Intracellular Metabolism and Action of Acyclic Nucleoside Phosphonates on DNA Replication

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SUMMARY

9-(2-phosphonylmethoxyethyl)guanine (PMEG) is an acyclic nucleoside phosphate derivative that has demonstrated significant anticancer activity in a number of in vitro and in vivo animal model systems. In this study, we compared the cellular metabolism of PMEG and 9-(2-phosphonylmethoxyethyl)adenine (PMEA), a clinically active anti-HIV and antihepatitis agent, and the inhibitory activities of their putative active diphosphate derivatives, PMEGpp and PMEApp, respectively, toward human cellular DNA polymerases. PMEG was significantly more cytotoxic than PMEA against a panel of human leukemic cells. The diphosphate derivatives were the major metabolites formed in cells on both these agents, with PMEGpp reaching cellular concentration approximately 4-fold higher than that achieved for PMEApp. These differences in cellular accumulation of the diphosphate derivatives were not, however, sufficient to account for the 30-fold difference in cytotoxicity between the two analogs. PMEGpp was also at least a 7-fold more effective inhibitor of in vitro simian vacuolating virus 40 DNA replication system than that of PMEApp ($IC_{50} = 4.6 \mu M$). Studies with a defined primed DNA template showed that PMEGpp was a potent inhibitor of both human polymerases $\alpha$ and $\delta$, two key enzymes involved in cellular DNA replication, whereas PMEApp inhibited these enzymes relatively poorly. From these studies, we can conclude that the factors that contribute to the enhanced antileukemic activity of PMEG derives both from its increased anabolic phosphorylation and the increased potency of the diphosphate derivative to target the cellular replicative DNA polymerases.

The ANP analogs display a broad spectrum of activity against a range of DNA viruses and retroviruses, including the human immunodeficiency virus (1, 2). The prototype compounds PMEA and PMPA (Fig. 1) inhibit viral replication in a number of animal model systems of acquired immunodeficiency syndrome (3–5). PMPA is rather unique in that it has shown potent efficacy in simian immunodeficiency virus-infected macaques and has been shown to completely suppress viremia and disease symptoms in treated animals (5). The mechanism for the antiretroviral activity of PMPA and PMEA may be accounted for by the inhibition of the viral DNA polymerases by the corresponding active diphosphate derivatives (6, 7). In addition to their antiviral activity, several of the ANPs are cytotoxic. PMEA and, particularly, the guanine derivative, PMEG, inhibit the growth of both murine (8) and human (9) leukemic cells. PMEG was shown to be the most cytotoxic of the ANP analogs studied (9). Studies in murine models have shown that PMEG increases the survival of mice with transplantable tumors (8). In addition, PMEG suppresses human condylomas from papilloma virus (HPV-11)-infected human foreskins in transplanted mice (10). The mechanism responsible for all these effects by PMEG is not known. Metabolic studies have shown that the ANP analogs are resistant to hydrolysis by purine nucleoside phosphorylase and other known catabolic enzymes such as phosphatase and nucleotidase, which gives these compounds prolonged metabolic stability. To be active, analogs need to be phosphorylated by intracellular kinases. The appearance of mono- and diphosphates of PMEG was demonstrated in murine and human cells and its effects on human DNA polymerases have not been examined. The present study was aimed at a comparison of the metabolism of PMEA and PMEG and their action against human cellular DNA polymerases, pol $\alpha$ and $\delta$.
HeLa cells (5 x 10^9) prepared as described previously (17). Briefly, asynchronously grown as described previously (15, 16). HeLa cell cytosolic extracts were obtained from Moravek Biochemicals (Brea, CA). The purity of the mM MgCl2, 1 mM DTT). Protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, and 0.2 mg/ml antipain) were then added, and the swollen cells were broken by 15–20 strokes in a dounce homogenizer. After adjusting the salt concentration to 200 mM, we cleared the extracts by centrifuging and dialyzing them then added, and the swollen cells were broken by 15–20 strokes in a dounce homogenizer. After adjusting the salt concentration to 200 mM, we cleared the extracts by centrifuging and dialyzing them.

