FEBS Letters 445 (1999) 92–97 FEBS 21576

Impact of 9-(2-phosphonylmethoxyethyl)adenine on (deoxy)ribonucleotide metabolism and nucleic acid synthesis in tumor cells

Sigrid Hatse[1], Erik De Clercq, Jan Balzarini*

Rega Institute for Medical Research, Minderbroedersstraat 10, K.U. Leuven, B-3000 Leuven, Belgium

Received 18 December 1998; received in revised form 18 January 1999

Abstract Following exposure to 9-(2-phosphonylmethoxyethyl)adenine (an inhibitor of the cellular DNA polymerases α, δ and ε), human erythroleukemia K562, human T-lymphoid CEM and murine leukemia L1210 cells markedly accumulated in the S phase of the cell cycle. In contrast to DNA replication, RNA synthesis (transcription) and protein synthesis (mRNA translation) were not affected by 9-(2-phosphonylmethoxyethyl)-adenine. The ribonucleoside triphosphate pools were slightly elevated, while the intracellular levels of all four deoxyribonucleoside triphosphates were 1.5–4-fold increased in 9-(2-phosphonylmethoxyethyl)adenine-treated K562, CEM and L1210 cells. The effect of 9-(2-phosphonylmethoxyethyl)adenine on de novo (thymidylate synthase-mediated) and salvage (thymidine kinase-mediated) dTTP synthesis was investigated using radiolabelled nucleoside precursors. The amount of thymidylate synthase-derived dTTP in the acid soluble pool was 2–4-fold higher in PMEA-treated than in untreated K562 cells, which is in accord with the 3–4-fold expansion of the global dTTP level in the presence of 9-(2-phosphonylmethoxyethyl)adenine. Strikingly, 2-derived dTTP accumulated to a much higher extent (i.e. 16–40-fold) in the soluble dTTP pool upon 9-(2-phosphonylmethoxyethyl)adenine treatment. In keeping with this finding, a markedly increased thymidine kinase activity could be demonstrated in extracts of 9-(2-phosphonylmethoxyethyl)adenine-treated K562 cell cultures. Also, in the presence of 200 μM 9-(2-phosphonylmethoxyethyl)adenine, 14-fold less thymidylate synthase-derived but only 3-fold less thymidine kinase-derived dTTP was incorporated into the DNA of the K562 cells. These data show that thymidine incorporation may be inappropriate as a cell proliferation marker in the presence of DNA synthesis inhibitors such as 9-(2-phosphonylmethoxyethyl)adenine. Our findings indicate that 9-(2-phosphonylmethoxyethyl)adenine causes a peculiar pattern of (deoxy)ribonucleotide metabolism deregulation in drug-treated tumor cells, as a result of the metabolic block imposed by the drug on the S phase of the cell cycle.

Key words: (Deoxy)ribonucleotide metabolism; Acyclic nucleoside phosphonate; Thymidine kinase; Cell cycle; DNA synthesis

*Corresponding author. Fax: (32) (16) 337340.

[1]Research Assistant of the 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (F.W.O.)'.

Abbreviations: Ara-C, 1-β-D-arabinofuranosylcytosine; CDK, cyclin-dependent kinase; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibitory concentration for cell proliferation; NTP, ribonucleoside 5′-triphosphate; PBS, phosphate-buffered saline; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEApp, the diphosphorylated metabolite of 9-(2-phosphonylmethoxyethyl)adenine; TCA, trichloroacetic acid; TK, thymidine kinase; TS, thymidylate synthase

1. Introduction

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) (Fig. 1) is the prototype congener of the class of acyclic nucleoside phosphonates [1]. The oral lipophilic ester prodrug of PMEA, bis(pivaloyloxymethyl)-PMEA, is currently being explored in advanced clinical trials for its efficacy against human immunodeficiency virus (HIV) and human hepatitis B virus (HBV) infections [2]. Not PMEA itself, but its diphosphorylated intracellular metabolite, PMEApp, is responsible for the antiviral activity of the drug [3]. Both adenylate kinase [4,5] and 5-phosphoribosyl-1-pyrophosphate synthetase ([3,6], and our unpublished data) have been suggested to play a role in the intracellular activation (phosphorylation) of PMEA. Based on its structural analogy with the natural DNA precursor dATP, PMEApp competitively inhibits viral DNA polymerases and reverse transcriptase. When incorporated into the nascent DNA strand, PMEApp inevitably causes DNA chain termination because of the lack of a hydroxyl group for further DNA chain elongation. At higher concentrations, PMEApp also interferes with the cellular replicative DNA polymerases α, δ and ε [7,8]. This results in a marked cytostatic activity of PMEA against a variety of tumor cell lines.

