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Different Inhibitory Potencies of Acyclic Phosphonomethoxyalkyl Nucleotide Analogs toward DNA Polymerases α, δ, and ε

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SUMMARY

Based on the powerful virostatic potency and cytostatic activity of adenine, 2,6-diaminopurine, and guanine derivatives of acyclic phosphonate nucleotide analog (*S*)-1-(3-hydroxy-2-phosphonomethoxypropyl) and 9-(2-phosphonomethoxyethyl) series, we examined the inhibitory potencies of their diphosphates [(*S*)-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine diphosphate (HPMPApp), 9-(2-phosphonomethoxyethyl)adenine diphosphate, 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine diphosphate (PMEDAPpp), and 9-(2-phosphonomethoxyethyl)guanine diphosphate, analogs of nucleoside 5′-triphosphates] toward cellular DNA polymerases α, δ, and ε (isolated from tumors of T cell spontaneous acute lymphoblastic leukemia in Sprague-Dawley inbred rats). Kinetic measurements (K_m, K_i, and V_{max}) of synthetic homopolymeric template primers have shown that HPMPApp is a selective and potent inhibitor of polymerase ε, whereas PMEDAPpp strongly inhibits polymerase δ. These two compounds may be useful for elucidating the roles of polymerases δ and ε. Of the nucleotide analogs tested, 9-(2-phosphonomethoxyethyl)guanine diphosphate is the most efficient inhibitor of polymerases α and ε, whereas the diphosphate of 9-(2-phosphonomethoxyethyl)adenine, the therapeutically important agent adefovir, inhibits polymerases α and ε relatively poorly and exerts only moderate inhibition of polymerase δ. These data are quite consistent with previously reported cytostatic activity of these nucleotide analogs. All of the enzymes studied catalyze the incorporation of 9-(2-phosphonomethoxyethyl)adenine, 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine, and (*S*)-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine into DNA chain. 9-(2-Phosphonomethoxyethyl)adenine diphosphate and PMEDAPpp were confirmed to be DNA chain terminators. On the other hand, HPMPApp formed poly(dT)/oligo(dA_{18})-[(*S*)-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine]$_{2-4}$ structures.

Acyclic phosphonate analogs (Fig. 1) of HPMP (1) and PME (2) series are some of the most promising novel antiviral substances. It has been shown in experimental models (infected cell cultures and animals) that, in particular, the adenine, 2,6-diaminopurine, cytosine, and guanine derivatives exhibit a strong antiviral potency against an unusually broad spectrum of DNA viruses as well as against retroviruses (see review in Ref. 3). The phosphonomethyl ether group [—O—CH_2—P(O)$(OH)_2$] simulates natural phosphate moiety [—CH_2—O—P(O)$(OH)_2$] and is resistant toward hydrolysis by cellular phosphatase or 5′-nucleotidase activity. Its presence in the molecule circumvents the phosphorylation step to nucleoside 5′-phosphate by virus-encoded TK, which is indispensable in the antiviral activity of classic nucleoside analogs (e.g., acyclovir, ganciclovir). Therefore, these analogs are also active against mutant (TK^-) viral strains or against viruses that do not encode their own TK; their stability against degradation results in their high intracellular half-life. Metabolic studies have revealed that these compounds are further phosphorylated by cellular enzymes to monophosphates and diphosphates, which act as analogs of diphosphates and triphosphates of natural nucleosides (4–7); the mechanism of their antiviral activity is accounted for by the inhibition of virus-induced DNA polymerase (e.g., herpes simplex virus, human cytomegalovirus) (8–10) or of reverse transcriptase (human immunodeficiency virus, avian myeloblastosis virus) (5, 11) by diphosphorylated analog.

However, these phosphonate analogs also exert cytostatic

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ABBREVIATIONS: HPMP, (*S*)-(3-hydroxy-2-phosphonomethoxypropyl); PME, 9-(2-phosphonomethoxyethyl); PMEA, 9-(2-phosphonomethoxyethyl)adenine; PMEApp, 9-(2-phosphonomethoxyethyl)adenine diphosphate; PMEGpp, 9-(2-phosphonomethoxyethyl)guanine diphosphate; PMEDAP, 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine; PMEDAPpp, 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine diphosphate; PMETpp, 1-(2-phosphonomethoxyethyl)thymine diphosphate; HPMPA, (*S*)-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine; HPMPApp, (*S*)-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine diphosphate; HPMPC, (*S*)-1-(3-hydroxy-2-phosphonomethoxypropyl)cytosine; PCNA, proliferating cell nuclear antigen; TK, thymidine kinase.

