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Evidence for Distinction of the Differentiation-Inducing Activities and Cytostatic Properties of 9-(2-Phosphonylmethoxyethyl)adenine and a Variety of Differentiation-Inducing Agents in Human Erythroleukemia K562 Cells

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SUMMARY

The acyclic nucleoside phosphonate derivative 9-(2-phosphonylmethoxyethyl) adenine (PMEA) is a strong inducer of differentiation in several tumor cell lines, including human erythroleukemia K562 cells. A PMEA-resistant K562 cell line (designated K562/PMEA-1) was selected in the presence of escalating PMEA concentrations. This cell line proved to be insensitive to the induction of erythroid differentiation by PMEA. It also was 108-fold resistant to the cytostatic effects of PMEA. The K562/PMEA-1 cells showed reduced sensitivity to the differentiation-inducing capacity and cytostatic activity of several closely related PMEA analogs. Furthermore, the mutant cells exhibited a decreased sensitivity to the differentiation-inducing activity of a wide variety of structurally nonrelated antimetabolites targeted at different enzymes of nucleotide biosynthesis. A 5–25-fold higher concentration of each of these compounds was required to obtain the same level of differentiation in the K562/PMEA-1 cells as in the wild-type cells. However, unlike the PMEA derivatives, the antimetabolites remained equally cytostatic for the mutant K562 cells and for the wild-type cells. Our results reveal two unique features of the K562/PMEA-1 cells: (i) specific resistance to both the differentiation-inducing and cytostatic effects of several acyclic nucleoside phosphonate analogs (which can be accounted for by a diminished cellular uptake and subsequent metabolism of the compounds) and (ii) nonspecific resistance to antimetabolites with regard to their differentiation-inducing, but not cytostatic, properties (which must reside in an unspecified alteration of a common site of the differentiation process that is shared by all of these antimetabolites).

Certain malignancies result from a block in the commitment of stem cells toward the mature stage. However, malignant transformation does not necessarily mean irreversible loss of differentiation capacity, and induction of terminal differentiation of neoplastic stem cells generally implies the loss of self-renewal capacity. Therefore, differentiation induction might provide an attractive additional approach to cancer treatment. The therapeutic use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia is a well-known example of the successful application of this concept (1).

A large series of structurally diverse natural and synthetic compounds are able to trigger tumor cell differentiation. Among these differentiation-inducing agents, many are known to interfere at certain stages of purine/pyrimidine metabolism and/or DNA synthesis. Also, modulation of oncogene expression (in particular, c-*myc* down-regulation) seems to be an important event accompanying the onset of cellular differentiation (2, 3). However, for the majority of differentiation inducers, the exact mechanism of action remains largely unknown. Furthermore, it is currently unclear whether differentiation induction is causally related to cytotoxicity of the particular compounds.

The human erythroleukemia K562 cell line, which was established from a patient with chronic myelogenous leukemia (4–6), is a widely used model for the *in vitro* study of

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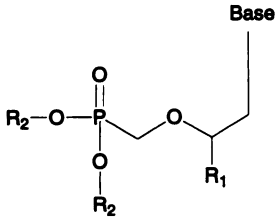
ABBREVIATIONS: Ara-C, 1-β-D-arabinofuranosylcytosine; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; mono(POM)PMEA, mono(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine; bis(POM)PMEA, bis(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine; PMEDAP, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine; HPMPA, 9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine; HPMPDAP, 9-(3-hydroxy-2-phosphonylmethoxypropyl)-2,6-diaminopurine; FPMPA, 9-(3-fluoro-2-phosphonylmethoxypropyl)adenine; HPMPG, 9-(3-hydroxy-2-phosphonylmethoxypropyl)guanine; HPMPC, 1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; AICA-riboside, 5-amino-4-imidazolecarboxamide riboside; MDR, multidrug resistance or resistant; HIV, human immunodeficiency virus; PMEG, 9-(2-phosphonylmethoxyethyl)guanine.

erythropoiesis and can be triggered to undergo differentiation by a variety of chemical agents, such as butyric acid, tiazofurin, and Ara-C (7, 8). In the presence of these compounds, K562 cells develop phenotypical characteristics similar to those of normal red blood cells, including red blood cell surface antigens, membrane-bound acetylcholinesterase production, and hemoglobin synthesis (9). The appearance of the latter erythroid marker can easily be followed through benzidine staining of the cells.

Recently, we reported the marked differentiation-inducing potential of PMEA in several tumor cell lines, including human promyelocytic HL-60 cells, rat choriocarcinoma RCHO cells, and the human K562 cell line (10). PMEA is the prototype congener of a class of acyclic nucleoside phosphonate derivatives endowed with potent activity against herpesviruses, hepatitis B virus, and retroviruses, including HIV (11–14). The antiviral activity of the acyclic nucleoside phosphonates is based on their structural resemblance to natural nucleoside-5′-monophosphates. Indeed, PMEA can be considered a synthetic analog of AMP, in which the phosphate group is directly linked to an acyclic nucleoside moiety by a stable carbon—phosphorus bond (Fig. 1).

PMEA is converted intracellularly into its monophosphorylated and diphosphorylated metabolites (PMEAp and PMEApp, respectively). The latter metabolite has been shown to be a strong inhibitor of retroviral reverse transcriptases, and therefore PMEApp is generally believed to be the active metabolite responsible for the inhibition of HIV replication (15). Based on its promising anti-HIV and anti-hepatitis B virus properties in cell culture and animal models, PMEA [in its oral prodrug form, bis(POM)PMEA] is currently investigated in phase II/III clinical trials in patients with acquired immune deficiency syndrome and hepatitis B virus-infected individuals. Furthermore, it was recently shown that unlike 3′-azido-2′,3′-dideoxythymidine, (*R*)-9-(2-phosphonylmethoxypropyl)adenine, a closely related structural analog of PMEA, was able to prevent simian immunodeficiency virus infection, even when drug administration was initiated at 24 hr after infection (16). These findings are suggestive of a prophylactic usefulness of this type of drug and justify further studies with this class of compounds. Therefore, insight into the biochemical/molecular basis underlying the differentiation-inducing effect of acyclic nucleoside phosphonates such as PMEA may be of great interest.

It is not known whether erythroid differentiation of K562 cells is triggered by PMEA or by one of its metabolites. To study the effects of PMEA on cellular metabolism and its molecular mode of action for induction of differentiation, we selected a PMEA-resistant cell line derived from the K562 cells. The sensitivity of the parental K562 cell line (K562/0) and its PMEA-resistant variant (K562/PMEA-1) to the cytostatic and differentiation-inducing effects of various acyclic nucleoside phosphonates and structurally nonrelated antimetabolites of purine/pyrimidine metabolism was investigated.



	R_1	R_2	Base
PMEA	-H	-H	adenine
Bis(POM)PMEA	-H	$-CH_2OCC(CH_3)_3$ (=O)	adenine
PMEDAP	-H	-H	2,6-diaminopurine
HPMPA	$-CH_2OH$	-H	adenine
HPMPDAP	$-CH_2OH$	-H	2,6-diaminopurine
FPMPA	$-CH_2F$	-H	adenine
PMEG	-H	-H	guanine
HPMPG	$-CH_2OH$	-H	guanine
HPMPC	$-CH_2OH$	-H	cytosine

Fig. 1. Structural formulae of acyclic nucleoside phosphonate analogs.

