9-(2-Phosphonylmethoxyethyl)adenine Induces Tumor Cell Differentiation or Cell Death by Blocking Cell Cycle Progression through the S Phase

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Abstract
In addition to its inhibitory activity against viral DNA polymerases and reverse transcriptase, the acyclic nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine (PMEA) also markedly inhibits the replicative cellular DNA polymerases α, δ, and ε. We have previously shown that PMEA is a strong inducer of differentiation in several in vitro tumor cell models and has marked antitumor potential in vivo. To elucidate the molecular mechanism of the differentiation-inducing activity of PMEA, we have now investigated the effects of the drug on cell proliferation and differentiation, cell cycle regulation, and oncogene expression in the human erythroleukemia K562 cell line. Terminal, irreversible erythroid differentiation of PMEA-treated K562 cells was evidenced by hemoglobin production, increased expression of glycophorin A on the K562 cell membrane, and induction of acetylcholinesterase activity. After exposure to PMEA, K562 cell cultures displayed a marked retardation of S-phase progression, leading to a severe perturbation of the normal cell cycle distribution pattern. Whereas no substantial changes in c-myc mRNA levels and p21, PCNA, cdc2, and CDK2 protein levels were noted in PMEA-treated K562 cells, there was a marked accumulation of cyclin A and, most strikingly, cyclins E and B1. A similar picture of cell cycle deregulation was also observed in PMEA-exposed human myeloid THP-1 cells. However, in contrast to the strong differentiation-inducing activity of PMEA in K562 cells, the drug completely failed to induce monocytic maturation of human myeloid THP-1 cells. On the contrary, THP-1 cells underwent apoptotic cell death in the presence of PMEA, as demonstrated by prelytic, intracellular DNA fragmentation and the binding of annexin V to the cell surface. We hypothesize that, depending on the nature of the tumor cell line, PMEA triggers a process of either differentiation or apoptosis by the uncoupling of normally integrated cell cycle processes through inhibition of DNA replication during the S phase.

Introduction
PMEA, the prototype congener of the acyclic nucleoside phosphonates, is a structural analogue of the natural nucleotides AMP and dAMP (Fig. 1). PMEA is endowed with potent activity against herpesviruses, hepatitis B virus, and human immunodeficiency virus (1). The drug has been shown to enter the cell by an endocytosis-like process (2) and is converted intracellularly to its diphosphorylated metabolite, PMEApp (3). This fraudulent dATP analogue is responsible for the antiviral activity of the drug through competitive inhibition of viral DNA polymerases and reverse transcriptase. Subsequent incorporation of PMEApp into the growing DNA strand inevitably results in DNA chain termination (3, 4). At higher concentrations, PMEA also interferes with the replicative cellular DNA polymerases α, δ, and ε (4–6). As a result, PMEA shows a marked antiproliferative activity against a variety of rapidly growing tumor cells.

We have recently reported the marked differentiation-inducing activity of PMEA in several tumor cell lines, including human erythroleukemia K562, human myeloid HL-60, and rat choriocarcinoma RCHO cells (7, 8). In addition, PMEA was shown to be very effective against differentiation-susceptible choriocarcinoma tumors in an in vivo rat model (9). The drug not only suppressed the development of choriocarcinoma tumors in WKAV/H rats engrafted under the kidney capsule with RCHO cells but was also able to cause regression of preexisting choriocarcinoma tumors. Moreover, the antitumor effect of PMEA persisted for extended time periods after termination of therapy (9). Given the highly aggressive character of the choriocarcinoma tumor, these findings open new perspectives for the potential application of PMEA and/or related acyclic nucleoside phosphonate analogues in the anticancer field.
Very few of the numerous agents that are known to be endowed with in vitro differentiation-inducing properties in cell cultures have entered clinical trials at present. PMEA, in its oral prodrug form bis(pivaloxyloxy)methyl-PMEA, is currently the subject of clinical trials for the treatment of human immunodeficiency virus and human hepatitis B virus infections (10, 11). Phase I clinical studies with PMEA have revealed that drug plasma levels of 10 μg/ml (35 μM) can be achieved in patients without severe toxic side effects (12). At this concentration, PMEA induces marked tumor cell differentiation in vitro (7, 8). Thus, the differentiation-inducing properties of PMEA may be of clinical relevance and may add to the therapeutic benefit of the drug for the treatment of patients with AIDS, who frequently develop malignancies like Kaposi’s sarcoma and lymphomas.

