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N^6-Cyclopropyl-PMEDAP: A Novel Derivative of 9-(2-Phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) with Distinct Metabolic, Antiproliferative, and Differentiation-Inducing Properties

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ABSTRACT. N^6-Cyclopropyl-PMEDAP (cPr-PMEDAP) is a novel derivative of the acyclic nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP). Its cytostatic activity was found to be 8- to 20-fold more pronounced than that of PMEDAP and equivalent to that of the guanine derivative 9-(2-phosphonylmethoxyethyl)guanine (PMEG) against a variety of tumor cell lines. Unlike PMEDAP, but like PMEG, cPr-PMEDAP was equally cytostatic to wild-type and 9-(2-phosphonylmethoxyethyl)adenine/PMEDAP-resistant variants of the human erythroleukemia K562 and the murine leukemia L1210 cell lines. Also, cPr-PMEDAP and PMEG proved to be equipotent inducers of K562 and rat choriocarcinoma RCHO cell differentiation, whereas the differentiation-inducing activity of PMEDAP was 5- to 25-fold less pronounced. Furthermore, compared to PMEDAP, cPr-PMEDAP and PMEG were 10- to 25-fold more potent in inhibiting the progression of K562 cells through the S phase of the cell cycle, resulting in a marked accumulation of the four 2′-deoxyribonucleoside 5′-triphosphate pools. The biological effects of cPr-PMEDAP, but not PMEDAP, were reversed by the adenylate deaminase inhibitor 2′-deoxycoformycin (dCF). Formation of the deaminated derivative of cPr-PMEDAP (i.e. PMEG) was demonstrated in crude extracts from K562 and L1210 cells and in metabolism studies with radiolabeled cPr-PMEDAP and PMEG. This is the very first example of an acyclic nucleoside phosphonate analogue that is susceptible to deamination. However, cPr-PMEDAP was not recognized as a substrate by purified adenosine deaminase or by adenylate deaminase. These findings might point to an as yet unidentified cellular enzyme, sensitive to dCF but different from the common adenosine and AMP deaminases. Our data demonstrate the superior antiproliferative and differentiation-inducing effects of cPr-PMEDAP on tumor cells, as compared to the parent compound PMEDAP, based on the unique metabolic properties of this novel compound. BIOCHEM PHARMACOL **58**;2:311–323, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. acyclic nucleoside phosphonates; tumor cell differentiation; cytostatic agents; N^6-cyclopropyl-PMEDAP; prodrugs, 2′-deoxycoformycin

The acyclic nucleoside phosphonates represent a unique class of potent broad-spectrum antiviral agents consisting of an adenine, guanine, or cytosine base, linked through an aliphatic moiety to a phosphonate by a highly stable carbon–phosphorus bond. PMEA‡, the prototype congener of the acyclic nucleoside phosphonates, contains an adenine base and can thus be considered as an acyclic derivative of adenosine monophosphate (Fig. 1). PMEA is endowed with strong antiviral activity against herpesviruses, hepatitis B virus (HBV), and retroviruses, including human immunodeficiency virus (HIV) [1–4]. The oral prodrug form of PMEA, bis(pivaloyloxymethyl)-PMEA (adefovir dipivoxil), is currently being evaluated in phase II/III clinical studies in HIV- and HBV-infected patients [5]. Recently, we reported that PMEA is a potent inducer of tumor cell differentiation in several cell lines [6]. These findings suggested that PMEA, in addition to its antiviral activity, may also possess antitumor potential.

PMEDAP (Fig. 1), another member of the acyclic nucleoside phosphonates, displays a spectrum of biological activities that closely resembles that of PMEA [7]. In contrast, the biological effects of PMEG, the guanine counterpart of PMEA (Fig. 1), clearly differ from those of PMEA and PMEDAP. PMEG has a much more pronounced cytostatic activity than the other acyclic nucleoside phosphonate derivatives. Consequently, the potential applications of PMEG may be situated in the field of cancer, rather than virus, chemotherapy [8, 9]. Acyclic

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‡ *Abbreviations:* ABC, abacavir (GW1592); dCF, 2′-deoxycoformycin; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; NTP, ribonucleoside 5′-triphosphate; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEDAP, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine; PMEG, 9-(2-phosphonylmethoxyethyl)guanine: cPr-PMEDAP, 9-(2-phosphonylmethoxyethyl)-N^6-cyclopropyl-2,6-diaminopurine; PMPA, (*R*)-enantiomer of 9-(2-phosphonylmethoxypropyl)adenine; PMPDAP, (*R*)-enantiomer of 9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine; and RCHO, rat choriocarcinoma.

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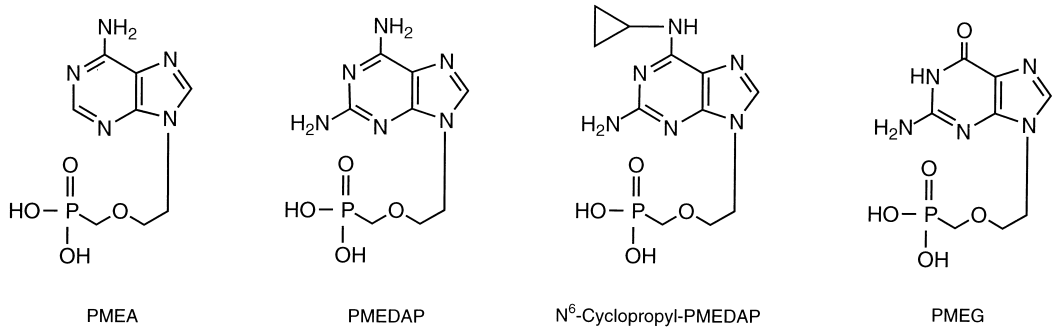


FIG. 1. Structural formulae of acyclic nucleoside phosphonate analogues.

nucleoside phosphonates (e.g. PMEA) are converted intracellularly by cellular enzymes to their mono- and diphosphorylated metabolites (e.g. PMEAp and PMEApp, respectively) [10]. PMEApp acts as a competitive inhibitor of viral and cellular DNA polymerases and may cause DNA chain termination during the DNA replication process [1, 11, 12]. Thus, PMEApp is the active metabolite of PMEA and is responsible for the antiviral and cytostatic properties of the compound. A close correlation has been established by Kramata *et al.* [13] between the cytostatic activities of the different acyclic nucleoside phosphonate analogues and the inhibitory effects of their diphosphates on cellular DNA polymerization. PMEGpp, the diphosphate of PMEG, shows the highest affinity for human DNA polymerases [1, 11, 12]. This is consistent with the fact that, among the acyclic nucleoside phosphonates, PMEG emerged as the strongest inhibitor of cell proliferation.

Here, we report on a novel derivative of PMEDAP, namely N^6-cyclopropyl-PMEDAP (cPr-PMEDAP) (Fig. 1). We compared its cytostatic activity with those of PMEA, PMEDAP, and PMEG in a variety of tumor cell lines, as well as its inhibitory effect on K562 cell cycle progression. In addition, cPr-PMEDAP was evaluated for its differentiation-inducing potential in human erythroleukemia K562 cells [14] and in the RCHO cell line [15]. We found that cPr-PMEDAP acts as a prodrug of PMEG, rather than of PMEDAP, in the biological systems examined. We also demonstrated that cPr-PMEDAP is deaminated to PMEG by a cellular enzyme different from AMP deaminase.

