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Inhibition of herpes simplex virus DNA polymerase by diphosphates of acyclic phosphonylmethoxyalkyl nucleotide analogues

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Summary

The inhibition of HSV-1 DNA polymerase and HeLa DNA polymerases α and β by diphosphoryl derivatives of acyclic phosphonylmethoxyalkyl nucleotide analogues was studied and compared with the inhibition by ACV-TP, araCTP, ddTTP and AZT-TP. In the series of phosphonylmethoxyethyl (PME-) derivatives of heterocyclic bases, the inhibitory effect of their diphosphates on HSV-1 DNA polymerase decreased in the order 2-amino-PMEApp (K_i = 0.03 μM) >> PMEGpp > PMEApp > PMETpp >> PMECpp >> n^8z^7PMEApp > PMEUpp. The diphosphate derivative of the antiherpes agent (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine (HPMPA) proved to be a relatively weak inhibitor of HSV-1 DNA polymerase (K_i = 1.4 μM). The inhibitors could be divided into three groups: (a) the diphosphoryl derivatives of acyclic nucleotide analogues (PME-type and HPMPA) and ACV-TP specifically inhibit HSV-1 DNA polymerase and DNA polymerase α and do not significantly inhibit DNA polymerase β; (b) AZT-TP and ddTTP are effective only against DNA polymerase β, and (c) araCTP inhibits all three enzymes. When dATP was omitted from the reaction mixture, the addition of HPMPApp stimulated DNA synthesis by HSV-1 DNA polymerase indicating that HPMPApp is an alternative substrate for in vitro DNA synthesis catalyzed by this enzyme.

DNA polymerase inhibition; Herpes simplex virus; Phosphonylmethoxyalkyl-purine; -pyrimidine; HPMPA; PMEA

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Introduction

(S)-9-(3-Hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA), 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and other related 2-phosphonylmethoxyethyl (PME-) nucleotide analogues derived from purine and pyrimidine bases were found to suppress a wide array of DNA viruses, including most types of herpesviruses. They exhibit the same activity against both wild types of herpesviruses and strains with altered or deficient thymidine kinase (De Clercq et al., 1986, 1987; Holý et al., 1989); unlike most nucleoside antivirals, these compounds do not depend on phosphorylation by viral thymidine kinase (Merta et al., in press; Votruba et al., 1987). HPMPA is accumulated and converted to mono- (HPMPAp) and diphosphoryl (HPMPApp) derivatives both in HSV-1-infected and mock-infected VERO and HEL cells (Votruba et al., 1987). HPMPA and PMEA are also phosphorylated by cellular kinases of L1210 cell-free extracts (Merta et al., 1990). HPMPA effectively inhibits the synthesis of viral DNA under conditions where de novo synthesis of cellular DNA is not affected. HPMPA is incorporated into cellular DNA to a low but significant extent. However, its incorporation into viral DNA has not yet been confirmed (Votruba et al., 1987).

The above findings suggested that the virus encoded DNA polymerase might be the target enzyme for the compounds mentioned. To obtain information useful for designing new selective inhibitors of viral DNA polymerase, we studied the inhibitory effects of diphosphoryl derivatives of HPMPA and of PMEA and its analogues with other heterocyclic bases (Fig. 1) on HSV-1 DNA polymerase and on cellular DNA polymerases α and β and compared the effects with those of 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP), 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate (AZT-TP), acyclovir triphosphate (ACV-TP) and 1-(β-D-arabinofuranosyl)cytosine 5′-triphosphate (araCTP).

Materials and Methods

Chemicals

The analogues of nucleoside triphosphates, *N*-(2-diphosphorylphosphonylmethoxyethyl) derivatives of heterocyclic bases (Fig. 1), were synthesized from the corresponding *N*-(2-phosphonylmethoxyethyl) derivatives by the morpholidate procedure in analogy to the reaction described for PMEA diphosphate (Holý and Rosenberg, 1987a). The same procedure was applied to the synthesis of 9-(S)-(3-hydroxy-2-diphosphorylphosphonylmethoxypropyl)adenine (Holý and Rosenberg, 1987b). ACV-TP, araCTP, ddTTP and AZT-TP were prepared by Moffatt's procedure (Moffatt, 1964). The compounds were homogeneous on PC, TLC, HPLC and paper electrophoresis.

