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9-(2-Phosphonylmethoxyethyl) Derivatives of Purine Nucleotide Analogs: A Comparison of Their Metabolism and Interaction with Cellular DNA Synthesis

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ABSTRACT

Incubation of CEM cells for 24 h with the guanine, 2,6-diaminopurine, and adenine nucleotide analogs of the 9-(2-phosphonylmethoxyethyl) series, 9-(2-phosphonylmethoxyethyl)guanine (PMEG), 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP), and 9-(2-phosphonylmethoxyethyl)adenine (PMEA), was found to inhibit DNA synthesis 50% at concentrations of 1, 6, and 25 μM, respectively. Possible reasons for the marked differences were investigated, including cellular transport of the analogs, different efficiencies of intracellular phosphorylation, differential effects on 2′-deoxynucleotide (dNTP) pools, and differences in the affinities of the cellular DNA polymerases for the diphosphate derivatives of the drugs. No significant differences in cellular uptake were found among the analogs; however, they did differ in the efficiency of phosphorylation, i.e., CEM cells were found to accumulate higher levels of PMEG-diphosphate (PMEGpp) than PMEDAP-diphosphate (PMEDAPpp) or PMEA-diphosphate (PMEApp). Treatment of cells with any of the nucleotide analogs resulted in increased dNTP pools, with PMEG producing the greatest increase. All three analogs had the greatest effect on the dATP pool size, whereas the dGTP pool size was not significantly affected. Comparison of the ratios of nucleotide analog diphosphates to their corresponding dNTPs under conditions where DNA synthesis is inhibited 50% suggested that cellular DNA polymerases were approximately twice as sensitive to PMEGpp than to PMEDAPpp and 5-fold more sensitive to PMEGpp than to PMEApp. Consistent with this hypothesis, examination of the efficiencies with which the replicative DNA polymerases α, δ, and ϵ incorporated the analogs showed that DNA polymerase δ, the most sensitive of the DNA polymerases, incorporated PMEGpp twice as efficiently as PMEDAPpp and 7-fold more efficiently than PMEApp.

The guanine, 2, 6-diaminopurine, and adenine derivatives of the 9-(2-phosphonylmethoxy**e**thyl) series [9-(2-phosphonylmethoxyethyl)guanine (PMEG), 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP), and 9-(2-phosphonylmethoxyethyl)adenine (PMEA)] belong to a class of acyclic nucleotide analogs containing a catabolically stable P-C bond in a phosphonylmethylether group that simulates a phosphate moiety (Fig. 1). Members of this class have been investigated for antiviral activity (De Clercq, 1991). The most extensively studied analog, PMEA, exhibits potent activity against both DNA viruses and retroviruses (Balzarini et al., 1991b; Heijtink et al., 1993; Naesens et al., 1994), and the oral prodrug of PMEA (adefovir dipivoxil) is currently being evaluated for the treatment of both HIV and hepatitis B virus infections. Several members of this class exhibit cytotoxicity toward proliferating cells. The cellular toxicity of PMEG eliminated this compound from further development as an antiviral drug (De Clercq et al., 1987); however, PMEG and its prodrug N^6-cyclopropyl PMEDAP (Compton et al., 1999) are currently being examined as potential antitumor agents. PMEG was found to have antiproliferative effects in vitro against human leukemic cells (Robbins et al., 1995a) as well as solid tumor cell lines (Paborsky et al., 1997) and in vivo against two types of mouse transplantable tumors (Rose et al., 1990). PMEG also suppressed the growth of papillomavirus-induced condylomas on human foreskin xenografts in mice (Krieder et al., 1990), and both PMEDAP and PMEA were found to prolong the mean survival time of rats bearing lymphomas (Otová et al., 1997).

It has been shown that both PMEG and PMEA are converted to mono- and diphosphates intracellularly (Balzarini et al., 1991a; Ho et al., 1992). Furthermore, the results of in vitro studies with purified or partially purified replicative DNA polymerases suggest that the diphosphorylated forms of PMEG and PMEA [PMEG-diphosphate (PMEGpp) and

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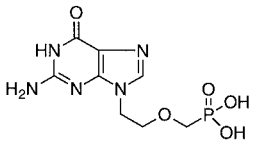
ABBREVIATIONS: PMEG, 9-(2-phosphonylmethoxyethyl)guanine; PMEDAP, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEGpp, PMEG-diphosphate; PMEApp, PMEA-diphosphate; PAGE, polyacrylamide gel electrophoresis; PMEDAPpp, PMEDAP-diphosphate; TBAHS, tetrabutylammonium hydrogen sulfate; ANPpp, acyclic nucleoside phosphonate diphosphate; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine.

PMEA-diphosphate (PMEApp)] effectively compete with the corresponding deoxynucleoside triphosphates (dGTP and dATP) for incorporation into DNA, and that PMEGpp is a more potent inhibitor of cellular DNA polymerases than PMEApp (Kramata et al., 1996; Pisarev et al., 1998). These studies suggest that the diphosphorylated forms of the acyclic nucleotide analogs might inhibit cellular DNA synthesis by a direct inhibition of replicative DNA polymerases. Acyclic nucleotide analogs of the PME series also may function as chain terminators after their incorporation into DNA due to the lack of a 3′-OH moiety in the molecule. Furthermore, interaction of the nucleotide analogs or their metabolites with enzymes involved in the synthesis of deoxyribonucleotides may cause an imbalance in cellular 2′-deoxynucleotide (dNTP) pools. This, in turn, could affect the ratio of nucleotide analog diphosphate to dNTP and thereby indirectly affect the ability of an analog diphosphate to compete for binding at a DNA polymerase active site.

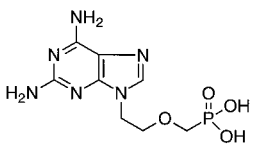
To understand the mechanism of antiproliferative action of acyclic nucleotides of the PME series, we examined the metabolism of PMEG, PMEDAP, and PMEA in CEM cells. We also determined the levels of dNTP pools in treated cells, the intracellular ratios of the diphosphorylated forms of the analogs to their corresponding deoxynucleoside triphosphates, and the efficiency of incorporation of the analog diphosphates by replicative DNA polymerases α, δ, and ϵ in vitro. In addition, we examined the correlation of these data with the inhibitory effects of the analogs on cellular DNA synthesis.