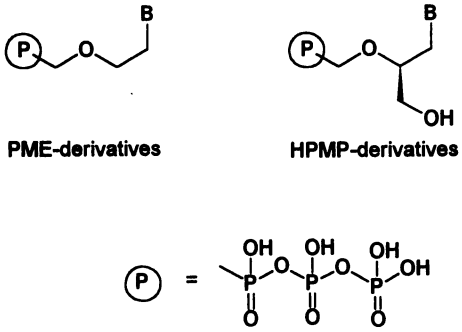

Fig. 1. Structure of acyclic nucleotide analogs: PMEApp (B. . . adenine), PMEDAPpp (B. . . 2,6-diaminopurine), PMEGpp (B. . . guanine), PMETpp (B. . . thymine), and HPMPApp (B. . . adenine).

and cytotoxic effects. PMEA, HPMPA, and, especially, PMEDAP affect the growth of L1210 mouse leukemia cells *in vitro* and their DNA synthesis (12); the cancerostatic activity of the guanine derivative 9-(2-phosphonomethoxyethyl)guanine has been demonstrated in two types of mouse tumors *in vivo* (13). Recently, the cytostatic effect of PMEA was observed in lymphoblastic leukemia KHP-Lw-I in Lewis rats (14) and in lymphoblastic leukemia of Sprague-Dawley rats (15), and the embryotoxicity of PMEA and HPMPC was demonstrated in chickens and rats (16). Chromosomal aberrations have been observed in human embryonic lung cells (16) after the application of PMEA and HPMPC.

The mechanism responsible for all of these effects is not yet known. It is possible that the compounds interact with cellular DNA polymerases during DNA replication; this hypothesis is supported not only by the analogy with their mode of antiviral activity (5, 9–11) but also by their genotoxicity (16), which could be a result of their incorporation into the cellular DNA. According to the present concept, three different DNA polymerases α, δ, and ϵ, participate in the process of eukaryotic genome replication. However, no study has been reported on the effect of acyclic phosphonate analogs on the activity of these enzymes except for data on the inhibition of DNA polymerases α (5, 9), β (9), β, and γ (17, 18).

For this reason, we decided to investigate the inhibitory effect of different acyclic phosphonate analogs *in vitro* on DNA polymerases α, δ, and ϵ isolated from Sprague-Dawley rat lymphomas. We analyzed kinetic data of reactions catalyzed by individual DNA polymerases on synthetic homopolymers in the presence of inhibitors, confirmed the ability of enzymes to incorporate analogs into DNA chain, and studied the possible occurrence of HPMP structures inside the DNA chains.

Materials and Methods

Compounds. All reagents were purchased from Sigma Chemical Co. unless otherwise noted. Nucleotides (dATP, dGTP, and dTTP), oligodeoxynucleotides [oligo(dA_{12-18}), oligo(dT_{12-18}), and oligo(dG_{12-18})], and Sephadex G-50 Medium were obtained from Pharmacia P-L Biochemicals. Oligo(dA_{18}) was synthesized in the Institute of Organic Chemistry and Biochemistry (Prague, The Czech Republic). Glycerol (99%) was obtained from Riedel-de Haen and redestilled in a glass apparatus. The radiolabeled nucleotides [8-^{3}H]dATP (888 GBq/mmol), [8-^{3}H]dGTP (481 GBq/mmol), [*methyl*-^{3}H]dTTP (1.5 TBq/mmol), [α-^{32}P]dATP (15 TBq/mmol), and [γ-^{32}P]ATP (110 TBq/mmol) were purchased from Amersham International. Acyclic analogs of nucleotides, PMEA, PMEDAP, and HPMPA were synthesized by Dr. A. Hol[undot]i and Dr. I. Rosenberg; the diphosphates (analogs of nucleoside 5′-triphosphates) were synthesized by Dr. M. Otmar (all from Institute of Organic Chemistry and Biochemistry, Academy of Sciences of The Czech Republic, Prague). Template primers [poly(dA)/oligo(dT_{12-18}), poly(dT)/oligo(dA_{12-18}), and poly(dC)/oligo(dG_{12-18})] were prepared through annealing an oligodeoxynucleotide to the corresponding polydeoxynucleotide (at 60° for 5 min with the subsequent cooling at room temperature; a base ratio of 1:10) in 10 mM Tris·HCl and 1 mM EDTA, pH 8.0.

[^{32}P]Oligo(dA_{18}) was prepared through 5′-OH end-labeling of oligo(dA_{18}) with [γ-^{32}P]ATP (110 TBq/mmol at a molar ratio 3:1) with T4 polynucleotide kinase (Sigma, Prague, The Czech Republic). The labeled product was separated from [γ-^{32}P]ATP on Sephadex G-50 columns (19).

DNA polymerases and PCNA. DNA polymerases α, δ, and ϵ were isolated from Sprague-Dawley rat compact transplantable lymphomas through a purification procedure described previously (20) except for the last glycerol gradient step. Specific enzyme activities were determined with V_{max} values. One enzyme unit of DNA polymerases is defined as the amount of enzyme that incorporates 1 nmol of the corresponding dNMP into acid-insoluble precipitate after 10 min at 37° under the optimized conditions (see below). PCNA was purified to homogeneity according to the method of Fien and Stillman (21) from *Escherichia coli* strain BL21/DE3 harboring a plasmid encoding the human PCNA cDNA sequence (22). The bacterial strain was kindly provided by Dr. B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

DNA polymerase assay. Reaction conditions for experiments on synthetic homopolymeric templates were optimized with regard to pH (6.5–8.0 range), Mg^{2+} concentration (0–20 mM $MgCl_2$), and ionic strength (0–100 mM KCl) and are summarized in Table 1. The reactions were carried out at 37° for 10, 20, and 30 min and stopped by spotting an appropriate aliquot onto a GF/A fiberglass disk (Whatman) that was then immersed in cold 5% trichloroacetic acid containing 20 mM $Na_4P_2O_7{\cdot}10H_2O$. Disks were extensively washed three times with the same solution and then with an excess of 96% ethanol and dried, and trichloroacetic acid-insoluble radioactivity was determined in a toluene-based scintillation fluid.