We have already described the differentiation-inducing properties of PMEA in previous reports (7, 8). However, the molecular mechanisms responsible for PMEA-induced tumor cell differentiation have remained largely unknown. Therefore, the present study was aimed at a detailed investigation of the mechanism of action of PMEA as a differentiation-inducing agent in the human erythroleukemia K562 cell model, which has been widely used to study in vitro tumor cell differentiation (13, 14). In addition to hemoglobin production, we have also measured glycophorin A expression and acetylcholinesterase activity as appropriate erythroid markers to monitor K562 cell differentiation (14). We have examined the effects of PMEA on the cell cycle distribution pattern and on the expression of crucial cell cycle regulators and growth-controlling oncogenes in K562 cell cultures. For comparison, the classical DNA synthesis inhibitors aphidicolin and ara-C were included in our study. To gain more information about the cell type specificity of PMEA-induced cell differentiation, we also evaluated the effects of PMEA on cellular functioning and on the appearance of monocytic differentiation markers in the human myeloid THP-1 cell line. Our results indicate that cell differentiation and apoptotic cell death are two alternative responses of tumor cells to PMEA-induced cell cycle arrest in the S phase.

### Results

#### Growth Inhibition and Viability of PMEA-exposed K562 Cells.

K562 cells were exposed for 4 days to PMEA at increasing concentrations from 5 μM to 1 mM. At a concentration of 5 μM, PMEA inhibited K562 cell proliferation by 20%. Cell growth inhibition further increased to 47%, 64%, 78%, and 94% at PMEA concentrations of 20, 50, and 150 μM and 1 mM, respectively. The IC_{50} value of PMEA for K562 cells, i.e., the compound concentration that inhibited cell proliferation by 50%, was 24 μM. The viability of the K562 cell cultures remained high (86–89%) after 4 days of PMEA exposure at concentrations of up to 1 mM, indicating that PMEA exerts a cytostatic rather than a cytotoxic activity against K562 cells.

#### Appearance of Erythroid-specific Markers in PMEA-exposed K562 Cells.

Benzidine staining allows easy and rapid distinction between differentiated hemoglobin-containing (benzidine-positive) and undifferentiated (benzidine-negative) K562 cells. In untreated K562 cell cultures, the background of benzidine-positive cells, resulting from spontaneous differentiation, was 4%. After a 4-day exposure of K562 cells to PMEA at increasing drug concentrations, the percentage of benzidine-positive (hemoglobin-containing) cells gradually increased to a plateau level of 50–60%, which was reached at a PMEA concentration of 50–100 μM (Table 1). Higher drug concentrations did not further increase the percentage of hemoglobin-positive K562 cells (Table 1).

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#### Treatment of K562 cells with PMEA also markedly stimulated the expression of the erythroid-specific membrane antigen glycophorin A, as demonstrated by flow cytometry. The glycophorin A-specific mean fluorescence values of K562 cell populations (Fig. 2, black histograms) after a 5-day exposure to PMEA at 0 (A), 10 (B) and 50 μM (C) were 18, 27, and 50 (relative units), respectively. The corresponding mean values of aspecific (background) fluorescence (Fig. 2, white histograms) were 4, 4, and 6 (relative units), respectively. In untreated K562 cell cultures stained with FITC-conjugated glycophorin A mAb, as many as 40% of the cells fell within the range of aspecific (background) fluorescence, compared to 15% in K562 cell cultures exposed to 10 μM PMEA and only 4% in K562 cell cultures exposed to 50 μM PMEA (Fig. 3).
which is generally assumed to trigger terminal (irreversible) differentiation as a reversible differentiation inducer (14), and ara-C, and butyrate, respectively. Combined expression of several key regulators of S-phase initiation, progression, and termination. For comparison, we included aphidicolin and ara-C in our experiments. K562 cells were exposed to 100 μM PMEA, 1 μM aphidicolin, and 0.2 μM ara-C. At these concentrations, which correspond to 4- to 6-fold the respective IC_{50} values for K562 cell proliferation, the three drugs afforded a comparably high degree of K562 cell differentiation (i.e., 50-70% benzidine-positive cells after a 5-day drug exposure) at 24, 48, and 72 h, protein extracts were prepared, and the protein levels of PCNA, p21, cdc2, and CDK2 and cyclins E, A, and B1 were investigated by immunoblotting. No significant changes in protein expression were found for p21 in drug-treated versus untreated cells (Fig. 5). The CDK2 protein level increased slightly in K562 cells in the presence of PMEA and butyrate had substantially recovered at 72 h after the drug had been removed at 48 h (Fig. 4, E and f).

**Effect of PMEA on the Expression of Cell Cycle-regulating Proteins in K562 Cells.** Because PMEA specifically interferes with DNA replication during the S phase of the cell cycle, we investigated its possible impact on the expression of several key regulators of S-phase initiation, progression, and termination. For comparison, we included aphidicolin and ara-C in our experiments. K562 cells were exposed to 100 μM PMEA, 1 μM aphidicolin, and 0.2 μM ara-C. At these concentrations, which correspond to 4- to 6-fold the respective IC_{50} values for K562 cell proliferation, the three drugs afforded a comparably high degree of K562 cell differentiation (i.e., 50-70% benzidine-positive cells after a 5-day drug exposure). At 24, 48, and 72 h, protein extracts were prepared, and the protein levels of PCNA, p21, cdc2, and CDK2 and cyclins E, A, and B1 were investigated by immunoblotting. No significant changes in protein expression were found for p21 in drug-treated versus untreated cells (Fig. 5). The CDK2 protein level increased slightly in K562 cells in the presence of PMEA and butyrate had substantially recovered at 72 h after the drug had been removed at 48 h (Fig. 4, E and f).