Materials and Methods

Compounds. The synthesis and antiretroviral activity of the acyclic nucleoside phosphonates used in this study have been described previously (12, 17, 18). PMEA; PMEDAP; the *S*-enantiomers of HPMPA [(*S*)-HPMPA], FPMPA [(*S*)-FPMPA], and HPMPDAP [(*S*)-HPMPDAP]; PMEG; and both *R*- and *S*-enantiomers of HPMPG [(*R*)-HPMPG and (*S*)-HPMPG] were generously supplied by Dr. A. Holy (Prague, Czech Republic). Mono(POM)PMEA, bis(POM)PMEA, and the (*S*)-enantiomer of HPMPC [(*S*)-HPMPC] were obtained from Dr. N. Bischofberger (Gilead Sciences, Foster City, CA). Tiazofurin and mycophenolic acid were kindly provided by Dr. D. G. Johns (National Cancer Institute, National Institutes of Health, Bethesda, MD), and EICAR was kindly supplied by Dr. A. Matsuda (Hokkaido University, Sapporo, Japan). Phosphonoformic acid (Foscarnet) was obtained from Astra Läkemedel (Södertälje, Sweden), and ribavirin was obtained from ICN Biomedicals (Cleveland, OH). Hydroxyurea, tubercidin, 3-deazauridine, butyric acid, colchicine, vinblastine, genistein, thymidine, and AICA-riboside were obtained from Sigma Chemical (St. Louis, MO). MTX was obtained from Lederle (Mont-St-Guibert, Belgium), and Ara-C was obtained from Upjohn (Puurs, Belgium). Verapamil and cyclosporin A were kindly provided by Dr. C. Roelant (Laboratory of Hematology, Katholieke Universiteit, Leuven, Belgium).

Cells. Human erythroleukemia K562 cells were obtained from the American Tissue Culture Collection (Rockville, MD) and were routinely cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (GIBCO, Paisley, UK), 2 mM glutamine (Flow Laboratories, Irving, UK), and 0.075% $NaHCO_3$ (Flow). Cells were allowed to grow for 3–4 days in a humidified, CO_2-controlled atmosphere.

The mutant K562/PMEA-1 cell line was selected as described in Results and was maintained in the same RPMI-based culture medium in the presence of PMEA at a final concentration of 1 mM. Before use in the experiments, the K562/PMEA-1 cells were cultured in PMEA-free medium for ≥4 days.

Light-scattering measurements (flow cytometry) for estimation of cell size and granularity. After being washed in phosphate-buffered saline, cell cultures were analyzed for forward scatter (indicative of the size of the cells) and side scatter (indicative of granularity) on a FACScan (Becton Dickinson, Le Pont de Claix,

France). To avoid morphological changes during preparation of the samples, cells were not fixed before analysis. To ensure statistical reliability, 50,000 cells were analyzed for each sample.

Differentiation assay. Exponentially growing cells were harvested and seeded onto 96-well microtiter plates (Falcon, Becton Dickinson) at a final density of 2×10^5 cells/ml in RPMI-based growth medium. Test compounds were added at the appropriate concentrations. In each well, the final volume was 200 μl. The cells were then incubated at 37° in a humidified CO_2-controlled atmosphere. After 5 days, 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 H_2O_2 30% in 2.5 ml of 0.2% benzidine in 0.5 M glacial acetic acid) was added. The staining reaction was allowed to proceed for 20 min at 37°, after which the percentage of blue-stained K562 cells was determined under the light microscope using a hemocytometer. At least 200 cells were counted. The benzidine-positive (blue) cells were those in which hemoglobin production had been induced by the test compound, whereas the nondifferentiated cells remained transparent. Untreated control cell cultures were included in the assays to estimate the background of spontaneously differentiated K562 cells (usually <5-10%).

Cytostatic assay (IC_{50} measurement). Exponentially growing cells were seeded onto 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 (for approximately three cell generations) at 37° in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted with a Coulter Counter (Coulter Electronics, Harpenden Herts, UK). The IC_{50} value was defined as the concentration of compound that inhibited cell proliferation by 50% compared with the untreated control.

Cell viability assay. Viability of K562 cell cultures was estimated by the trypan blue dye exclusion method. To 50 μl of cell suspension, 50 μl of a 0.2% trypan blue solution in phosphate-buffered saline was added. The cell cultures were then incubated for 5–10 min at 37°. Then, the number of viable (transparent) and nonviable (blue) cells was counted under the light microscope using a hemocytometer.

Uptake and metabolism of radiolabeled PMEA as a function of time. Exponentially growing cells were seeded onto 5-ml culture flasks at a final density of 2.5×10^5 cells/ml in RPMI-based growth medium. The next day, 20 μCi (0.46 μM) of [2,8-^{3}H]PMEA (Moravek Biochemicals, Brea, CA) was added, and the cell cultures were further incubated at 37° in a humidified CO_2-controlled atmosphere for several time periods (i.e., 6, 12, 24, and 48 hr). At the appropriate time points, cells were harvested, and extracellular [2,8-^{3}H]PMEA was carefully removed by three subsequent washing steps. The cell pellets were extracted with 400 μl of ice-cold 75% methanol. At each time point, an aliquot (200 μl) of the culture medium was also extracted with two volumes (400 μl) of ice-cold methanol. High performance liquid chromatography analysis of the cell and medium extracts was performed on a Whatman Partisphere SAX ion exchange column (4.6 × 125 mm) eluted with a linear gradient of 0.002 M ammonium phosphate buffer, pH 5.0, to 0.25 M ammonium phosphate buffer containing 0.5 M KCl, pH 5.0. Radiolabeled peaks were identified by chromatography in the presence of authentic standards. Radioactivity of the eluted fractions was determined by liquid scintillation counting.

Efflux of radiolabeled PMEA and PMEA metabolites. Exponentially growing cells were seeded onto 5-ml culture flasks at a final density of 2×10^6 cells/ml in RPMI-based growth medium, and 25 μCi (0.57 μM) of [2,8-^{3}H]PMEA (Moravek Biochemicals) was added. The cell cultures were incubated at 37° in a humidified CO_2-controlled atmosphere. After 24 hr, the cells were harvested, and extracellular [2,8-^{3}H]PMEA was carefully removed by three subsequent washing steps at 4°. Then, the cells were resuspended in warm RPMI-based growth medium at a final density of 10^6 cells/100 μl and incubated at 37°. At various time points (0, 5, 15, 30, 60, 120, 240, and 360 min), a 100-μl aliquot was removed and immediately cooled on ice. Cells were separated from the medium through centrifugation at 4°, and total radioactivity in the cell-free medium was determined by liquid scintillation counting. To the cell pellets, 50 μl of 1% Triton X-100 in water was added to disrupt cell membranes, and total radioactivity in the crude cell extracts was also measured.

Results

Selection of PMEA-resistant cells. Selection for PMEA resistance was carried out through exposure of the K562/0 cell cultures to increasing concentrations of PMEA, starting at 50 μM, which is 2-fold the IC_{50} value of PMEA for K562/0 cell proliferation. As soon as the cells recovered from the growth-inhibitory effect of PMEA, the drug concentrations were gradually increased. After several months (~50 subcultivations), a culture able to grow in the presence of 1 mM PMEA was established and designated K562/PMEA-1. After microscopical evaluation, which revealed no differences between wild-type and mutant cells, the morphological properties of both cell lines were further investigated by flow cytometry. Values obtained for forward scatter, which is widely used for estimation of cell size and is expressed in relative units, were almost identical (458 and 444 relative units for K562/0 and K562/PMEA-1 cells, respectively). Likewise, values for side scatter (226 and 250 relative units) for K562/0 and K562/PMEA-1 cells, respectively) revealed no significant differences in cell granularity. Also, the generation times were comparable for both cell lines (~24 hr). However, compared with wild-type, K562/PMEA-1 cells were 108-fold less sensitive to the cytostatic activity of PMEA. The IC_{50} value increased from 27 μM for the parental cells to 2.9 mM for the mutant cells. The PMEA resistance of the K562/PMEA-1 cells remained stable for ≥2 months after removal of PMEA from the culture medium.