MATERIALS AND METHODS

Compounds

The synthesis and biological activities of the N^6-substituted acyclic nucleoside phosphonates have been described recently [16]. PMEDAP was supplied by Dr. A. Holý (Czech Academy of Sciences, Prague, Czech Republic). CPr-PMEDAP, PMEA, PMEG, and the (*R*)-enantiomers of PMPA and PMPDAP were obtained from Dr. N. Bischofberger (Gilead Sciences). dCF was provided by Dr. D. G. Johns (National Cancer Institute). Abacavir (ABC; GW1592) was a kind gift from Dr. S. Daluge (Glaxo Wellcome).

Cells

Human erythroleukemia K562 cells, human lymphoid MOLT4/C8, Raji and CEM cells, and murine leukemia L1210 cells were obtained from the American Type Culture Collection. Murine B-mix K-44/6 cells, devoid of adenosine deaminase activity [17], were kindly provided by Dr. J. C. Drach. The cell lines were cultured in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 2 mM glutamine (GIBCO), and 0.075% $NaHCO_3$ (GIBCO). Subcultivations were performed every 3–4 days. The RCHO cell line was established from choriocarcinoma-bearing WKA/H rats [15] and was routinely cultured in tissue culture flasks coated with 0.1% gelatin (Sigma Chemical) and containing RPMI-1640 medium (GIBCO), supplemented with 10% fetal bovine serum (GIBCO), 1 mM sodium pyruvate (GIBCO), 2 mM L-glutamine (GIBCO), 2 ng/mL epidermal growth factor (GIBCO), and 5×10^{-5} M β-mercaptoethanol (UCB). *In vitro* passages were performed every 2–3 days by digestion with dispase grade II (Boehringer). All cell cultures were maintained at 37° in a humidified, CO_2-controlled atmosphere.

Cytostatic Assay (IC_{50} Measurement)

Exponentially growing K562, CEM, MOLT4/C8, Raji, or L1210 cells were seeded in 96-well microtiter plates (Falcon, Becton Dickinson) at a final density of 2.5×10^5 cells/mL in RPMI-based growth medium. Test compounds were added at 1:5 serial dilutions in the appropriate concentration range. In each well, the final volume was 200 μL. The cells were then allowed to proliferate for 72 hr (approximately 3 cell generations). At the end of the incubation period, the cells were counted in a Coulter Counter (Coulter Electronics Ltd.). The IC_{50} (50% inhibitory concentration for cell proliferation) was defined as the compound concentration required to inhibit cell proliferation by 50%, as compared to the untreated control.

Differentiation Assay in Human Erythroleukemia K562 Cells

Exponentially growing K562 cells were seeded in 96-well microtiter plates (Falcon, Becton Dickinson) as described

above. Test compounds were added at the appropriate concentrations. In each well, the final volume was 200 μL. After 5 days of incubation of the cell cultures at 37°, differentiation was measured by benzidine staining of the drug-treated cell cultures. To each well, 20 μL of a freshly prepared staining solution (10 μL of hydrogen peroxide 30% in 2.5 mL of 0.2% benzidine (Sigma Chemical) in 0.5 M glacial acetic acid) was added. The staining reaction was allowed to proceed for 20 min at 37°, whereafter the percentage of blue-stained K562 cells was determined by light microscopy using a hemocytometer. At least 200 cells were counted. The benzidine-positive (blue-colored) cells were those in which hemoglobin production had been induced by the test compound, whereas the non-differentiated cells remained transparent. Untreated control cell cultures were included in the assays to estimate the background of spontaneously differentiated K562 cells (usually below 5 to 10%).

Differentiation Assay in RCHO Cells

RCHO cells were seeded at 2.5×10^3 cells/well into non-coated 96-well microtiter plates (Falcon, Becton Dickinson) in RPMI-based growth medium. Test compounds were added at the appropriate concentrations. After incubation of the cells at 37° for 96 hr, the following assays were performed on the drug-treated RCHO cell cultures.

TOTAL PROTEIN MEASUREMENT. Since extensive morphological and metabolic changes occur when RCHO cytotrophoblast cells differentiate into giant cells, no method was found suitable to determine and compare cell numbers of untreated control cell cultures and drug-treated, differentiated cell cultures. Therefore, total protein concentration was used as a parameter to measure the growth-inhibitory effect of the test compounds on RCHO cell cultures. To each microplate well, 100 μL water was added and the cells were lysed by repeated freezing (−70°) and thawing (37°). The cell lysates of identical wells were mixed together. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories) and expressed as percentages of untreated control cultures.

ALKALINE PHOSPHATASE ASSAY. Differentiation of RCHO cytotrophoblasts into non-proliferating giant cells is accompanied by an increased activity of the enzyme alkaline phosphatase, which we used as an appropriate parameter to assess the differentiation stage of drug-treated RCHO cell cultures. After incubation for 96 hr in the presence of the test compounds, the culture supernatant was aspirated and the cells were lysed in the microplate wells by repeated freezing and thawing. Then, to each well 100 μL substrate was added (2 mg/mL disodium *p*-nitrophenyl phosphate [Sigma Chemical] in 50 mM Tris–HCl buffer pH 9.5 containing 0.1% Tween-20). After incubation at 37° for 90 min, the optical density was measured at 405 nm and at a reference wavelength of 620 nm. The data of each independent experiment represent the average values for 3 identical wells. The data have been normalized to equal amounts of cell material using the results of the total protein measurements.

Flow Cytometric Cell Cycle Analysis of Drug-Treated K562 Cells

Exponentially growing K562 cells were seeded in 5-mL culture flasks at 3×10^5 cells/mL in RPMI-based growth medium. The different test compounds were added at concentrations that were 7 to 12 times higher than their respective IC_{50} values. After 48 hr of incubation at 37°, the cells were stained with the CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson). The cell cycle distribution of drug-treated K562 cell cultures was assessed by flow cytometry on a FACScan equipped with CellQuest software (Becton Dickinson).

Determination of Intracellular dNTP Pools

Exponentially growing K562 cells were seeded in 5-mL culture flasks as described above. CPr-PMEDAP was added at a final concentration of 10 μM. After incubation for 48 hr at 37°, the cells were harvested and washed once with RPMI-1640 medium without serum. The cell pellets were resuspended in ice-cold 0.5 N perchloric acid (2×10^6 cells per 100 μL) and incubated on ice for 10 min. After centrifugation at 23,000 *g* at 4° for 5 min, the supernatant was neutralized by addition of an equal volume of tri-*n*-octylamine (Sigma Chemical) in Freon (Merck) (1:4 by v/v) and vigorous shaking for 20 min. The cell extracts were centrifuged at 4° for 5 min at 23,000 *g*, whereafter the upper aqueous phase was carefully collected and stored at −20° until further analysis.

Reaction mixtures for the enzymatic dNTP assays contained 50 mM Tris–HCl pH 8.3, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.25 mg/mL BSA, 0.05 A_{260} units alternating copolymer template (i.e. poly(dA-dT) · poly(dA-dT) (Pharmacia Biotech) for dATP and dTTP determinations and poly(dI-dC) · poly(dI-dC) (Pharmacia) for dGTP and dCTP determinations), 1.1 μM complementary [^{3}H]dNTP (i.e. [2,8-^{3}H]dATP, [methyl-^{3}H]dTTP, [8-^{3}H]dGTP, and [5-^{3}H]dCTP (Moravek Biochemicals) for determinations of dTTP, dATP, dCTP, and dGTP, respectively) at a specific activity of 9.1 Ci/mmol, 10 μL of dNTP standard (0–40 pmol) (Pharmacia) or cell extract (undiluted or 1/2 or 1/4 diluted) and 0.2 units of *Escherichia coli* DNA polymerase I Klenow fragment (Pharmacia). The total assay volume was 100 μL. Reactions were started by addition of the enzyme and were carried out at 37°. After 80 or 100 min, 20-μL aliquots of the reaction mixtures were spotted onto DE81 filter discs (Whatman). The dried filters were washed three times for 5 min with 5% Na_2HPO_4 and rinsed once with distilled water and once with 70% ethanol. After drying, the radioactivity on the filter discs was measured by liquid

scintillation counting, using UltimaGold counting fluid (Packard).