Kinetic experiments. Kinetic constants (K_m, K_i, and V_{max}) were determined from the both Lineweaver-Burk and Dixon plots using five concentrations of an analog tested and four concentrations of the corresponding substrate (dNTP). Data were evaluated with the nonlinear regression method based on results from four independent experiments. Reactions were carried out at 37° for 10 min.

Polyacrylamide gel electrophoresis. Products of DNA polymerase reactions were analyzed on denaturing polyacrylamide gels. Enzymes were incubated (under the optimized conditions; for details, see Table 1) with 20 μg/ml poly(dT)/oligo(dA_{12-18}) (10:1) in the presence of 20 μM [α-^{32}P]dATP (74 GBq/mmol) and nucleotide analog (1–20 μM) and/or with 20 μg/ml poly(dT)-[5′-^{32}P]oligo(dA_{18}) (10:1; 32 TBq/mmol of 5′-ends) and nucleotide analog (0.5, 5, and 50 μM) only. After incubation for 30 min at 37°, the reaction was stopped by the addition of EDTA (pH 8.0) to the final concentration of 20 mM. The reaction mixture was then treated with 1 volume of phenol/chloroform/isoamylalcohol (25:24:1) and centrifuged, and DNA was precipitated (0.3 M sodium acetate, pH 5.2) from the aqueous phase by two volumes of ethanol, washed with 70% ethanol, and then dissolved in 98% deionized formamide containing 10 mM EDTA, 0.2% bromphenol blue, and 0.2% xylene cyanole FF. The solubilized samples were heated at 90° for 5 min, cooled in an ice bath, and loaded onto polyacrylamide gels (8% or 20%) containing Tris/Borate/EDTA buffer (1× = 89 mM Tris-borate and 2 mM EDTA) and 7 M urea. Denatured [5′-^{32}P]X-174DNA *Hae*III digest was used as marker. After the electrophoresis (1600 V, 3 hr), the gels were fixed on glass plates in 10% methanol (v/v) and 10% acetic acid (v/v) for 10 min, rinsed with water, wrapped in plastic wrap (Saran Wrap), and autoradiographed.

Poly(dT)/[5′-^{32}P]oligo(dA_{18})/$(HPMPA)_3$ was prepared in 500 μl of a reaction volume that contained 20 mM Tris·HCl, pH 7.4, 0.4 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 200 μg/ml bovine serum albumin, 10% glycerol, 20 μg/ml poly(dT)/[5′-^{32}P]oligo(dA_{18}) (32 TBq/mmol 5′-ends), 50 μM HPMPApp, and 1.5 units of DNA/polymerase α. The reaction was carried out for 1 hr at 37° and then processed in the same way as described above. Products were fractionated by electrophoresis through denaturating 20% polyacrylamide gel. The ^{32}P-labeled oligonucleotide bands were located through autoradiography of the wet gel, and the portion with [5′-^{32}P]oligo(dA_{18})/$(HPMPA)_3$ was excised. The gel was homogenized, and the oligonucleotide was eluted with water, purified with chromatography on DEAE-Sephacel, and annealed to poly(dT) (at the molar ratio of 1:10). The resulting template primer was then concentrated through the conventional ethanol precipitation, washed, solubilized in water, and used for a polymerase reaction.

Protein assay. Concentration of proteins was done according to the method of Bradford (23) with serum bovine albumin as the standard.

Results

DNA polymerases. Cellular DNA polymerases α, δ, and ε were isolated from lymphomas of spontaneous rat T cell leukemia to a high purity. They were identified through estimation of the activity of associated enzymes (DNA primase, 3′-5′-exonuclease), identified further with differing sensitivity toward known DNA polymerase inhibitors, and finally identified through interaction with PCNA, which is a specific auxiliary protein of polymerase δ. These results are described elsewhere (20).

Kinetic analysis of DNA polymerase reactions on synthetic homopolymeric templates and inhibitory effects of acyclic nucleotide analogs. The determination of the inhibitory potency of purine acyclic phosphonate analogs of the HPMP and PME series on polymerases α, δ, and ε was carried out with synthetic template primers poly(dA)/oligo(dT_{12-18}), poly(dT)/oligo(dA_{12-18}), and poly(dC)/oligo(dG_{12-18}). The activities of the individual DNA polymerases on these template primers differ considerably and strongly depend on the reaction conditions that are used (20). Therefore, their parameters (buffer, pH, Mg^{2+} ion concentration, and ionic strength) were estimated separately for each enzyme and template primer. The optimum values of these parameters are shown in Table 1. Under these conditions, the kinetic data (K_m and V_{max}) were obtained for the incorporation of dNTPs (Table 2), and K_i values were obtained for PMEApp, PMEDAPpp, HPMPApp, and PMEGpp. For comparison, the diphosphate of biologically ineffective phosphonate analog PMETpp and the triphosphate of the known biologically active nucleoside analog ddA (ddATP) were also studied. The inhibitory potency of individual nucleotide analogs is expressed with the use of the K_i/K_m ratio.

