agut, H., Collandre, H., Aubin, J.-T., Guétard, D., Favier, V., Ingrand, D., Montagnier, L. and Huraux, J.-M. (1989a) In vitro sensitivity of human herpesvirus-6 to antiviral drugs. Res. Virol. 140, 219–228.

Agut, H., Aubin, J.-T. and Huraux, J.-M. (1991) Homogeneous susceptibility of distinct human herpesvirus 6 strains to antivirals in vitro. J. Infect. Dis. 163, 1382–1383.

Andrei, G., Snoeck, R., Schols, D., Goubau, P., Desmyter, J. and De Clercq, E. (1991) Comparative activity of selected antiviral compounds against clinical isolates of human cytomegalovirus. Eur. J. Clin. Microbiol. Infect. Dis. 10, 1026–1033.

Balzarini, J., Naesens, L., Herdewijn, P., Rosenberg, I. Holý, A., Pauwels, R., Baba, M., Johns, D.G. and De Clercq, E. (1989) Marked in vivo antiretrovirus activity of 9-(2-phosphonylmethoxyethyl)adenine, a selective anti-human immunodeficiency virus agent. Proc. Natl. Acad. Sci. USA 86, 332–336.

Balzarini, J. Naesens, L., Slachmuylders, J., Niphuis, H., Rosenberg, I., Holý, A., Schellekens, H. and De Clercq, E. (1991) 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) effectively inhibits retrovirus replication in vitro and simian immunodeficiency virus infection in rhesus monkeys. AIDS 5, 21–28.

Balzarini, J., Holý, A., Jindřich, J., Dvořáková, H., Hao, Z., Snoeck, R., Herdewijn, P., Johns, D.G. and De Clercq, E. (1991a) 9-[(2*RS*)-3-fluoro-2-phosphonylmethoxypropyl] derivatives of purines: a class of highly selective antiretroviral agents in vitro and in vivo. Proc. Natl. Acad. Sci. USA 88, 4961–4965.

Balzarini, J., Holý, A., Jindřich, J., Naesens, L., Snoeck, R., Schols, D. and De Clercq, E. (1993) Differential antiherpesvirus and antiretrovirus effects of the (*S*) and (*R*) enantiomers of acyclic nucleoside phosphonates: potent and selective in vitro and in vivo antiretrovirus activities of (*R*)-9-(2-phosphonomethoxypropyl)-2,6-diaminopurine. Antimicrob. Agents Chemother. 37, 332–338.

Bapat, A.R., Bodner, A.J., Ting, R.C.Y. and Cheng, Y.-C. (1989) Identification and some properties of a unique DNA polymerase from cells infected with human B-lymphotropic virus. J. Virol. 63, 1400–1403.

Burns, W.H. and Sandford, G.R. (1990) Susceptibility of human herpesvirus 6 to antivirals in vitro. J. Infect. Dis. 162, 634–637.

Corbellino, M., Lusso, P., Gallo, R.C., Parravicini, C., Galli, M. and Moroni, M. (1993) Disseminated human herpesvirus 6 infection in AIDS. Lancet 342, 1242.

De Clercq, E. (1991) Broad-spectrum anti-DNA virus and anti-retrovirus activity of phosphonylmethoxyalkylpurines and -pyrimidines. Biochem. Pharmacol. 42, 963–972.

De Clercq, E. (1993) Therapeutic potential of HPMPC as an antiviral drug. Rev. Med. Virol. 3, 85–96.

De Clercq, E., Sakuma, T., Baba, M., Pauwels, R., Balzarini, J., Rosenberg, I. and Holý, A. (1987) Antiviral activity of phosphonylmethoxyalkyl derivatives of purines and pyrimidines. Antiviral Res. 8, 261–272.

Dewhurst, S. (1994) Newly discovered human herpesviruses (HHV-6 and HHV-7). Curr. Opinion Infect. Dis. 7, 238–245.

Di Luca, D., Katsafanas, G., Schirmer, E.C., Balachandran, N. and Frenkel, N. (1990) The replication of viral and cellular DNA in human herpesvirus 6-infected cells. Virology 175, 199–210.