We have recently shown that PMEA strongly induces differentiation in several in vitro tumor cell models, including human erythroleukemia K562 cells and rat choriocarcinoma RCHO cells [9–11]. Moreover, PMEA effectively inhibits rat choriocarcinoma tumor growth in an in vivo model [12], pointing to the antitumor potential of PMEA and of related acyclic nucleoside phosphonate analogues. Induction of tumor cell differentiation by antimetabolites of purine and pyrimidine nucleotide metabolism, such as the IMP dehydrogenase inhibitor tiazofurin, the dihydrofolate reductase inhibitor methotrexate and the classical DNA synthesis inhibitors 1-β-D-arabinofuranosylcytosine (ara-C) and aphidicolin, is a well documented phenomenon [13]. The ultimate inhibition of cellular DNA replication, common to all these drugs, is assumed to be the primary trigger for the onset of the differentiation program in drug-exposed tumor cells [13]. In addition, it cannot be excluded that (deoxy)ribonucleotide pool imbalances, caused by these agents, also play a role in antimetabolite-induced tumor cell differentiation. PMEA, as a direct inhibitor of the DNA polymerization process, clearly fits into this picture.

Although the metabolism of PMEA has been intensively studied in several cell lines, no detailed investigations have been performed on the antimetabolic action of the drug. Therefore, we have now examined the effects of PMEA on cell cycle distribution, nucleic acid synthesis and de novo and salvage (deoxy)ribonucleotide metabolism in human erythro-

PII: S0014-5793(99)00104-0

leukemia K562, human T-lymphoid CEM and murine leukemia L1210 cells. Our investigations have disclosed novel information on the (de)regulation of (deoxy)ribonucleotide metabolism in the presence of PMEA and on the biochemical context in which PMEA, and other agents that directly inhibit DNA synthesis, may exert its tumor cell differentiation inducing activity.

2. Materials and methods

2.1. Compounds

PMEA was synthesized and generously supplied by Dr. A. Holy (Czech Academy of Sciences, Prague, Czech Republic) and Dr. N. Bischofberger (Gilead Sciences, Foster City, CA, USA). Ara-C and aphidicolin were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. Cell culture

Human erythroleukemia K562, human T-lymphoid CEM and murine leukemia L1210 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured in a humidified, CO_2-controlled atmosphere in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine and 0.075% $NaHCO_3$ (Flow Laboratories, Irving, UK). Subcultivations were performed every 3–4 days.

2.3. Flow cytometric cell cycle analysis

Exponentially growing K562, CEM and L1210 cells were exposed to PMEA at concentrations corresponding to 2–3 and 20–30 times the respective 50% inhibitory concentration for cell proliferation values (IC_{50}) of the compound in the different cell lines (i.e. 24 μM, 70 μM and 7.5 μM for K562, CEM and L1210 cells, respectively). At 24 h, the DNA of the cells was stained with propidium iodide using the CycleTest plus DNA reagent Kit (Becton Dickinson, Le Pont de Claix, France). The DNA content of the stained K562 cell cultures was assessed by flow cytometry on a FACScan equipped with CellQuest software (Becton Dickinson). Cell debris and cell clumps were excluded from the analysis by conventional dot plot gating.

2.4. Measurement of RNA and protein synthesis via incorporation of radiolabelled uridine and leucine

Exponentially growing K562, CEM and L1210 cells were seeded in 96-well microtiter plates at a final density of 1×10^5 cells/200 μl well and PMEA was added at various concentrations (i.e. 0, 1.6, 8, 40, 200 and 1000 μM). After a 24 h incubation at 37°C, 1 μCi of either [5-^{3}H]uridine or [4,5-^{3}H]leucine (Moravek Biochemicals, Brea, CA, USA) was added per microplate well. The cell cultures were then incubated for another 4 h. Thereafter, the 200 μl cell suspension was spotted onto Whatman GF/C glass microfiber filter discs mounted on a Millipore manifold. After washing the filters with PBS, the acid insoluble cellular material was precipitated on the filters with ice-cold 10% TCA, washed once with ice-cold 10% TCA, twice with ice-cold 5% TCA and finally twice with 70% ethanol. The radioactivity on the filter discs was measured using UltimaGold liquid scintillation counting fluid (Packard, Meriden, CT, USA).

2.5. Enzymatic determination of intracellular 2′-deoxyribonucleoside-5′-triphosphate pools

Exponentially growing K562, CEM and L1210 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 at 2×10^6 cells/100 μl and incubated on ice for 10 min. After centrifugation (15 000 rpm) at 4°C for 5 min, the supernatant was neutralized by addition of an equal volume of tri-*n*-octylamine/freon-TF solution (1:4, v/v) and vigorous shaking during 20 min. The cell extracts were centrifuged at 4°C for 5 min at 15 000 rpm, whereafter the upper aqueous phase was carefully collected and stored at −70°C until further analysis. Reaction mixtures for the enzymatic dNTP assays [14,15] contained 50 mM Tris-HCl pH 8.3, 1 mM 1,4-dithiothreitol, 5 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, 0.05 A_{260} units of alternating copolymer template (i.e. poly(dA-dT).poly(dA-dT) (Pharmacia Biotech, Uppsala, Sweden) 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 radiospecificity 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 U 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°C. After 80 and 100 min, 20 μl aliquots of the reaction mixtures were spotted onto Whatman DE81 filter discs. After drying, the filters were washed three times for 5 min with 5% Na_2HPO_4, rinsed once with distilled water and once with 70% ethanol. The radioactivity on the dried filters was measured by liquid scintillation counting using UltimaGold counting fluid (Packard).