Materials and Methods

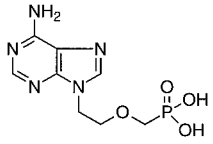
Compounds. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. PMEG, PMEDAP, and PMEA, along with their mono- and diphosphates, were synthesized at Gilead Sciences (Foster City, CA). [8-^{3}H]PMEG (15.5 Ci/mmol), [8-^{3}H]PMEDAP (18 Ci/mmol), [2,8-^{3}H]PMEA (35 Ci/mmol), [2,8-^{3}H]dATP (16.8 Ci/mmol), and [*methyl*-^{3}H]deoxyribothymidine 5′-triphosphate ([*methyl*-^{3}H]dTTP) (60 Ci/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA). The purity of radioactively labeled nucleotide analogs was >97%. Inulin[^{14}C]carboxylic acid (6.4 mCi/mmol), tritiated water (1 mCi/ml), [*methyl*-^{3}H]thymidine (59 Ci/mmol), [γ-^{32}P]ATP (6000 Ci/mmol), and unlabeled dNTPs were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). Deoxyribonuclease I (DNase I), proteinase K, alkaline phosphatase, and the protease inhibitor cocktail were obtained from Boehringer Mannheim (Indianapolis, IN). Phosphodiesterase I was obtained from Worthington Biochemical (Lakewood, NJ), and RNace-It ribonuclease cocktail was from Stratagene (La Jolla, CA). All oligonucleotides used in the study were synthesized and polyacrylamide gel electrophoresis (PAGE)-purified by Genosys (The Woodlands, TX).



PMEG



PMEDAP



PMEA

Fig. 1. The structures of the acyclic nucleotide analogs.

DNA Polymerases. DNA polymerases α and ϵ from CCRF-CEM cells and DNA polymerase δ from calf thymus were isolated according to previously published procedures (Ng et al., 1991; Kramata et al., 1995, 1998). Proliferating cell nuclear antigen was prepared from calf thymus as described (Tan et al., 1986). Protein concentrations were determined according to the previously published method of Bradford (1976) with BSA as standard.

Cells. The human T-lymphoblastoid cell line CCRF-CEM (ATCC, CCL 119) was maintained in suspension culture in exponential growth in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (Irvine Scientific) and 2 mM L-glutamine (Irvine Scientific) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were counted by hemocytometer, and cell volume was estimated as described with [^{3}H]H_2O and inulin[^{14}C]carboxylic acid (Wohlhueter and Plagemann, 1989). Cell viability was determined by a standard dye exclusion assay with trypan blue.

Determination of Cellular DNA Synthesis. After 24-h incubation with 1 to 1000 μM nucleotide analogs, cells were harvested by centrifugation, washed twice in warm, fresh medium, and seeded at a density of 2×10^5/ml. After 30 min, the cells were incubated with [*methyl*-^{3}H]thymidine (1 μCi/ml) for 30 min, harvested by centrifugation, washed in PBS, and extracted on ice with 0.4 N $HClO_4$. After centrifugation, the acid-insoluble material was washed twice in ice-cold 0.4 N $HClO_4$ and dissolved in dimethyl sulfoxide. Radioactivity was determined by liquid scintillation counting. To determine the specific activity of the [^{3}H]dTTP cellular pool, the acid-soluble extract was neutralized with trioctylamine in 1,1,2-trichlorotrifluoroethane, centrifuged, and the water phase was separated with a Partisil-10 SAX column (4.6 × 250 mm) eluted at 1 ml/min with a 30-min linear gradient of 20 to 200 mM KPO_4, pH 5.0. The incorporation of [^{3}H]dTMP in DNA was calculated as the counts per minute detected in the acid-insoluble material divided by counts per minute per nanomoles of cellular[^{3}H]dTTP.

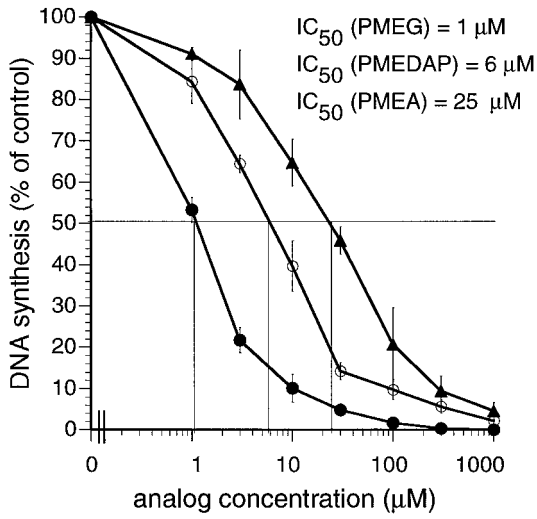


Fig. 2. Effect of nucleotide analogs on DNA synthesis in whole cells. Exponentially growing cells were incubated with the indicated concentrations of nucleotide analogs for 24 h at 37°C, washed in fresh medium, and labeled with [*methyl*-^{3}H] thymidine. DNA synthesis was quantitated as described in *Materials and Methods*. Results are expressed relative to the control (no drug). Data are means of three separate experiments; bars, S.D.; ●, PMEG; ○, PMEDAP; ▲, PMEA.

Measurement of dNTP Pools. After a 24-h incubation with 1 to 100 μM nucleotide analogs, 0.75 to 1.5 $\times$ 10^6 cells were harvested by centrifugation, washed twice in cold PBS, counted, and extracted on ice with 0.4 N $HClO_4$. The acid-soluble extract was neutralized with trioctylamine in 1,1,2-trichlorotrifluoroethane, centrifuged, and the water phase (100 μl) was frozen and stored at −80°C. The dNTP pools were determined by adding 2.5 μl of the extract to a 25-μl reaction mixture containing oligonucleotide template primers of defined sequence, [^{3}H]dTTP or [^{3}H]dATP, and DNA polymerase I (Klenow fragment, 3′-5′ exo$^-$; New England Biolabs, Beverly, MA) as described in Sherman and Fyfe (1989). Inclusion of PMEGpp, PMEDAPpp, or PMEDAP-diphosphate (PMEApp) up to 1 μM in the assay did not affect the polymerase reaction, indicating that the nucleotide analog diphosphates formed during the incubation of cells with PMEG, PMEDAP, or PMEA would not interfere in the dNTP pool size determination.