Dvořáková, H. and Holý, A. (1993) Synthesis and biological effects of N-(2-phosponomethoxyethyl)derivatives of deazapurine bases. Collect. Czech. Chem. Commun. 58, 1419–1429.

Dvořáková, H., Holý, A. and Alexander, P. (1993) Synthesis and biological effects of 9-((3-hydroxy-2-phosphonylmethoxypropyl)derivatives of deazapurine bases. Collect. Czech. Chem. Commun. 58, 1403–1418.

Efstathiou, S., Gompels, U.A., Craxton, M.A., Honess, R.W. and Ward, K. (1988) DNA homology between a novel human herpesvirus (HHV-6) and human cytomegalovirus. Lancet i, 63–64.

Ensoli, B., Lusso, P., Schachter, S., Josephs, S.F., Rappaport, J., Negro, F., Gallo, R.C. and Wong-Staal, F. (1989) Human herpes virus-6 increases HIV-1 expression in co-infected T cells via nuclear factors binding to the HIV-1 enhancer. EMBO J. 8, 3019–3027.

Gompels, U.A., Nicholas, J., Lawrence, G., Jones, M., Thomson, B.J., Martin, M.E.D., Efstathiou, S., Craxton, M. and Macaulay, H.A. (1995) The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. Virology 209, 29–51.

Ho, H.-T., Woods, K.L., Bronson, J.J., De Boeck, H., Martin, J.C. and Hitchcock, M.J.M. (1991) Intracellular metabolism of the antiherpes agent (*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine. Mol. Pharmacol. 41, 197–202.

Holý, A. (1993) Synthesis of enantiomeric N-(3-hydroxy-2-phosphonomethoxypropyl)derivatives of purine and pyrimidine bases. Collect. Czech. Chem. Commun. 58, 649–674.

Holý, A., Rosenberg, I. and Dvořáková, H. (1989) Synthesis of (3-hydroxy-2-phosphonylmethoxypropyl)derivatives of heterocyclic bases. Collect. Czech. Chem. Commun. 54, 2470–2501.

Horvat, R.T., Wood, C. and Balachandran, N. (1989) Transactivation of human immunodeficiency virus promoter by human herpesvirus 6. J. Virol. 63, 970–973.

Jindřich, J., Holý, A. and Dvořáková, H. (1993) Synthesis of N-(3-fluoro-2-phosphonomethoxypropyl)derivatives of heterocyclic bases. Collect. Czech. Chem. Commun. 58, 1645–1667.

Josephs, S.F., Salahuddin, S.Z., Ablashi, D.V., Schachter, F., Wong-Staal, F. and Gallo, R.C. (1986) Genomic analysis of the human B-lymphotropic virus (HBLV). Science 234, 601–603.

Krueger, G.R.F. and Sander, Ch. (1989) What's new in human herpesvirus-6? Clinical immunopathology of the HHV-6 infection. Pathol. Res. Pract. 185, 915–929.

Lusso, P., Ensoli, B., Markham, P.D., Ablashi, D.V., Salahuddin, S.Z., Tschachler, E., Wong-Staal, F. and Gallo, R.C. (1989) Productive dual infection of human $CD4^+$ T lymphocytes by HIV-1 and HHV-6. Nature 337, 370–373.

Lusso, P., De Maria, A., Malnati, M., Lori, F., DeRocco, S.E., Baseler, M. and Gallo, R.C. (1991) Induction of CD4 and susceptibility to HIV-1 infection in human CD8+T lymphocytes by human herpesvirus 6. Nature 349, 533–535.

Naesens, L., Balzarini, J., Rosenberg, I., Holý, A. and De Clercq, E. (1989) 9-(-2-Phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP): a novel agent with anti-human immunodeficiency virus activity in vitro and potent anti-Moloney murine sarcoma virus activity in vivo. Eur. J. Clin. Microbiol. Infect. Dis. 8, 1043–1047.