Cytostatic activity of other acyclic nucleoside phosphonates and various unrelated compounds on wild-type versus PMEA-resistant K562 cells. A series of PMEA-related and structurally unrelated test compounds were examined for their cytostatic activity against both cell lines. The relative resistance of K562/PMEA-1 and K562/0 cells to the test compounds is expressed as the ratio of the IC_{50} values for the mutant versus the wild-type cells (Table 1). Several subclasses of acyclic nucleoside phosphonates, containing different bases (adenine, guanine, cytosine) and/or different aliphatic side chains, were included to evaluate the impact of structural modifications on the cytostatic effects of PMEA-related compounds on wild-type versus mutant K562 cells. Among the unrelated test compounds, agents targeted at different cellular enzymes that are involved in purine and pyrimidine nucleotide metabolism were chosen to compare cellular metabolism of K562/0 and K562/PMEA-1 cells.

The mutant cells were 13- and 47-fold resistant to the lipophilic ester prodrugs mono(POM)PMEA and bis(POM)PMEA, respectively. Partial cross-resistance was observed for PMEDAP, (*S*)-HPMPA, and (*S*)-FPMPA, which were 5.8-, 4.2-, and >9-fold, respectively, less cytostatic to the mutant than to the wild-type cells (Table 1). For (*S*)-HPMPDAP, only a slight increase (2.4-fold) in IC_{50} was observed. Interestingly, wild-type and mutant K562 cells showed equal sensi-

TABLE 1

Cytostatic activity of test compounds against wild-type and PMEA-resistant K562 cells

Compound	IC_{50}[a]		Relative resistance[b]
	K562/0	K562/PMEA-1	
	μM		
PMEA	27 ± 8	2918 ± 1053	108
Bis(POM)PMEA	0.53 ± 0.20	25 ± 11	47
Mono(POM) PMEA	41 ± 5	544 ± 86	13
PMEDAP	14 ± 5	81 ± 16	5.8
(*S*)-HPMPA	71 ± 22	298 ± 32	4.2
(*S*)-HPMPDAP	147 ± 2	353 ± 33	2.4
(*S*)-FPMPA	278 ± 43	>2500	>9
Foscarnet	751 ± 171	1120 ± 124	1.5
PMEG	1.1 ± 0.3	1.3 ± 0.3	1.2
(*S*)-HPMPG	55 ± 9	81 ± 2	1.5
(*R*)-HPMPG	180 ± 8	307 ± 64	1.7
(*S*)-HPMPC	111 ± 4	239 ± 88	2.2
Tiazofurin	21 ± 5	84 ± 22	4.0
Ribavirin	86 ± 31	68 ± 7	0.8
Mycophenolic acid	1.8 ± 0.4	1.70 ± 0.03	0.9
EICAR	1.7 ± 0.2	2.8 ± 0.5	1.6
AICA-riboside	223 ± 51	1301 ± 22	5.8
Tubercidin	0.35 ± 0.12	0.41 ± 0.21	1.2
Ara-C	0.040 ± 0.016	0.021 ± 0.014	0.53
Thymidine	94 ± 36	63 ± 12	0.7
3-Deazauridine	0.88 ± 0.35	2.09 ± 0.01	2.4
MTX	0.012 ± 0.005	0.013 ± 0.005	1.1
Hydroxyurea	101 ± 21	81 ± 30	0.8
Butyric acid	562 ± 243	726 ± 354	1.3
Colchicine	0.0137 ± 0.0033	0.0223 ± 0.0004	1.6
Vinblastine	0.003 ± 0.001	0.005 ± 0.001	1.7

[a] The IC_{50} values represent the concentration of compound required to inhibit cell proliferation by 50% compared with untreated control cells. The IC_{50} values were determined after 3 days of incubation. Each result represents the mean ± standard deviation of at least two independent experiments, performed in duplicate.

[b] The relative resistance was defined as the ratio of the IC_{50} in K562/PMEA-1 cells to the IC_{50} in K562/0 cells.

tivity to phosphonoformic acid and to the acyclic nucleoside phosphonates (*S*)-HPMPC, PMEG, and (*S*)- and (*R*)-HPMPG (Table 1).

Also evaluated in K562/0 versus K562/PMEA-1 cells were the cytotoxicities of a series of antimetabolites known to induce differentiation, such as the IMP dehydrogenase inhibitors tiazofurin (19), ribavirin, EICAR (20), and mycophenolic acid; the CTP synthetase inhibitor 3-deazauridine; the ribonucleotide reductase inhibitor hydroxyurea; the dihydrofolate reductase inhibitor MTX; and thymidine, Ara-C, AICA-riboside, and butyric acid. Except for tiazofurin and AICA-riboside, which were 4–6-fold less inhibitory to K562/PMEA-1 than to K562/0 cell proliferation, all of these antimetabolites, as well as colchicine and vinblastine, showed similar toxicity against both cell lines (Table 1).

All IC_{50} values were calculated from cell concentrations determined through automated cell counting. However, because the Coulter Counter method does not discriminate between viable and dead cells, for the most relevant compounds we compared the IC_{50} value obtained by the Coulter Counter method with the IC_{50} value determined by the trypan blue staining method, which discriminates between living and nonliving cells. As shown in Table 2, no significant differences were observed between the IC_{50} values resulting from both methods, suggesting that these compounds inhibit K562 cell proliferation without inducing considerable cell death. Furthermore, Table 2 shows that there is no marked difference between IC_{50} values determined after 3 or 5 days. These findings are important because differentiation experiments were carried out by exposing K562 cells to the differentiation-inducing compounds over 5 days. Compared with K562 cells exposed to differentiation-inducing compounds for 3 days, K562 cells exposed to these drugs for 5 days showed only slightly reduced viability. However, a comparable decrease in viability was noted for untreated control cultures, indicating that nutrient starvation rather than cytotoxic effects of the differentiation-inducing compounds caused the observed decrease in cell viability (Table 2).

Induction of differentiation in K562/0 versus K562/PMEA-1 cells by acyclic nucleoside phosphonates. To evaluate the differentiation-inducing potential of a series of PMEA-related and structurally unrelated test compounds in both wild-type and mutant K562 cell lines, the degree of erythroid differentiation in the drug-treated cell cultures was estimated from the percentage of benzidine-positive (hemoglobin-containing) cells after 5 days of drug exposure. The results are shown in Fig. 2.

In a first set of experiments, adenine/diaminopurine-containing acyclic nucleoside phosphonates were tested (Fig. 2A). In untreated control cultures, 8.3% and 5.2% of the cells

TABLE 2

IC_{50} values after 3 and 5 days determined by the Coulter Counter method and the trypan blue dye exclusion method

Compound	IC_{50} values measured by the Coulter Counter method[a]				IC_{50} values estimated by trypan blue dye exclusion[b]			
	K562/0		K562/PMEA-1		K562/0		K562/PMEA-1	
	3 days	5 days	3 days	5 days	3 days	5 days	3 days	5 days
	μM							
PMEA	38	55	>2000	>2000	31 (88)	56 (84)	>2000	>2000
PMEG	0.87	0.98	2.4	1.9	0.69 (83)	0.83 (81)	2.5 (95)	1.6 (88)
ARA-C	0.04	0.04	0.11	0.09	0.03 (87)	0.03 (83)	0.10 (92)	0.07 (86)
MTX	0.011	0.010	0.011	0.010	0.010 (88)	0.008 (76)	0.011 (91)	0.008 (74)
EICAR	1.3	1.4	2.5	2.3	1.2 (87)	1.5 (83)	2.4 (92)	2.0 (84)

[a] IC_{50} values were calculated from cell numbers measured by automated cell counting after 3 or 5 days of incubation. This method does not discriminate between living and dead cells.

[b] IC_{50} values were calculated from the number of viable (transparent) cells at day 3 or 5, as estimated under the light microscope after trypan blue staining of the cell cultures. This method accounts for cytotoxicity because only viable cells are considered. Values in parentheses represent the percentage of viable cells corresponding to the IC_{50} value, as estimated by interpolation from the dose-response curve for viability in function of compound concentration. Viability of untreated control cells was 90% and 80% for K562/0 cells at days 3 and 5, respectively, and 93% and 89% for K562/PMEA-1 cells at days 3 and 5, respectively. At 2 mM PMEA, viability of K562/PMEA-1 cells was 93% and 95% at days 3 and 5, respectively.