TABLE 1

Assay conditions of DNA polymerases α, δ, and ε

All reaction mixtures (25 μl) contained 20 μM [^{3}H]dNTP (250–400 cpm/pmol), 20 μg/ml template primer (10:1), 200 μg/ml bovine serum albumin 1 mM DTT, and 10% (v/v) glycerol.

DNA polymerase	Template primer	Concentration of reaction components			
		Buffer[a]	pH	$MgCl_2$	KCl
					mM
α	$(dA)_n/(dT)_{12-18}$	Tris·HCl	7.5	5.0	0.0
	$(dT)_n/(dA)_{12-18}$	Tris·HCl	7.5	0.2	40.0
	$(dC)_n/(dG)_{12-18}$	Tris·HCl	8.0	15.0	0.0
δPCNA[b]	$(dA)_n/(dT)_{12-18}$	HEPES (K^+)	7.0	8.0	20.0
	$(dT)_n/(dA)_{12-18}$	HEPES (K^+)	7.0	8.0	40.0
	$(dC)_n/(dG)_{12-18}$	N[c]	N[c]	N[c]	N[c]
ε	$(dA)_n/(dT)_{12-18}$	HEPES (K^+)	7.5	5.0	75.0
	$(dT)_n/(dA)_{12-18}$	HEPES (K^+)	7.5	0.2	75.0
	$(dC)_n/(dG)_{12-18}$	Tris·HCl	7.8	2.0	25.0

[a] 20 mM Tris·HCl and 40 mM HEPES (K^+) were used.
[b] 18 μg/ml of PCNA was added.
[c] No enzyme activity on poly (dC)/oligo $(dG)_{12-18}$ was detected.

TABLE 2

Apparent kinetic constants of DNA polymerases

DNA polymerase	Template primer	dNTP	V_{max}	K_m
			pmol/min/μg	μmol/liter
α	$(dA)_n/(dT)_{12-18}$	dTTP	466 ± 42	15.6 ± 1.4
	$(dT)_n/(dA)_{12-18}$	dATP	610 ± 45	10.0 ± 1.1
	$(dC)_n/(dG)_{12-18}$	dGTP	598 ± 50	21.4 ± 1.9
δ/PCNA	$(dA)_n/(dT)_{12-18}$	dTTP	51.8 ± 5.9	1.86 ± 0.17
	$(dT)_n/(dA)_{12-18}$	dATP	3.1 ± 0.3	0.71 ± 0.08
	$(dC)_n/(dG)_{12-18}$	dGTP	N[a]	N[a]
ε	$(dA)_n/(dT)_{12-18}$	dTTP	147 ± 5	1.42 ± 0.13
	$(dT)_n/(dA)_{12-18}$	dATP	20.5 ± 1.9	0.72 ± 0.07
	$(dC)_n/(dG)_{12-18}$	dGTP	42.3 ± 3.5	14.3 ± 1.5

[a] No reaction.

The most powerful inhibitor of the tested substances was PMEGpp (Table 3), which is a competitive inhibitor of dGTP incorporation. Its K_i values were determined for polymerases α and ε; no reaction was observed for polymerase δ on the poly(dC)/oligo(dG_{12-18}) template. Among PMEApp, PMEDAPpp, and HPMPApp structures, differences were found in the inhibition of the individual DNA polymerases (Tables 4 and 5). PMEApp moderately inhibits polymerase δ and has the same affinity to polymerases α and ε as dATP. In contrast, the effect of 2,6-diaminopurine derivative PMEDAPpp is very selective; it is a strong inhibitor of polymerase δ, it has a moderate potency against polymerase α, and its affinity toward polymerase ε is identical to that of the natural substrate. Theoretically, PMEDAPpp also could act as a competitive inhibitor of dGTP incorporation into poly(dC)/oligo(dG_{12-18}). This alternative, however, in the case of polymerases α and ε was excluded (data not shown). Fig. 2 shows products of polymerase α, δ, and ε reactions on poly(dT)/oligo(dA_{12-18}) in the presence of [α-^{32}P]dATP and of different concentrations of PMEDAPpp (1–20 μM); Dixon plots of kinetic data for DNA polymerase reactions in the presence of PMEDAPpp are given in Fig. 3. Both PMEApp and PMEDAPpp competitively inhibit dATP incorporation catalyzed by each of the enzymes tested.

We also observed, in case of HPMPApp, selectivity of action. This compound is a very strong inhibitor of polymerase ε and, in comparison with dATP, has 4-fold higher affinity to polymerases δ and >2-fold lower affinity to polymerase α. The interaction with polymerase α is clearly competitive, but reactions catalyzed by polymerases δ and ε have a somewhat different character in the presence of HPMPApp. At concen-

TABLE 3

Inhibition of DNA polymerases by PMEGpp on poly (dC)/oligo $(dG)_{12-18}$

DNA polymerase	K_i (PMEGpp)	K_i/K_m[a]
	μmol/liter	
α	0.55 ± 0.06	0.026
ε	0.059 ± 0.006	0.004

[a] For the corresponding K_m values, see Table 2.