2.6. Determination of intracellular ribonucleotide pools by HPLC analysis

Exponentially growing K562, CEM and L1210 cells were harvested, washed twice with cold RPMI 1640 medium without serum and lysed with ice-cold 75% methanol. After incubation on ice for 10 min, the cell extracts were centrifuged at 4°C for 5 min at 15 000 rpm and the supernatant was stored at −20°C until HPLC analysis. The supernatants were separated on an anion exchange Whatman Partisphere SAX column (particle size: 10 μm, column dimensions: 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 rate: 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, 5 min equilibration at the same buffer conditions. The eluting nucleotides were detected by UV absorbance at 260 nm and quantitated by the use of standard calibration curves. The retention times were 21, 23, 25 and 32 min for UTP, CTP, ATP and GTP, respectively.

2.7. Incorporation of [methyl-^{3}H]thymidine and [6-^{3}H]deoxyuridine into DNA and thymine deoxyribonucleotide pools

Exponentially growing K562 and L1210 cells were pre-exposed during 16 h to PMEA, ara-C or aphidicolin at the appropriate concentrations and were then loaded during 6 h with 1 μCi/ml [methyl-^{3}H]dThd (radiospecificity: 65 Ci/mmol) or 1 μCi/ml [6-^{3}H]dUrd (radiospecificity: 15.2 Ci/mmol) (Moravek Biochemicals). Thereafter, methanol extracts were prepared and analyzed by HPLC as described above. Determination of radiolabelled thymine deoxyribonucleotides was accomplished by liquid scintillation counting of the corresponding eluted fractions (i.e. 6–7 min, 13–14 min and 21–23 min for dTMP, dTDP and dTTP, respectively), using OptiPhase 'HiSafe' 3 counting fluid (Wallac, Turku, Finland). The radioactivity in the thoroughly washed DNA pellets of the cells was also determined.

2.8. Determination of thymidine kinase (TK) activity in crude K562 cell extracts

Drug-exposed K562 cells were washed twice with PBS and once with 100 mM Tris-HCl pH 7.8 containing 20 mM β-mercaptoethanol. Then, the cells were lysed by sonication (two times 5 s) in Tris buffer. After centrifugation (4°C, 15 min, 15 000 rpm), the supernatants were stored at −70°C until further analysis. The reaction mixtures for the TK assay contained 50 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 10 mM 1,4-dithiothreitol, 1 mg/ml bovine serum albumin, 2.5 mM ATP, 10 mM NaF and 1 μM [methyl-^{3}H]thymidine (radiospecificity: 2.4 Ci/mmol) (Moravek Biochemicals). The reactions were started by addition of 5 μl cell extract. The total reaction volume was 50 μl. At several time points (i.e. 0, 10, 20 or 30 min), 45 μl of the reaction mixtures was spotted onto Whatman DE81 filter discs. After drying, the filters were washed three times 5 min with 1 mM ammonium formate and thereafter once with distilled water and once with 70% ethanol. The radioactivity on the dried filters was measured using UltimaGold liquid scintillation counting fluid (Packard).

2.9. Immunoblotting

Protein extracts of drug-exposed K562 cells were prepared in cold PBS containing 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS and freshly added protease inhibitors. 50 μg of protein was electrophoresed on 4–15% Tris-HCl Ready Gels (Bio-Rad, Hercules, CA, USA) and subsequently blotted onto a Hybond-ECL nitrocellulose membrane (Amersham-Pharmacia, Uppsala, Sweden). Aspecific binding sites were blocked in 5% dried milk in PBS-T (i.e. PBS containing

0.1% Tween-20). After rinsing, the membrane was incubated overnight at 4°C with monoclonal mouse anti-human cyclin-dependent kinase (CDK)2 (clone D-12) (Calbiochem-Novabiochem, San Diego, CA, USA), cyclin A (clone BF683) or cyclin E (clone HE12) (PharMingen-Becton Dickinson), diluted to 0.5–2.5 μg/ml in PBS-T containing 2% dried milk. After washing, the membrane was incubated with peroxidase-linked sheep anti-mouse Ig antibody (Amersham) diluted in PBS-T containing 2% dried milk. After thorough washing of the membrane, protein bands were visualized by enhanced chemiluminescence detection (Amersham).