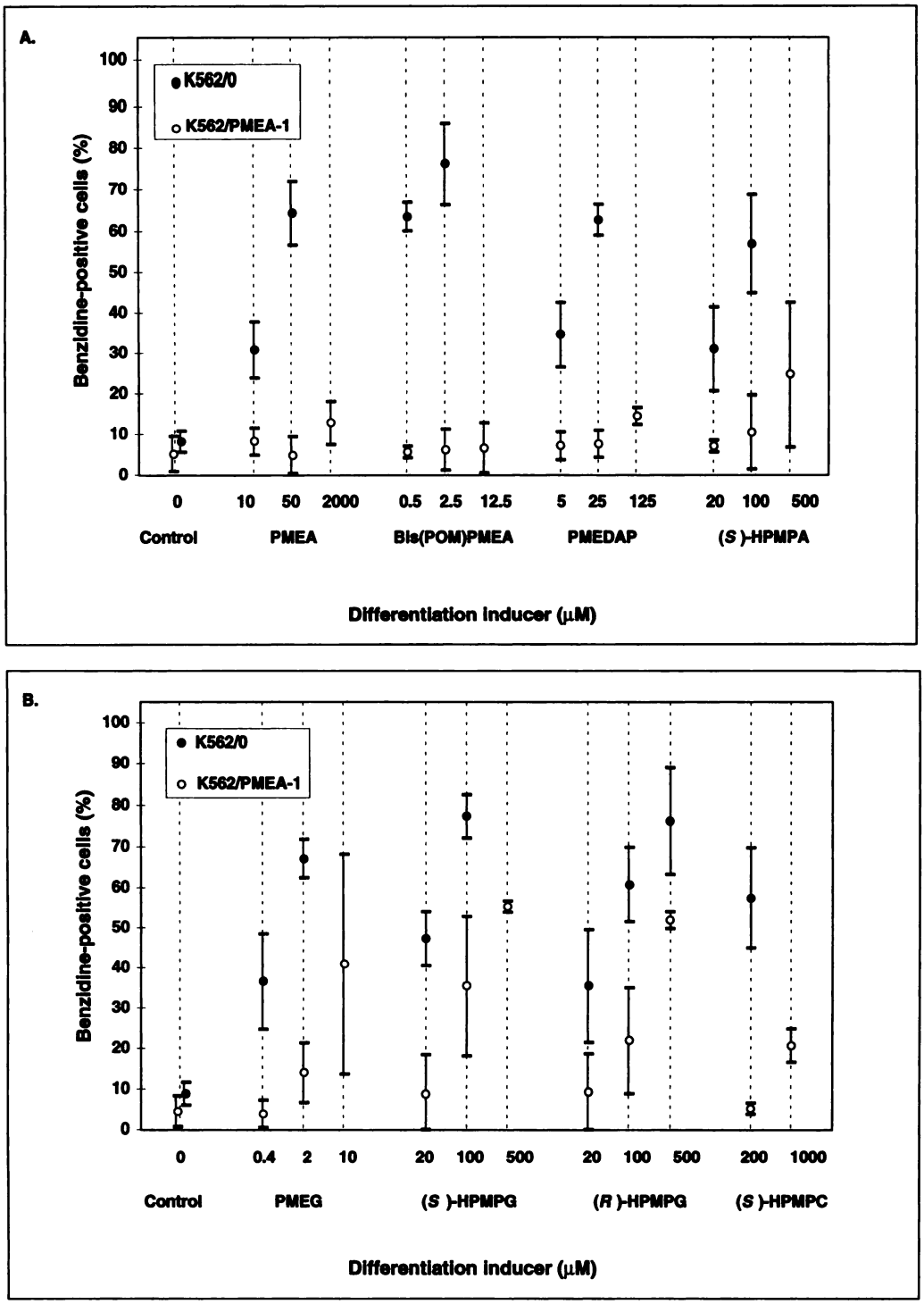
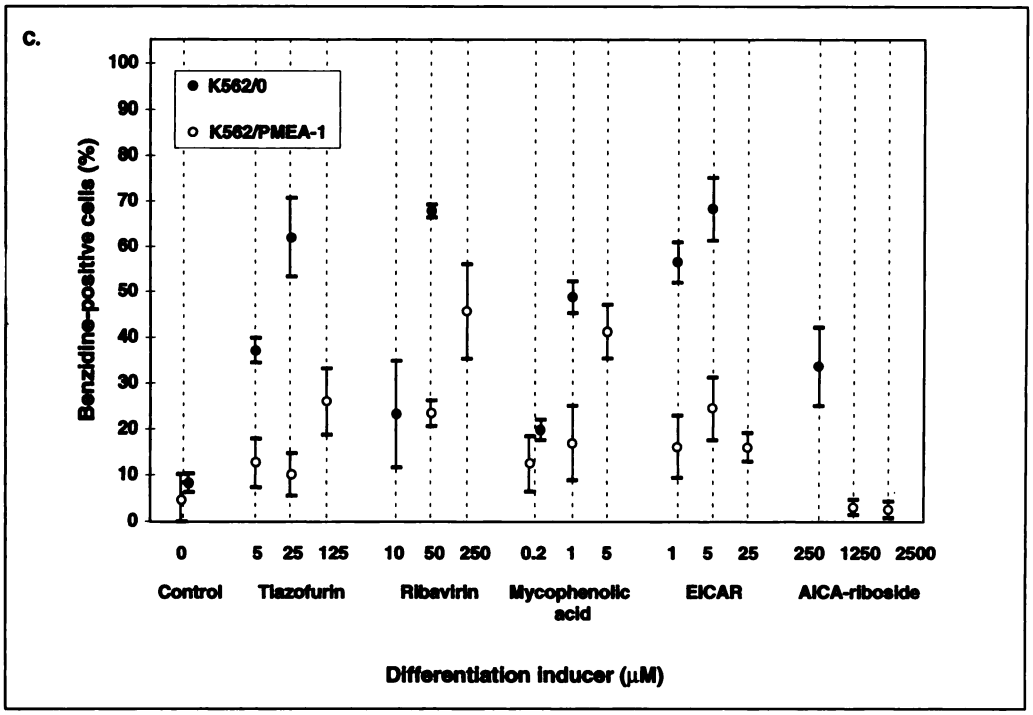


Fig. 2. Induction of erythroid differentiation in K562/0 (●) and K562/PMEA-1 (○) cells by various differentiation-inducing compounds, as estimated by the percentage of benzidine-positive cells. A, Adenine/diaminopurine-containing acyclic nucleoside phosphonates. B, Guanine/cytosine-containing acyclic nucleoside phosphonates.

were positive for benzidine for the wild-type and the mutant, respectively. Exposure of wild-type K562 cells to PMEA at concentrations of 10 and 50 μM resulted in 31% and 64% benzidine-positive cells, respectively, whereas differentiation remained at background levels in the PMEA-treated K562/PMEA-1 cell cultures (Fig. 2A). At a PMEA concentration as high as 2 mM, no marked differentiation was achieved in the mutant cells (13%). Also, 64% and 76% of the parental cells but only 5.7% and 6.2% of the mutant cells were benzidine positive after treatment with 0.5 and 2.5 μM bis(POM)PMEA, respectively. PMEDAP, administered at concentrations of 5 and 25 μM, triggered differentiation in 35% and 63%, respectively, of the wild-type cells compared with 7.2% and 7.7%, respectively, of the PMEA-resistant cells. For (*S*)-HPMPA at 20 and 100 μM, a similar tendency was observed: 31% and 57%, respectively, of the K562/0 cells but only 7.1% and 11%, respectively, of the K562/PMEA-1 cells could be induced to differentiate. Even drug concentrations as high as 12.5, 125, and 500 μM for bis(POM)PMEA, PMEDAP, and (*S*)-HPMPA, respectively, did not achieve the high degree of differentiation that was measured at much lower compound concentrations in the wild-type cells.