[5-Methyl-^{3}H]dTTP (1100 GBq/mmol) was obtained from Institute for Research, Production and Uses of Radioisotopes, Prague. [8-^{3}H]dATP (570 GBq/mmol), [5-^{3}H]dCTP (1040 GBq/mmol) and [5-^{3}H]dGTP (685 GBq/mmol)

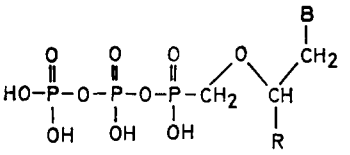

Fig. 1. Inhibitor structures

PMEApp:	B = adenine	R = H
2-amino-PMEApp:	B = 2-aminoadenine	R = H
n^8z^7PMEApp:	B = 8-aza-7-deaza-adenine	R = H
PMEGpp:	B = guanine	R = H
PMECpp:	B = cytosine	R = H
PMETpp:	B = thymine	R = H
PMEUpp:	B = uracil	R = H
HPMPApp:	B = adenine	R = CH_2OH

were purchased from Amersham. 2′-Deoxynucleoside 5′-triphosphates and DNA cellulose (native) were from Pharmacia, DEAE-cellulose DE-52 and phosphocellulose P11 were obtained from Whatman.

DNA polymerases

Herpes simplex virus type-1 induced DNA polymerase was purified by DEAE-cellulose, phosphocellulose and native DNA cellulose chromatography from HSV-1 (KOS) infected HeLa cells by the slightly modified method of Knopf (1979). Cellular DNA polymerases α and β were partially purified by DEAE cellulose and phosphocellulose chromatography from HeLa cells according to Ono (1985). Cellular polymerases α and β were clearly separated from each other and from DNA polymerase γ; we did not attempt at isolating DNA polymerase δ. The specific activities of HSV-1 DNA polymerase and DNA polymerases α and β were 3700, 50 and 40 U/mg of protein, respectively. One unit is defined as the amount of enzyme that incorporates 1 nmol of [^{3}H]dAMP per hour at 37°C under the conditions specific for each particular enzyme with 5 μM [^{3}H]dATP and 100 μM remaining 2′-deoxynucleoside 5′-triphosphates (dNTP).

Activity of DNA-polymerases

Reaction mixture (40 μl) for HSV-1 DNA polymerase activity estimation consisted of 20 mM Tris-HCl buffer (pH 8.0), 3 mM $MgCl_2$, 0.3 mM DTT, 140 mM ammonium sulphate, 2.5% glycerol, 0.5 mg/ml heat inactivated BSA, 0.1 mg/ml activated calf thymus DNA, 0.1 mM each of non-competing dNTPs, labelled competing dNTP (0.5 to 5.0 μM) and various concentrations of the test compounds. DNA was activated according to Baril et al. (1977) with the DNAse concentration of 10 ng/ml. Reaction was started with 0.05 U of HSV-1 DNA polymerase and after 10 min of incubation time at 37°C, 30-μl aliquots were precipitated in 1 ml of ice-cold 5% trichloroacetic acid. After 30 min the precipitates were collected on

nitrocellulose filters BA85 (Schleicher & Schuell, Germany), washed five times with ice-cold 5% trichloroacetic acid and the acid-insoluble radioactivity was measured in toluene-based scintillator.

The reaction mixture for DNA polymerase α was the same as for HSV-1 DNA polymerase, except that ammonium sulphate was omitted and the competing substrate was used at concentrations of 1.0 to 10.0 μM. Reaction was started with 0.08 U of DNA polymerase α and incorporation of radioactivity was evaluated after 10-min incubation as with HSV-1 DNA polymerase.

