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Potent Differentiation-inducing Properties of the Antiretroviral Agent 9-(2-Phosphonylmethoxyethyl) Adenine (PMEA) in the Rat Choriocarcinoma (RCHO) Tumor Cell Model

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ABSTRACT. 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and its closely related structural analogue (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) are potent inhibitors of retroviruses and hepatitis B virus. In its oral prodrug form (adefovir dipivoxil), PMEA is currently the subject of advanced phase II/III clinical trials for the treatment of HIV infections. PMEA has also been shown to be a potent differentiation-inducing agent. In the present study, PMEA was found to have a strong differentiation-inducing effect on rat choriocarcinoma (RCHO) cells, comparable to that of methotrexate, which is the drug of choice for the chemotherapy of choriocarcinoma in humans. PMEA induced differentiation of choriocarcinoma trophoblast cells in a concentration-dependent manner within the 2- to 50-μM concentration range, as ascertained by giant cell formation, alkaline phosphatase induction, progesterone secretion, and the disappearance of a cytotrophoblast-specific surface antigen. PMEA had to be exposed to the rat choriocarcinoma cell cultures for at least 2–3 days to achieve optimal growth inhibition and differentiation of the tumor cells. Unlike PMEA, (*R*)-9-(2-phosphonylmethoxypropyl)adenine failed to induce differentiation of proliferating cytotrophoblasts into nonproliferating, hormonally active giant cells. This points to the specificity of PMEA as an inducer of choriocarcinoma cell differentiation. BIOCHEM PHARMACOL **56**;7:851–859, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. antiretrovirals; nucleotide analogs; choriocarcinoma; tumor cell differentiation; methotrexate

The acyclic nucleoside phosphonates represent a unique class of broad-spectrum antiviral agents with strong activity against herpesviruses, hepatitis B virus and retroviruses, including HIV [1, 3–6]. PMEA (Fig. 1) is the prototype congener of the acyclic nucleoside phosphonates, which, in its oral prodrug form bis(POM)-PMEA‡ (adefovir dipivoxil), is presently being evaluated in phase II/III clinical trials in HIV-infected individuals and in phase I/II studies in hepatitis B virus-infected patients [7]. In a previous paper, we reported the remarkable differentiation-inducing potential of PMEA in several tumor cell lines, including human erythroleukemia K562 and promyelocytic HL-60 cells and RCHO cells [2]. Given the high incidence of several types of cancer, including Kaposi's sarcoma, in patients with AIDS, the tumor cell differentiation-inducing properties of PMEA may be of particular interest for these patients. Here, we describe the different parameters of the differentiation-inducing effect of PMEA on RCHO cells.

Choriocarcinoma is a highly aggressive post-gestation tumor that may spontaneously arise in the uterus after malignant transformation of the trophoblast. This tumor is composed of proliferating trophoblast cells which may spontaneously differentiate into hormonally active giant cells. Teshima *et al.* artificially induced choriocarcinomas in rats (WKA/H strain) by fetectomy and displacement of the visceral yolk sac [8]. To investigate in detail the biologic characteristics of the choriocarcinoma cells and the factors that influence the growth and differentiation of the cytotrophoblasts, a continuous *in vitro* cell line of the rat choriocarcinoma, designated RCHO, was established by Verstuyf *et al.* [9]. This RCHO cell line displays the same morphological, biologic and immunologic properties as the choriocarcinoma from which it is derived and, therefore, provides a useful *in vitro* model to study the action of differentiation-inducing compounds.

We have now developed several appropriate differentiation markers to monitor cell differentiation in this *in vitro* RCHO model. The hormonal activity of the differentiated giant cells, due to induction of Δ^5-3β-hydroxysteroid dehydrogenase activity, is evidenced by the production and secretion of progesterone into the culture supernatant [10]. Furthermore, alkaline phosphatase activity increases when

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‡ *Abbreviations:* bis(POM)-PMEA, bis(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX, methotrexate; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; and PMPA, (*R*)-9-(2-phosphonylmethoxypropyl)adenine.