Analysis of Nucleotide Analog Metabolites by HPLC. After a 24-h incubation with 1 to 100 μM ^{3}H-labeled nucleotide analogs ([8-^{3}H]PMEG, 15.5–0.155 Ci/mmol; [8-^{3}H]PMEDAP, 18–0.18 Ci/mmol; [2,8-^{3}H]PMEA, 35–0.35 Ci/mmol), cells were harvested by centrifugation, washed twice in cold PBS, counted, and extracted with 60% methanol. The extract was evaporated, dissolved in water, and analyzed by HPLC with a Separon SGX C_{18} reversed-phase column (Melcor Technologies, Sunnyvale, CA) with counter ion [tetrabutylammonium hydrogen sulfate (TBHAS)]. Mono- and diphosphates of nucleotide analogs were separated by a 30-min linear gradient of acetonitrile (0–20%) in 50 mM KH_2PO_4/K_2HPO_4, pH 6.8, 3 mM TBAHS at a flow rate of 1 ml/min. After each run the column was saturated for 5 min with 50 mM KH_2PO_4/K_2HPO_4, pH 6.8, 20 mM TBAHS. Radioactivity of collected fractions (1 ml) was measured by liquid scintillation counting. Radioactively labeled metabolites were identified with nonlabeled mono- and diphosphates of nucleotide analogs as internal standards.

DNA Primer Extension Assay. A primer (18-mer) was 5′-^{32}P-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs), the labeled products were separated on Quick Spin columns (Boehringer Mannheim) and annealed in a 1.5:1 ratio to the respective templates in 10 mM TE, pH 7.5. The following template primers (T · P) were used: T · P-1: 3′-ACTGGTACATTGTCTCTCAAA**C**AAGGAA-5′; 5′-TGACCATGTAACAGAGAG; T · P-2: 3′-ACTGGTACATTGTCTCTCAAA**T**AAGGAA-5′; 5′-TGACCATGTAACAGAGAG.

Reaction mixtures (10 μl) for DNA polymerase α, δ, or ϵ contained 0.5 μM dTTP and buffer α (40 mM HEPES-KOH, pH 7.0, 10% glycerol, 1 mM dithiothreitol, 5 mM $MgCl_2$, 200 mg/ml BSA), buffer δ (buffer α + 1 μg of proliferating cell nuclear antigen/ml) or buffer ϵ (buffer α with pH 7.5), respectively. In addition, either 0.1 μM ^{32}P-labeled T · P-1 along with various concentrations of PMEGpp or dGTP were added (PMEG and dGMP incorporation) or 0.1 μM ^{32}P-labeled T · P-2 along with various concentrations of PMEDAPpp, PMEApp, or dATP were added (PMEDAP, PMEA, and dAMP incorporation). After a 15-min incubation at 37°C, the reactions were stopped by the addition of an equal volume of 98% formamide containing 10 mM EDTA, 0.2% bromophenol blue, and 0.2% xylene cyanol FF. Products were separated by 20% denaturing PAGE, the gels were scanned, and the radioactivity was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). K_m and V_{max} values for incorporation of dNTPs and acyclic nucleoside phosphonate diphosphates (ANPpps) were calculated from the Michaelis-

TABLE 1

Intracellular concentrations of nucleotide analogs and their metabolites after a 24-h incubation with the drugs

Exponentially growing cells were incubated with the indicated concentrations of nucleotide analogs for 24 h at 37°C and extracted. Intracellular concentrations of nucleotide analogs and their mono- and diphosphates were determined as described in *Materials and Methods*. Results are means of two separate experiments.

	PMEG			PMEDAP			PMEA		
	[PMEG]	[PMEGp]	[PMEGpp]	[PMEDAP]	[PMEDAPp]	[PMEDAPpp]	[PMEA]	[PMEAp]	[PMEApp]
					μM				
1	0.12 ± 0.00	0.13 ± 0.03	0.48 ± 0.10	0.46 ± 0.06	0.06 ± 0.01	0.15 ± 0.02	0.35 ± 0.05	0.09 ± 0.02	0.11 ± 0.00
3	0.32 ± 0.04	0.61 ± 0.09	2.1 ± 0.3	1.6 ± 0.2	0.25 ± 0.04	0.62 ± 0.10	1.3 ± 0.2	0.43 ± 0.04	0.50 ± 0.08
10	0.76 ± 0.06	2.0 ± 0.4	8.0 ± 1.0	5.8 ± 0.4	0.87 ± 0.10	3.0 ± 0.6	4.5 ± 0.7	1.8 ± 0.3	2.2 ± 0.7
30	3.9 ± 0.3	7.0 ± 0.9	27.5 ± 2.2	24.6 ± 3.0	4.1 ± 0.5	11.6 ± 2.4	19.7 ± 1.7	6.8 ± 1.0	9.3 ± 1.9
100	9.6 ± 1.3	19.3 ± 2.1	91.5 ± 7.5	71.3 ± 8.5	15.3 ± 2.8	44.0 ± 6.3	54.7 ± 4.8	27.0 ± 3.7	36.9 ± 3.4