Determination of Intracellular NTP Pools

Exponentially growing K562 and CEM cells were seeded in 5-mL flasks as described above and test compounds were added at the appropriate concentrations. The cell cultures were incubated at 37° for 24 or 48 hr. Then, the cells were harvested and washed twice with RPMI-1640 medium without serum. The cell pellets were resuspended in 500 μL of ice-cold 75% methanol. After incubation on ice for 10 min, the cell extracts were centrifuged at 4° for 5 min at 23,000 *g*. HPLC analysis of the supernatants was performed as described below.

Incubation of cPr-PMEDAP with Crude Cell Extracts of K562 and L1210 Cells

Extracts of exponentially growing K562 and L1210 cells were prepared in 50 mM potassium phosphate buffer pH 7.4 (1–2.5 × 10^8 cells/mL) by sonication (3 × 10 sec) and two subsequent centrifugations at 23,000 *g* for 20 min at 4°. From these cell extracts, 50 μL was incubated overnight at 37° with or without 400 μM cPr-PMEDAP in 50 mM potassium phosphate buffer pH 7.4 in a final volume of 0.5 mL. Thereafter, proteins were precipitated by addition of 1 mL of pure, cold methanol and incubation on ice for 10 min. After centrifugation at 4° for 15 min at 23,000 *g*, the supernatant was analyzed by HPLC as described below.

Incubation of K562 Cells with Radiolabeled cPr-PMEDAP or PMEG in the Presence or Absence of dCF

The metabolism of radiolabeled [8-^{3}H]cPr-PMEDAP (radiospecificity: 14.1 Ci/mmol) and [8-^{3}H]PMEG (radiospecificity: 15.5 Ci/mmol) (Moravek Biochemicals) was monitored as follows. Exponentially growing K562 cells were seeded as described above and incubated with 0.1 μM [8-^{3}H]cPr-PMEDAP or 0.1 μM [8-^{3}H]PMEG in the presence or absence of 10 μg/mL dCF. At different time intervals (i.e. 5, 24, and 48 hr for cPr-PMEDAP and 24 hr for PMEG), cells were centrifuged at 4°, thoroughly washed with ice-cold RPMI-1640 medium (without serum), and precipitated with 75% ice-cold methanol. After centrifugation, HPLC analysis of the supernatants was performed on a Whatman Partisphere SAX ion-exchange column (4.6 × 125 mm). The buffer gradient system was as follows: 5 min at 5 mM $(NH_4)H_2PO_4$ pH 5.0 (flow: 2 mL/min); 15-min linear gradient to 0.3 M $(NH_4)H_2PO_4$ pH 5.0; 20 min at 0.3 M $(NH_4)H_2PO_4$ pH 5.0; 5-min linear gradient to 5 mM $(NH_4)H_2PO_4$ pH 5.0; and 5-min equilibration at the same buffer conditions. The retention times for the metabolites detected in cPr-PMEDAP-exposed K562 cells were 10, 18–19, and 34–35 min, and were identical to the retention times of PMEG, PMEGp, and PMEGpp, respectively, obtained from PMEG-exposed K562 cells. Quantitation of radiolabeled metabolites was accomplished by liquid scintillation counting of the eluted fractions using OptiPhase 'HiSafe' 3 counting fluid (Wallac).

Enzymatic Assays with Adenosine Deaminase and AMP Deaminase

The enzymes and test compounds were diluted in 50 mM potassium phosphate buffer (pH 7.4). Adenosine and cPr-PMEDAP were tested as substrates for adenosine deaminase from bovine spleen (Sigma Chemical) by adding 0.1 mL of enzyme solution, containing 0.05 and 0.5 units of adenosine deaminase, to 0.9 mL of 100 μM solutions of adenosine and cPr-PMEDAP, respectively. The deamination of AMP by 5′-adenylic acid deaminase from rabbit muscle (Sigma Chemical) was demonstrated by adding 0.1 mL of enzyme solution containing 0.5 units of AMP deaminase to 0.9 mL of 100 μM AMP. When cPr-PMEDAP was tested as a substrate for AMP deaminase, 0.2 mL of enzyme solution containing 5 units of the enzyme was added to 0.8 mL of 100 μM cPr-PMEDAP. The reactions were carried out at 25° and monitored spectrophotometrically as a function of time. The optical density was monitored at 260 nm for the conversions of adenosine into inosine and AMP into IMP, and at 290 nm for the formation of PMEG from cPr-PMEDAP. At these wavelengths, the spectra of the reaction substrates (adenosine, AMP, or cPr-PMEDAP) and their respective reaction products (inosine, IMP, or PMEG) showed a maximal divergence.

RESULTS

Inhibition of Tumor Cell Proliferation by cPr-PMEDAP and Related Analogues in the Presence or Absence of dCF

The cytostatic properties of cPr-PMEDAP against several tumor cell lines were evaluated and compared to those of PMEA, PMEDAP, PMEG, PMPA, and PMPDAP. In the four human cell lines (i.e. human leukemia K562, MOLT4/C8, CEM, and Raji cells), the cytostatic activity of cPr-PMEDAP was found to be 8- to 20-fold higher than that of PMEDAP and, surprisingly, equivalent to that of PMEG (Table 1). The compound concentrations required to inhibit cell proliferation by 50% (IC_{50}) ranged from 0.7 to 1.9 μM for cPr-PMEDAP and PMEG, compared to 13 to 16 μM for PMEDAP. For PMEA, IC_{50} values of 28 to 94 μM were noted in the different human cell lines. Unlike human cell lines, murine L1210 cells were markedly less (18-fold) sensitive to the cytostatic activity of cPr-PMEDAP (IC_{50}: 10 μM) than to PMEG (IC_{50}: 0.6 μM). PMEDAP was 6-fold more cytostatic to murine L1210 cells than was cPr-PMEDAP (Table 1). In contrast to PMEA and PMEDAP, their closely related structural derivatives PMPA and PMPDAP showed only a marginal cytostatic effect in all five tumor cell lines (Table 1).

Simultaneous exposure of the tumor cells to 10 μM of

TABLE 1. Cytostatic activity of acyclic nucleoside phosphonates against tumor cell lines

Compound	IC_{50}* (μM)									
	K562		MOLT4/C8		Raji		CEM		L1210	
	–	+10 μM dCF	–	+10 μM dCF	–	+10 μM dCF	–	+10 μM dCF	–	+10 μM dCF
cPr-PMEDAP	1.37 ± 0.62	59.4 ± 5.3	0.74 ± 0.15	123 ± 53	1.92 ± 0.81	≥250	1.16 ± 0.49	19.7 ± 6.5	10.2 ± 6.5	>250
PMEG	0.79 ± 0.27	0.59 ± 0.26	0.92 ± 0.31	1.04	1.91	1.86	1.74 ± 0.73	0.94 ± 0.03	0.57 ± 0.02	0.65 ± 0.01
PMEDAP	12.5 ± 5.8	13.0	16.3 ± 6.8	15.5 ± 6.1	15.8 ± 3.2	15.7 ± 3.3	14.6 ± 2.6	16.3 ± 0.6	1.73 ± 0.56	1.98 ± 0.84
PMEA	26.5 ± 7.9	34.8 ± 6.1	52.4 ± 4.4	57.9 ± 0.4	28.3 ± 0.03	33.4 ± 16.6	94.3 ± 73.8	55.5 ± 11.3	7.45 ± 0.67	8.38 ± 2.04
PMPDAP	>250	>250	>250	>250	>1250	>1250	>250	>250	243 ± 9	223 ± 37
PMPA	502 ± 33	607 ± 137	>250	>250	>1250	>1250	>1250	>1250	>250	>250

*50% Inhibitory concentration or compound concentration required to inhibit cell proliferation by 50%.