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ence of PMEA and aphidicolin (Fig. 5). Minor accumulations were also noted for PCNA and cdc2 in PMEA-exposed K562 cells (Fig. 5). Most strikingly, cyclin E, cyclin A, and cyclin B1 accumulated to supranormal levels in PMEA-exposed K562 cells, as compared to untreated control cells. A comparable pattern of cyclin accumulation was also observed with aphidicolin- and ara-C-treated K562 cells (Fig. 5).

**Effect of PMEA on c-myc mRNA Expression in K562 Cells.** The c-myc mRNA levels of drug-treated and untreated K562 cells were compared by a unique, newly developed method based on semiquantitative RT-PCR and subsequent HPLC analysis of the PCR reaction products (17, 18). Total RNA was isolated from the cells and reverse-transcribed into cDNA, which was used as the template for the PCR reaction. As a constant internal reference, a β-actin DNA fragment was coamplified with the c-myc DNA fragment. To validate the quantitative character of the RT-PCR mRNA assay, the duplex PCR reaction was performed on serial dilutions of cDNA obtained from untreated control cells. Fig. 6 demonstrates that the reaction is within the exponential range of the amplification curve for both c-myc and β-actin. Thus, for PCR reactions yielding comparable HPLC peaks for the β-actin fragment, the peak areas of the c-myc fragment reflect the relative amounts of c-myc mRNA initially present in the K562 cells. As a positive control for c-myc down-regulation, K562 cells were treated for 4 h with 1.8% DMSO (19). The RT-PCR mRNA assay revealed that the c-myc mRNA level was indeed 4-fold decreased in DMSO-exposed cells compared to the untreated control (Fig. 6). For the c-myc DNA fragment, peak areas of $23 \times 10^3$ and $92 \times 10^3 \mu$Vdt/sec were recorded for DMSO-treated and untreated K562 cells, respectively, whereas the β-actin PCR yield was even slightly higher for the drug-exposed cells (peak area, $223 \times 10^3 \mu$Vdt/sec) than for the control (peak area, $200 \times 10^3 \mu$Vdt/sec) cells (Fig. 6).

In K562 cells incubated for 30 h with 50 μM, 500 μM, and 5 μM PMEA, no significant changes in the c-myc mRNA level were observed as compared to the untreated control. Peak areas of the c-myc PCR fragment were $100 \times 10^3$, $77 \times 10^3$, $88 \times 10^3$ mVdt/sec and $64 \times 10^3 \mu$Vdt/sec for untreated control and 50 μM, 500 μM, and 5 μM PMEA, respectively. The corresponding β-actin PCR yields were $157 \times 10^3$, $151 \times 10^3$, $139 \times 10^3$, and $150 \times 10^3 \mu$Vdt/sec respectively (data not shown). Even after a PMEA (50 μM) exposure as long as 12 days, no c-myc down-regulation
could be demonstrated. When cDNA dilutions yielding comparable β-actin peak areas were compared (i.e., 1:4 for control versus 1:2 for PMEA; 1:8 for control versus 1:4 for PMEA, 1:16 for control versus 1:8 for PMEA; Table 2), equal c-myc PCR yields were noted for 50 μM PMEA and control samples (Table 2). The results obtained with this method were confirmed by standard Northern blotting and hybridization procedures. 5

Effect of PMEA on Monocytic Differentiation of Human Myeloid THP-1 Cells. To find out whether or not differentiation induction by PMEA is a universal phenomenon that applies to all differentiation-susceptible tumor cell lines, we also examined the capability of PMEA to induce monocytic differentiation in human myeloid THP-1 cells. Differentiation of THP-1 cells can be easily demonstrated by the appearance of monocyte-specific surface antigens, such as CD14 (20). THP-1 cells were incubated in the presence of 50 μM PMEA for 4 days. As a positive control for induction of monocytic differentiation, 1,25-dihydroxyvitamin D3 (0.1 μM) was also included. After drug exposure, the expression of the monocytic marker CD14 on the THP-1 cells was assessed by flow cytometry. Fig. 7 shows that PMEA, unlike 1,25-dihydroxyvitamin D3, did not induce CD14 expression. The mean fluorescence intensities of the CD14-stained THP-1 cell cultures were 4.5, 5.9, and 133 (relative units) for control, PMEA, and 1,25-dihydroxyvitamin D3, respectively. Accordingly, microscopic inspection of the drug-treated cell cultures re-