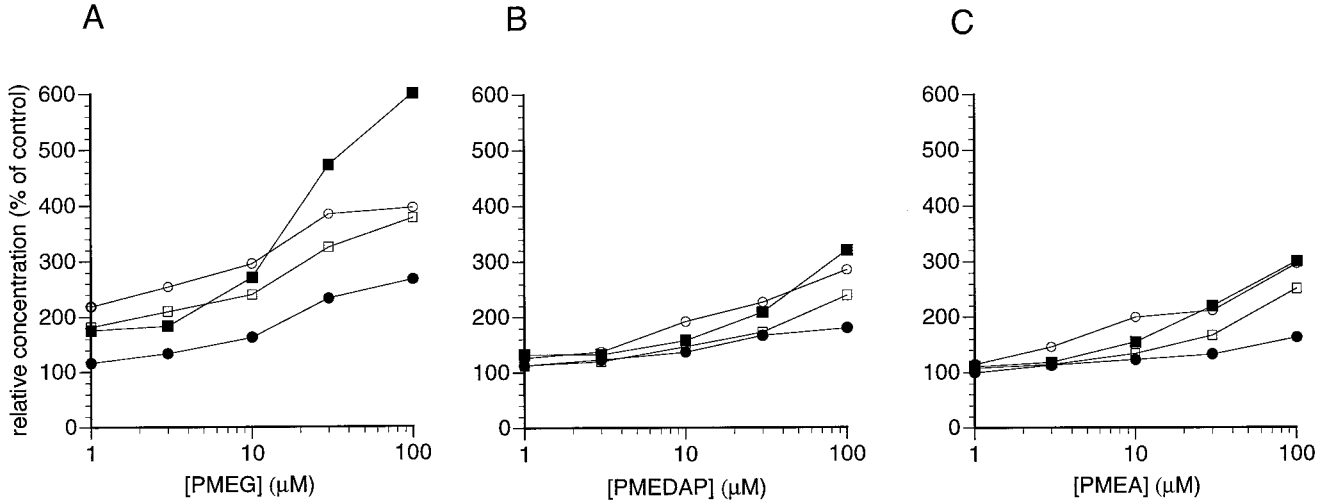


Fig. 3. Effects on dNTP pools of treatment with A, PMEG; B, PMEDAP; and C, PMEA. Exponentially growing cells were incubated with the indicated concentrations of nucleotide analogs for 24 h at 37°C. Cells (0.75–1.5 $\times$ 10^6/assay) were extracted and the concentration of a particular dNTP in extracts was determined by an enzymatic assay as described in *Materials and Methods*. One hundred percent represents 45 μM dATP (■), 30 μM dGTP (●), 83 μM dTTP (□), and 26 μM dCTP (○). The intracellular concentrations were calculated by dividing the quantity of nucleotide determined per million cells by their volume (10^6 cells represents a volume of 0.37 ± 0.1 μl). The calculation assumes a uniform distribution of nucleotides in total cellular water.

Menten equasion with Lineweaver-Burk plots and the KinetAsyst computer program.

Isolation of Cellular DNA and HPLC Analysis of DNA Digest. Exponentially growing CEM cells were incubated with 1 μM [8-^{3}H]PMEG (15.5 Ci/mmol) or 6 μM [8-^{3}H]PMEDAP (18 Ci/mmol) for 24 h at 37°C, harvested, and extracted with methanol as described above. The insoluble material from 1.5×10^7 cells was resuspended in 10 mM Tris-HCl, pH 7.5, 0.5% SDS, 100 mM NaCl, and 25 mM EDTA, and incubated overnight at 50°C with 0.2 mg/ml proteinase K. The material was twice extracted by phenol/chloroform/isoamyl alcohol (25:24:1) and once by chloroform. After ethanol precipitation, RNA was removed by a 3-h incubation with 100 U/ml of RNace-It ribonuclease cocktail (Boehringer Mannheim) at 37°C. The phenol extraction and ethanol precipitation steps were repeated. The resulting DNA (~200 μg) was digested for 2 h at 25°C with DNase I (500 U/mg DNA) in buffer containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$ and 2 mM $CaCl_2$, with addition of fresh DNase I after 1 h. The pH of the solution was then adjusted to 9, and the material was divided into halves. One part was digested to deoxyribonucleoside monophosphates by a 1-h incubation with 2 U of phophodiesterase I at 37°C. The second part was digested to deoxyribonucleosides by a 1-h incubation with 2 U of phophodiesterase I and 5 U of alkaline phosphatase. To identify the phosphonates in the DNA digests, the samples were loaded onto a Partisil-10 SAX (4.6 × 250 mm) column and eluted at 1 ml/min for 30 min with a gradient of 10 to 50 mM KH_2PO_4/K_2HPO_4, pH 3.7. Radioactivity in each 1-ml fraction was determined by liquid scintillation counting. Retention time of a particular labeled phosphonate was compared with that of an unlabeled standard.

Results

Effects of PMEG, PMEDAP, and PMEA on Cellular DNA Synthesis in CEM Cells. The ability of the nucleotide analogs to inhibit cellular DNA synthesis was determined by measuring [^{3}H]thymidine incorporation into DNA after treatment of CEM cells with various concentrations of the drugs (1–1000 μM) for 24 h (Fig. 2). The concentration of drug that inhibited cellular DNA synthesis 50% (IC_{50}) was found to be ~1 μM for PMEG, 6 μM for PMEDAP, and 25 μM for PMEA. The marked differences in the activities of these drugs as inhibitors of DNA synthesis could be the result of differences in drug metabolism and/or differences in the affinities of DNA polymerases for the diphosphate derivatives of the drugs.

Intracellular Metabolism of PMEG, PMEDAP, and PMEA in CEM Cells. The levels of PMEG, PMEDAP, and PMEA and their mono- and diphosphate derivatives were determined after a 24-h treatment of CEM cells with varying concentrations of the drugs. As shown in Table 1, we found that the accumulation of analog diphosphates was linearly dependent on the extracellular concentration of analog in the tested range (1–100 μM). PMEGpp accumulated to significantly higher levels (2- to 4-fold) than PMEDAPpp and PMEApp, with the greatest differences seen at lower analog concentrations. In accord with the previous observation of Compton et al. (1999), neither PMEG nor its phosphorylated metabolites were detected in cells treated with PMEDAP, suggesting that PMEDAP is not a substrate for cellular adenosine or adenylate deaminases.

No significant differences were seen among the nucleotide analogs in the total level of all of their intracellular metabolites; however, the fraction of total intracellular drug metabolized to the mono- and diphosphate derivative varied. For example, at a 10 μM extracellular concentration of the analogs, the intracellular distribution of parental analog and its mono- and diphosphate derivatives was found to be 7, 19, and 74% for PMEG; 60, 9, and 31% for PMEDAP; and 53, 21, and 26% for PMEA, respectively (Table 1). Consistent with a previous report that the uptake of PMEA into CEM cells apparently proceeds by fluid-phase endocytosis and is nonconcentrative and nonsaturable (Olšanská et al., 1997), we did not observe saturation of any of the nucleotide analogs in the tested concentration range. These results suggest that the differences in the levels of the diphosphates of PMEG, PMEDAP, and PMEA in CEM cells reflect differences in the efficiencies with which cellular enzymes phosphorylate the parental analogs.