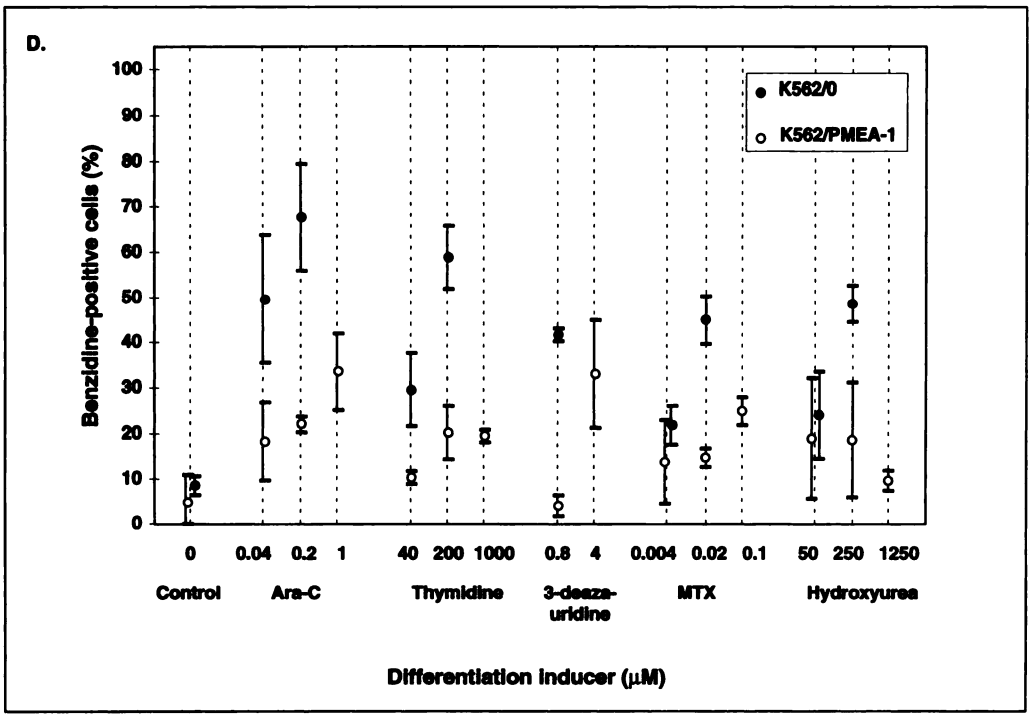


Fig. 2. C, Antimetabolites of purine metabolism. D, Antimetabolites of pyrimidine metabolism. Data represent average value ± standard deviation of two to five independent experiments.

As observed for the adenine/diaminopurine-containing acyclic nucleoside phosphonates, the guanine/cytosine-containing acyclic nucleoside phosphonates were also less potent differentiation-inducing agents in K562/PMEA-1 cells compared with K562/0 cells. PMEG at 0.4 and 2 μM induced differentiation in 37% and 67%, respectively, of the K562/0 cells but in only 3.9% and 14% of the K562/PMEA-1 cells (Fig. 2B). At 10 μM PMEG, 41% benzidine-positive K562/PMEA-1 cells were recorded, a percentage that is comparable to 37% in the wild-type cell culture at 0.4 μM PMEG. Also, the *S*- and *R*-enantiomers of HPMPG, as well as (*S*)-HPMPC, required a 5–25-fold higher concentration in K562/PMEA-1 cell cultures to afford a degree of differentiation comparable to that seen in K562/0 cells (Fig. 2B), notwithstanding the equal cytostatic activity of the guanine/cytosine-containing nucleoside phosphonates in both wild-type and mutant K562 cells (Table 1).

Induction of differentiation in K562/0 and K562/PMEA-1 cells by other differentiation-inducing agents with varying cellular targets. To examine whether the drop in sensitivity to differentiation-inducing agents ob-

served in the mutant cells was restricted to acyclic nucleoside phosphonate derivatives or common to other well-established differentiation inducers (regardless of their cellular target), a series of PMEA-unrelated differentiation-inducing agents were included in our investigation (Fig. 2, C and D).

As shown in Fig. 2C, each of the IMP dehydrogenase inhibitors tiazofurin, ribavirin, mycophenolic acid, and EICAR was a ≥5-10-fold less potent differentiation-inducing agent in K562/PMEA-1 cells than in K562/0 cells. For example, tiazofurin at 5 and 25 μM promoted hemoglobin synthesis in 37% and 62%, respectively, of the wild-type cells (background of spontaneous differentiation, 8.4%) but only in 13% and 10% of the mutant cells (background, 4.7%). Even at a 5-fold higher drug concentration (i.e., 125 μM), only 26% of the K562/PMEA-1 cells were benzidine positive. Also, for mycophenolic acid, a 5-fold lower concentration was sufficient in the wild-type cell cultures to obtain the same extent of differentiation obtained for the mutant cell cultures. The exposure of K562/0 cells to 0.2 and 1 μM mycophenolic acid resulted in 20% and 49% of differentiated cells, respectively, whereas in the mutant, 17% and 41% of the cells were benzidine positive after exposure to 1 and 5 μM mycophenolic acid, respectively. When administered at 250 μM, the intermediate of purine nucleotide metabolism AICA-riboside initiated the differentiation process in 34% of the parental K562 cells. In contrast, differentiation remained at the background level in the PMEA-resistant cells exposed to AICA-riboside at a concentration as high as 2.5 mM. Thus, for AICA-riboside, markedly >10-fold higher drug concentrations were required to afford an equal degree of cell differentiation in K562/PMEA-1 cells than in K562/0 cells.

Fig. 2D shows the data obtained for diverse antimetabolites of pyrimidine metabolism. When administered at 0.2 μM, Ara-C triggered hemoglobin synthesis in 68% of the wild-type cells but only 22% of the mutant cells. The degree of differentiation in K562/PMEA-1 cell cultures treated with Ara-C at 1 μM (i.e., 34%) was markedly lower than that in K562/0 cells treated with Ara-C at a 25-fold lower concentration (i.e., 50% benzidine-positive K562/0 cells at 0.04 μM Ara-C). In general, the other antimetabolites that were evaluated, such as 3-deazauridine, MTX, hydroxyurea, and the natural nucleoside thymidine, were 5–25-fold less potent differentiation-inducing agents in the mutant K562/PMEA-1 than in the wild-type K562/0 cells (Fig. 2D).

The viability of drug-treated and -untreated cells, as estimated by trypan blue dye exclusion, usually ranged between 75% and 85% at the time of the differentiation measurements (day 5) (data not shown). Only at the highest concentrations of some of the differentiation-inducing compounds tested in the mutant cells was lower viability of the cell cultures observed.

Induction of differentiation in a low PMEA-resistant mutant K562 cell line obtained at an intermediate stage of the PMEA resistance selection process. After ~40 subcultivations, a culture that was able to grow slowly in the presence of 500 μM PMEA was obtained. The IC_{50} value of PMEA was ~250 μM in this cell culture, being 10-fold higher than that in the parental K562/0 cells. We also compared the differentiation-inducing potential of several other compounds in this low PMEA-resistant mutant and in the wild-type K562 cell line (Table 3). For each of the compounds tested, a 5–10-fold increase in drug concentration was required in the low PMEA-resistant mutant cells to obtain a degree of differentiation comparable to that in the wild-type cells. Apparently, this intermediate mutant already displays the general decrease in sensitivity to differentiation induction that was found in the K562/PMEA-1 cell line. However, the low-resistant mutant easily reverted to the wild-type phenotype; therefore, we focused our further investigations on the high-resistant K562/PMEA-1 cell line, which proved to be more stable.