5 A.B.P. van Kullenburg, personal communication.
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Flow Cytometric Analysis of the DNA Content of PMEA-exposed THP-1 Cells. PMEA was found to be a strong inducer of differentiation in K562 cells but not THP-1 cells, although differentiation could be easily triggered in the latter cell line by other agents such as 1,25-dihydroxyvitamin D3. To find out whether this differential behavior of PMEA in K562 versus THP-1 cells might be related to different cell cycle effects of the drug in both cell lines, we also investigated the cell cycle distribution of PMEA- and aphidicolin-exposed THP-1 cell cultures as a function of drug exposure time (Fig. 8A). The inhibition of cell cycle progression through the S phase, as observed in PMEA-exposed K562 cells, was also found in THP-1 cells after exposure to 20 μM PMEA, which is 2.5-fold the IC50 of PMEA for THP-1 cell proliferation (Fig. 8, A and B). However, under more stringent conditions (i.e., 200 μM PMEA or 2 μM aphidicolin), there was a striking increase in the proportion of sub-G1 THP-1 cells (Fig. 8, A and B). The percentages of THP-1 cells in the sub-G1 phase at 72 h were 2%, 4%, 58%, and 42% for control, 20 μM PMEA, 200 μM PMEA, and 2 μM aphidicolin, respectively (Fig. 8B).

Effect of PMEA on the Expression of Cell Cycle-regulating Proteins in THP-1 Cells. THP-1 cells were exposed to 40 μM PMEA for 24 h, and then protein extracts were prepared, and the protein levels of cyclins E, A, and B1 were investigated by immunoblotting. In analogy with the observations made in K562 cells, each of the three cyclins markedly accumulated in PMEA-exposed THP-1 cells, as compared to untreated control cells (Fig. 9).

Intracellular DNA Fragmentation in PMEA-exposed THP-1 Cells. Because DNA fragmentation occurs before plasma membrane lysis during the apoptotic process, the amount of DNA fragments detected intracellularly can be considered as a measure of the proportion of apoptotic but not necrotic cells in the cell culture. After prelabeling the genomic DNA with BrdUrd, THP-1 cell cultures were exposed to 20 or 200 μM PMEA or 2 μM aphidicolin. After 72 h, cell lysates were prepared, and the intracellular amounts of BrdUrd-labeled DNA fragments were measured by the use of a photometric ELISA. We found a markedly increased abundance of BrdUrd-labeled DNA fragments in the cytoplasmic fraction of PMEA- and aphidicolin-exposed cells compared to control THP-1 cells (Fig. 10).

Discussion
The acyclic nucleoside phosphonate PMEA (adefovir), a potent antiretroviral drug, also exhibits strong differentiation-inducing properties in several in vitro tumor cell lines (7, 8). Thus, in addition to its outstanding potential as an antiviral drug, PMEA may also be endowed with antitumor potential.
The present study was undertaken to gain more insight into the molecular basis underlying the differentiation-inducing activity of PMEA.

Terminal erythroid differentiation of PMEA-exposed human leukemia K562 cells was evidenced by three different markers, namely, induction of hemoglobin synthesis, increased glycophorin A expression on the cell membrane, and elevated acetylcholinesterase activity. We have previously shown that continuous exposure of tumor cells to PMEA for at least 2–3 days is required to reveal the marked

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**Fig. 7.** Effect of PMEA on monocytic differentiation of THP-1 cells. Monocytic differentiation was estimated by flow cytometric measurement of the monocytic-specific surface antigen CD14 on drug-exposed cells. White histograms, staining with the FITC-conjugated isotype control mAb; black histograms, staining with FITC-conjugated anti-CD14 mAb. A, control; B, 50 μM PMEA; C, 0.1 μM 1,25-dihydroxyvitamin D3.

**Fig. 8.** A, cell cycle distribution of THP-1 cell cultures exposed to PMEA or aphidicolin (APC). After 4, 24, 48, 72, and 96 h of drug exposure, the DNA content of the THP-1 cells was analyzed by PI staining and flow cytometry. The black, gray, dark gray, and white sections represent the percentages of sub-G1, G1, S-phase, and G2-M-phase cells, respectively. In untreated THP-1 cell cultures, the percentage of cells residing in the sub-G1, G1, S phase, and G2-M phase was 2%, 55%, 26%, and 17%, respectively. B, DNA content frequency histograms of THP-1 cell cultures exposed to PMEA or aphidicolin (APC) for 72 h. The marker Apo spans the sub-G1 region, which comprises apoptotic cells exhibiting a subdiploid DNA content.
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PMEA parallel those of ara-C, which induces irreversible, differentiation-inducing activity of the drug (7). In this respect, PMEA contrasts with the phorbol ester phorbol 12-myristate 13-acetate, which triggers megalakrocyclic differentiation of K562 cells within very short exposure times via direct activation of a signal transduction cascade involving protein kinase C (22). Apparently, differentiation induction represents a delayed effect of PMEA, which may result from the block imposed on DNA replication by the diphosphorylated drug metabolite PMEApp. This idea is supported by the fact that the DNA synthesis inhibitors aphidicolin and ara-C also strongly induce erythroid differentiation of K562 cells (data not shown). Conversely, (R)-9-(2-phosphonomethoxypyrazoxyl)adenine, a closely related structural analogue of PMEA, as well as the nucleoside analogues 3′-azido-2′,3′-dideoxythymidine and 2′,3′-dideoxycytidine, which are poor inhibitors of the replicative cellular DNA polymerases (5), are only marginally effective in inducing K562 cell differentiation (data not shown).