Effects of Nucleotide Analogs on Intracellular Levels of dNTPs. Treatment of cells with increasing concentrations of the nucleotide analogs was found to result in increased levels of all four dNTPs (Fig. 3). Changes in dNTP levels were much more dramatic in cells treated with PMEG (Fig. 3A) than in cells treated with PMEDAP (Fig. 3B) or PMEA (Fig. 3C). All three nucleotide analogs produced the greatest change in the level of dATP followed by dCTP and dTTP; the level of dGTP changed relatively little.

The data in Table 1 and Fig. 3 were used to calculate the intracellular ratios of the nucleotide analog diphosphates to their corresponding dNTPs after a 24-h exposure to various concentrations of drugs (Fig. 4). Comparable extracellular concentrations of the analogs generated a 3.8- to 5.6-fold higher ratio of [PMEGpp]/[dGTP] than [PMEDAPpp]/[dATP] and a 3.9- to 6.4-fold higher ratio of [PMEGpp]/[dGTP] than [PMEApp]/[dATP]. At an extracellular concentration that inhibits DNA synthesis 50%, i.e., 1, 6, and 25 μM for PMEG, PMEDAP, and PMEA, respectively (Fig. 2), the ratio of [PMEGpp]/[dGTP], [PMEDAPpp]/[dATP], and [PMEApp]/[dATP] corresponded to values of 0.014, 0.025, and 0.07, respectively (Fig. 4). If the affinities of the DNA replication enzymes for the nucleotide analog diphosphates were the same, the values of these ratios should be identical. However, the results indicate that PMEGpp is 1.8-fold more efficient than PMEDAPpp and 5-fold more efficient than PMEApp in

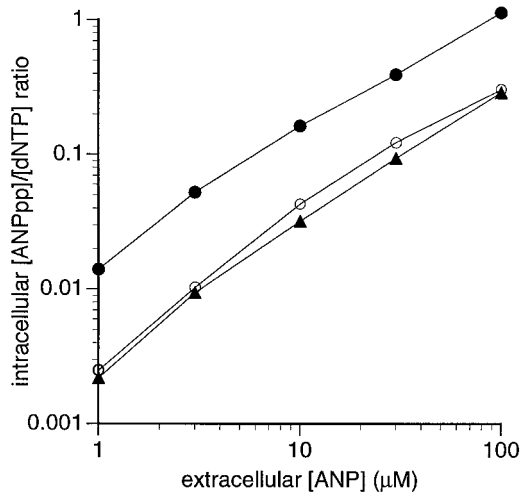


Fig. 4. Intracellular ratios of nucleotide analog diphosphates to their corresponding dNTPs. Exponentially growing cells were incubated with the indicated concentrations of nucleotide analogs for 24 h at 37°C. Nucleotide analog metabolites and dNTPs were extracted and analyzed as described in *Materials and Methods*. ●, [PMEGpp]/[dGTP]; ○, [PMEDAPpp]/[dATP]; ▲, [PMEApp]/[dATP].

inhibiting DNA synthesis in whole cells. Thus, the inhibition of cellular DNA synthesis by the acyclic nucleotide analogs is probably a function of both the intracellular ratios of [ANPpp]/[dNTP] and the affinities of the cellular DNA polymerases for the nucleotide analogs.

Efficiency of Incorporation of PMEG, PMEDAP, and PMEA Diphosphates by DNA Polymerase α, δ, and ϵ. To determine the efficiency with which the replicative DNA polymerases α, δ, and ϵ incorporate the nucleotide analog diphosphates into DNA, a primer extension assay with oligonucleotide template primers (T · P) was used (Fig. 5). The template primers, with otherwise identical sequences, contained a single incorporation site for either dGMP (T · P-1) or dAMP (T · P-2), located at the fourth position following the primer 3′ terminus. The K_m and V_{max} values for the incorporation of PMEGpp, PMEDAPpp, and PMEApp were obtained from Lineweaver-Burk plots as shown in Fig. 5 for DNA polymerase δ. Because incorporated nucleotide analogs function as chain terminators due to the lack of a 3′-OH moiety, extension of the primer beyond the incorporated analog (Fig. 5, arrow) did not occur. However, in control experiments with dATP or dGTP as a substrate, extension of the primer beyond the incorporated dAMP or dGMP did occur and thus the evaluation of control experiments required inclusion of three product bands for the analysis (data not shown).

For each replicative DNA polymerase, the K_m and V_{max} values for incorporation of dGTP and PMEGpp are shown in Table 2 and those for incorporation of dATP, PMEDAPpp, and PMEApp are shown in Table 3. Also shown in Tables 2 and 3 are the incorporation efficiencies, $f_{INC}(\%) = 100 \times (V_{max}/K_m)_{PMEXpp}/(V_{max}/K_m)_{dNTP}$ (Petruska et al., 1988), which reflect the abilities of the enzymes to discriminate between the analog diphosphate and the corresponding dNTP for incorporation into DNA. PMEGpp exhibited the highest substrate affinity for all three DNA polymerases: 2.5-, 2.0-, and 1.4-fold higher than PMEDAPpp and 18.3-, 7.4-, and 10.4-fold higher than PMEApp, as determined with DNA polymerases α, δ, and ϵ, respectively. Among the DNA polymerases, DNA polymerase δ was found to have the highest affinity for the analogs. The incorporation efficiencies were 40.2% for PMEGpp, 20.5% for PMEDAPpp, and 5.4%