The IC_{50} values were determined after 3 days of incubation. The initial cell numbers at day 0 were 0.5×10^5, 0.6×10^5, 0.7×10^5, 0.6×10^5, and 0.6×10^5 cells per microplate well (200 μL) for K562, MOLT4/C8, Raji, CEM, and L1210 cells, respectively. At day 3, the corresponding cell numbers in the untreated control cell cultures were 2.2×10^5, 2.6×10^5, 3.0×10^5, 2.4×10^5, and 3.5×10^5 cells per microplate well, respectively, in the absence of dCF, and 2.1×10^5, 2.6×10^5, 2.9×10^5, 2.4×10^5, and 3.4×10^5 cells per microplate well, respectively, in the presence of dCF. The results represent the means ± standard deviations for two to five independent experiments.

the adenylate deaminase inhibitor dCF [18] and the different test compounds at the appropriate concentrations did not influence the IC_{50} values of PMEA, PMEDAP, PMEG, PMPA, and PMPDAP (Table 1). In striking contrast, dCF markedly affected the cytostatic activity of cPr-PMEDAP, which became 17- to >130-fold less inhibitory against K562, MOLT4/C8, CEM, Raji, and L1210 cell proliferation in the presence of 10 μM dCF (Table 1). For a PMEA-resistant variant of the human K562 cell line, K562/PMEA-1 (which is described below in more detail), the cytostatic activity of cPr-PMEDAP decreased even more than 200-fold in the presence of dCF (data not shown).

PMEA-resistant variants of the human erythroleukemia K562 and the murine leukemia L1210 cell lines had been selected previously by culturing wild-type K562/0 and L1210/0 cells in the presence of escalating PMEA concentrations [19, 20]. Compared to the respective wild-type cells, the mutant K562/PMEA-1 and L1210/PMEA-1 cell lines were 115-fold and >300-fold, respectively, less sensitive to the cytostatic activity of PMEA. Both PMEA-resistant cell lines showed only a marginal cross-resistance to cPr-PMEDAP. The ratios of the IC_{50} values of cPr-PMEDAP for PMEA-resistant versus wild-type cells were 2.2 (K562 cells) and 11 (L1210 cells) (Table 2). These resistance ratios closely corresponded to those noted for PMEG (i.e. 2.3 and 13 for K562 and L1210 cells, respectively) (Table 2). In contrast, cross-resistance of K562/PMEA-1 and L1210/PMEA-1 cells to PMEDAP was markedly more pronounced (i.e. 8.8- and 82-fold, respectively) (Table 2). Thus, as also observed in the diverse wild-type human and murine tumor cell lines (Table 1), cPr-PMEDAP behaved much more similarly to PMEG than to PMEA or PMEDAP in the two PMEA/PMEDAP-resistant tumor cell lines (Table 2).

Inhibition of Cell Proliferation and Induction of Cell Differentiation in RCHO Cells by cPr-PMEDAP and Related Analogues in the Presence or Absence of dCF

The RCHO cell line consists of proliferating cytotrophoblast cells which can be stimulated by acyclic nucleoside phosphonates to differentiate into non-proliferating, hor-

TABLE 2. Cross-resistance of the PMEA-resistant L1210/PMEA-1 and K562/PMEA-1 cell lines to PMEDAP, PMEG, and cPr-PMEDAP

Compound	Resistance factor*	
	L1210/PMEA-1 / L1210/0	K562/PMEA-1 / K562/0
PMEA	>300	115
PMEDAP	82	8.8
cPr-PMEDAP	11	2.2
PMEG	13	2.3

*The resistance factor was defined as the ratio of the 50% inhibitory concentration (IC_{50}) for PMEA-resistant cells to the IC_{50} for wild-type cells, determined after 3 days of incubation.

monally active giant cells [21]. Therefore, the RCHO cell line was used as an additional *in vitro* tumor cell model, not only to compare the growth-inhibitory effect of cPr-PMEDAP with that of other acyclic nucleoside phosphonates, but also to evaluate the differentiation-inducing properties of cPr-PMEDAP.

After drug exposure, RCHO cell cultures showed extensive, differentiation-related morphological and metabolic changes that did not allow accurate determination and comparison of cell numbers in drug-treated (differentiated) versus untreated (non-differentiated) RCHO cell cultures by standard methods, such as automated cell counting and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays. Moreover, selective loss of less adhesive, proliferating cytotrophoblasts easily occurs during processing of drug-treated RCHO cell cultures in standard cell proliferation assays and largely compromises the reliability of the results. Also, thymidine incorporation was not suitable to estimate the proliferative status of drug-exposed versus untreated RCHO cells, since we have found that DNA synthesis inhibitors such as PMEA influence the thymidine salvage pathway [22]. Attempts to assess DNA replication by propidium iodide staining and flow cytometry were unsuccessful as well, because the highly differentiated giant cells were disrupted by passage through the nozzle. Therefore, the inhibitory effect of the test compounds on RCHO cell proliferation was evaluated by comparing the increase in the total amount of protein (as a measure of the increase in the total amount of cell material) over 96 hr in drug-treated versus untreated RCHO cell cultures. However, it should be kept in mind that the antiproliferative activity of differentiation-inducing compounds against RCHO cells may be underestimated by protein determination, since protein synthesis and metabolism may remain at a high level in differentiated giant cells.

When exposed to cPr-PMEDAP at 0.2, 1, or 2 μM, the increase in the total amount of cell material in the RCHO cell cultures was 86%, 58%, and 28%, respectively, of the increase in the amount of protein observed in untreated control cell cultures over the same incubation period (Fig. 2A, black bars). This reflects an inhibition of RCHO cell proliferation by at least 14%, 42%, and 72% for cPr-PMEDAP at 0.2, 1, and 2 μM, respectively. Interestingly, cPr-PMEDAP proved nearly equipotent to PMEG, which inhibited RCHO cell growth (as estimated by the total amount of cell material) by 25%, 66%, and 81% when evaluated at equal drug concentrations (i.e. 0.2, 1, or 2 μM) (Fig. 2A, black bars). In contrast, ~10-fold higher concentrations were required for PMEDAP to attain a comparable decrease in total RCHO cell material as afforded by cPr-PMEDAP. PMEDAP at 2, 5, and 10 μM inhibited RCHO tumor cell growth by at least 17%, 34%, and 62%, respectively (Fig. 2A, black bars). Moreover, PMEA was 50- to 100-fold less potent in inhibiting RCHO cell growth (as estimated by the total amount of cell material) than was