TABLE 3

Induction of erythroid differentiation in wild-type K562 cells and in the low PMEA-resistant mutant K562 cells by various differentiation-inducing compounds

Compound	Concentration	Benzidine-positive cells[a]	
		Wild-type	Low-resistant mutant
	μM	%	
PMEA	20	27	3
	50	47	8
	250		41
PMEG	0.4	27	11
	2	58	44
	10		63
ARA-C	0.04	35	26
	0.4	58	43
	4		50
MTX	0.004	6	6
	0.02	27	17
	0.1		28
EICAR	1	48	21
	5	56	50
Tiazofurin	1	2	3
	10	41	10
	100	63	38

[a] Differentiation was measured after exposure of the cell cultures to the compounds over 5 days. Backgrounds of spontaneous differentiation were <10% and have been accounted for by subtraction. Data represent the mean of two independent experiments.

Assessment of the K562/PMEA-1 cell line for expression of the MDR phenotype. The above findings show that the K562/PMEA-1 cell line, which was selected for resistance to the differentiation-inducing agent PMEA, became partially cross-resistant to the differentiation-inducing effects of a large range of drugs with different structures and cellular targets. The possible involvement of a MDR mechanism in the decreased sensitivity of the PMEA-resistant cells to differentiating compounds should be considered. Therefore, we compared in the two cell lines the IC_{50} value of PMEA in the absence of an MDR inhibitor with the IC_{50} value of PMEA in the presence of 8 or 40 μM verapamil (IC_{50} for verapamil = ~50 μM for both wild-type and mutant K562 cells), 2.5 or 12.5 μM cyclosporin A (IC_{50} for cyclosporin A = ~12.5 μM in both cell lines), or 3.7 or 18.5 μM genistein (IC_{50} for genistein = ~18.5 μM in both cell lines). In K562/0 cells, the IC_{50} value of PMEA ranged between 23 and 34 μM for control (no MDR inhibitor), verapamil, cyclosporin A, and genistein. In K562/PMEA-1 cells, the IC_{50} value of PMEA ranged between 0.90 and 2.75 mM, which is ~40-fold higher than that for wild-type cells. Apparently, none of the MDR-reversing agents examined were able to abolish the resistance of K562/PMEA-1 cells to the cytostatic effects of PMEA.

Uptake and metabolism of [2,8-^{3}H]PMEA. Because the K562/PMEA-1 cell line was selected for resistance to PMEA, transport and metabolism of PMEA may be considered possible sites for a mutation leading to PMEA resistance. There-

fore, we compared uptake of radiolabeled PMEA in wild-type and mutant K562 cells, as well as intracellular conversion to the phosphorylated metabolites PMEAp and PMEApp. In two independent experiments, K562/0 and K562/PMEA-1 cell cultures were exposed for several time periods to [2,8-^{3}H]-PMEA at an extracellular concentration of 0.46 μM. At several time points (i.e., 6, 12, 24, and 48 hr), the amount of intracellular radiolabeled PMEA and PMEA metabolites was determined and quantified in cell extracts of the K562/0 and K562/PMEA-1 cells by high performance liquid chromatography analysis. In wild-type K562 cells, average amounts of 0.8, 0.8, 0.5, and 0.3 pmol of PMEA/million cells were found at 6, 12, 24, and 48 hr, respectively (Fig. 3A). At each of these time points, 10–20-fold less PMEA was detected in cell extracts of K562/PMEA-1 cells (Fig. 3A). Also, a proportional decrease in the amount of PMEApp was observed in K562/PMEA-1 cells: compared with the wild-type cells, 15–>20-

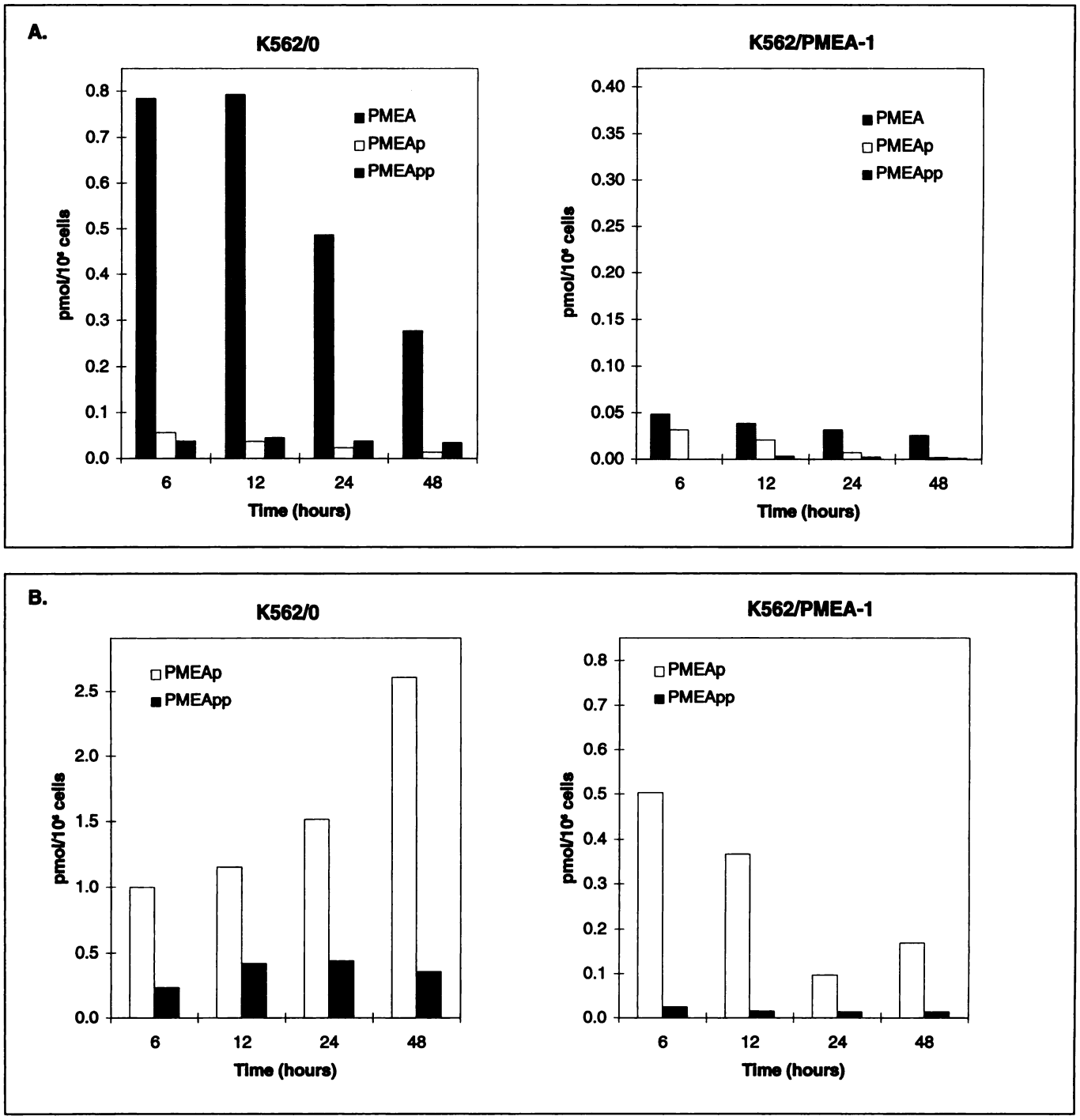


Fig. 3. A, Intracellular amounts of radiolabeled PMEA and PMEA metabolites in K562/0 and K562/PMEA-1 cells after incubation of the cells for 6, 12, 24, or 48 hr with [2,8-^{3}H]PMEA at an extracellular concentration of 0.46 μM. B, Extracellular amounts of PMEAp and PMEApp after incubation of wild-type and mutant K562 cells for 6, 12, 24, or 48 hr with radiolabeled PMEA at 0.46 μM. Data represent the mean of two independent experiments and are expressed in pmol/10^6 cells.

fold lower levels of PMEApp were detected in K562/PMEA-1 cells, being close to the detection limit (Fig. 3A).