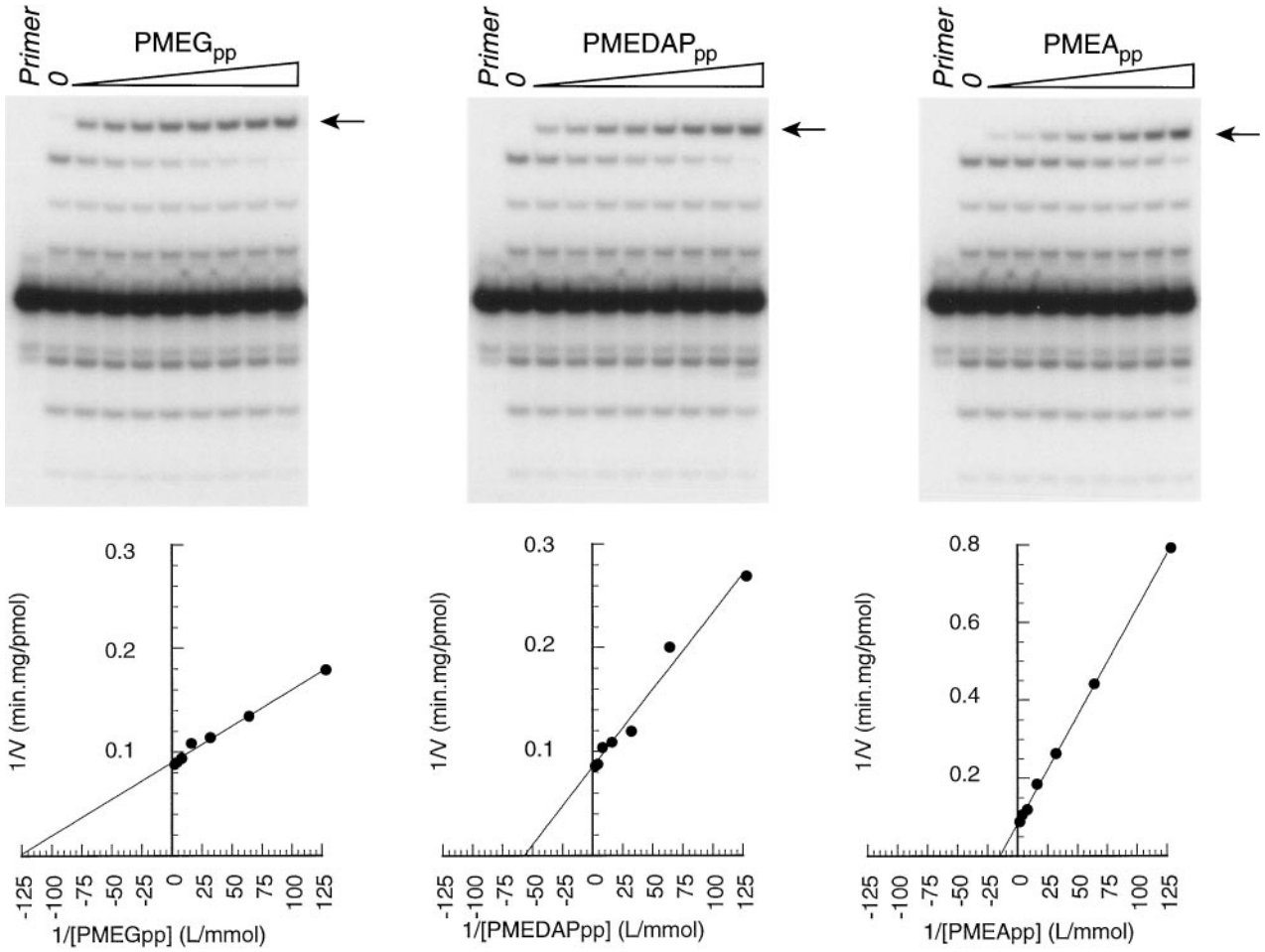


Fig. 5. Incorporation of A, PMEGpp; B, PMEDAPpp; and C, PMEApp by DNA polymerase δ. Pol δ (0.07 U) was incubated with 0.1 μM ^{32}P-labeled T · P-1 (PMEGpp) or T · P-2 (PMEDAPpp or PMEApp), and 0.5 μM dTTP in 10-μl reaction mixtures as described in *Materials and Methods*. Reaction mixtures also contained the following concentrations of nucleotide analog diphosphates: 0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, and 5 μM. After 15 min, reactions were stopped by the addition of an equal volume of 98% formamide containing 10 mM EDTA, 0.2% bromophenol blue, and 0.2% xylene cyanol FF. Products were separated by 20% denaturing PAGE, and the gel was scanned. The radioactivity present in each lane was quantitated with a PhosphorImager. Top panels, primer, nonextended primer; 0, the primer extended with dTTP only. Arrow, position of the primer extended with dTTP and terminated with the analog. Bottom panels, Lineweaver-Burk plots of the data from the corresponding top gels. Kinetic constants were calculated with the KinetAsyst computer program.

for PMEApp. These results suggest that among the replicative cellular DNA polymerases, DNA polymerase δ is a preferred target for the action of the nucleotide analog diphosphates. Moreover, the substrate affinities of PMEGpp, PMEDAPpp, and PMEApp for DNA polymerase δ correspond well with the sensitivities of cellular DNA replication to the three nucleotide analogs.

Incorporation of PMEG and PMEDAP into Cellular DNA. To determine the levels of PMEG and PMEDAP incorporated into DNA under conditions where these analogs cause 50% inhibition of cellular DNA synthesis, cells were labeled with 1 μM [^{3}H]PMEG (Fig. 6, A and B) or 6 μM [^{3}H]PMEDAP (Fig. 6, C and D) for 24 h, followed by isolation of labeled cellular DNA. Aliquots of purified DNA were then digested to either deoxynucleotides (Fig. 6, A and C) or deoxynucleosides (Fig. 6, B and D). Analysis of deoxynucleotides in the digests with an ion-exchange column revealed small radioactive peaks separated from four deoxynucleotides with a retention time corresponding to PMEG (27 min; Fig. 6A) and PMEDAP (10 min; Fig. 6C). The majority of the radioactivity coeluted with deoxynucleotides, probably due to labeling of cellular dNTPs with a free purine base present as a minor impurity (<3%) in samples of labeled nucleotide analogs. Consistent with this hypothesis, the radioactivity eluted from an ion-exchange column in the void volume along with deoxynucleosides after treatment with alkaline phosphatase (Fig. 6, B and D). Because the phosphonate bond is resistant to treatment with alkaline phosphatase, the analogs retain their charged phosphonate group and are bound by the ion-exchange resin. Approximately the same amounts of incorporated analogs were detected in both systems, i.e., 0.32 pmol of PMEG and 0.25 pmol of PMEDAP/mg DNA. The data demonstrate that both of the analogs are incorporated into cellular DNA to a similar level following a 24-h incubation with an extracellular concentration of analog corresponding to the IC_{50} for cellular DNA synthesis.