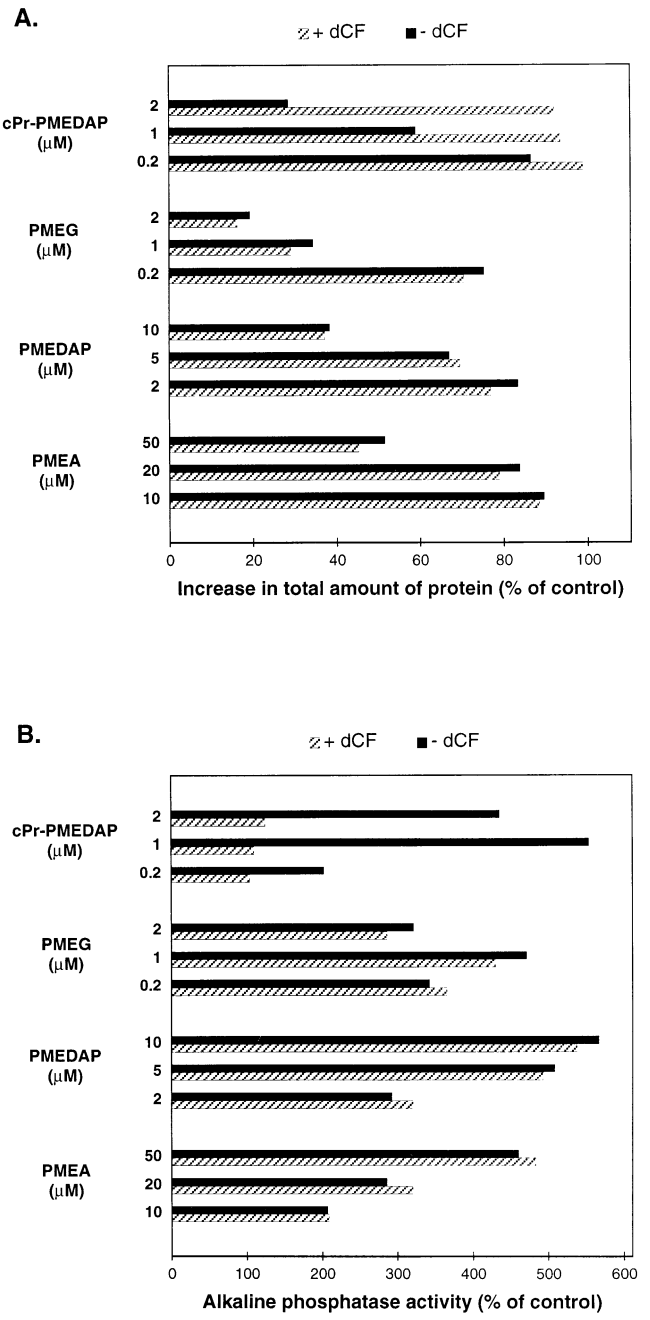


FIG. 2. Effects of acyclic nucleoside phosphonates on proliferation and differentiation of RCHO cells. (A) Increase in total amount of protein over a 96-hr incubation period in RCHO cell cultures exposed to the different acyclic nucleoside phosphonates in the presence (shaded bars) or in the absence (black bars) of dCF. The results are expressed as percentages of the increase in protein concentration noted in untreated control cell cultures over the 96-hr period (i.e. from 9.0 μg to 46.2 μg and 43.7 μg per microplate well in the presence and absence of dCF, respectively). The data represent the means for two independent experiments. (B) Alkaline phosphatase activity in RCHO cell cultures after 96-hr exposure to the test compounds in the presence (shaded bars) or the absence (black bars) of dCF. The data are normalized to equal amounts of cell material and are expressed as the percentage of the alkaline phosphatase activity found in untreated (non-differentiated) control cell cultures. The data represent the means for two independent experiments.

cPr-PMEDAP: cPr-PMEDAP at 0.2 and 1 μM was as effective as PMEA at 20 and 50 μM.

The inhibitory effect of cPr-PMEDAP on RCHO cell proliferation was strongly abrogated by dCF. When combined with 10 μM dCF, cPr-PMEDAP at 0.2, 1, and 2 μM inhibited RCHO cell proliferation by only 1%, 6%, and 8%, respectively, compared to 14%, 42%, and 72% in the absence of dCF. On the other hand, dCF had no effect on the cytostatic effects of PMEA, PMEDAP, and PMEG (Fig. 2A, shaded bars).

Differentiation of proliferating cytotrophoblasts into non-proliferating giant cells upon exposure of the RCHO cell cultures to acyclic nucleoside phosphonates can be easily monitored microscopically. When exposed to cPr-PMEDAP at 1 μM, RCHO cell cultures showed 60–70% differentiated giant cells (data not shown). A PMEG concentration as low as 0.2 μM resulted in ~50–60% giant cells. PMEDAP at 2 μM and PMEA at 20 μM both gave ~40% giant cell formation (data not shown). At their highest concentrations (i.e. 50 μM for PMEA, 10 μM for PMEDAP, and 2 μM for PMEG and cPr-PMEDAP), all four nucleoside phosphonate derivatives caused morphological differentiation in the vast majority (>85%) of the RCHO cells. Although an equally high degree of differentiation was observed with all four drugs, the cell density was significantly higher in RCHO cell cultures exposed to 50 μM PMEA and 10 μM PMEDAP than in those exposed to 2 μM PMEG or cPr-PMEDAP. The percentage of giant cells remained at the background level (<10%) in RCHO cell cultures that were simultaneously exposed to 10 μM dCF and cPr-PMEDAP at 0.2, 1, or 2 μM, whereas dCF had no effect on PMEA-, PMEDAP-, and PMEG-induced giant cell formation (data not shown).

In addition to giant cell formation, we also determined the increase in alkaline phosphatase activity, another appropriate marker to monitor RCHO cell differentiation, in drug-exposed RCHO cells. CPr-PMEDAP and PMEG, both at a concentration as low as 1 μM, afforded an increase in alkaline phosphatase activity to 472% and 554%, respectively, compared to the untreated control (Fig. 2B, black bars). A comparable increase in alkaline phosphatase activity was achieved by PMEDAP at 5 μM and by PMEA at 50 μM (Fig. 2B, black bars). Thus, PMEG and cPr-PMEDAP showed a 5-fold greater differentiation-inducing potential in RCHO cells than PMEDAP, and a 50-fold greater differentiation-inducing potential than PMEA. Unlike PMEA, PMEDAP, and PMEG, cPr-PMEDAP completely lost its ability to induce alkaline phosphatase activity in RCHO cells when combined with 10 μM dCF (Fig. 2B, shaded bars).

Induction of Erythroid Differentiation in K562/0 Cells by cPr-PMEDAP and Related Analogues

The differentiation-inducing potential of cPr-PMEDAP was also evaluated in the human erythroleukemia K562 cell model. Under the influence of differentiation-inducing agents, K562 cells are triggered to produce hemoglobin, which can be easily demonstrated by benzidine staining of the drug-treated K562 cell culture [14]. K562 cells were exposed to the test compounds at the appropriate concentrations for 5 days, whereafter differentiation was measured by determining the percentage of benzidine-positive cells. As shown in Table 3, 2 μM cPr-PMEDAP, 2 μM PMEG, 25 μM PMEDAP, and 50 μM PMEA all gave a comparable result, 65%–70% of the K562 cells in the drug-exposed cell cultures being benzidine-positive. Also, exposure of K562 cells to cPr-PMEDAP at 0.4 and 1 μM triggered hemoglobin production in 27% and 55%, respectively, of the cells, as compared to 37% and 65% for PMEDAP at 5 and 25 μM, respectively (Table 3). Thus, cPr-PMEDAP was equipotent to PMEG and 5- to 25-fold more potent than PMEDAP in inducing K562 cell differentiation. For PMEA, a 25- to 50-fold higher concentration than for cPr-PMEDAP was required to yield a comparable percentage of differentiated cells in the drug-treated K562 cell cultures (Table 3). The data for the K562 differentiation model confirm the relative potency of the acyclic nucleoside phosphonates as differentiation-inducing agents as found in the RCHO differentiation model: PMEA ≪ PMEDAP < PMEG ~ cPr-PMEDAP, in order of increasing potency.