In the same experiments, we also studied the appearance of phosphorylated PMEA metabolites in the culture medium as a function of time. Surprisingly, considerable amounts of PMEAp (1.0, 1.2, 1.5, and 2.6 pmol/million cells at 6, 12, 24, and 48 hr, respectively) and PMEApp (0.2–0.4 pmol/million cells at 6, 12, 24, and 48 hr) were excreted by K562/0 cells (Fig. 3B). Consistent with the lower intracellular levels of PMEAp and PMEApp detected in K562/PMEA-1 cells compared with K562/0 cells, proportionally fewer PMEA metabolites were found in the extracellular medium of the mutant cells. The ratio for extracellular PMEAp of wild-type over mutant cells varied from 2 to 15 over the 48-hr time period, which is in full agreement with the 2–7-fold lower intracellular PMEAp levels in K562/PMEA-1 cells compared with the wild-type. Likewise, the 9–30-fold lower extracellular amounts of PMEApp found over the 48-hr time period for K562/PMEA-1 compared with K562/0 cells closely reflect the 15–30-fold lower intracellular PMEApp levels in the mutant cell line. Other than radiolabeled PMEAp and PMEApp, no metabolites were detected in the culture medium.

Efflux of radiolabeled PMEA and PMEA metabolites from wild-type versus mutant K562 cells after loading with [2,8-^{3}H]PMEA for 24 hr. To compare initial efflux velocities of PMEA and its metabolites by K562/0 and K562/PMEA-1 cells, both cell types were loaded with radiolabeled PMEA over 24 hr. After careful wash-out of extracellular radioactivity at 4°, the cells were resuspended in warm, drug-free medium. Decrease in total intracellular radioactivity and increase in total extracellular radioactivity in the culture medium were recorded as a function of time. As shown in Fig. 4, no increased efflux could be observed for K562-PMEA-1 compared with wild-type cells.

Discussion

Previously, we reported the remarkable differentiation-inducing capacity of PMEA in three different tumor cell systems (i.e., the human leukemia HL-60 and K562 cell lines and the rat choriocarcinoma RCHO cell line) (10). However, the nature of the signal that triggers the cascade of events leading to maturation of the tumor cells exposed to PMEA has remained unclear. Therefore, it was decided to select a K562 cell line that shows resistance to the differentiation-inducing properties of PMEA. Comparison of the sensitivity of parental and PMEA-resistant tumor cells to various compounds interfering at diverse levels of the cellular biochemical pathways and investigation of the uptake and metabolism of PMEA in the PMEA-resistant K562 cells might reveal new information on the site of the differentiating action of PMEA. The K562 cell line that was selected in the presence of escalating concentrations of PMEA displayed unaltered biological characteristics, such as cell size, granularity, and generation time, but proved to be highly resistant to both the cytostatic and differentiation-inducing properties of PMEA. On the other hand, the PMEA-resistant K562 cell line showed cross-resistance to the differentiation-inducing, but not cytostatic, effects of other antimetabolites, thus providing evidence that the cytostatic and differentiation-inducing potentials of these compounds can be clearly dissected.

Resistance to the cytostatic action was highly specific for PMEA and structurally related nucleoside phosphonates such as PMEDAP, (*S*)-FPMPA, (*S*)-HPMPA, mono(POM)-PMEA, and bis(POM)PMEA. It was most pronounced for PMEA and its lipophilic prodrug bis(POM)PMEA, in which the negative charges of the phosphonyl moiety are masked by alkylation with two lipophilic pivaloyloxymethyl chains (Fig. 1) to improve cellular uptake and bioavailability (21). Enhanced cellular uptake of the bis(POM) derivative of PMEA, compared with free PMEA, most likely accounts for the 51-fold lower IC_{50} value of bis(POM)PMEA in K562/0 cells (0.53 μM compared with 27 μM for PMEA). Interestingly, cross-resistance for cytostatic activity did not apply to other (guanine and cytosine) nucleoside phosphonates and phosphonoformic acid, indicating a highly specific mechanism of decreased sensitivity to PMEA. Indeed, we found a 15-fold decrease in intracellular PMEA in K562/PMEA-1 cells compared with K562/0 cells after incubation with radiolabeled PMEA for 24 hr. The proportional 15-fold decrease of the PMEApp levels in the mutant cells compared with wild-type cells together with the fact that the proportion of phosphorylated PMEA metabolites formed from intracellular PMEA (10–20%) was not significantly different in the K562/0 and K562/PMEA-1 cell lines argue against an alteration at the level of PMEA phosphorylation. Furthermore, the lower amounts of PMEA metabolites appearing in the culture medium of the mutant compared with the wild-type cells were in proportion with the lower intracellular levels in the mutant cells. In agreement with these findings, the appearance of radioactivity in the culture medium after loading of the cell cultures with [2,8-^{3}H]PMEA and subsequent wash-out of extracellular radioactivity was proportionally similar for both wild-type and mutant K562 cells. Taken together, our results are strongly suggestive of a defect at the level of PMEA uptake rather than an altered intracellular drug metabolism or increased efflux as a possible mechanism of PMEA resistance in the mutant cells. In this respect, it is interesting to note that the corresponding guanine derivative PMEG did not show different cytostatic properties in the two cell lines, indicating another mechanism of uptake of the guanine derivatives versus adenine (and 2,6-diaminopurine) derivatives. Thus, the cross-resistance characteristics of the PMEA-resistant K562 cell line clearly differ from those observed in a PMEA-resistant variant of the human lymphoid CEM cell line, established by Robbins *et al.* (22), which was found to be cross-resistant to PMEG. An alteration in the cellular efflux of PMEA was suggested as the major basis for the PMEA-resistant phenotype of that cell line.

Except for tiazofurin and AICA-riboside, no marked cross-resistance in cytostatic activity was found for a series of antimetabolites that are targeted at a variety of enzymes in the purine and/or pyrimidine nucleotide pathways. However, evaluation of the differentiation-inducing activity of this wide variety of compounds in both wild-type and mutant K562 cells revealed a remarkable phenomenon: for every compound tested, a 5–25-fold shift in differentiation-inducing potential was noted in K562/PMEA-1 versus K562/0 cells regardless of the metabolic site of action of the compound. Indeed, diverse acyclic nucleoside phosphonates, several IMP dehydrogenase inhibitors, the PRPP synthetase inhibitor AICA-riboside, the DNA synthesis inhibitor Ara-C, the CTP synthetase inhibitor 3-deazauridine, the dihydrofolate reductase inhibitor MTX (which indirectly interferes with the for-