Discussion

The acyclic nucleotide analogs PMEG, PMEDAP, and PMEA were found to differ dramatically in their ability to inhibit cellular DNA synthesis in CEM cells. Possible reasons were investigated, including differences in cellular transport of the analogs, different efficiencies of intracellular phosphorylation, and differences in the affinities of the cellular DNA polymerases for the diphosphate derivatives of the drugs.

No significant differences in cellular uptake were found among the analogs; however, they did differ in the efficiency of phosphorylation by cellular enzymes. CEM cells were found to accumulate higher levels of PMEGpp than PMEDAPpp or PMEApp. Previous studies reported that GMP kinase catalyzes the phosphorylation of PMEG (Ho et al., 1992) and that mitochondrial and cytosolic isoenzymes of AMP kinase catalyze phosphorylation of PMEDAP and PMEA (Merta et al., 1992; Robbins et al., 1995b). Thus, more efficient phosphorylation of the acyclic guanine analog than the acyclic adenine analogs in CEM cells is probably due to a higher substrate specificity of GMP kinase for PMEG (Ho et al., 1992) compared with the substrate specificity of AMP kinase for PMEA or PMEDAP (Merta et al., 1992; Robbins et al., 1995).

Treatment of CEM cells with any of the acyclic nucleotide analogs was found to result in increased dNTP pools. PMEG produced the greatest increase and all three analogs had the greatest effect on the dATP pool size. Levels of dGTP were not significantly affected by treatment with any of the nucleotide analogs. Recently, Hatse et al. (1999) reported a general increase in dNTP pool size after a treatment of human erythroleukemia cells (K562) with PMEA. However, in contrast to the present results, this group observed the highest elevation in intracellular concentration of dTTP and the lowest change in the dATP pool size. It is possible that changes in levels of a particular dNTP after cellular treatment with acyclic nucleotide analogs are dependent on cell type.

The increase in dNTP pools following treatment with the acyclic nucleotide analogs contrasts with the effects of the clinically effective nucleoside analogs fludarabine (Gandhi and Plunkett, 1989; Huang et al., 1990) and gemcitabine (Huang et al., 1991) as well as 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (Xie and Plunkett, 1996) and

TABLE 2
Incorporation efficiency of PMEGpp by replicative DNA polymerases with a running-start primer extension assay

	dGTP		PMEGpp		
	V_{max}	K_m	V_{max}	K_m	f_{INS} (%)[a]
	pmol/min/mg	*nM*	*pmol/min/mg*	*nM*	
Pol α	3.6 ± 0.2	25 ± 4	0.93 ± 0.05	101 ± 7	6.4
Pol δ	20.9 ± 1.0	6 ± 1	11.2 ± 0.9	8 ± 1	40.2
Pol ε	0.60 ± 0.02	9 ± 1	0.48 ± 0.03	122 ± 16	5.9

[a] Insertion efficiency was calculated according to the following formula:
f_{INS} (%) = 100 × $(V_{max}/K_m)_{PMEGpp}/(V_{max}/K_m)_{dGTP}$ (Petruska et al., 1988).

TABLE 3
Incorporation efficiency of PMEDAPpp and PMEApp by replicative DNA polymerases with a running-start primer extension assay

	dATP		PMEDAPpp			PMEApp		
	V_{max}	K_m	V_{max}	K_m	f_{INS}(%)[a]	V_{max}	K_m	f_{INS}(%)[a]
	pmol/min/mg	*nM*	*pmol/min/mg*	*nM*		*pmol/min/mg*	*nM*	
Pol α	3.3 ± 0.2	21 ± 3	0.96 ± 0.05	238 ± 12	2.6	0.83 ± 0.08	1510 ± 230	0.35
Pol δ	13.8 ± 0.9	4.0 ± 0.6	12.0 ± 0.5	17 ± 2	20.5	12.5 ± 0.50	67 ± 8	5.4
Pol ε	0.56 ± 0.02	15 ± 1	0.58 ± 0.02	370 ± 16	4.2	0.42 ± 0.03	1960 ± 150	0.57

[a] See Table 2.