3. Results

3.1. Accumulation of PMEA-exposed tumor cells in the S phase of the cell cycle

Fig. 2 shows the cell cycle distribution of human erythroleukemia K562, human T-lymphoid CEM and murine leukemia L1210 cell cultures, exposed to PMEA for 24 h. The percentage of drug-treated K562 cells residing in the S phase gradually increased with increasing PMEA concentrations (i.e. 45%, 49%, 58% and 65% at 0, 5, 50 and 500 μM PMEA, respectively) while the percentage of K562 cells in the G_1 phase reciprocally decreased (i.e. 44%, 37%, 27% and 21%, respectively). The proportion of K562 cells in the G_2/M phase remained virtually constant (i.e. 11–15%) in the presence of the different PMEA concentrations (Fig. 2A). A similar trend was observed for PMEA-exposed CEM and L1210 cell cultures (Fig. 2B,C).

3.2. Effect of PMEA on RNA and protein synthesis

The effects of PMEA on RNA and protein synthesis were investigated by measuring the incorporation of radiolabelled uridine and leucine, respectively, in the acid insoluble fraction of PMEA-exposed (1.6, 8, 40, 200 or 1000 μM for 24 h) versus control K562, CEM and L1210 cells. No appreciable inhibitory activity of PMEA was noted under these experimental conditions. The 50% inhibitory concentration of PMEA was >1 mM for both uridine and leucine incorporation in all three tumor cell lines (data not shown). Thus, it was concluded that, in contrast to DNA synthesis, RNA and protein synthesis were not markedly inhibited in PMEA-treated K562, CEM and L1210 cells.

3.3. Accumulation of intracellular dNTP and NTP pools in PMEA-exposed tumor cells

K562, CEM and L1210 cells were exposed to PMEA at ~ 2 and ~ 10 times its IC_{50} in the different cell lines. After 24 h, the intracellular dNTP pools were measured by an enzymatic assay using the Klenow fragment of *E. coli* DNA polymerase I and alternating copolymer templates. All four dNTPs markedly accumulated intracellularly in the presence of PMEA. The intracellular levels of dATP, dCTP, dGTP and dTTP increased from 100% (control) to 224%, 261%, 288% and 330%, respectively, in K562 cells exposed to 50 μM PMEA and to 293%, 299%, 354% and 446%, respectively, in the pres-

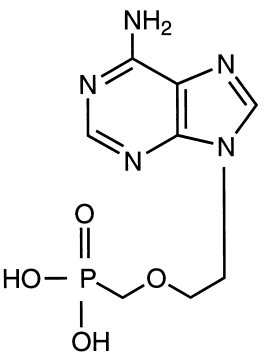

Fig. 1. Structural formula of PMEA.