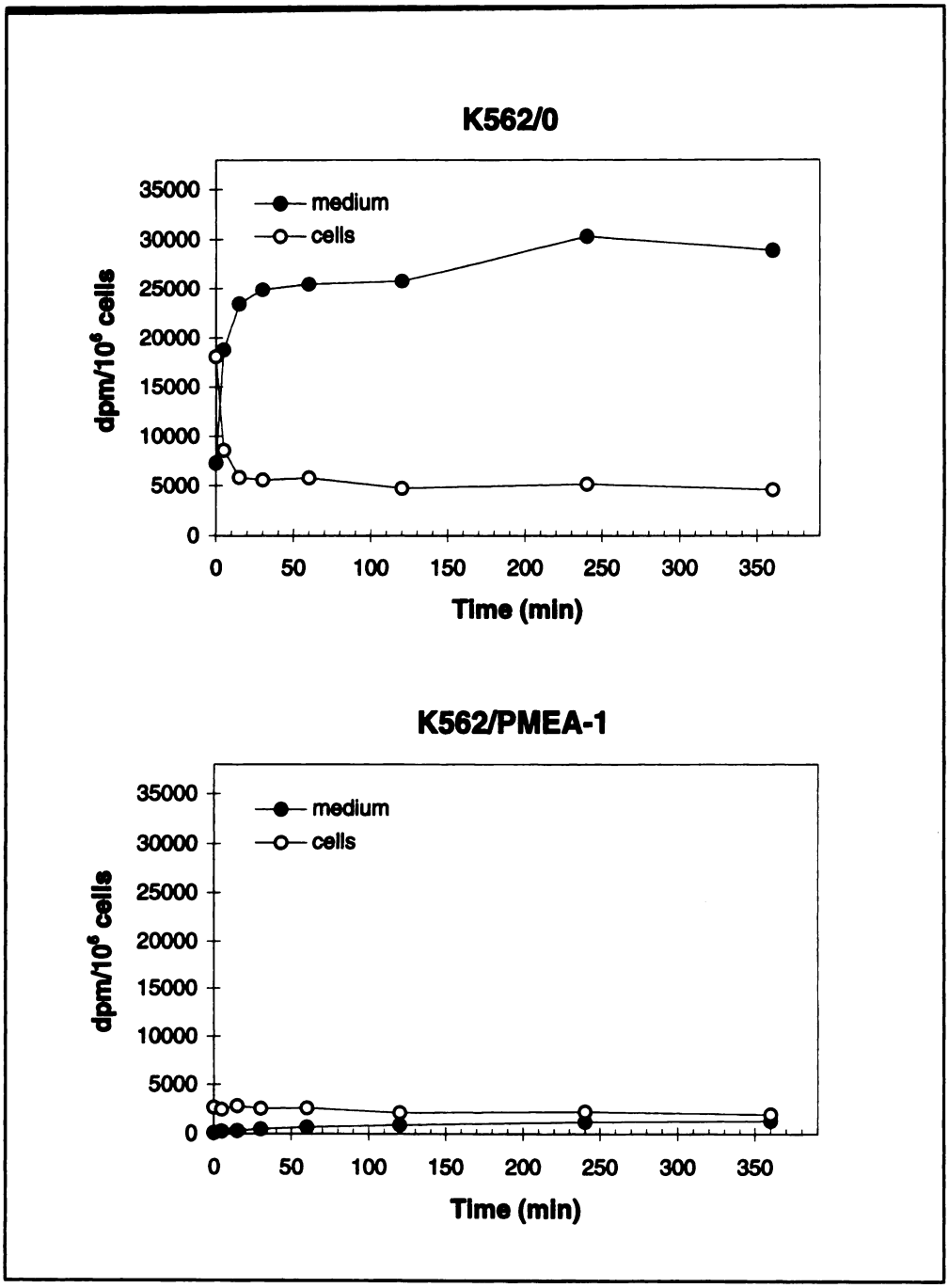


Fig. 4. Efflux of PMEA, PMEAp, and PMEApp from K562/0 and K562/PMEA-1 cells after loading with 25 μCi of [2,8-^{3}H]PMEA (0.57 μM) for 24 hr and subsequent careful wash-out of extracellular radioactivity. Values represent total radioactivity (i.e., PMEA, PMEAp, and PMEApp), expressed in dpm/10^6 cells. These data are from a typical experiment, which was repeated with similar results.

mation of dTMP from dUMP) (23), the ribonucleotide reductase inhibitor hydroxyurea, and even thymidine (which is thought to cause a dCTP deficiency through inhibition of the ribonucleotide reductase at the CDP/dCDP enzyme site) (23) were less-potent inducers of erythroid differentiation in the PMEA-resistant K562 cell line than in the wild-type cells.

Even at highly toxic concentrations, many of these compounds proved unable to afford a comparable degree of differentiation in the K562/PMEA-1 cells as detected for the K562/0 cells at subtoxic concentrations. These observations are surprising and clearly point to a distinction between the cytostatic (antimetabolic) and differentiation-inducing properties of these compounds. Indeed, initiation of cellular differentiation is generally associated with G1 arrest and withdrawal from the cell cycle (24). Because many differentiation inducers exert their differentiating effect at concentrations close to or exceeding their IC_{50} value, the contribution of cytotoxicity to the cell cycle arrest preceding the onset of differentiation is difficult to estimate, and cytotoxic and differentiating effects often seem to be closely related. In this regard, our observations that the pronounced loss of differentiating capacity in the mutant K562 cell line was also found for compounds with unaltered cytostatic activity in wild-type versus mutant K562 cells provide evidence for the existence of distinction between cytostatic activity and differentiation-inducing potential at the molecular level.

As mentioned, wild-type and PMEA-resistant cells proved to be equally sensitive to the cytotoxic effects of colchicine

and vinblastine (Table 1), two agents that generally lose their toxicity in cell lines exhibiting an MDR phenotype. Further evidence for the absence of MDR in the K562/PMEA-1 cell line was provided by experiments with MDR-reversing agents. The calcium channel blocker verapamil is known to be a down-regulator of *mdr-1* gene transcription and was previously found to cause a marked decrease in P-glycoprotein mRNA levels when administered at 15 μM to MDR K562 cells (25). Cyclosporin A also reverses P-glycoprotein-based MDR by inhibiting the pump function of P-glycoprotein. A second mechanism of MDR is based on increased expression of the transmembrane drug-efflux pump MRP (MDR-associated protein), which is inhibited by the isoflavinoid genistein (26). The inability of these agents to reverse the resistance of the K562/PMEA-1 cells to PMEA argues against the involvement of a classic MDR mechanism. Furthermore, in our experiments with radiolabeled PMEA, no proportional increase in PMEA-efflux could be detected in K562/PMEA-1 cells compared with K562/0 cells, as discussed above.

Thus, presumably, two phenomena play a role in the eventual decreased sensitivity of K562/PMEA-1 cells to the differentiation-inducing activity of PMEA: (i) markedly decreased uptake of the test compound in the K562/PMEA-1 cells and (ii) moderate resistance of K562/PMEA-1 cells to differentiate on exposure to differentiation-inducing agents with different sites of antimetabolic action. In this respect, it may be of interest to mention our observations in a low PMEA-resistant mutant K562 cell line obtained at an intermediate stage of the PMEA resistance selection process. This intermediate mutant K562 cell line was only 10-fold less sensitive to the cytostatic activity of PMEA, whereas the K562/PMEA-1 cell line is >100-fold resistant. On the other hand, the intermediate mutant cell line already showed a 5–10-fold decreased sensitivity to the differentiation-inducing properties of all of the compounds tested. These findings might indicate that the nonspecific resistance to differentiation induction appeared at an early stage during the selection process of the K562 cells for PMEA resistance. However, due to instability, we were not able to carry out extensive metabolism and differentiation studies on this low PMEA-resistant mutant cell line, and it was decided to focus on the K562/PMEA-1 cells. Apparently, further selection only improved the specific resistance to the cytostatic activity of PMEA, possibly by selecting for an additional defect at the level of PMEA uptake.

In conclusion, we have established a PMEA-resistant erythroleukemia K562 cell line that displays a decreased sensitivity to various differentiation-inducing agents and partial cross-resistance to the cytostatic effects of some but not all compounds. Unlike the resistance in cytostatic activity, which is specific for the adenine/2,6-diaminopurine nucleoside phosphonates, the resistance to differentiation induction is not confined to certain compounds. Although the molecular basis of the cross-resistance of the K562/PMEA-1 cell line to the differentiating effects of the antimetabolites remains to be resolved, it is clear that the defect in the K562/PMEA-1 cells must be located at a common site in the converging differentiation pathways initiated by modulators of diverse cellular processes. The biochemical/molecular mechanisms underlying the specific resistance to the cytostatic activity of PMEA (and some of its congeners) and the nonspecific resistance to the differentiation-inducing properties of acyclic nucleoside phosphonates and various structurally nonrelated antimetabolites are under investigation. Furthermore, it will be of great interest to address the question of whether interaction of PMEA with genes active in the early stages of cell maturation plays a role in PMEA-induced tumor cell differentiation.

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