Our observation that the pronounced accumulation of PMEA-treated K562 cells in the S phase of the cell cycle only became apparent after exposure times longer than 4 h further supports the hypothesis that the drug needs to be converted to an active metabolite (presumably the diphosphorylated form PMEApp) to afford its biological effects. Inhibition of S-phase progression became more pronounced with increasing PMEA concentrations, which is in full agreement with our earlier observation that the uptake of PMEA and its subsequent phosphorylation to PMEApp by K562 cells is dose dependent and not yet saturated at an extracellular concentration as high as 2.5 µM (23). Moreover, PMEA-induced S-phase arrest of K562 cells was not reversed upon drug removal. This is presumably related to the fact that PMEApp, when incorporated instead of dATP into the nascent DNA strand, inevitably causes DNA chain termination due to the lack of the hydroxyl group required for further DNA chain elongation. In this context, it should be mentioned that the differentiation-inducing properties of PMEA parallel those of ara-C, which induces irreversible, terminal differentiation of K562 cells (16). In contrast, butyrate-induced K562 cell differentiation proved to be reversible (Ref. 14; data presented here). The effect of butyrate on histone acetylation, which is thought to be the major cellular target of the compound, is indeed reversed upon removal of the drug (24).

The protein levels of cyclins E, A, and B1 were considerably elevated after treatment of K562 cells with PMEA, aphidicolin, or ara-C. These findings are in accord with the observations of Gong et al. (25), who reported that inhibitors of DNA synthesis induce growth imbalance and altered expression of cyclins E, A, and B1 in human MOLT-4 cells. The uncoupling of DNA replication from RNA and protein synthesis is a typical feature of cells treated with DNA synthesis inhibitors, which may lead to perturbation of the orchestrated schedule of periodic expression of crucial cell cycle regulators (25). Indeed, we have previously found that unlike DNA replication, DNA transcription (mRNA synthesis) and mRNA translation (protein synthesis) are not inhibited by PMEA (26). Despite the accumulation of the mitotic cyclins A and B1, tumor cells arrested in the S phase by DNA synthesis inhibitors, such as PMEA, did not proceed prematurely to mitosis, indicating that the regulatory mechanism that preserves the temporal order of completion of DNA replication and initiation of the M phase is still fully functional under these circumstances (27).

The slight increases in cdc2, PCNA, and especially CDK2 protein levels noted in K562 cells exposed to PMEA (and aphidicolin and ara-C) presumably simply reflect the higher number of S-phase cells in drug-treated cultures compared to untreated cell cultures. Indeed, cdc2, PCNA, and CDK2 are known to be regulated in a cell cycle-dependent manner (28, 29).

The constitutive expression of p21 in K562 cells, in which p53 is inactivated by a frameshift mutation (30), can be explained by the fact that transcription of the p21WAF1/CIP1 gene is regulated by both p53-dependent and p53-independent mechanisms (31). PMEA-induced K562 cell differentiation was not associ-
ated with increased p21 protein expression or down-regulation of the c-myc mRNA level, two effects that have frequently been reported to accompany terminal differentiation of tumor cells induced by a variety of agents (32–34). Induction of p21 and/or down-regulation of c-myc may be required to provoke G1 arrest and cell cycle exit and thus may constitute crucial early events in the differentiation program activated by agents that do not by themselves obstruct cell cycle progression. Conversely, PMEA may block cell proliferation by directly interfering with DNA synthesis. Hence, PMEA may induce differentiation without a need to trigger additional negative growth signals. Moreover, several studies have revealed that c-myc down-regulation is not obligatory for tumor cell differentiation or even for growth arrest and strongly depends on the nature of the inducing agent (35–37).

Inhibition of DNA replication represents a common, principal factor in the induction of differentiation by diverse antimitabolites of purine and pyrimidine nucleotide metabolism (38). Moreover, unlike exponentially growing cells exhibiting extensive DNA biosynthesis, murine erythroleukemia cells in stationary phase (i.e., showing diminished DNA replication activity) were found to be unable to undergo DMSO-induced differentiation (39). Apparently, duplication of the cellular genome in the S phase of the cell cycle is a critical event during which the cells are highly susceptible to differentiation induction. Consistent with this concept, differentiation can be induced in K562 cells by compounds that affect nucleotide metabolism and, consequently, DNA replication, but not by the mitosis inhibitor vinblastine (16) nor by agents that interfere with RNA or protein synthesis (40).