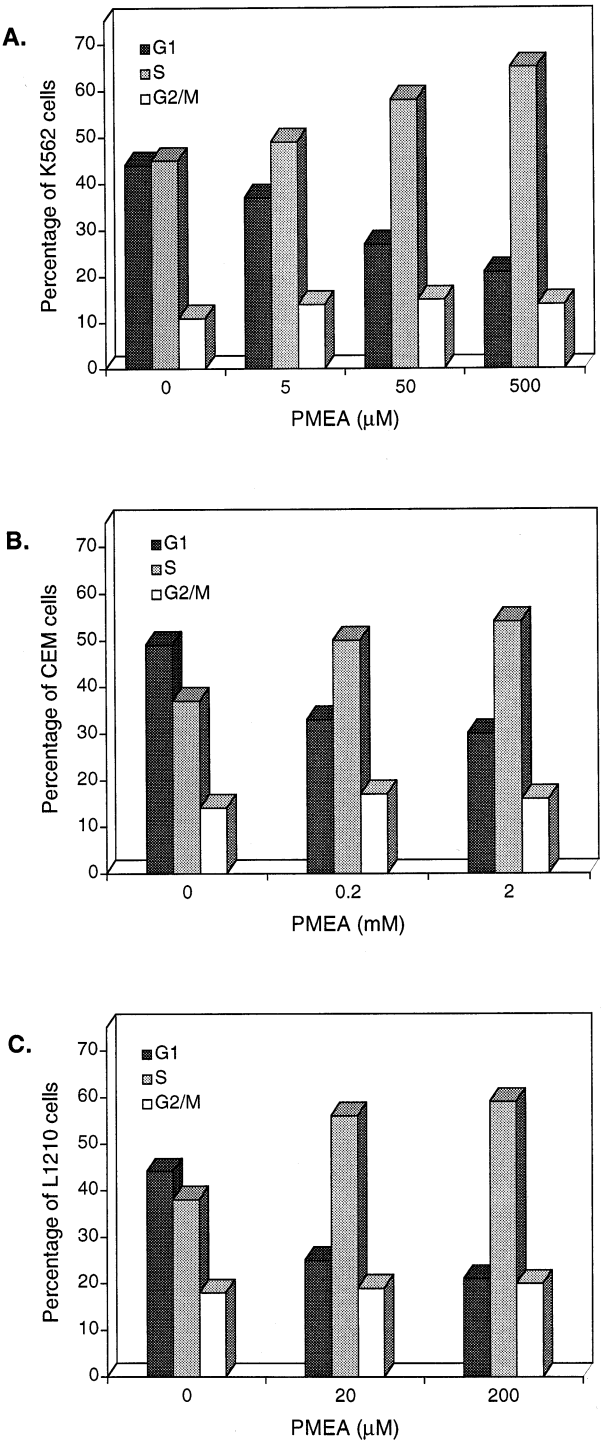

Fig. 2. Effect of PMEA on the cell cycle distribution of K562 cell cultures. K562 cells were exposed to PMEA at varying concentrations for 24 h. Thereafter, the DNA content of drug-treated and untreated K562 cells was analyzed by propidium iodide staining and flow cytometry.

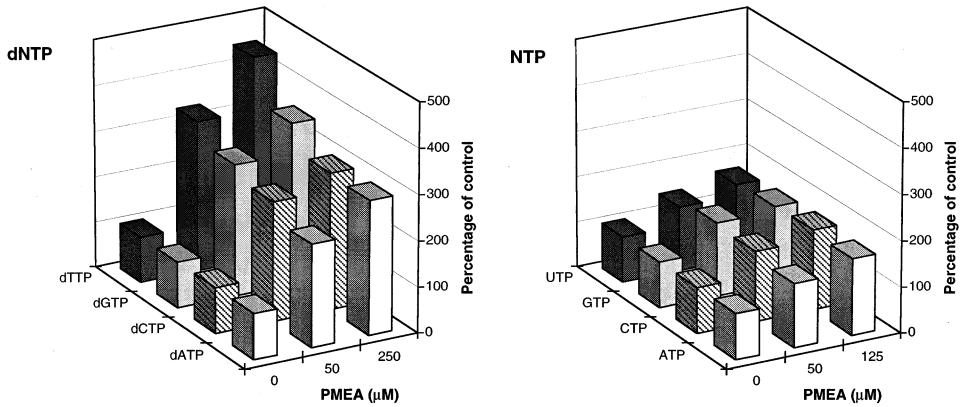

Fig. 3. Intracellular accumulation of (deoxy)ribonucleotides in K562 cells exposed to PMEA. The 2′-deoxyribonucleoside 5′-triphosphates (left panel) were quantitated by an enzymatic assay. The absolute amounts of dATP, dCTP, dGTP and dTTP in untreated control cells were 34, 32, 8 and 52 pmol/10^6 cells. The ribonucleoside 5′-triphosphates (right panel) were measured by HPLC analysis. The absolute amounts of ATP, CTP, GTP and UTP in untreated control cells were 7620, 724, 1333 and 3842 pmol/10^6 cells. All data are expressed as percentages of the pool sizes measured in untreated control cells and represent the means of two independent experiments.

ence of 250 μM PMEA (Fig. 3, left panel). Likewise, the four dNTPs accumulated to 1.5–4-fold their respective control values in CEM cells treated with 100 or 500 μM PMEA and to 1.5–3-fold their respective control values in L1210 cells treated with 20 or 100 μM PMEA (data not shown).

We also examined the effect of PMEA on the intracellular ribonucleoside triphosphate levels. After 24 h exposure to 50 μM PMEA, K562 cells showed NTP pool size elevations from 100% (control) to 139%, 153%, 161% and 141% for ATP, CTP, GTP and UTP, respectively. In K562 cells exposed to 250 μM PMEA, the ATP, CTP, GTP and UTP levels increased to 167%, 175%, 172% and 166%, respectively (Fig. 3, right panel). A similar modest accumulation (1.4–2-fold) of all four NTPs was also observed in PMEA-treated CEM and L1210 cells (data not shown).

3.4. Incorporation of de novo TS-derived thymine deoxyribonucleotides in PMEA-exposed K562 cells

K562 cells were pre-incubated with PMEA for 16 h and subsequently exposed to [6-^{3}H]deoxyuridine (dUrd) for another 6 h in the continued presence of PMEA. The amounts of dUrd-derived thymine deoxyribonucleotides that had been incorporated in the cellular DNA of K562 cells exposed to 50 and 200 μM PMEA were respectively 4- and 14-fold lower than in untreated control cells (Table 1). In the presence of PMEA, 2–4-fold accumulations of dUrd-derived dTMP, dTDP and dTTP were noted (Table 1).