TABLE 4
Inhibition of DNA polymerases by adenine nucleotide analogues on poly (dT)/oligo $(dA)_{12-18}$

DNA polymerase	K_i			
	HPMPApp	PMEApp	PMEDAPpp	ddATP
	μmol/liter			
α	22.9 ± 2.0	11.7 ± 1.2	2.57 ± 0.23	39.3 ± 3.2
δ/PCNA	0.18 ± 0.02	0.41 ± 0.03	0.059 ± 0.006	81.4 ± 6.3
ε	0.05[a]	0.67 ± 0.08	0.9 ± 0.1	46.9 ± 5.3

[a] The character of inhibition differs from purely competitive inhibition.

TABLE 5
Inhibitory potency of adenine nucleotide analogues toward DNA polymerases on poly (dT)/oligo $(dA)_{12-18}$

DNA polymerase	K_i/K_m[a]			
	HPMPApp	PMEApp	PMEDAPpp	ddATP
α	2.29	1.17	0.26	3.93
δ/PCNA	0.25	0.58	0.08	114.60
ε	0.07	0.93	1.25	65.10

[a] For the corresponding K_m and K_i values, see Tables 2 and 4.

trations of dATP < K_m, the reaction rate is lower than expected for competitive inhibition. The decreased rate of reaction is not connected with 3′-5′-exonuclease activity of both enzymes activated by the low concentrations of substrate. Such a decrease was also observed when 2 mM GMP (exonuclease inhibitor) was added (data not shown).

The kinetic analysis of DNA polymerase inhibition caused by adenine and 2,6-diaminopurine analogs was compared with the data on ddATP. Our results confirm previous observations that replicative eukaryotic DNA polymerases are completely resistant toward the inhibition by dideoxynucleotides.

Last, we performed an analogous kinetic analysis of PMETpp as a representative of a pyrimidine acyclic nucleotide analog tested in the current study. Its weak but significant interaction with polymerases δ and ε is rather surprising (Table 6) due to its lack of cytostatic and/or antiviral activity.

Incorporation of PMEA and PMEDAP into DNA. The structure of PME, whose side chain does not contain the —OH group, may serve as a chain terminator after its incorporation into DNA, whereas the presence of the —OH group in the acyclic part of the HPMP structure may result in further elongation (i.e., the ability of the analog to become part of the DNA chain). To examine the ability of DNA polymerases to incorporate acyclic nucleotide analogs into DNA chain, we used poly(dT) with oligo(dA_{18}) as primer labeled with ^{32}P at the 5′-end; dATP in the reaction mixture was exchanged for nucleotide analog (0.5, 5, and 50 μM; for details, see Materials and Methods). The results of these experiments are shown in Fig. 4.

PMEApp is a poor substrate of polymerases; we observed its incorporation with polymerase α, a very weak incorporation with polymerase δ, and a negligible reaction with polymerase ε (Fig. 4A). In contrast, PMEDAPpp is incorporated relatively strongly with polymerase ε, but only at the highest (50 μM) concentration (Fig. 4B) [the weak band visible above the oligo(dA_{18}) band, especially in Fig. 4A, was identified as a minor contaminant of oligo(dA_{19}), whereas the series of bands in the lower portion of the of gels represent minor quantities of short oligo(dA_n), which result from 3′-5′-exonuclease activity of polymerases δ and ε].

Incorporation of HPMPA into DNA and the ability of poly(dT)/oligo$(dA)_{18}$/$(HPMPA)_3$ to serve as template primer. HPMPA not only is well accepted as substrate by each of the three enzymes studied but also forms chains of concentration-dependent length. As shown in Fig. 4C, polymerases α, δ, and ε form chains on poly(dT)/oligo(dA_{18}) templates that contain two to four HPMPA units.

To investigate the ability of polymerases α, δ, and ε to use HPMPA chain as a DNA primer for the DNA synthesis, we isolated poly(dT)/oligo(dA_{18})/$(HPMPA)_3$ on a preparative scale with the use of polymerase α (see Materials and Methods), and we used it as a template primer in reaction mixtures containing 20 μM dATP or 20 μM HPMPApp and other reaction components (Table 1). Fig. 5 indicates that polymerases α and ε can use such a structure as a template primer for the synthesis of poly(dA) strand; the simultaneous occurrence of oligo(dA)/$(HPMPA)_3$/$(dA)_{1-2}$ suggests a partially abortive character of this synthesis. Both enzymes are able to prolong original primer by adding one to two molecules of HPMPA. In contrast to polymerases α and ε, polymerase δ is much less efficient in its use of this template primer for the synthesis of poly(dA) strand.

Discussion

The diphosphoryl derivatives of acyclic phosphonate nucleotide analogs are inhibitors of DNA polymerases α, δ, and ε with a considerable selectivity that depends on the character of the base and the side chain. PMEApp, PMEDAPpp, and HPMPApp are also substrates for all DNA polymerases that we investigated. Therefore, most of the acyclic phosphonate nucleotide analogs should probably be characterized as substrate inhibitors. Unfortunately, unavailability of the radioactively labeled analogs makes impossible a precise estimation of their interaction with DNA polymerases (i.e., determination of k_{cat} and K_m for nucleotide analogs and comparison with natural substrates). Nevertheless, because the measured K_i values and K_i/K_m ratios reflect the overall competitive inhibition of DNA polymerase-catalyzed incorporation of natural nucleotides, the acyclic nucleotide analogs can be classified as their inhibitors.