Materials and Methods

Compounds. Reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Nonlabeled nucleotides were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Human pol α-primase complex, pol δ, and PCNA were prepared as described previously (15, 16). HeLa cell cytosolic extracts were prepared as described previously (17). Briefly, asynchronously grown HeLa cells (5 x 10^9) were washed twice with phosphate-buffered saline and resuspended in hypotonic buffer (20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid-KOH, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 1 mM DTT). Protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, and 0.2 mg/ml antipain) were then added, and the swollen cells were broken by 15–20 strokes in a dounce homogenizer. After adjusting the salt concentration to 200 mM, we cleared the extracts by centrifuging and dialyzing them against buffer (50 mM Tris-HCl pH 7.8, 10% glycerol, 1 mM DTT, 0.5 mM EDTA) containing 25 mM NaCl. A unit of activity was defined as the incorporation of 1 nmol of dNTP/hr into primed DNA template under the conditions previously described for the enzymes (15). Oligonucleotides, 12-mer (primer) and 30-mer (template), were synthesized by the Midland Certified Reagent Company (Midland, TX) with more than 95% purity. ANP analogs (see Fig. 1) were kindly provided by Dr. Norbert Bischofberger, Gilead Sciences (Foster City, CA). 8-[3H]PMEG (17.6 Ci/mmol) and 2,8-[3H]PMEA (17 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA).

Metabolism of [3H]PMEG and [3H]PMEA. Exponentially growing CEMss cells were harvested by centrifugation, resuspended at 1 x 10^9/ml in fresh medium, and incubated at 37°C with [3H]PMEG or [3H]PMEA. At different time points, aliquots of the cells were removed and centrifuged through Nyosil 50 (W.P. Nye, New Bedford, MA) at 13,000 x g for 60 sec at 4°C. The cell pellet was then extracted with 70% ice-cold methanol and 15 mM Tris, pH 7.0, and the aqueous phase was collected and analyzed for compounds and their metabolites by using an HPLC Partisil 10 SAX column (18, 19). Mono- and diphosphates of PMEG and PMEA were separated by a linear gradient run over 47 min at 1.5 ml/min starting at 100% buffer A (5 mM NH4H2PO4, pH 4.0) to 100% buffer B (0.6 M NH4H2PO4, pH 4.0) with the optimal regimen as described previously (20). The radioactivity associated with the PMEG and PMEA metabolites was measured by using a Beckman scintillation counter (Beckman Instruments, Palo Alto, CA).

SV40 DNA replication in vitro. The reactions were carried out as described previously (16) with some modifications. In brief, the reaction mixtures (40 µl) contained 40 mM creatine phosphate-diphosphate-Tris salt (pH 7.7); 40 µg/ml creatine kinase; 7.0 mM MgCl2; 0.5 mM DTT; 4 mM ATP; 5 µg/ml SV40 origin-containing plasmid (pZ 189 or pUCori+); 33 µM UTP, GTP, and CTP; 25 µM dATP, dGTP, dCTP, and [3H]dTTP (200–300 cpm/pmol); 0.6 µg SV40 T-antigen; HeLa cell cytosolic extract (150 µg); and the indicated amounts of diphosphates of PMEA and PMEG. The reactions ran at 37°C for the time indicated, and the trichloroacetic acid precipitable radioactive material was collected for quantitation. For product analysis, replication reactions were carried out using [33P]dATP (25,000 cpm/pmol) instead of [3H]dTTP. The reactions were stopped by the addition of 80 µl of a solution containing 20 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 µg/ml Escherichia coli tRNA. Aliquots of the reaction mixture were then withdrawn for glass filter assay. DNA was isolated from the remaining reaction mixture (21) and electrophoretically separated on 0.8–0.9% alkaline agarose gels containing 40 mM NaOH and 1 mM EDTA at 2 V/cm for 12–14 hr. The gels were dried and exposed to x-ray film (Eastman Kodak, Rochester, NY).