Induction of tumor cell differentiation by PMEA was not universal but appeared to be cell type specific. For instance, PMEA was shown to trigger neuronal differentiation in LA-N-5 cells but not in SK-N-SH neuroblastoma cells. Moreover, unlike 1,25-dihydroxyvitamin D$_3$, PMEA proved unable to trigger monocytic differentiation in human myeloid THP-1 cells. However, we have demonstrated that the impact of PMEA at the biochemical/molecular level (i.e., accumulation of cells in the S phase of the cell cycle and deregulation of cyclin expression) is similar in differentiating K562 cells and nondifferentiating THP-1 cells. On the other hand, PMEA strongly induced apoptotic cell death in the THP-1 cell line, as demonstrated by intracellular DNA fragmentation and annexin V binding. Similar observations were made in PMEA-treated human T-lymphoid CEM cells and murine leukemia L1210 cells (data not shown). Apparently, the severe perturbation of cellular functioning by PMEA may cause differentiation in certain tumor cell types, whereas it triggers apoptosis in other cell types. The fact that PMEA does not induce marked apoptosis in the K562 cell line is consistent with the well-known resistance of K562 cells to drug-induced apoptosis. The lack of apoptotic response in K562 cells has been attributed to the activity of the chimeric Bcr/Abl tyrosine kinase present in this tumor cell line (41). In addition, K562 cells have been shown to express high levels of the antiapoptosis protein Bcl-x$_L$, a member of the Bcl-2-related family of apoptosis modulators (42).

In conclusion, tumor cells arrested in the S phase of the cell cycle after exposure to the DNA synthesis inhibitor PMEA can undergo differentiation and/or apoptotic cell death, depending on the tumor cell type. We assume that differentiation and apoptosis represent alternative escape mechanisms from an aberrant situation, created by PMEA through the dissociation of normally tightly coordinated events, i.e., through the separate inhibition of DNA replication relative to the continuation of mRNA synthesis and protein synthesis (26). The mechanism that determines whether and when either the differentiation program or the apoptotic pathway or possibly both processes are activated after exposure of an individual tumor cell line to PMEA remains unclear. The genetic background (i.e., mutations in oncoregions, tumor suppressor genes, and apoptosis-modulating genes) of the cell line studied most likely plays a decisive role.

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* A. Giacomello, personal communication.
in the eventual behavior (differentiation or apoptosis) of the tumor cell upon exposure to PMEA.

Materials and Methods

Chemicals

The synthesis and antiviral activity of PMEA have been described previously (1, 43). Ara-C, butyrate, and aphidicolin were purchased from Sigma Chemical Co. (St. Louis, MO), and 1,25-dihydroxyvitamin D3 was obtained from Solvay Duphar (Weesp, the Netherlands).

Cell Culture

Human erythroleukemia K562 and myeloid THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and routinely cultured in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% FCS (Life Technologies, Inc.), 2 mM glutamine, 0.5% nonessential amino acids and 0.075% NaHCO3 (Life Technologies, Inc.). Cell cultures were maintained at 37°C in a humidified, CO2-controlled atmosphere, and subcultivated with 10% FCS (Life Technologies, Inc.), and 2 mM glutamine, 0.075% NaHCO3 (Life Technologies, Inc.). Cell cultures were maintained at 37°C in a humidified, CO2-controlled atmosphere, and subcultivations were performed every 3–4 days.

Cytostatic Assay

Exponentially growing K562 and THP-1 cells were seeded at a final density of 2.5 x 105 cells/ml in RPMI 1640-based growth medium, and test compounds were added at 1:5 serial dilutions. The cells were then allowed to proliferate for 72–96 h (approximately three cell generations), and then the cells were counted in a Coulter Counter (Coulter Electronics, Harpenden Herts, United Kingdom).

Drug-treated K562 cells were washed once with PBS and resuspended in PBS at 5 x 106 cells/ml. Ten µl of the appropriate FITC-conjugated antibody was added to 200 µl of cell suspension. As a negative control for specific background staining, cells were stained in parallel with Simultest Control γ1/γ2 (Becton Dickinson, Erembodegem, Belgium). After incubation on ice for 30 min, the cells were washed twice in PBS and fixed with 1% paraformaldehyde in PBS. The fluorescence of the cells was then measured on a FACScan flow cytometer equipped with CellQuest software (Becton Dickinson). Cell debris was excluded from the analysis by conventional gating of forward scatter versus side scatter dot plots.