TABLE 3. Induction of erythroid differentiation of K562 cells by acyclic nucleoside phosphonates

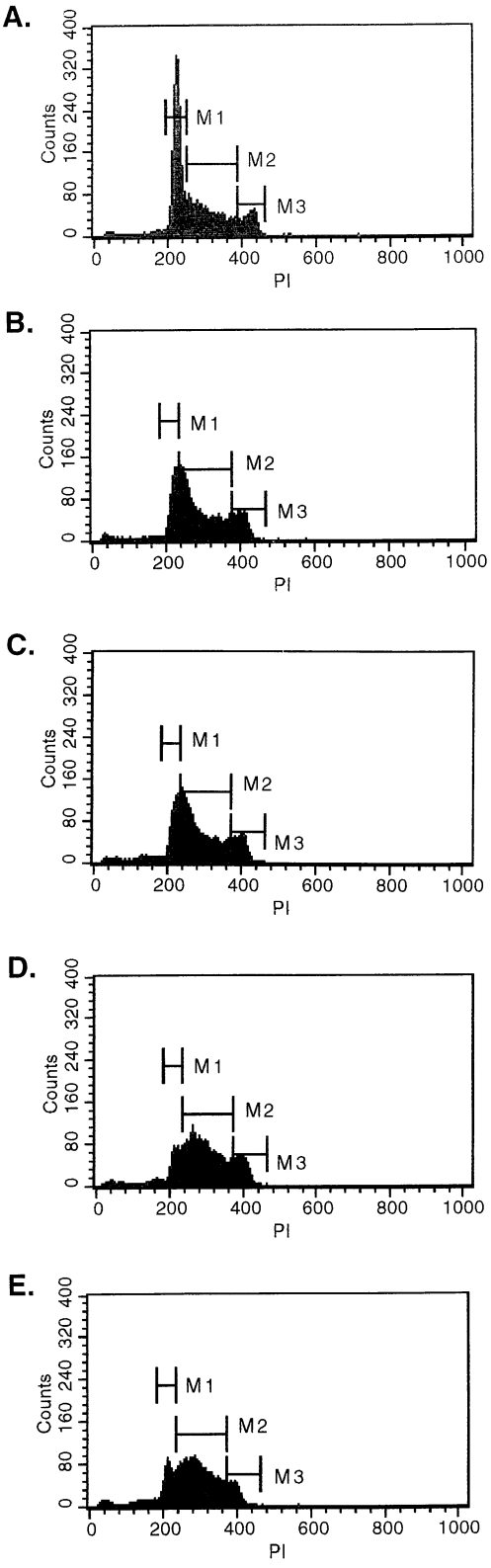
Compound	Concentration (μM)	Percentage of benzidine-positive cells*
Control	0	7 ± 2
cPr-PMEDAP	0.1	10 ± 1
	0.4	27 ± 4
	1	55 ± 8
	2	70 ± 3
PMEG	0.4	31 ± 8
	1	41
	2	65 ± 3
PMEDAP	5	37 ± 8
	25	65 ± 1
PMEA	10	26 ± 7
	50	66 ± 2

*Differentiated, hemoglobin-containing K562 cells appear blue under the light microscope after benzidine-staining, whereas undifferentiated cells remain transparent.

Differentiation was measured after 5 days of incubation. The data represent the means ± standard deviations for at least two independent experiments.

Effect of cPr-PMEDAP and Related Analogues on Cell Cycle Distribution of Human Erythroleukemia K562 Cells

Since the diphosphorylated metabolites of acyclic nucleoside phosphonates are known to act as DNA polymerase inhibitors [11–13], we investigated the effect of high concentrations of cPr-PMEDAP, PMEG, PMEDAP, and PMEA on the cell cycle distribution of K562 cells by analyzing their DNA content by flow cytometry. In untreated K562 cell cultures, 48% of the cells were in the G1 phase, 38% of the cells in the DNA-replicating phase (S



Control

Marker	% of cells
M1	48
M2	38
M3	11

cPr-PMEDAP 10 μM

Marker	% of cells
M1	26
M2	56
M3	15

PMEG 10 μM

Marker	% of cells
M1	24
M2	59
M3	13

PMEDAP 100 μM

Marker	% of cells
M1	15
M2	66
M3	15

PMEA 250 μM

Marker	% of cells
M1	18
M2	67
M3	10

FIG. 3. Cell cycle distribution of untreated K562 cells (A) and K562 cells exposed to 10 μM cPr-PMEDAP (B), 10 μM PMEG (C), 100 μM PMEDAP (D), and 250 μM PMEA (E). The DNA content of the cells was analyzed by propidium iodide (PI) staining, using the CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson). The M1, M2, and M3 markers represent G1, S, and G2/M cells, respectively. Histogram plots were gated to exclude cell debris and cell clumps.

phase), and 11% of the cells in the G2/M phase (Fig. 3A). Exposure of the K562 cells to acyclic nucleoside phosphonates at high concentrations (i.e. 7- to 12-fold their IC_{50} values [see Table 1]) for 48 hr completely perturbed the normal cell cycle pattern. CPr-PMEDAP and PMEG at 10 μM both conferred a similar effect: S phase entry and progression were strongly impeded, resulting in the vast majority of the cells being arrested at the early S stage (Fig. 3, B and C). A marked accumulation of S phase cells (66–67%, compared to 38% in the untreated K562 cell

TABLE 4. dNTP levels in cell extracts of untreated and drug-treated K562 cells

	Intracellular dNTP pool size (pmol/10^6 cells)			
	dATP	dGTP	dCTP	dTTP
Control	23	6.4	27	45
cPr-PMEDAP 10 μM	110	23	155	301

cultures) also occurred in K562 cells exposed to 100 μM PMEDAP and 250 μM PMEA, as illustrated in Fig. 3, D and E.

Effect of cPr-PMEDAP on Intracellular dNTP Pools in Human Erythroleukemia K562 Cells

To examine whether the inhibition of K562 cell cycle progression by cPr-PMEDAP had an effect on intracellular dNTP levels, we compared dATP, dGTP, dCTP, and dTTP pool sizes of untreated K562 cells versus K562 cells exposed to 10 μM cPr-PMEDAP for 48 hr. As shown in Table 4, dNTP pool sizes of drug-treated K562 cells had increased 4.8-fold, 3.6-fold, 5.7-fold, and 6.7-fold for dATP, dGTP, dCTP, and dTTP, respectively, as compared to the untreated control.

Effect of cPr-PMEDAP and Related Analogues on Intracellular Nucleoside NTP Pools in Human Erythroleukemia K562 and Lymphoid CEM Cells

We also investigated whether the acyclic nucleoside phosphonates could influence the intracellular NTP pools as well. Already after 24 hr of exposure of K562 cells to cPr-PMEDAP, PMEG, PMEDAP, or PMEA, marked accumulation of all four intracellular nucleoside triphosphates could be observed. In K562 cells exposed to 2 μM cPr-PMEDAP for 24 hr, NTP pools had increased to 143%, 135%, 147%, and 130% of control values for UTP, CTP, ATP, and GTP, respectively (data not shown). At the same concentration (i.e. 2 μM), PMEG did not significantly influence NTP pool sizes, but at a 5-fold higher PMEG concentration (i.e. 10 μM), NTP levels were elevated 1.5- to 1.8-fold as compared to the control (data not shown). Exposure of K562 cells to PMEDAP at 20 μM resulted in intracellular NTP levels that were 1.6-fold to 1.7-fold higher than in untreated cells. PMEA at a concentration as high as 250 μM afforded a moderate increase in intracellular NTP pools of K562 cells (data not shown). Essentially the same observations were made in CEM cells, where the NTP accumulation was more pronounced (pool sizes increased up to 2-fold under certain conditions) and already apparent at lower concentrations for all four acyclic nucleoside phosphonates (i.e. 50 μM for PMEA, 5 μM for PMEDAP, and 0.5 μM for both PMEG and cPr-PMEDAP) (data not shown).