It is probable that the influence of these compounds on the synthesis of cellular nuclear DNA differs according to the character of their interaction with individual DNA polymerases that play different roles during the DNA replication. According to the data for the simian virus 40 DNA replication model *in vitro*, polymerases α and δ are the only DNA polymerases that function in eukaryotic DNA replication fork (24). Polymerase α, which is tightly connected with DNA

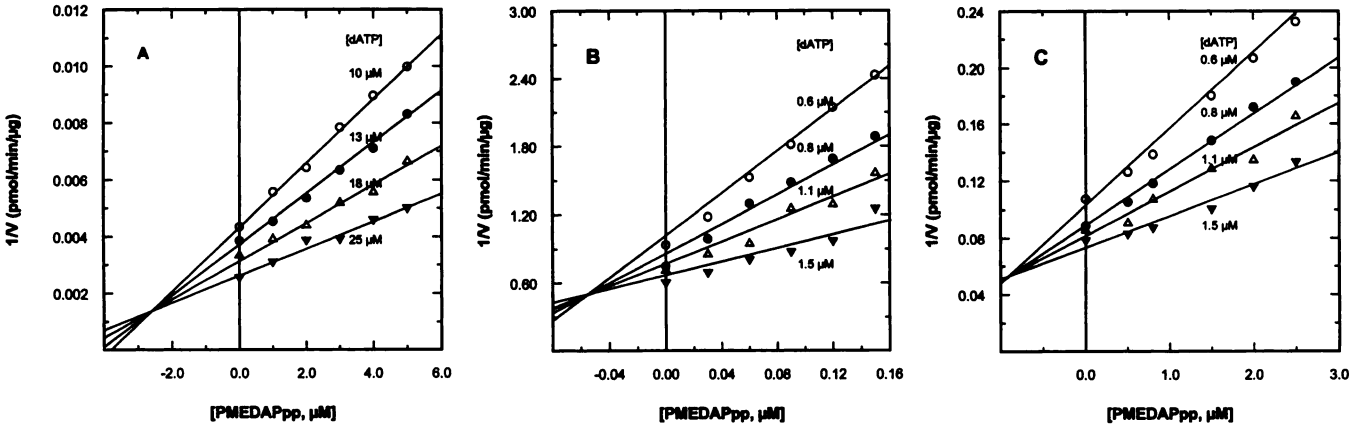

Fig. 2. Dixon plot of DNA-polymerase inhibition by PMEDAPpp on the template poly(dT)/oligo(dA_{12-18}). A, Polymerase α (0.03 unit). B, Polymerase δ/PCNA (0.003 unit). C, Polymerase ε (0.003 unit). Reactions were carried out under optimized conditions (see Table 1) for 10 min at 37°.

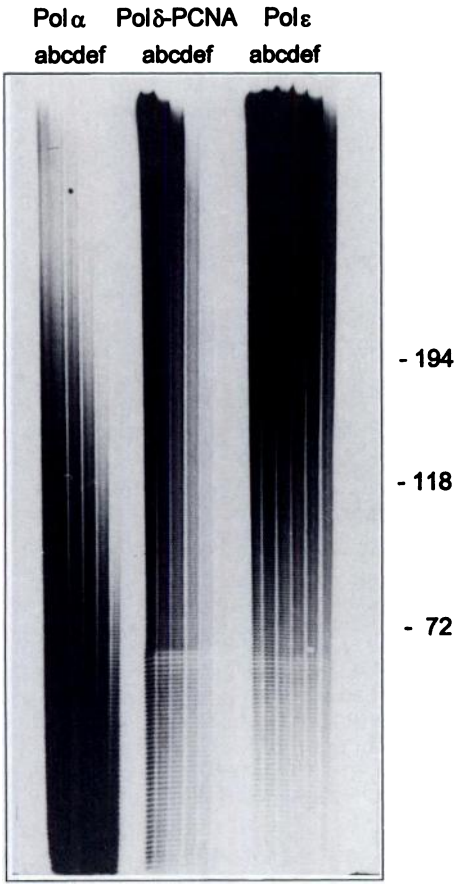

Fig. 3. Inhibition of the DNA chain growth by PMEDAPpp. Analysis of DNA products synthesized on poly(dT)/oligo(dA_{12-18}) in the presence of polymerases α, δ, and ε were done on an 8% denaturating polyacrylamide gel. Reaction mixtures containing 20 μM [α-^{32}P]dATP (74 GBq/mmol), 0.03 unit of the corresponding polymerase, and various concentrations of the nucleotide analog (*a*, none; *b*, 1 μM; *c*, 2 μM; *d*, 5 μM; *e*, 10 μM; *f*, 20 μM) were incubated for 30 min at 37° under optimized conditions (see Table 1).