3.5. Effect of PMEA on the salvage of exogenous radiolabelled thymidine

K562 and L1210 cell cultures were pre-incubated with PMEA for 16 h and subsequently exposed to radiolabelled thymidine for another 6 h in the continued presence of PMEA. In contrast to the observations made with dUrd, the incorporation of exogenous radiolabelled dThd into DNA was unaltered in the presence of 50 μM PMEA (Table 2). At 200 μM, the amount of radiolabelled dThd that was incorporated into DNA (i.e. 705 pmol/10^9 cells) was only 3-fold lower than in untreated K562 cells (i.e. 2099 pmol/10^9 cells) (Table 2). Following PMEA treatment, dThd-derived (i.e. TK-derived) thymine deoxyribonucleotides accumulated to a much higher extent in the acid soluble pools of K562 cells (i.e. 25–32-fold for dTMP, 9–12-fold for dTDP and 23–31-fold for dTTP) (Table 2) than did dUrd-derived thymine deoxyribonucleotides (i.e. only 2–4-fold) (Table 1). Such pronounced stimulation of dThd incorporation into the acid soluble thymine deoxyribonucleotide pools (i.e. 14–43-fold for each dTMP, dTDP and dTTP) was also observed in PMEA-treated CEM and L1210 cells (data not shown).

These findings prompted us to examine the effect of PMEA on the global activity of the salvage enzyme TK in drug-treated cell cultures. We found a gradual increase of TK activity in K562 cell cultures exposed to increasing PMEA concentrations. The amount of radiolabelled thymidine that was converted to thymine deoxyribonucleotides within 10 min by crude K562 cell extracts was 0.16 nmol/10^6 cells for control K562 cell cultures and 0.20, 0.31, 0.47 and 0.57 nmol/10^6 cells

Table 1
Incorporation of deoxyuridine-derived thymine deoxyribonucleotides in K562 cells exposed to PMEA

PMEA (μM)	pmol/10^9 cells[a]			
	dTMP	dTDP	dTTP	DNA
0	10	14	346	7005
50	39	62	1452	1820
200	25	32	831	507

[a]The data were calculated from the radioactivity measured in the DNA and in the thymine deoxyribonucleotide pools of drug-exposed versus untreated K562 cells, loaded with [6-^{3}H]deoxyuridine. The results represent the means for two independent experiments.

Table 2
Incorporation of thymidine-derived thymine deoxyribonucleotides in K562 cells exposed to PMEA

PMEA (μM)	pmol/10^9 cells[a]			
	dTMP	dTDP	dTTP	DNA
0	1.1	4.4	40	2099
50	27	40	935	2047
200	35	51	1244	705

[a]The data were calculated from the radioactivity measured in the DNA and in the thymine deoxyribonucleotide pools of drug-exposed versus untreated K562 cells, loaded with [methyl-^{3}H]thymidine. The results represent the means for two independent experiments.

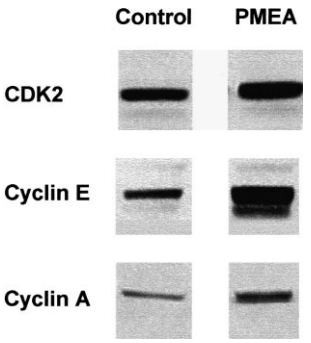

Fig. 4. Accumulation of CDK2, cyclin E and cyclin A in K562 cells exposed to PMEA for 24 h. The protein bands were visualized by immunological staining of Western blots and enhanced chemiluminescence detection. CDK2, cyclin E and cyclin A migrated at molecular weigths of 33, 50 and 60 kDa, respectively. The blots from one representative experiment are shown.

for K562 cell cultures incubated for 24 h with PMEA at 2, 10, 50 or 250 μM, respectively.

3.6. *Effect of PMEA on cyclin E, cyclin A and CDK2 expression in K562 cells*

Cyclins associate with and regulate the protein kinase activity of CDKs during the cell cycle. Since the CDK2/cyclin E and CDK2/cyclin A complexes are known to play crucial roles in S phase initiation and S phase progression, respectively, we decided to measure the protein levels of CDK2 and cyclins E and A in untreated K562 cells versus K562 cells exposed to 100 μM PMEA for 24 h. Striking increases of the cyclin E and cyclin A protein levels were noted in PMEA-exposed K562 cells, as compared to the control (Fig. 4). Also, the CDK2 protein level was slightly elevated in the presence of PMEA (Fig. 4). The DNA synthesis inhibitors aphidicolin and ara-C afforded similar cyclin and CDK2 accumulations (data not shown).

4. Discussion

The antiretroviral properties and intracellular metabolism of the acyclic nucleoside phosphonate analogue PMEA are well documented [1–8]. In contrast, the antimetabolic effects elicited by the drug at the cellular level have remained largely unknown. Therefore, we have now studied the impact of PMEA on cell cycle distribution and (deoxy)ribonucleotide metabolism in the human erythroleukemia K562, human T-lymphoid CEM and murine leukemia L1210 tumor cell lines. Because the K562 cell line has been extensively used in our laboratory to investigate the differentiation inducing properties of PMEA [9,10], the present report is focused on the results obtained with the K562 cells. However, it should be noted that the experiments with CEM and L1210 cells consistently led to similar conclusions.

