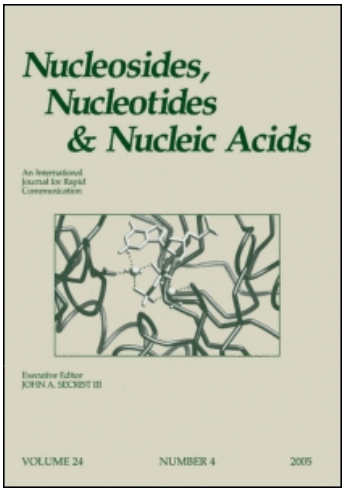

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INDUCTION OF ERYTHROID DIFFERENTIATION OF HUMAN LEUKEMIA K562 CELLS BY THE ACYCLIC NUCLEOSIDE PHOSPHONATE 9-(2-PHOSPHONYLMETHOXYETHYL)ADENINE (PMEA)

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ABSTRACT : 9-(2-Phosphonylmethoxyethyl)adenine (PMEA), the prototype compound of a well-defined structural class of potent and selective anti-retroviral agents, was found to be endowed with long-lasting and dose-dependent differentiation-inducing properties in human erythroleukemia K562 cell cultures.

INTRODUCTION

The majority of currently available anticancer drugs exert their action through cytotoxicity and/or inhibition of cell growth. However, the concept of selective tumor cell killing does not always seem to be a feasible goal for succesful cancer therapy. Therefore, there is an increasing need for new chemotherapeutic approaches. Since tumor cell proliferation is strongly limited or even completely arrested upon induction of cell differentiation, differentiation-induction therapy might provide an attractive additional approach to cancer treatment. Acyclic nucleoside phosphonates represent a unique class of antiviral agents (1,2). We recently found that the prototype compound of this class, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), is a potent inducer of differentiation of rat choriocarcinoma RCHO cells, human promyelocytic HL-60 cells and human erythroleukemia K562 cells (3). The K562 cell line (4,5) is a widely used system for erythroid differentiation and can be induced by various agents to produce hemoglobin (6,7). In this study, we focus on PMEA-induced differentiation of K562 cells.

MATERIALS AND METHODS

Compound - The synthesis (8) and antiretroviral activity (1,2) of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) have been previously described.

Cells and culture conditions - The human erythroleukemia K562 cell line was obtained from the American Tissue Culture Collection (ATCC) (Rockville, MD). The cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% foetal calf serum (GIBCO, Paisley, UK), 2 mM glutamine

(Flow Laboratories, Glasgow, Scotland) and 0.075% $NaHCO_3$ (Flow Laboratories). The cells were subcultured every 3-4 days.

Benzidine-staining - Erythroid differentiation was assessed by microscopical counting of hemoglobin-containing cells after staining with benzidine as described previously (3).

Drug treatment - In a first set of experiments, K562 cells were seeded at 2.5 x 10^5 cells per ml growth medium in 5 ml culture flasks. The next day, PMEA was added at a final concentration of 0, 50 or 75 μM. At days 1, 2, 3, 4 and 5 the cell cultures were assessed for differentiation by benzidine-staining. In a second set of experiments, PMEA was administered as described above, but the cells were allowed to grow in the presence of PMEA for different time periods (i.e. 1, 2, 3, 4 or 5 days). Then, drug-exposed cell cultures were thoroughly washed with culture medium and the cells were resuspended in drug-free medium and further incubated until day 5. At day 5, all K562 cell cultures were benzidine-stained. In a third set of experiments, K562 cells were exposed to PMEA at a final concentration of 0, 50, 100, 250 or 500 μM for 24 hours, whereafter the drug was removed by thorough washing. Then, the cells were resuspended in drug-free medium and the percentage of benzidine-positive cells was determined at days 3, 5, 7 and 10/11 after drug removal.

RESULTS AND DISCUSSION

Differentiation of K562 cells in function of time upon continuous exposure to PMEA - K562 cells were continuously exposed to PMEA at 50 or 75 μM. The number of benzidine-positive cells was determined daily. Untreated control cells were also stained to estimate the background of differentiation. At both PMEA concentrations, initiation of differentiation (~ 13-14%) became apparent at day 2 and further increased to ~ 30% at day 3 (Table I). Until day 3, the different PMEA concentrations (50 and 75 μM) afforded a similar degree of differentiation of the K562 cell cultures, but at days 4 and 5, the effect of PMEA was more pronounced at 75 μM than at 50 μM : 57% *versus* 45% at day 4, and 66% *versus* 50% at day 5, for 75 μM and 50 μM, respectively (Table I). Thus, PMEA induces a marked degree of differentiation after 4 days of continuous drug exposure. Longer exposure times cause a relatively minor further increase in the percentage of differentiated cells.