TABLE 6

Inhibition of DNA polymerases by PMETpp on poly (dA)/oligo ($dT)_{12-18}$

DNA polymerase	K_i (PMETpp)	K_i/K_m[a]
	μmol/liter	
α	44.6 ± 5.2	2.86
δ/PCNA	0.41 ± 0.04	0.22
ε	0.63 ± 0.10	0.44

[a] For the corresponding K_m values, see Table 2.

primase (25), initiates the synthesis of both strands of the replication fork and repeatedly forms DNA primers on the lagging strand. Both strands are subsequently prolonged by the simultaneous action of polymerase δ. The inhibitory effect of PMEGpp on polymerase α (Table 3) and of PMEDAPpp on both polymerases α and δ (Tables 4 and 5) could result in substantial inhibition of cellular DNA replication. In contrast to these diphosphates, PMEApp, which is a relatively weak inhibitor of both enzymes, obviously affects this process only marginally. This is corroborated by a low substrate affinity of PMEApp for the incorporation into DNA by polymerases α and δ (Fig. 4A), whereas PMEDAPpp is very effectively incorporated into DNA by both enzymes (Fig. 4B). Questions remain as to the efficiency of the excision of incorporated acyclic nucleotide analogs from cellular DNA and to what extent proofreading 3′-5′-exonuclease activity associated with the preferential removal by polymerases δ and ε of mispaired nucleotides (26, 27) participates in this process.

Polymerase ε is an enzyme that is necessary for cell viability (28); nevertheless, its role in DNA replication is not quite understood. It has been proposed to act as a second DNA polymerase for lagging strand completion (29–31); it participates in DNA repair (32) and DNA recombination (33). HPMPApp is a very powerful inhibitor *in vitro* (K_i/K_m = 0.07); it is incorporated by polymerase ε in the absence of dATP into DNA under "internucleotide" bond formation, which can be further prolonged by another analog unit (Fig. 4C). Pols α and δ have similar properties (Fig. 4C); however, because in the presence of a natural nucleotide (dATP) the resulting inhibitory effect of HPMPApp is considerably lower ($K_i/K_m^{\text{polymerase }\alpha}$ = 2.29, $K_i/K_m^{\text{polymerase }\delta}$ = 0.25), the weak effect of this analog *in vivo* on polymerases α and δ is predictable.

Our observation that poly(dT)/oligo(dA_{18})/$(HPMPA)_3$ serves as an effective template primer for polymerases ε and α (Fig. 5) means that the fragment $(HPMPA)_3$ can substitute $(dA)_3$ at the end of primer building complementary pairs HPMPA/dTMP. Polymerase δ/PCNA recognizes this template primer with considerably lower efficiency. From the model of the ordered sequential interaction of polymerase δ,

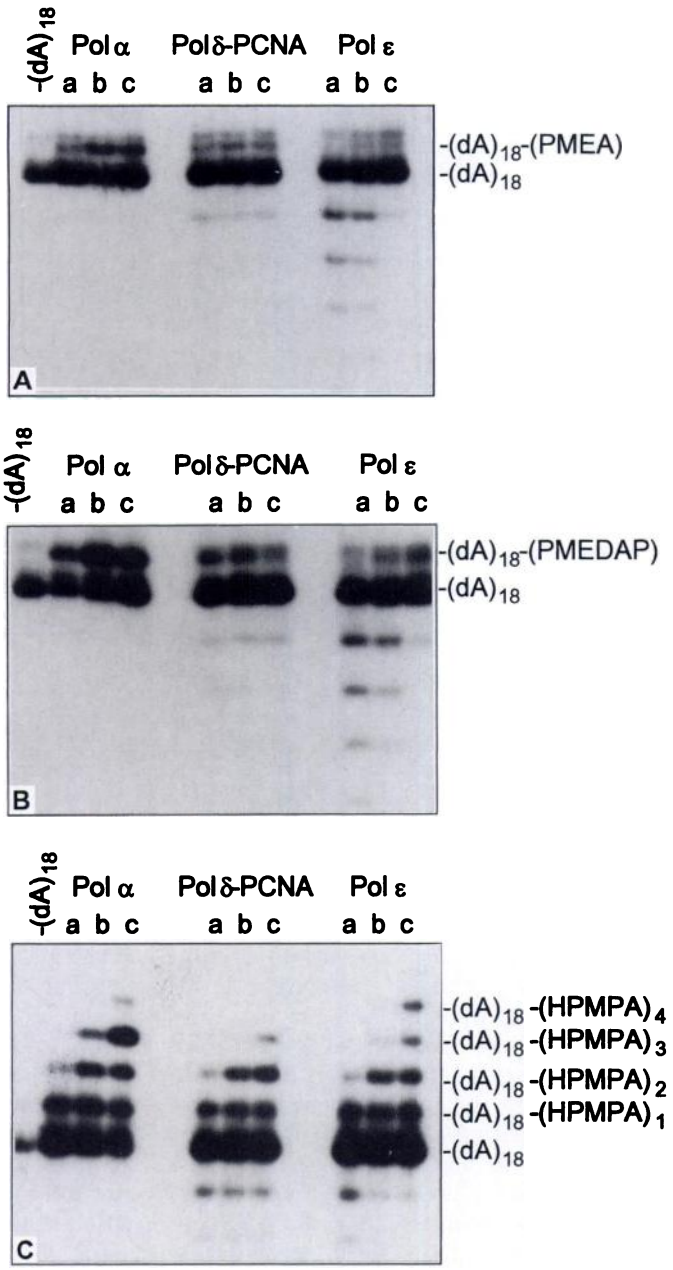

Fig. 4. Incorporation of phosphonomethoxyalkyl derivatives of adenine into poly(dT)/oligo(dA_{18}) template catalyzed by polymerases α, δ, and ϵ. Reaction mixtures containing (instead of dATP) a nucleotide analog [PMEApp (A), PMEDAPpp (B), or HPMPApp (C); *a*, 0.5 μM; *b*, 5 μM; *c*, 50 μM] and 0.03 unit of the corresponding polymerase were incubated for 30 min at 37° under optimized conditions (see Table 1). Reaction products were analyzed in 20% denaturing polyacrylamide gel.