The reaction mixture for DNA polymerase β contained 85 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 30 mM KCl, 10% glycerol, 0.5 mg/ml heat inactivated BSA, 0.1 mg/ml activated calf thymus DNA, 0.1 mM each of noncompeting dNTPs, labelled competing dNTP (1.0 to 10.0 μM) and various concentrations of inhibitors. The acid insoluble radioactivity was measured after a 20-min incubation period with 0.03 U of enzyme as with HSV-1 DNA polymerase.

The reaction mixtures for examination of HPMPApp and PMEApp as alternative substrates of HSV-1 DNA polymerase were the same as the standard reaction mixture, except that dNTP concentrations were 100 μM dGTP, 100 μM dCTP, 10 μM [^{3}H]dTTP and various concentrations of dATP, HPMPApp or PMEApp. The time course of the reaction was monitored with 0.6 U of DNA polymerase in a total volume of 200 μl; concentration dependence was monitored with 0.13 U of DNA polymerase in 40 μl after a 20-min incubation. In both reactions, acid-insoluble radioactivity was evaluated as described above.

Results

Inhibition of DNA polymerases by N-(diphosphoryl phosphonylmethoxyalkyl)nucleotides

The inhibition of DNA synthesis catalyzed by HSV-1 DNA polymerase and cellular DNA polymerases α and β by diphosphoryl derivatives of the PME-series and of HPMPA is summarized in Table 1 in terms of apparent inhibition constants (K_i). The most potent inhibitor of both the viral enzyme and DNA polymerase α is 2-amino-PMEApp. The inhibitory effect of the PME-type compounds on the viral enzyme decreases in the order: 2-amino-PMEApp >> PMEGpp > PMEApp > PMETpp > PMECpp >> n^8z^7PMEApp > PMEUpp; the inhibitory pattern of these compounds on DNA polymerase α is very similar. The K_i values for HSV-1 DNA polymerase for most of these triphosphate analogues are 6- to 13-fold lower than their K_i values for DNA polymerase α. With PMECpp the ratio of these inhibition constants was only 2.4. In contrast, with HSV-1 DNA polymerase and DNA polymerase α, DNA polymerase β was not significantly inhibited by any of these compounds (Table 1).

Also HPMPApp, in analogy with the PME-derivatives, inhibits HSV-1 DNA polymerase more efficiently than DNA polymerase α (K_i = 1.4 and 6.4 μM, respectively) and does not affect DNA polymerase β. The ratio of the inhibition of

TABLE 1

Apparent kinetic constants of inhibition of DNA polymerases

Inhibitor	Competing substrate	HSV-1 DNA polymerase		DNA polymerase α			DNA polymerase β	
		K_i (μM)	K_m/K_i	K_i (μM)	K_m/K_i	K_i^{α}/K_i^{HSV}	K_i (μM)	K_m/K_i
dATP (K_m)		0.73		2.05			1.47	
dTTP (K_m)		1.25		1.65			2.97	
dGTP (K_m)		0.70		0.88			1.69	
dCTP (K_m)		0.90		1.48			1.69	
PMEApp	dATP	0.105	6.95	0.87	2.36	8.3	370	0.004
PMETpp	dTTP	1.01	1.24	8.36	0.20	8.3	820	0.004
PMEUpp	dTTP	5.90	0.21	47.3	0.035	8.0	>500	<0.006
PMEGpp	dGTP	0.090	7.78	1.15	0.77	12.8	130	0.013
PMECpp	dCTP	1.27	1.41	3.04	0.49	2.4	140	0.012
2-amino-PMEApp	dATP	0.029	25.1	0.18	11.5	6.2	>250	<0.006
n^8z^7PMEApp	dATP	5.03	0.15	27.6	0.074	5.5	>250	<0.006
HPMPApp	dATP	1.42	0.51	6.4	0.32	4.6	>250	<0.006
ddTTP	dTTP	21.0	0.059	38.5	0.043	1.8	1.7	1.75
AZT-TP	dTTP	330	0.004	116	0.014	0.35	1.1	2.70
araCTP	dCTP	0.30	3.02	2.56	0.58	8.6	0.71	2.38
ACV-TP	dGTP	0.12	5.83	2.53	0.35	21.8	350	0.005

HSV-1 DNA polymerase and DNA polymerases α and β by PMEApp and HPMPApp is shown in Fig. 2A. PMEApp is more potent inhibitor of both HSV-1 DNA polymerase and DNA polymerase α than HPMPApp.