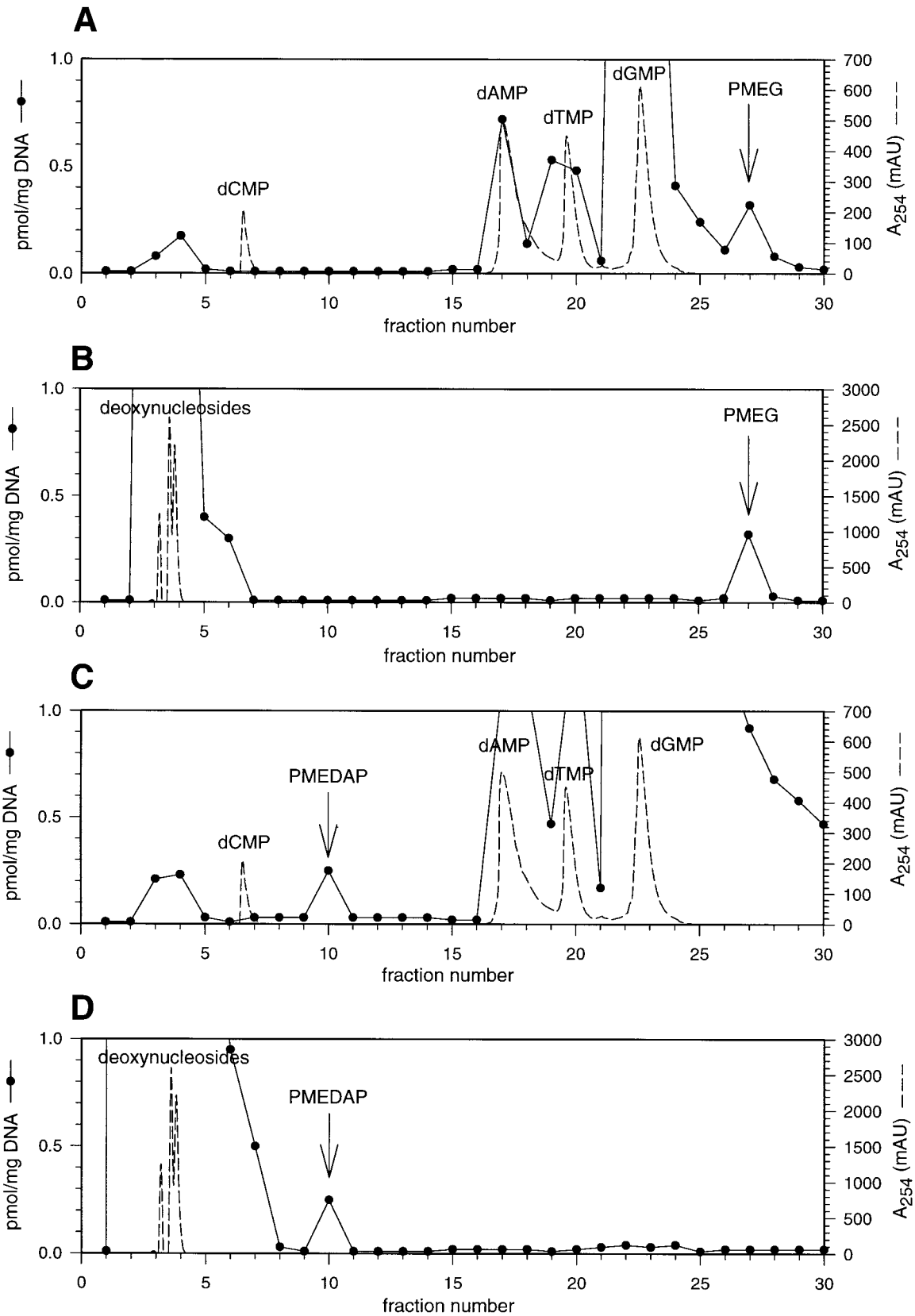


Fig. 6. HPLC separation of PMEG (A and B) or PMEDAP (C and D) from dNMPs and deoxynucleosides in digested cellular DNA. Cells were incubated with 1 μM [^{3}H]PMEG or 6 μM [^{3}H]PMEDAP for 24 h at 37°C. DNA was isolated, and one-half digested to dNMPs and one-half digested to deoxynucleosides as described in *Materials and Methods*. Each digest was applied to a Partisil-10 SAX column and eluted at 1 ml/min for 30 min with a gradient of 10 to 50 mM KH_2PO_4/K_2HPO_4, pH 3.7. The position of a nucleotide analog was identified with a nonlabeled standard. (●), the level of incorporated material in picomoles per milligram DNA.

2′,2′-difluorodeoxyguanosine (Gandhi et al., 1995). The antiproliferative action of these drugs was shown to be the result of both a reduction in dNTP pools, due to inhibition of ribonucleotide reductase, and incorporation of the analogs into DNA by DNA polymerases. Thus, depletion of cellular dNTP pools increases the effectiveness of the triphosphate derivatives of these drugs as competitors with dNTPs for binding to a DNA polymerase catalytic site. In contrast, the increased levels of dNTPs in cells treated with analogs of the PME series may diminish the inhibitory effects of their diphosphates on cellular DNA synthesis.

Comparison of the ratios of nucleotide analog diphosphates to their corresponding dNTPs under conditions where DNA synthesis was inhibited 50% suggested that DNA replicative enzymes were approximately twice as sensitive to PMEGpp than to PMEDAPpp and 5-fold more sensitive to PMEGpp than to PMEApp. Examination of the incorporation efficiencies of these analogs in vitro showed that DNA polymerase δ, the most sensitive of the replicative DNA polymerases, incorporated PMEGpp twice as efficiently as PMEDAPpp and 7-fold more efficiently than PMEApp. In accord with the present results, a previous study reported that DNA polymerase δ had a 7.6-fold lower IC_{50} for PMEGpp than for PMEApp and was more sensitive to the nucleotide analogs than DNA polymerase α (Pisarev et al., 1998). Because DNA polymerase δ not only cooperates with DNA polymerases α and ϵ at the replication fork (Bambara et al. 1997) but also participates in nucleotide excision repair as a gap filling enzyme (Sancar, 1995), it is possible that nucleotide analogs may be incorporated into DNA during both DNA replication and repair.

Although both PMEG and PMEDAP were detected in DNA of cells treated with these analogs, their levels were unexpectedly low. In cells where DNA synthesis was inhibited 50%, only 0.32 pmol of PMEG and 0.25 pmol of PMEDAP/mg of DNA was found. In contrast, 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate (F-ara-A), a DNA chain terminator, was detected at a level of 30 pmol/mg DNA in CEM cells under similar conditions (Huang et al., 1990). It is possible that cellular proofreading and/or repair mechanisms have markedly different abilities to remove the acyclic nucleotide analogs versus 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate from 3′ termini of DNA chains. Although little is known about cellular repair of nucleoside or nucleotide analogs incorporated into DNA, it has been demonstrated that F-ara-A-terminated DNA is not a substrate for the 3′-5′ exonuclease of DNA polymerase ϵ and inhibits the enzyme by formation of a "dead end complex" (Kamiya et al., 1996). In contrast, our previous studies showed not only that the 3′-5′ exonuclease activity of DNA polymerase ϵ could remove PMEG from the 3′ ends of DNA but also that the DNA polymerase activity of the enzyme was able to elongate PMEG-terminated primers (Kramata et al., 1998). The ability of both DNA polymerases δ and ϵ to excise PMEA from the 3′ ends of DNA chains in vitro also was demonstrated by Birkuš et al. (1999). The in vivo significance of these activities of the DNA polymerases remains to be elucidated.

The results of the present studies suggest that differences in inhibition of cellular DNA synthesis by PMEG, PMEDAP, and PMEA may be explained by different intracellular ratios of the analog diphosphates to their corresponding deoxynucleoside triphosphates and different affinities of DNA polymerases, primarily DNA polymerase δ, for the nucleotide analog diphosphates. However, we cannot exclude the possibility that the mechanism of antiproliferative action of the acyclic nucleotide analogs also involves some unidentified targets.

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