Polymerase activity. Reaction mixtures (10 µl) contained 50 mM Tris, pH 7.5, 5 mM MgCl2, 5–100 µM dNTPs, 2 mM DTT, 0.05 mg/ml BSA, and 0.5 pmol DNA. The reactions were initiated by the addition of 0.5 units of pol α or 0.2 units of pol δ plus 35 ng of PCNA. After a 30-min incubation at 37°C, the reactions were quenched by the addition of 0.2 volume of 6 x gel loading buffer, which contained 300 mM NaOH, 6 mM EDTA, 18% Ficoll 400 in water, 0.15% bromocresol green, and 0.25% xylene cyanol (21). To measure radioactivity in the samples, [α-32P]dATP (105 cpm/pmol) or a 12-mer primer labeled with [γ-32P]ATP at its 5’ end (21) was used. After polyclaramide gel electrophoresis through 15–18% acrylamide/8 M urea, the DNA products were analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The IC50 value for each compound was determined by using SigmaPlot for 29- to 30-mer oligonucleotides, which were the final products of this reaction.

Results

Cytotoxicity and metabolism of PMEG and PMEA. PMEG and PMEA were compared for their inhibitory effect...
on the growth of the human T cell and B cell lines, CEMss and WIL-2, respectively. We found that the cytotoxicity of PMEG was approximately 30 times higher than that of PMEA. Thus, the IC₅₀ value for cell growth after 72 hr of culture with PMEG was 0.3–0.6 μM compared with 12–15 μM for PMEA. Both PMEG and PMEA require phosphorylation to the diphosphate derivative for their cytotoxicity (11, 22). We examined their intracellular metabolism in the human CEMss T cells, which are sensitive to growth inhibition by ANPs. Exponentially growing CEMss cells were incubated with 10 μM [³H]PMEG or [³H]PMEA for 24 hr, and the various intracellular metabolites were quantitated after their separation by HPLC. The levels of intracellular PMEG and mono- and diphosphates were 0.4, 1.35, and 3.8 pmol/10⁶ cells, respectively, whereas the corresponding levels for PMEA and its two anabolites were 2.6, 0.47, and 0.89 pmol/10⁶ cells, respectively. These differences in the intracellular profiles of PMEG and PMEA metabolites were irrespective of the extracellular concentration of the two compounds used (1–10 μM) or time (3, 6, or 24 hr) of cell cultivation (data not shown). These results, therefore, show that PMEG is more effectively converted to its respective metabolites than is PMEA; however, the difference in nucleotide accumulation between these two agents was only approximately 4-fold, which could not explain their approximately 30-fold difference in cytotoxicity. It could be suggested that a significant difference may exist between ANPs at the level of targeting of DNA replication machinery.

Inhibition of SV40 DNA replication by ANPs. The SV40 DNA replication system is widely used for studies of eukaryotic DNA replication because, with the exception of virally encoded Tag, it uses host replication factors, such as DNA polymerases α and δ, replication protein A, also called human single-stranded DNA binding protein, PCNA, replication factor C, also called activator 1, and topoisomerases I and II (15–17). We examined the effects of the diphosphates of PMEG and PMEA on in vitro replication of plasmid pUCori² of SV40 origin using cytosolic HeLa cell extract as a source of human replication factors (17) in the presence of dNTPs and rNTPs. The addition of increasing concentrations of the diphosphates of the ANPs inhibited the incorporation of [³H]dTMP into DNA with an IC₅₀ value of 4.6 μM and 75 μM for PMEGpp and PMEApp, respectively. We also examined the effect of these compounds on the size distribution of labeled products separated by PAGE. The specificity of pUCori² plasmid replication was confirmed by the total absence of replication in incubation mixtures without Tag (Fig. 2, lane 1) and by the appearance of specific pUCori² plasmid final replication products of 5–6 kbp, ssc and ssl (Fig. 2, lane 2). As shown in Fig. 2, PMEGpp was a more potent inhibitor of SV40 DNA replication than PMEApp or the derivative PMPApp. Thus, at 10 μM, PMEGpp caused almost complete inhibition of DNA replication, whereas PMEApp did so at 100 μM and PMPApp exhibited little effect, at least up to 1 mm. There was also a diminution in product size with increasing concentrations of the PMEGpp and PMEApp. The accumulation of DNA products less than 2000 bp long indicated that the analogs affected DNA chain elongation rather than chain initiation. This was examined further in time course experiments. Fig. 3 depicts the appearance of the DNA replication products at different time points during the replication of the same plasmid. Time-dependent accumulation of DNA products sized between 300 and 2000 bp in length after action of PMEGpp or PMEApp suggests that the initiation of DNA synthesis is not markedly affected by the diphosphates of the ANPs. In another set of experiments, pZ189 plasmid DNA and cell extract were preincubated in the presence of rNTP and dNTP, which allowed the initiation of DNA replication without DNA synthesis. The addition of PMEGpp with dNTPs after incubation led to inhibition of dNMP's incorporation into the growing chain (Fig. 4). This further demonstrated that PMEGpp strongly inhibits DNA chain elongation mediated by HeLa cell extract replication factors.