The parent compounds PMEA, HPMPA and their monophosphoryl derivatives PMEAp and HPMPAp do not inhibit either HSV-1 DNA polymerase or DNA polymerase α (data not shown).

The mode of inhibition of DNA polymerases α and the enzyme from HSV-1 was examined using Lineweaver-Burke plots; the typical plots demonstrating HSV-1 DNA polymerase inhibition by PMEApp are shown in Fig. 3. All triphosphate analogues showed competitive inhibition of both enzymes with respect to the normal substrates (Table 1).

Inhibition of DNA polymerases by ACV-TP, araCTP, ddTTP and AZT-TP

Also ACV-TP, that strongly inhibited viral enzyme (K_i = 0.12 μM) and to a lesser extent also DNA polymerase α, was also completely inefficient toward DNA polymerase β (Fig. 2B, Table 1). Unlike ACV-TP, ddTTP and, particularly, AZT-TP did not effectively inhibit either HSV-1 or α DNA polymerase. On the other hand, both compounds were potent inhibitors of DNA polymerase β (Table 1, Fig. 2C). Only araCTP was effective against all three enzymes. It was the most efficient DNA polymerase β inhibitor (K_i = 0.71 μM) of all the compounds tested (Table 1, Fig. 2D).

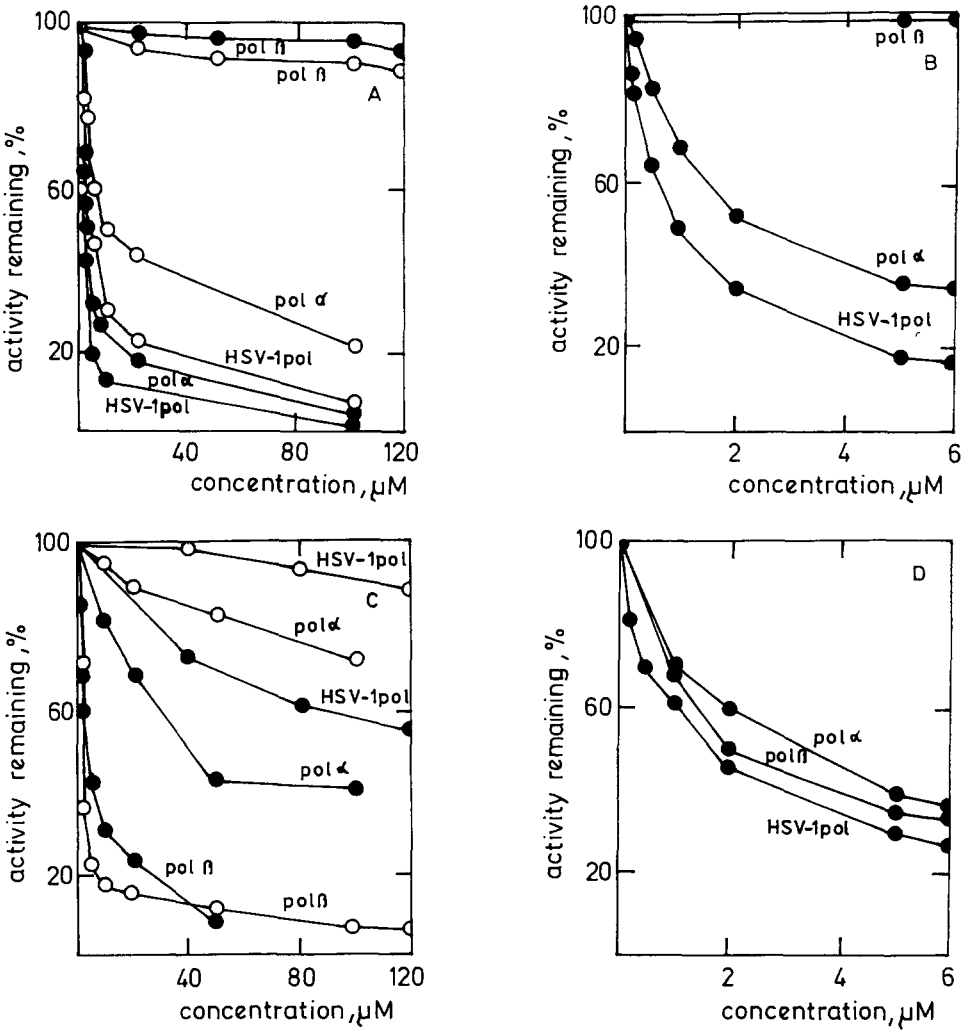

Fig. 2. Concentration-dependence of the inhibition of HSV-1 DNA polymerase and DNA polymerases α and β by PMEApp (•) and HPMPApp (○) (panel A), ACV-TP (panel B), ddTTP (•) and AZT-TP (○) (panel C) and by araCTP (panel D).

HPMPApp and PMEApp as alternate substrates for HSV-1 DNA polymerase

The ability of HPMPApp and PMEApp to support DNA synthesis by HSV-1 DNA polymerase was examined in the absence of the competing substrate (dATP) (Fig. 4A). Although DNA polymerase requires all four natural 2′-deoxynucleoside 5′-triphosphates for its optimal activity, a significant DNA synthesis was observed when dATP was replaced by HPMPApp. The course of the DNA synthesis with HPMPApp was linear during the period under study whereas with dATP it was levelling off at the end of the incubation period (Fig. 4A). Unlike HPMPApp,

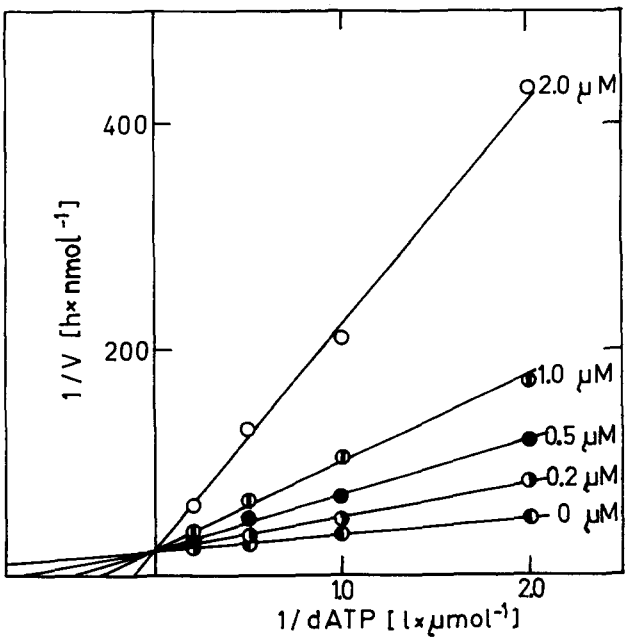

Fig. 3. Lineweaver-Burke plot of HSV-1 DNA polymerase inhibition by PMEApp.