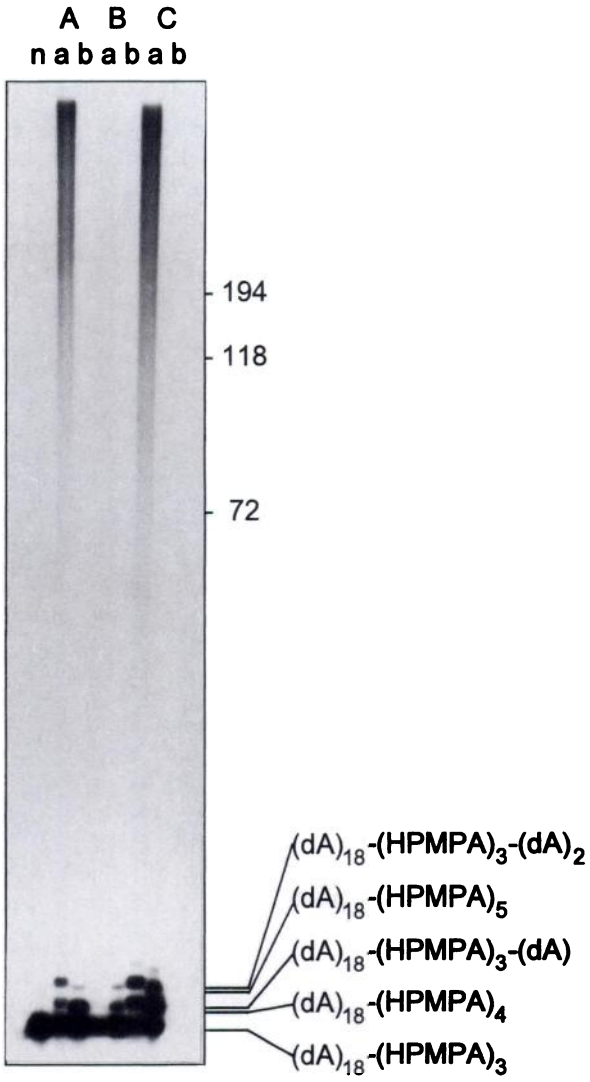

Fig. 5. Poly(dT)/oligo(dA_{18})/$(HPMPA)_3$ as template primer. Analysis of DNA products synthesized on poly(dT)/[5′-^{32}P]oligo(dA_{18})/$(HPMPA)_3$ in the presence of polymerase α (A), polymerase δ/PCNA (B), or polymerase ϵ (C) were done in 10% denaturing polyacrylamide gel. Reaction mixtures containing 20 μM dATP (*a*) or 20 μM HPMPApp (*b*) and 0.03 unit of the corresponding polymerase were incubated for 30 min at 37° under optimized conditions (see Table 1). Poly(dT)/[5′-^{32}P]oligo(dA_{18})/$(HPMPA)_3$ was prepared with polymerase α (for details, see Materials and Methods). *n*, No enzyme was added.

PCNA, template primer, and dNTP (34), it can be deduced that the fragment $(HPMPA)_3$ either hinders the formation of the complex of polymerase δ with template primer or prevents the stabilization of the complex by PCNA. The incorporation of HPMPA into DNA of the cells treated with this analog, which has been suggested (4), may thus be due primarily to its interaction with polymerase ϵ. It can affect DNA duplex structure, stability, protein binding, and rate and fidelity of DNA synthesis during subsequent cycle, RNA transcription, and so on.

We believe to that the fact that PMEDAPpp, a strong inhibitor of polymerase δ, is minimally active against polymerase ϵ, which in contrast is strongly inhibited by HPMPApp structure, may have general significance. For PMEDAPpp, as for ganciclovir triphosphate (35), the affinities to polymerases δ and ϵ are principally different. These substances may be regarded as leading structures for the development of very specific substrate inhibitors of polymerases δ and ϵ, which may be helpful for further investigation of the role of both enzymes in DNA replication *in vivo*.

The comparison of the overall effect of the study compounds results in the following order of decreasing inhibitory activity: PMEGpp > PMEDAPpp > HPMPApp > PMEApp. It is consistent with data on the cytostatic effect of 9-(2-phosphonomethoxyethyl)guanine > PMEDAP > HPMPA > PMEA in cell cultures (36). This indicates that the inhibition of replicative DNA polymerases significantly participates in

the cytostatic effects of these substances. However, although PMETpp is a more potent inhibitor of polymerases δ and ϵ than PMEApp (Table 6), its parent compound 1-(2-phosphonomethoxyethyl)thymine does not exhibit any cytostatic effects (37, 38). This apparent inconsistency may be explained by multiple factors, which probably include efficiency of plasma membrane transport (39), intracellular phosphorylation differences, and the level of their activated derivatives (monophosphoryl and diphosphoryl derivatives) in the cell.

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