Cell Viability Assay

An equal volume of a 0.2% trypan blue solution in PBS was added to a 100-µl suspension of drug-treated K562 cells, and the cells were incubated at 37°C. After 10 min, the percentage of blue-stained (nonviable) cells was determined under the light microscope.

Measurement of Erythroid Differentiation of Drug-exposed K562 Cells

Benzidine Staining.

Twenty µl of a freshly prepared staining solution (10 µl of 1 M H2O2 (30%) in 2.5 ml of 0.2% benzidine in 0.5 M glacial acetic acid) were added to a 200-µl cell suspension. After incubation at 37°C for 20 min, the percentage of blue-green-stained K562 cells was determined under the light microscope. At least 200 cells were counted for each sample. The benzidine-positive (colored) cells were those in which hemoglobin production had been induced by the test compound, whereas the nondifferentiated cells remained transparent. The background of spontaneously differentiated K562 cells in untreated cell cultures was approximately 5%.

Glycophorin A Expression.

Drug-treated K562 cells were stained with FITC-conjugated mouse antihuman glycophorin A mAb (clone JC159; DAKO, Glostrup, Denmark) and analyzed by flow cytometry as described below.

Acetylcholinesterase Activity.

The method used was modified from that of Elman et al. (15). Drug-treated K562 cells were washed once with PBS and resuspended in 0.1 M potassium phosphate buffer (pH 8.0) at 0.5 x 106 cells/930 µl. The reaction tubes contained 930 µl of cell suspension (0.5 x 106 cells) and 60 µl of a 10 mM stock solution of 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M potassium phosphate buffer (pH 7.0) containing 1.5 mg/ml sodium bicarbonate. After the addition of 10 µl of a 75 mM aqueous solution of acetylthiocholine iodide substrate, the tubes were transferred to a 37°C incubator. The cell-free supernatant obtained after centrifugation (3000 rpm, 5 min) of 930 µl of cell suspension was also incubated at 37°C together with the two reagents in an additional tube. This blank mixture was used as the reference in the spectrophotometric measurements. After 0, 15, 30, 45, 60, 75, 90, and 105 min, one tube of each series was centrifuged at 4°C (3000 rpm, 5 min). The absorbance of the supernatant versus the corresponding blank was measured spectrophotometrically at 405 nm.

Flow Cytometric Assessment of Cell Surface Antigen Expression

Drug-exposed cells were washed twice with PBS and resuspended in PBS at 5 x 106 cells/ml. Ten µl of the appropriate FITC-conjugated antibody was added to 200 µl of cell suspension. As a negative control for specific background staining, cells were stained in parallel with Simultest Control γ1/γ2 (Becton Dickinson, Erembodegem, Belgium). After incubation on ice for 30 min, the cells were washed twice in PBS and fixed with 1% paraformaldehyde in PBS. The fluorescence of the cells was then measured on a FACScan flow cytometer equipped with CellQuest software (Becton Dickinson). Cell debris was excluded from the analysis by conventional gating of forward scatter versus side scatter dot plots.

Flow Cytometric Cell Cycle Analysis

Exponentially growing K562 and THP-1 cells were exposed to the test compounds at the appropriate concentrations. After 4, 24, 48, 72, and 96 h, the DNA of the cells was stained with PI using the CycleTEST PLUS DNA Reagent Kit (Becton Dickinson). The DNA content of the stained cell cultures was assessed on a FACScan flow cytometer equipped with CellQuest software (Becton Dickinson). Cell debris and cell clumps were excluded from the analysis by convenient gating of peak width versus peak area fluorescence contour plots. Due to the severe perturbation of the cell cycle distribution pattern of PMEA-exposed cell cultures, the specialized cell cycle analysis software package ModFit LT (Verity) was not suitable to analyze our experimental data. Therefore, appropriate region markers defining the different cell cycle phases (sub-G1, G1, S phase, and G2-M phase) were arbitrarily set on the DNA content frequency histograms of untreated control cell cultures and consistently applied to each of the drug-treated samples. The G2-M phase region was centered around a fluorescence value that was exactly twice as high as the G1 center value. The percentages of sub-G1 cells exhibiting a subdiploid DNA content (44) and G1, S phase, and G2-M phase cells were calculated by CellQuest software.