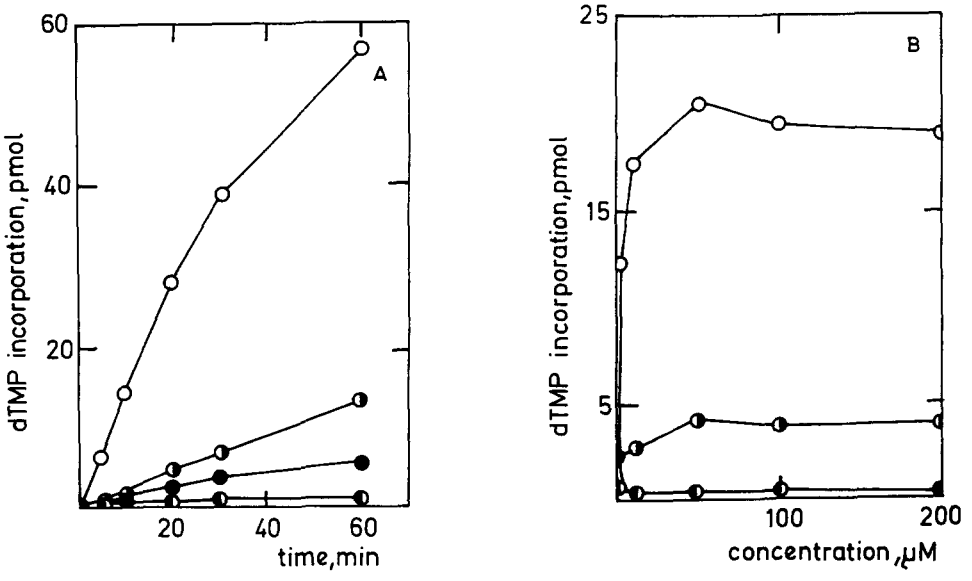

Fig. 4. Examination of HPMPApp and PMEApp as alternative substrates for HSV-1 DNA polymerase. Time course of the reaction in the presence of 100 μM dATP (○), 100 μM HPMPApp (◑), 10 μM PMEApp (◐) or with the fourth nucleotide omitted (●) (panel A). Dependence of the reaction on dATP (○), HPMPApp (◑) and PMEApp (◐) (panel B).

PMEApp did not support DNA synthesis in the absence of dATP and even suppressed the limited [^{3}H]dTMP incorporation which was taking place under these conditions. The DNA synthesis in the presence of HPMPApp is concentration-dependent and reaches its maximum at a concentration of approximately 50 μM (Fig. 4B). At this concentration, PMEApp inhibits DNA synthesis almost completely.

Discussion

In previous reports we demonstrated that HSV-1 DNA polymerase is inhibited by the diphosphates of several purine and pyrimidine phosphonylmethoxyalkyl derivatives (Merta et al., 1988). In this study we investigated in detail the inhibitory effects of these compounds on HSV-1 DNA polymerase and cellular DNA polymerases α and β. In the series of PME-derivatives, 2-amino-PMEApp appears to be the strongest inhibitor of both HSV-1 DNA polymerase and DNA polymerase α; it is followed by PMEGpp, PMEApp, PMETpp and PMECpp. Also PMEUpp and n^8z^7PMEApp inhibit both enzymes; however, the effect is much less pronounced. HSV-1 DNA polymerase has a higher affinity for all compounds of this group than has cellular DNA polymerase α; in general, the effects of all these compounds on DNA polymerase β are only marginal. These analogues can thus be considered as inhibitors of HSV-1 DNA polymerase and DNA polymerase α, with preferential affinity for the viral enzyme.

The order of efficiency of DNA polymerase inhibition by 2-amino-PMEApp, PMEGpp and PMEApp followed approximately the same pattern as the antiviral potency of the parent phosphonates (De Clercq et al., 1987). Consequently, it can be assumed that HSV-1 DNA polymerase may be regarded as a possible target of the antiviral activity of the phosphonylmethoxyethyl derivatives of heterocyclic bases. However, the corresponding pyrimidine derivatives, as well as n^8z^7PMEA, do not exhibit any antiviral activity, although their diphosphates also inhibit DNA polymerases.