Formation of PMEG from cPr-PMEDAP by K562 and L1210 Cell Extracts

All the results described above suggest that cPr-PMEDAP acts as a prodrug of PMEG. We were indeed able to demonstrate the formation of small amounts of PMEG when cPr-PMEDAP was incubated overnight at 37° in the presence of crude extracts of K562 or L1210 cells. After incubation, HPLC analysis of the reaction mixtures (after protein precipitation with methanol) revealed a small peak that, based on its retention time and UV spectrum, could be unambiguously identified as PMEG (data not shown).

Metabolism of Radiolabeled cPr-PMEDAP and PMEG in the Presence or Absence of dCF in Human Erythroleukemia K562 Cells

K562 cells were incubated with 0.1 μM [8-^{3}H]cPr-PMEDAP or 0.1 μM [8-^{3}H]PMEG in the presence or absence of 10 μg/mL dCF. Surprisingly, the radiolabeled intracellular metabolites that were predominantly detected in cPr-PMEDAP-exposed cells represented the mono- and diphosphorylated PMEG metabolites PMEGp and PMEGpp (Table 5). Interestingly, the conversion of cPr-PMEDAP to PMEGp and PMEGpp was time-dependent and was markedly (i.e. 5- to 10-fold) suppressed in the presence of the AMP deaminase inhibitor dCF (Table 5).

TABLE 5. Intracellular formation of PMEG metabolites in K562 cells incubated with 0.1 μM [8-^{3}H]cPr-PMEDAP in the presence or absence of 10 μg/mL dCF

	pmol/10^6 cells*							
	Fraction 5–7 min† (cPr-PMEDAP)		Fraction 9–10 min† (PMEG)		Fraction 19–20 min† (PMEGp)		Fraction 35–36 min† (PMEGpp)	
Time (hr)	−	+10 μg/mL dCF	−	+10 μg/mL dCF	−	+10 μg/mL dCF	−	+10 μg/mL dCF
5	0.037	0.034	0.0012	0.0007	0.0017	0.0036	0.0047	0.0012
24	0.065	0.093	0.0056	0.0015	0.0040	0.0018	0.0120	0.0014
48	0.071	0.087	0.0053	0.0038	0.0070	0.017	0.0190	0.0020

*The data represent the means for two independent experiments.

†In the buffer gradient system used for HPLC analysis of the K562 cell extracts, the retention times of cPr-PMEDAP, PMEG, PMEGp, and PMEGpp were within 5–7 min, 9–10 min, 19–20 min, and 35–36 min, respectively.

The eventual levels of PMEGp and PMEGpp in cPr-PMEDAP-exposed K562 cells at 24 hr were at least 50% of the PMEGp and PMEGpp levels found in K562 cells exposed to PMEG at an equimolar extracellular concentration (data not shown).

Incubation of cPr-PMEDAP with Purified Adenosine Deaminase and AMP Deaminase

To find out whether cellular adenosine deaminase or AMP deaminase could be responsible for the deamination of cPr-PMEDAP to PMEG, we evaluated cPr-PMEDAP as a substrate for bovine spleen adenosine deaminase and rabbit muscle AMP deaminase. Whereas deamination of 90 nmol of adenosine to inosine was achieved within 5 min with 0.05 units of adenosine deaminase, the enzyme completely failed to convert cPr-PMEDAP into PMEG, even when a 10-fold higher amount of enzyme (i.e. 0.5 units) was added to the reaction mixture for several hours (data not shown). Furthermore, cPr-PMEDAP was not deaminated to PMEG by 5 units of rabbit muscle AMP deaminase over an extended time period (up to 8 hrs), while 90 nmol of the natural enzyme substrate, AMP, was totally converted to IMP within 10 min with 0.5 units of AMP deaminase (data not shown).

Cytostatic Activity of cPr-PMEDAP and Related Analogues against Adenosine Deaminase-Deficient Murine B-mix K-44/6 Cells in the Presence or Absence of dCF

Murine B-mix K-44/6 cells, which have been shown to be devoid of adenosine deaminase activity [17], were exposed to the test compounds in the presence or absence of 10 μM dCF. The IC_{50} values of the test compounds, as determined after 3 days of incubation, were 1.3, 0.06, 11, and 188 μM for cPr-PMEDAP, PMEG, PMEDAP, and PMEA, respectively, in the absence of dCF. The corresponding values in the presence of dCF were 34, 0.03, 8, and 75 μM, respectively. Thus, while dCF did not markedly influence the IC_{50} values of PMEG, PMEDAP, and PMEA, the cytostatic activity of cPr-PMEDAP was 26-fold reduced by dCF in the adenosine deaminase-deficient cells.

DISCUSSION

In this paper, we report on the antiproliferative and tumor cell differentiation-inducing potential of a novel, N^6-cyclopropyl-substituted derivative of the acyclic nucleoside phosphonate PMEDAP, designated cPr-PMEDAP. Notwithstanding the close structural analogy between both molecules, cPr-PMEDAP behaved quite differently from PMEDAP in a variety of biological assay systems. Also, its biological effects were far more pronounced than those of the parent compound. In fact, the biological properties of cPr-PMEDAP closely paralleled those of PMEG.

CPr-PMEDAP and PMEG were markedly and equally cytostatic to a variety of human and rat tumor cell lines. The aberrant behavior of cPr-PMEDAP in murine leukemia L1210 cells, which were 6- to 18-fold less sensitive to the cytostatic effect of cPr-PMEDAP than PMEDAP and PMEG, may possibly be ascribed in part to the peculiar uptake characteristics of the acyclic nucleoside phosphonate derivatives in this cell line. Indeed, previous studies on the uptake and metabolism of acyclic nucleoside phosphonates have shown that PMEA influx in murine leukemia L1210 cells occurs 60-fold more efficiently than in human lymphocyte CEM and H9 cells and 20-fold more efficiently than in murine carcinoma FM3A cells [23; and our unpublished data]. Moreover, our recent study on a mutant L1210 cell line (L1210/PMEA-1) with high-level (>300-fold) resistance to PMEA and cross-resistance to PMEDAP (>80-fold) but not PMEG points to the existence of a PMEA/PMEDAP-specific transport carrier in L1210 cells [20]. We have suggested a defect in this carrier system as the molecular basis for the diminished PMEA/PMEDAP uptake by the mutant L1210/PMEA-1 cells. The presence of such a specific carrier system on the membrane of L1210 cells may, at least in part, account for the considerably lower IC_{50} values of PMEA and PMEDAP in murine L1210 cells as compared to human K562, MOLT4/C8, CEM, and Raji cells.

Thus, our present findings indicate that cPr-PMEDAP is not recognized by the PMEA/PMEDAP-specific transport carrier protein present on the plasma membrane of L1210 cells. This is further confirmed by our observations that cPr-PMEDAP, like PMEG, is only ~10-fold less cytostatic to the PMEA-resistant L1210/PMEA-1 cell line than to the wild-type L1210/0 cell line, whereas the IC_{50} of PMEDAP is >80-fold higher in the mutant cells. Furthermore, the behavior of cPr-PMEDAP again paralleled that of PMEG, rather than PMEDAP, in a PMEA-resistant variant of the human erythroleukemia K562 cell line, which is impaired in both PMEA phosphorylation and PMEA transport [19].

We have also demonstrated that cPr-PMEDAP is endowed with a marked tumor cell differentiation-inducing potential, equivalent to that of PMEG and superior to that of PMEDAP and PMEA, in both human erythroleukemia K562 and RCHO cells. Our data on the induction of K562 and RCHO cell differentiation reflect the same order of (increasing) potency of the acyclic nucleoside phosponates (i.e. PMEA < PMEDAP < PMEG ~ cPr-PMEDAP) as found for the cytostatic activity of these compounds in K562, CEM, MOLT4/C8, Raji, and RCHO cells. Interestingly, we have also noted a marked antitumor activity with cPr-PMEDAP *in vivo* in choriocarcinoma-bearing rats*.