Effect of PMEA exposure time on the percentage of differentiated cells at day 5 of the experiment - K562 cells were exposed to PMEA at 50 and 75 μM during different time periods (i.e. 1, 2, 3, 4 or 5 days). At day 5, all the cell cultures were benzidine-stained. In the K562 cell cultures that had been incubated with PMEA for only 1 day, 12% and 24% of the cells showed differentiation at day 5, after they had been exposed to 50 and 75 μM PMEA, respectively (Table II). A 2-day drug exposure time resulted in differentiation percentages up to 39% and 46% for 50 and 75 μM PMEA, respectively (Table II). Longer exposure times only slightly raised the differentiation level. These results suggest that exposure of K562 cells to 50 μM PMEA during 2-3 days is sufficient to afford marked differentiation at day 5. However, the fact that PMEA needs to be present for at least 2 days to be fully active might point to the role of a PMEA metabolite in the onset of the differentiation process. Apparently, it takes several days for the cells to fully develop their differentiated phenotype (hemoglobin production) (Tables I and II). Indeed,

TABLE I : Differentiation of K562 cells upon continuous exposure to PMEA

	Differentiation measured at day				
	1	2	3	4	5
Control	3.9 ± 1.0	4.1 ± 0.2	3.3 ± 0.6	3.3 ± 0.9	4.2 ± 1.0
PMEA 50 μM	6.5 ± 1.0	13.6 ± 0.9	29.6 ± 8.4	45.4 ± 7.3	50.4 ± 3.1
PMEA 75 μM	7.9 ± 0.9	14.1 ± 5.5	32.1 ± 5.5	57.4 ± 6.2	66.2 ± 7.2

All data represent average values of 3 independent experiments ± standard deviation

TABLE II : Differentiation of K562 cells at day 5 upon limited exposure to PMEA

	PMEA exposed to the cells for ... days				
	1	2	3	4	5
PMEA 50 μM	12.4 ± 7.4	39.3 ± 11.7	51.3 ± 5.7	51.7 ± 10.6	45.1 ± 3.1
PMEA 75 μM	24.2 ± 13.5	46.3 ± 9.4	46.1 ± 5.9	54.9 ± 7.1	58.5 ± 13.5

All data represent average values of 3 independent experiments ± standard deviation

PMEA exposure during 1 day resulted in 6.5% and 7.9% benzidine-positive cells for 50 and 75 μM PMEA, respectively, when differentiation was measured at day 1 (Table I). In contrast, a 1-day drug exposure resulted in 12.4% and 24.2% differentiation for 50 and 75 μM PMEA, respectively, when differentiation was measured at day 5 (Table II). For a 2-day drug exposure period, the importance of further incubation after drug exposure was even more pronounced (Tables I and II). Thus, further incubation in drug-free medium of K562 cell cultures that had been exposed to PMEA for only 1 or 2 days significantly increased the percentage of benzidine-positive cells at day 5.

Dose-dependent differentiation after a short exposure of K562 cells to PMEA - K562 cells were treated with PMEA at various concentrations (i.e. 0, 50, 100, 250 and 500 μM) for 24 hours. Subsequently, the cells were shifted to drug-free growth medium and benzidine-positive cells were recorded at days 3, 5, 7 and 10/11 after drug removal. Fig. 1 clearly demonstrates the dose-dependent effect of PMEA on the differentiation of the K562 cells. Differentiation was approximately 3-fold more pronounced in K562 cell cultures exposed to 500 μM PMEA than in cultures exposed to 50 μM PMEA. This difference remained constant irrespective of the time at which differentiation was recorded (i.e. days 3, 5, 7 and 10/11 after drug removal) (Fig. 1). Apparently, intracellular PMEA accumulation is required to achieve optimal differentiation. Presumably, the higher the extracellular drug concentration, the more compound enters into the cells, resulting in higher intracellular levels of the active PMEA metabolite(s). This assumption is in agreement with earlier findings that the uptake and metabolism of PMEA by human lymphocytes is dose-dependent and not saturated at a concentration as high as 300 μM (9). Five days after drug removal, the differentiation reached its maximum level (Fig. 1), but even after 10/11 days of K562 cell incubation in drug-free medium, the effect of PMEA treatment was still pronounced (Fig. 1). From these

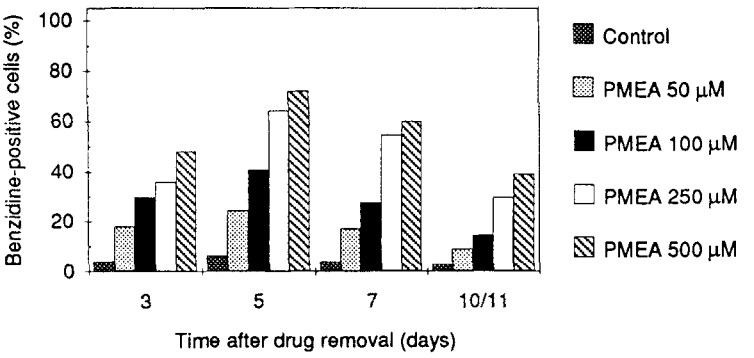

FIG. 1 : Differentiation of K562 cells after 24 hrs exposure to PMEA

observations we may conclude that PMEA is endowed with long-term differentiation-inducing properties. Such a long-lasting effect has also been observed for the anti-retroviral activity of PMEA *in vitro* and *in vivo* (10).

In conclusion, the acyclic nucleoside phosphonate PMEA, which has previously been shown to exert a potent and selective anti-retroviral action, also appears to be a strong inducer of erythroid differentiation of human erythroleukemic K562 cells in a dose- and time-dependent fashion. Whether the impact of PMEA on cell metabolism finally results in terminal, irreversible differentiation or whether it only causes a reversible change of the maturation state of the cells, remains to be determined. Also, the molecular mechanism of PMEA-induced differentiation needs to be further investigated, as well as the possible implementations of the present findings in terms of cancer treatment.

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