Sensitivity of human pol α and pol δ-mediated DNA chain elongation to ANPs. It was shown recently that SV40 replication is mainly catalyzed by polymerases α and δ (23). To further investigate the action of these ANPs on DNA replication, we decided to study effects on a complimentary experimental setup using purified human DNA pol α and pol δ. The synthetic DNA substrate of a defined sequence was used in these studies (Fig. 5). Using this 12-mer primer 30-mer template DNA₁₂/₃₀, 0.5 units of pol α incorporated 0.26 pmol of dNTPs into 0.5 pmol of DNA per 30 min. Initial experiments showed that in the presence of PCNA, this synthetic DNA also served as a substrate for pol δ, whereas without PCNA, no activity of the enzyme was present. We designed our DNA₁₂/₃₀ so that adenine and guanosine nucleotides were excluded from the end of the extended primer. In this way, we could use phosphorimagery of the 29- to 30-mer oligonucleotide to study the comparative actions of PMEGpp and PMEApp. The presence of critical C-sites for PMEGpp and T-sites for PMEApp allowed us to visualize the exact nucleotides at which the compounds terminate chain elongation. Importantly, because inhibition of DNA chain elonga-
tion was measured on the same template with both polymers, differences in the potency of this inhibition were not the result of differences in the templates used.

PAGE and phosphorimagery demonstrated that PMEGpp was more potent than PMEApp in inhibiting pol α-mediated DNA chain elongation (Fig. 6). From the 29- to 30-mer bands, we obtained IC₅₀ values of 6.0 μM for PMEGpp and 90.0 μM for PMEApp (Table 1). Similar differences between these compounds were obtained with the pol δ/PCNA-mediated elongation reaction (Table 1).

As shown in Fig. 6, diminution of 29- to 30-mer DNA products correlated with accumulation of the specific oligonucleotides. Phosphorimagery confirmed that chain elongation was interrupted at the T positions of the polynucleotide chain for PMEApp, and at the C positions of DNA₁₂/₃₀ for PMEGpp. This is direct evidence that both PMEApp and PMEGpp are site-specific absolute DNA chain terminators.

Competitive inhibition of DNA chain elongation. To determine whether PMEGpp and PMEApp are competitive inhibitors of DNA chain elongation, we used a 12-mer oligonucleotide primer labeled with ³²P at the 5' end and annealed it to its 30-mer template. As shown in Fig. 7A, by increasing the dGTP concentration in the pol α-mediated reaction, we could abrogate the effect of PMEGpp on the accumulation of shorter oligomer products. Changing the concentration of dNTPs other than dGTP had no effect on PMEApp’s inhibition of chain elongation (data not shown). The PAGE profiles of the DNA after PMEApp action revealed that the effect of PMEApp was abrogated by dATP concentrations but not by other dNTPs (Fig. 7B and data not shown). Similar PAGE profiles were obtained in the pol δ/PCNA-mediated reactions (data not shown). Taken together, these data demonstrate that PMEGpp and PMEApp compete with dGTP and dATP, respectively, in pol α- and pol δ-directed DNA polymerization.