Flow cytometric analysis of the DNA content of K562, CEM and L1210 cells in function of time revealed a marked retardation of S phase progression in the presence of PMEA, leading to the accumulation of S phase cells and a severe perturbation of the normal cell cycle distribution pattern. The observation that this phenomenon became increasingly apparent at higher drug concentrations is in agreement with the fact that the active drug metabolite PMEApp inhibits cellular DNA polymerases in a competitive manner [3]. Moreover, we have previously shown that the cellular uptake of PMEA by K562 cells and the subsequent conversion to its metabolically active form PMEApp occurs proportionally with the extracellular PMEA concentration up to 2 mM [16]. Thus, the detrimental effect of PMEA on the tumor cells should increase with increasing drug concentrations.

The marked increases (2–4-fold) in the dNTP pool sizes noted in PMEA-treated K562 cells are in agreement with the observations of Nicander and Reichard that the de novo synthesis of DNA precursors continues when DNA synthesis is blocked by aphidicolin [17]. Consequently, the dNTP substrates for the DNA polymerisation reaction accumulate in the presence of aphidicolin and PMEA due to their decreased consumption. Since we found that PMEA does not act as an inhibitor of RNA synthesis, the moderate accumulation of NTPs in PMEA-exposed cells can be attributed to feedback inhibition of ribonucleotide reductase by excess of dATP [18].

DNA synthesis in PMEA-exposed K562 cells was estimated by the use of radiolabelled nucleoside precursors. Consistent with the inhibition of DNA polymerases α, δ and ε by PMEApp (IC_{50} of PMEApp against purified mammalian DNA polymerase α: 1.8 μM [3]), markedly less dUrd-derived dTTP, synthesized via the de novo thymidylate synthase pathway, was consumed for DNA polymerisation in the presence of PMEA. The 2–4-fold increase of dUrd incorporation in the dTTP pool of PMEA-exposed, compared to control, K562 cells is consistent with the overall 3–4-fold expansion of the total (non-labelled) dTTP pool, as measured by the enzymatic dNTP assay after 24 h of PMEA exposure.

A different picture was obtained when radiolabelled dThd was used to measure thymine deoxyribonucleotide metabolism and DNA synthesis. The incorporation of exogenous thymidine into DNA was only≤3-fold lower in PMEA-exposed than in control K562 cells. Thus, the use of dThd as a radiolabelled precursor instead of dUrd resulted in an overestimation of DNA synthesis by about 4-fold. Also, the dramatic accumulation (i.e. 16–40-fold) of dThd-derived dTTP in the acid soluble pool of PMEA-treated K562, CEM and L1210 cells could not be explained by the increased dTTP pool size alone. Apparently, the salvage pathway of dTTP supply was highly active in PMEA-exposed cells. This was quite unexpected, since dTTP has been shown to inhibit purified TK at an IC_{50} value of 10 μM in a cell free assay (our unpublished data). In PMEA-exposed K562 cells, dTTP accumulated to intracellular concentrations up to approximately 90 μM, and thus, TK activity would be predicted to be substantially decreased under these conditions. We have ascertained that PMEA does not directly stimulate the enzymatic activity of purified cytosolic TK (our unpublished data).

Our findings that a comparably high accumulation of salvage-derived dTTP also occurred upon exposure of K562 cells to aphidicolin and ara-C (data not shown) indicate that this phenomenon must be related to the inhibition of DNA synthesis, which is common to all three compounds. Since TK is a cell cycle-dependent (S phase-specific) enzyme [19,20], its activity might be artificially increased in cell cultures exposed to agents (e.g. PMEA) that block cell cycle progression through the S phase. We could indeed demonstrate a 3.5-fold elevated TK activity in crude cell extracts from PMEA-exposed (S phase-enriched) compared to untreated K562 cell cultures. Thus, the dTTP-mediated feedback inhibition of TK may be counterbalanced by the higher expression level of TK,

caused by the drug-induced accumulation of S phase cells. This may explain why the total amount of exogenous radiolabelled dThd that was incorporated in cellular constituents (i.e. soluble dTMP, dTDP and dTTP pools and DNA) did not decrease in the presence of PMEA.

It is worth mentioning that PMEApp is a poor inhibitor of the human DNA polymerase β, which is responsible for DNA excision repair [21]. Moreover, it has been suggested that the salvage pathways of deoxyribonucleotide synthesis play an important role in providing the substrates for DNA repair and the mitochondrial DNA replication [22]. Hence, it is possible that a potential stimulation of DNA repair in PMEA-treated cells accounts, at least in part, for the observed salvage activity and incorporation of radiolabelled dThd into DNA.

We have also found a pronounced accumulation of cyclin E and modest increases in cyclin A and CDK2 protein levels in K562 cells exposed to each of the three DNA synthesis inhibitors PMEA, aphidicolin and ara-C. Interestingly, CDK2 and cyclins A and E are part of nuclear protein complexes with the transcription factor E2F, which bind to the promoter region of the TK gene and act as positive regulators of the S phase-specific TK gene transcription [23]. Thus, the increased abundance of CDK2 and cyclins A and E may also contribute to the observed stimulation of TK activity in PMEA-exposed K562 cells.