The diphosphorylated metabolites of the acyclic nucleoside phosphonates are structural analogues of the natural deoxyribonucleoside 5′-triphosphates and, thus, may be recognized as alternative substrates for DNA synthesis by cellular DNA polymerases [11, 13]. Moreover, incorpora-

*Naesens L, Hatse S, Segers C, Verbeken E, De Clercq E, Waer M and Balzarini J, manuscript submitted for publication.

tion of PMEApp, PMEDAPpp, and PMEGpp, which do not provide a hydroxyl group for further chain elongation, inevitably causes DNA chain termination [12]. Hence, it should be expected that the acyclic nucleoside phosphonates at sufficiently high concentrations may cause cell cycle arrest or at least accumulation of the drug-treated cells in the S phase. We were indeed able to demonstrate that S phase progression is strongly inhibited in K562 cells exposed for 48 hr to the four different phosphonate derivatives at concentrations affording a comparable cytostatic effect. From our cell cycle experiments, PMEG and cPr-PMEDAP again emerged as the most potent inhibitors of cell proliferation. In agreement with these findings, Pisarev *et al.* [11] have reported that PMEGpp is about 15-fold more potent than PMEApp in inhibiting DNA synthesis mediated by polymerase α, while PMEDAPpp has an intermediate inhibitory effect on DNA synthesis [13].

When DNA synthesis is inhibited, dNTPs may be expected to accumulate within the cells. We have demonstrated that all four dNTP pool levels were indeed increased after exposure of K562 cells to cPr-PMEDAP at a concentration that strongly inhibited cell proliferation. Although this effect is secondary to the primary action of cPr-PMEDAP (i.e. the inhibition of DNA replication by the active metabolite PMEGpp), it could be expected to interfere with the cytostatic action of cPr-PMEDAP through accumulation of the natural counterpart of PMEGpp, dGTP. Nonetheless, cPr-PMEDAP strongly inhibited tumor cell proliferation, indicating that PMEGpp is indeed a very potent inhibitor of the replicative cellular DNA polymerases.

Moreover, already after a 24-hr exposure of K562 and CEM cells to the acyclic nucleoside phosphonates at high concentrations, we observed a moderate accumulation of all four ribonucleoside 5′-triphosphates (NTP). Since we have recently shown that RNA synthesis is not affected by the acyclic nucleoside phosphonates [22], the NTP accumulation presumably results from feedback inhibition of ribonucleotide reductase by the elevated dATP levels [24]. The decline in the dTTP level that usually results from inhibition of ribonucleotide reductase is probably masked by the pronounced dTTP accumulation in the presence of the acyclic nucleoside phosphonates.

Taken together, all our data, including the reversal of the cytostatic and differentiation-inducing effects of cPr-PMEDAP by dCF, strongly suggest that cPr-PMEDAP acts as a prodrug of PMEG. To validate this hypothesis, we have demonstrated PMEG formation from cPr-PMEDAP in crude K562 and L1210 cell extracts. Moreover, comparative studies with radiolabeled cPr-PMEDAP and PMEG further evidenced the exclusive conversion of cPr-PMEDAP into PMEG and its metabolites. These findings are most surprising, since it has generally been assumed that acyclic nucleoside phosphonates such as PMEA and PMEDAP are not susceptible to deamination. Metabolic studies on PMEA and PMEDAP, whether performed with crude cell extracts or intact (tumor) cells, have always failed to demonstrate any traces of deaminated products [10]. Thus, to the best of our knowledge, this is the first demonstration of an acyclic nucleoside phosphonate derivative that can be converted to another nucleobase derivative upon deamination. Also, release of the nucleobase from the acyclic nucleoside phosphonates does not occur under physiological conditions. This is particularly relevant in the case of cPr-PMEDAP, since it has been reported by Thedford *et al.* [25] that N^6-cycloalkylated adenine analogues exert pronounced cytotoxic effects. Thus, with respect to its metabolic properties, cPr-PMEDAP behaves entirely differently from PMEDAP and PMEA and should in fact be considered as a prodrug of PMEG.

The question as to which cellular enzyme is responsible for the deamination of cPr-PMEDAP to PMEG still remains to be resolved. However, we have several reasons to assume that PMEG formation from cPr-PMEDAP is mediated by the same cytosolic enzyme that has recently been reported by Falettto *et al.* [26] to be involved in the activation of the novel anti-HIV agent abacavir (ABC; (−)-(1*S*, 4*R*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol). Indeed, ABC monophosphate has been found to be deaminated by a distinct cytosolic enzyme that is different from the well-known enzymes adenosine deaminase and adenylate deaminase, although it is also sensitive to inhibition by dCF [18]. In fact, the monophosphate form of ABC shows a striking structural analogy with cPr-PMEDAP, since both molecules are 2,6-diaminopurine nucleoside 5′-monophosphate analogues containing a cyclopropyl substituent at the N^6-position of the 2,6-diaminopurine ring. In analogy with the selective prevention of ABC monophosphate deamination by dCF, we have also found that the cytostatic and differentiation-inducing properties of cPr-PMEDAP are substantially reversed in the presence of dCF. Moreover, like ABC monophosphate, cPr-PMEDAP was not recognized as a substrate by purified adenosine deaminase and adenylate deaminase. However, it cannot be excluded that the commercially available enzymes that we used in the cell-free enzyme assays might show a slightly different substrate specificity than the homologous enzymes residing in the different tumor cell lines in which the antiproliferative activity of cPr-PMEDAP was evaluated. Our finding that the IC_{50} value of cPr-PMEDAP in adenosine deaminase-deficient murine B-mix K-44/6 cells was comparable to the IC_{50} values of the compound in K562, MOLT4/C8, Raji, and CEM cells also argues against a role of adenosine deaminase in the metabolic activation of cPr-PMEDAP. The fact that the cytostatic potential of cPr-PMEDAP was 5-fold reduced in the presence of 50 to 100 μM ABC (data not shown) is consistent with the hypothesis that cPr-PMEDAP and ABC compete for the same cellular enzyme to become activated (deaminated). CPr-PMEDAP was most cytostatic against MOLT4/C8 cells (IC_{50}: 0.74 μM), slightly less cytostatic against K562 cells (IC_{50}: 1.37 μM), and markedly less cytostatic against L1210 cells (IC_{50}: 10.2 μM). Interestingly, although 30- to 40-fold less pro-

nounced, the growth inhibitory effect of ABC mirrored that of cPr-PMEDAP in the different cell lines (i.e. most pronounced against MOLT4/C8 cells and least pronounced against L1210 cells). Since both compounds may share the same activating (deaminating) enzyme, these findings may point to different levels of this enzyme in the diverse tumor cell lines. Taken together, our observations provide convincing evidence that cPr-PMEDAP is inactive as such, but needs to be metabolized to PMEG by a dCF-sensitive enzyme to exercise its biological effects.

In conclusion, we have shown that cPr-PMEDAP, a novel structural derivative of PMEDAP, is superior to the parent drug with regard to its cytostatic and tumor cell differentiation-inducing properties. Surprisingly, cPr-PMEDAP acts as a prodrug of the cytostatic agent PMEG. The therapeutic potential of cPr-PMEDAP as an antitumor drug is currently being evaluated in several *in vivo* tumor models, including rat choriocarcinoma, xenografts in nude mice, and papilloma-related tumors in rabbits. We are also investigating whether this prodrug approach is advantageous, in terms of efficacy and selectivity, over the direct use of PMEG.

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