HPMPA is one of the most potent anti-HSV agents among the phosphonylmethoxyalkyl derivatives studied so far: it is as potent as 2-amino-PMEA (De Clercq et al., 1987; Holý et al.,1989). Nonetheless, the K_i value of 1.4 μM for the inhibition of HSV-1 DNA polymerase by HPMPApp is about 50-fold higher than the K_i for 2-amino-PMEApp. Also the respective K_m/K_i ratios indicate that the inhibition of HSV-1 DNA polymerase by HPMPApp is much less pronounced than the effect of 2-amino-PMEApp. Although PMEA is inferior to HPMPA in its anti-HSV-1 activity (De Clercq et al., 1987; Holý et al., 1989), its diphosphate is a stronger inhibitor of HSV-1 DNA polymerase than HPMPApp. The apparent discrepancy between the high antiviral potency of HPMPA and the comparatively low inhibitory effect of its diphosphate on HSV-1 DNA polymerase suggest that additional mechanisms might be involved in the antiviral effects of HPMPA.

One of the factors that might influence the antiviral potency of acyclic nucleoside analogues is the rate and extent of their phosphorylation within the cells. For instance, phosphorylation of HPMPA to HPMPApp with the crude extract of

mouse cells L1210 is roughly five times faster than the corresponding transformation of PMEA (Merta et al., in press). Thus, if this were true also for other cells, the different extent of phosphorylation might partly explain the enhanced antiviral potency of HPMPA compared to PMEA.

The mechanism of inhibition of DNA synthesis by the phosphonylmethyl ethers of acyclic nucleoside analogues has not been satisfactorily elucidated. Our experiments suggest that HPMPApp can support DNA synthesis catalyzed by HSV-1 DNA polymerase and that it is most likely incorporated into DNA. This should be possible because of the presence of a 3′-hydroxyl group in the HPMPA molecule which may enable the formation of an 'internucleotide' linkage. The absence of such a hydroxyl group in PMEA precludes the continuation of the DNA chain growth. It remains to be established whether PMEApp only acts as a competitive inhibitor of DNA polymerase by binding to its active site, or whether it is incorporated into the 3′-end of DNA thus terminating the DNA elongation. Incorporation of HPMPA into viral DNA of HSV-1-infected cells was not observed (Votruba et al., 1987), probably because the HPMPA concentrations used caused an inhibition of viral DNA synthesis and, at lower concentrations, the specific radioactivity of ^{14}C-labelled HPMPA did not allow its detection. On the other hand, the incorporation of HPMPA into cellular DNA of both mock- and HSV-1-infected Vero cells has been demonstrated (Votruba et al., 1987).

Acyclovir (ACV), 1-(β-D-arabinofuranosyl)cytosine (AraC),2′,3′-dideoxythymidine (ddThd) and 3′-azido-2′,3′-dideoxythymidine (AZT) are phosphorylated in the infected cells to their triphosphates; also in these cases, viral DNA polymerase is supposed to be the target enzyme for their antiviral effects (Ruth and Cheng, 1981; Germershausen et al., 1983; Furman et al., 1986). On comparison of the effects of these triphosphates on DNA polymerases with the effects of the acyclic nucleoside triphosphate analogues, we found that both the diphosphoryld-erivatives of the acyclic nucleotide analogues (PME-series and HPMPA) and ACV-TP specifically inhibit HSV-1 and α DNA polymerases and do not significantly affect DNA polymerase β; AZT-TP and ddTTP are effective only against cellular DNA polymerase β, whereas araCTP inhibits all three enzymes. While the inhibition of HSV-1 DNA polymerase and DNA polymerase α (expressed by the respective K_i ratio) is comparable for all the above acyclic nucleotides (except for PMEGpp), the action of ACV-TP is more specifically directed toward the viral enzyme. HPMPApp is a weaker HSV-1 DNA polymerase inhibitor than ACV-TP.

Our findings for HPMPApp are in contrast to those reported by Terry et al. (1988), who found that the diphosphate of 9-(*RS*)-(3-hydroxy-2-phosphonylmethoxypropyl)guanine (HPMPGpp) is a 70-fold less active inhibitor of HeLa DNA polymerase α than of HSV-1 DNA polymerase and that the selectivity of HPMPGpp and ACV-TP for the viral enzyme is similar. However, it should be emphasized that these data were obtained with the racemic mixture of (*S*)- and (*R*)-HPMPG diphosphates.

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