Discussion

In this study, we examined the effects of three ANPs with different cytostatic capabilities on in vitro DNA replication, specifically human replicative DNA polymerases. Using the SV40 DNA replication system and the diphosphoryl derivatives of the acyclic nucleotide analogs PMEApp, PMEGpp, and PMPApp, we showed that DNA chain elongation rather than initiation was sensitive to the action of the compounds tested (Figs. 2 and 3). The anti-replicative activities of the
compounds were of the order PMEGpp \(\gg\) PMEApp \(\gg\) PMEA. The same order of activity was obtained when we tested the ability of these compounds to inhibit cell growth (data not shown). Because the levels of active PMEA and PMEG mono- and diphosphoryl metabolites in human lymphoblast CEMss T cells differed by only 4-fold, it is very unlikely that these differences could account for the 30-fold difference in potency with which these compounds inhibit cell growth. It is more likely that the differences in potency are due to differential sensitivities of the ANP target enzyme(s). To confirm this, we performed our experiments using the primer-template extension assay with DNA12/30 and purified \(\alpha\) and \(\delta\) polymerases. The same differences between PMEG and PMEA as those found using the DNA replication assay were apparent using this system (Fig. 6). These data are in agreement with the recent results of Kramata et al. (12), who found a 20-fold difference between PMEGpp- and PMEApp-induced inhibition of pol \(\alpha\)-activity by using poly(dC)/oligo(dG) and poly(dT)/oligo(dA) DNA as their substrates, respectively. However, they used (dC)/(dG)12–18 DNA as a substrate for the pol \(\delta\)/PCNA reaction, which functioned too poorly for them to obtain data on the effect of PMEGpp on this reaction. The structure of DNA12/30 (Fig. 5) allowed us to compare the activities of PMEG and PMEA on pol \(\delta\)-mediated primer extension (Table 1). Thus, using our system, the inhibition of DNA synthesis could be measured on the same primed DNA template with both compounds and both polymerases, thereby ensuring that any difference in the potency of inhibition was not the result of differences in template composition. We obtained slightly lower \(IC_{50}\) values from the pol \(\delta\)/PCNA-mediated reactions than from the pol \(\alpha\)-directed DNA chain elongation reaction. This probably reflects the greater sensitivity of pol \(\delta\) to ANPs, as has been shown for some other acyclic nucleoside derivatives (24). These \(IC_{50}\) values for inhibition of cell growth were close to those obtained for ANP inhibition of SV40 DNA replication and for ANP-induced interruption of pol \(\alpha\)/pol \(\delta\)-mediated DNA chain elongation (Table 1). Taken together, these results show that PMEGpp and PMEApp arrest cell proliferation by inhibiting the main replicative DNA polymerases.

The mechanism of polymerase inhibition by nucleotide analogs may involve incorporation of the phosphorylated compounds into the growing DNA strand. The structure of the diphosphoryl ANPs allows their incorporation into DNA, where they could serve as absolute chain terminators because they lack 3′-OH groups. Our PAGE data and phosphorimagery analysis indicated that both PMEApp and PMEGpp terminated DNA chain elongation (Fig. 6). An additional band appeared for each specific T site in the reactions containing PMEApp (Fig. 6b). This is strong evidence that the inhibition of DNA synthesis by the ANPs is site-specific.

In summary, our data show that the enhanced anti-leukemic activity of PMEG is derived both from its enhanced anabolic phosphorylation and from the enhanced potency of the analog diphosphate for DNA polymerases. Moreover, our study shows that the human HeLa cell extract traditionally used for the study of SV40 DNA replication (13, 15, 16) is a
useful in vitro model system for studying the mechanism of antiviral nucleotide analogs on DNA replication.

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References


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