Naesens, L. Balzarini, J. and De Clercq, E. (1994) Therapeutic potential of PMEA as an antiviral drug. Rev. Med. Virol. 4, 147–159.

Neyts, J., Snoeck, R., Schols, D., Balzarini, J. and De Clercq, E. (1990) Selective inhibition of human cytomegalovirus DNA synthesis by (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(*S*)-HPMPC] and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG). Virology 179, 41–50.

Neyts, J., Andrei, G., Snoeck, R., Jähne, G., Winkler, I., Helsberg, M., Balzarini, J. and De Clercq, E. (1994) The N-7-substituted acyclic nucleoside analog 2-amino-7-[1,3-dihydro-2-propoxy)methyl]purine is a potent and selective inhibitor of herpesvirus replication. Antimicrob. Agents Chemother. 38, 2710–2716.

Russler, S.K., Tapper, M.A. and Carrigan, D.R. (1989) Susceptibility of human herpesvirus 6 to acyclovir and ganciclovir. Lancet ii, 382.

Salahuddin, S.Z., Ablashi, D.V., Markham, P.D., Josephs, S.F., Sturzenegger, S., Kaplan, M., Halligan, G., Biberfeld, P., Wong-Staal, F., Kramarsky, B. and Gallo, R.C. (1986) Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science 234, 596–601.

Salahuddin, S.Z., Kelley, A.S., Krueger, G.R.F., Josephs, S.F., Gupta, S. and Ablashi, D.V. (1993) Human herpes virus-6 (HIV-6) in diseases. Clin. Diagn. Virol. 1, 81–100.

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

Spira, T.J., Bozeman, L.H., Sanderlin, K.C., Warfield, D.T., Feorino, P.M., Holman, R.C., Kaplan, J.E., Fishbein, D.B. and Lopez, C. (1990) Lack of correlation between human herpesvirus-6 infection and the course of human immunodeficiency virus infection. J. Infect. Dis. 161, 567–570.

Starrett, J.E. Tortolani, D.R., Hitchcock, M.J.M., Martin, J.C. and Mansuri, M.M. (1992) Synthesis and in vitro evaluation of a phosphonate prodrug (bis(pivaloyloxymelthyl)-9-(2-phosphonylmethoxyethyl)adenine. Antiviral Res. 19, 267–274.

Streicher, H.Z., Hung, C.L., Ablashi, D.V., Hellman, K. Saxinger, C., Fullen, J. and Salahuddin, S.Z. (1988) In vitro inhibition of human herpesvirus-6 by phosphonoformate. J. Virol. Methods 21, 301–304.

Sullivan, V., Biron, K.K., Talarico, C., Stanat, S.C., Davis, M., Pozzi, L.M. and Coen, D.M. (1993) A point mutation in the human cytomegalovirus DNA polymerase gene confers resistance to ganciclovir and phosphonylmethoxyalkyl derivatives. Antimicrob. Agents Chemother. 37, 19–25.

Thompson, J., Choudhury, S., Kashanchi, F., Doniger, J., Berneman, Z., Frenkel, N. and Rosenthal, L.J. (1994) A transforming fragment within the direct repeat region of human herpesvirus type 6 that transactivates HIV-1. Oncogene 9, 1167–1175.

Wang, J., Jones, C., Norcross, M., Bohnlein, E. and Razzaque, A. (1994) Identification and characterization of a human herpesvirus 6 gene segment capable of transactivating the human immunodeficiency virus type 1 long terminal repeat in an Sp1 binding site-dependent manner. J. Virol. 68, 1706–1713.

Yalcin, S., Karpuzoglu, T., Suleymanlar, G., Mutlu, G., Mkai, T., Yamamoto, T., Isegawa, Y. and Yamanishi, K. (1994) Human herpesvirus 6 and human herpesvirus 7 infections in renal transplant recipients and healthy adults in Turkey. Arch. Virol. 136, 183–190.

Yamanishi, K., Okuno, T., Shiraki, K., Takahashi, M., Kondo, T., Asano, Y. and Kurata, T. (1988) Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet ii, 1065–1067.