Immunoblotting of Cellular Protein Extracts

Crude protein extracts from drug-exposed K562 and THP-1 cells were prepared in cold PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and freshly added enzyme inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 30 µg/ml aprotinin, and 1 mM sodium orthovanadate). Protein concentrations of the extracts were determined by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Electrophoresis of 50 µg of protein was accomplished on 4–15% Tris-HCl Ready Gels (Bio-Rad), using Rainbow-colored protein molecular weight markers (Amersham-Pharmacia Biotech, Uppsala, Sweden). The separated proteins were transferred to a Hybond-ECL nitrocellulose membrane (Amersham-Pharmacia Biotech) by electroblotting. Aspecific binding sites were blocked by immersing the membrane in 5% dried milk in PBS-T for 1 h at room temperature. After rinsing in PBS-T, the membrane was incubated for 1 h at room temperature or overnight at 4°C with monoclonal mouse antihuman p21 (clone 187), CDK2 (clone D-12), cyclin B1 (clone GNS1; Santa Cruz Biotechnology, Santa Cruz, CA), cdc2 (clone Ab-2), PCNA (clone Ab-1; Calbiochem-Novabiochem, San Diego, CA), cyclin D1 (clone BF883), or cyclin E (clone HE12; PharMingen-Becton Dickinson). The primary antibodies were diluted in PBS-T containing 2% dried milk to final concentrations ranging between 0.5 and 2.5 µg/ml. After washing with PBS-T, the membrane was incubated for 30 min at room temperature with peroxidase-linked sheep antimeurinoglobulin antibody (Amersham-Pharmacia Biotech) diluted 1:2000 in PBS-T containing 2% dried milk. After thorough washing five times; 10 min) in PBS-T, protein bands were visualized by enhanced chemiluminescence detection (Amersham-Pharmacia Biotech).

c-myc mRNA Determination by Semiquantitative RT-PCR and Measurement of the Reaction Products by HPLC Analysis

Drug-treated K562 cells (105 to 106) were dissolved in 1 ml Trizol reagent (Life Technologies, Inc.). After incubation at room temperature for 5 min, 200 µl of chloroform were added. The samples were thoroughly shaken and incubated at room temperature for 2–3 min. After centrifugation for 15 min at 12,000 rpm (4°C), the aqueous phase was isolated and incubated for 10 min at room temperature with 500 µl of isopropanol. After centrif-
igation for 10 min at 12,000 rpm (4°C), the RNA pellet was washed once with 1 ml of 75% ethanol and vacuum-dried for 1–5 min. The RNA was then dissolved in 50–100 μl of RNase-free water, and the concentration was measured by determining the A_{260nm}. RNA (1 μg) was reverse-transcribed into cDNA by 3.6 units of Rous-associated virus 2 reverse transcriptase (Amersham-Pharmacia Biotech) in a reaction mixture (50 μl) containing reaction buffer (supplied with the enzyme); 0.25 mm each of dATP, dGTP, dCTP, and dTTP (Life Technologies, Inc.); 0.18 A_{260nm} units random hexanucleotides primers (Life Technologies, Inc.); 5 μl 1:4-1:TT; and 100 units of human placental RNase inhibitor (HPRI; Amersham-Pharmacia Biotech). The reaction was allowed to proceed for 80 min at 42°C. After an additional incubation at 95°C for 5 min, the reaction mixtures were immediately cooled on ice, aliquoted, and stored at −20°C. To compensate for differences in individual RT and PCR reaction efficiencies and for small variations in the initial amount of template cDNA added above, the cells residing in the sub-G1 compartment (exhibiting a sub-G1 DNA content and for small variations in the initial amount of template cDNA added above, the cells residing in the sub-G1 compartment (exhibiting a sub-G1 DNA content and representative of apoptosis, an early event in the apoptotic process (44–46), had been initiated.

**Detection of Intracellular BrdUrd-labeled DNA Fragments by ELISA.** Exponentially growing THP-1 cell cultures (4 × 10^5 cells/ml) were incubated with 10 μM BrdUrd for 8 h. The cells were washed with cold PBS, and the cellular BrdUrd-containing medium was carefully removed, and the cells were seeded into 96-well round-bottomed microwells in 20°C. After centrifugation (1000rpm, 10 min), the cell pellets were incubated for 30 min at room temperature with 200 μl of lysis buffer. After centrifugation (2000 rpm, 10 min), 100 μl of cell lysate was transferred to a 96-well, flat-bottomed microplate that had been coated with mouse anti-DNA antibody and preincubated with blocking solution to minimize aspecific binding. After a 90-min incubation at ambient temperature and subsequent washing, the DNA in the microplate was denatured (supplied with mouse irradiation and immediate cooling for 10 min at −20°C. Then, peroxidase-linked mouse anti-BrdUrd antibody was added. After a 90-min incubation at room temperature and subsequent washing, the substrate solution (TMB) was added, and the staining reaction was allowed to proceed for 30 min. The absorbance was measured at 370 nm and at 492 nm (reference wavelength) in a microplate reader, and the difference between both values (A_{492–370}) was calculated.

**Flow Cytometric Measurement of Annexin V Binding.** Drug-exposed THP-1 cells were washed once with cold PBS and resuspended at 10^6 to 10^7 cells/100 μl in annexin V incubation reagent (Gentzyme, Cambridge, MA) containing binding buffer, PI, and annexin V-FITC conjugate (21). After incubation at room temperature for 15 min in the dark, 400 μl of binding buffer were added, and the cells were analyzed by flow cytometry.

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**References**