In conclusion, (the active metabolite of) PMEA has profound effects on (deoxy)ribonucleotide metabolism and pool levels, resulting from the inhibition of the DNA polymerisation process and the accumulation of drug-exposed cells in the S phase of the cell cycle. Most particularly, increased dNTP pool sizes and markedly elevated activity of the S phase-specific salvage enzyme TK were observed following PMEA treatment of K562 cell cultures. Our data also indicate that caution should be exercised when using thymidine incorporation into DNA as a parameter to evaluate the anti-proliferative effects of S phase-specific agents like PMEA.

Acknowledgements: These investigations were supported by Grants from the Fonds voor Wetenschappelijk Onderzoek (F.W.O.) Vlaanderen (G.0104.98), the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (3.0180.95), the A.S.L.K. Cancer Fund and the Flemish Geconcerteerde Onderzoeksacties (95/5). We are grateful to Mrs. Lizette van Berckelaer and Mrs. Ria Van Berwaer for the excellent technical assistance. We also thank Dr. Lieve Naesens for the helpful discussions.

References

[1] De Clercq, E., Holy, A., Rosenberg, I., Sakuma, T., Balzarini, J. and Maudgal, P.C. (1986) Nature 323, 464–467.
[2] Barditch-Crovo, P., Toole, J., Hendrix, C.W., Cundy, K.C., Ebeling, D., Jaffe, H.S. and Lietman, P.S. (1997) J. Infect. Dis. 176, 406–413.
[3] Balzarini, J., Hao, Z., Herdewijn, P., Johns, D.G. and De Clercq, E. (1991) Proc. Natl. Acad. Sci. USA 88, 1499–1503.
[4] Merta, A., Votruba, I., Jindrich, J., Holy, A., Cihlár, T., Rosenberg, I., Otmar, M. and Herve, T.Y. (1992) Biochem. Pharmacol. 44, 2067–2077.
[5] Robbins, B.L., Greenhaw, J., Connelly, M. and Fridland, A. (1995) Antimicrob. Agents Chemother. 39, 2304–2308.
[6] Balzarini, J. and De Clercq, E. (1991) J. Biol. Chem. 266, 8686–8689.
[7] Cherrington, J.M., Allen, S.J.W., Bischofberger, N. and Chen, M.S. (1995) Antiviral Chem. Chemother. 6, 217–221.
[8] Kramata, P., Votruba, I., Otová, B. and Holy, A. (1996) Mol. Pharmacol. 49, 1005–1011.
[9] Balzarini, J., Verstuyf, A., Hatse, S., Goebels, J., Sobis, H., Vandeputte, M. and De Clercq, E. (1995) Int. J. Cancer 61, 130–137.
[10] Hatse, S., Balzarini, J. and De Clercq, E. (1995) Nucleosides Nucleotides 14, 649–652.
[11] Hatse, S., Naesens, L., De Clercq, E. and Balzarini, J. (1998) Biochem. Pharmacol. 56, 851–859.
[12] Hatse, S., Naesens, L., DegrËve, B., Segers, C., Vandeputte, M., Waer, M., De Clercq, E. and Balzarini, J. (1998) Int. J. Cancer 76, 595–600.
[13] Hatse, S., De Clercq, E. and Balzarini, J. (1998) Biochem. Pharmacol. (in press).
[14] North, T.W., Bestwick, R.K. and Mathews, C.K. (1980) J. Biol. Chem. 255, 6640–6645.
[15] Hunting, D. and Henderson, J.F. (1981) Can. J. Biochem. 59, 723–727.
[16] Hatse, S., De Clercq, E. and Balzarini, J. (1998) Mol. Pharmacol. 54, 907–917.
[17] Nicander, B. and Reichard, P. (1985) J. Biol. Chem. 260, 5376–5381.
[18] Bjursell, G. and Skoog, L. (1980) Antibiot. Chemother. 28, 78–85.
[19] Dou, Q.P., Zhao, S., Levin, A.H., Wang, J., Helin, K. and Pardee, A.B. (1994) J. Biol. Chem. 269, 1306–1313.
[20] Bianchi, V., Borella, S., Rampazzo, C., Ferraro, P., Calderazzo, F., Bianchi, L.C., Skog, S. and Reichard, P. (1997) J. Biol. Chem. 272, 16118–16124.
[21] Cihlar, T. and Chen, M.S. (1997) Antiviral Chem. Chemother. 8, 187–195.
[22] Arnér, E.S.J. and Eriksson, S. (1995) Pharmacol. Ther. 67, 155–186.
[23] Chang, Z.F., Huang, D.Y. and Lai, T.C. (1995) J. Biol. Chem. 270, 27374–27379.