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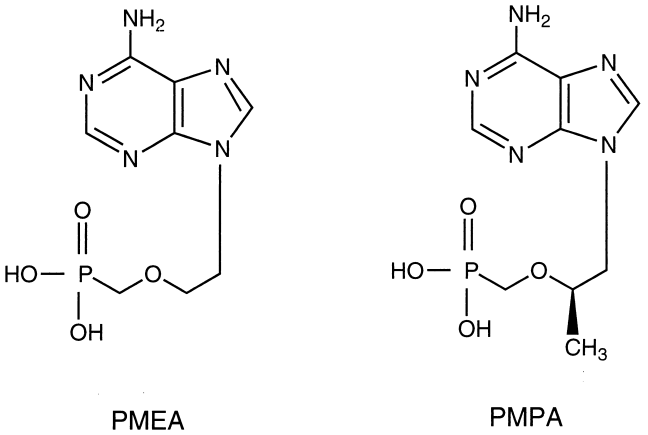

FIG. 1. Structural formulae of the acyclic nucleoside phosphonates PMEA and PMPA.

cytotrophoblast cells proceed to more differentiated stages [11, 12]. Moreover, a monoclonal antibody, 22H6, that reacts specifically with an antigen present on cytotrophoblasts but not giant cells has been developed [13]. Another monoclonal antibody, 28G4, reacts with both cell types. Hence, the ratio of 22H6 to 28G4 binding reflects the differentiation stage of the RCHO cells in the culture [13].

In the present study, we evaluated the effect of PMEA on the different differentiation parameters of the *in vitro* choriocarcinoma RCHO model. In addition, we compared the differentiation-inducing effect of PMEA in RCHO cell cultures with that of methotrexate and PMPA, a closely related structural analogue of PMEA (Fig. 1) which was found to be able to prevent simian immunodeficiency virus infection in macaques [14] and which is, like PMEA, under clinical development for the treatment of HIV infections.

MATERIALS AND METHODS

Compounds

The synthesis and antiretroviral activity of the acyclic nucleoside phosphonates have been described previously [3, 6, 15]. PMEA and PMPA were obtained from Dr. N. Bischofberger (Gilead Sciences). MTX was obtained from Lederle.

Cell Culture

RCHO were routinely cultured in tissue culture flasks coated with 0.1% gelatin (Sigma) and containing RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS) (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 2 mM L-glutamine (Life Technologies), 2 ng/mL of epidermal growth factor (Life Technologies) [16], and 5×10^{-5} M β-mercaptoethanol (UCB). Cultures were maintained at 37° in a humidified, CO_2-controlled atmosphere. *In vitro* passages were performed every 2–3 days by digestion with dispase grade II (Boehringer Mannheim).

Treatment of RCHO Cells with Test Compounds

RCHO cells were seeded (2.5×10^3 cells/well) into non-coated 96-well microtiter plates (Falcon, Becton Dickinson) in RPMI-1640-based growth medium.

Exposure of RCHO Cells to PMEA for Different Time Periods

To each microplate well, PMEA was added to a final concentration of 20 or 50 μM. Then, the cells were incubated at 37° according to the exposure time schedules indicated in Table 1. At each time point, total protein concentrations of PMEA-treated and untreated RCHO cell cultures were determined as described below. In addition, the PMEA-containing culture medium was aspirated from identically treated RCHO cell cultures and replaced by drug-free growth medium. These cell cultures were then further incubated and examined for cell growth (total protein concentration) at different time points (see Table 1).

Comparative Study of Induction of RCHO Cell Differentiation by PMEA, PMPA and MTX

Test compounds were added to the microplate wells at the appropriate concentrations and the cells were incubated at 37° for 96 hr. After exposure, the assays described below were performed on the microplates.

Combined Administration of PMEA and MTX to RCHO Cells

PMEA and MTX were simultaneously added at final concentrations of 2.5, 5 or 10 μM for PMEA and 0.001, 0.002, 0.004 or 0.008 μM for MTX. The cells were incubated at 37° for 96 hr. After this incubation period, cell differentiation was examined microscopically. Also, alkaline phosphatase activity and total protein concentration were determined as described below.

Assays Performed on Drug-Treated RCHO Cell Cultures

After exposure of the RCHO cell cultures to the test compounds, the culture medium was aspirated. To avoid selective loss of the less adhesive, nondifferentiated cytotrophoblasts, the cultures were not washed prior to the assays.

Cytology

The RCHO cell cultures were fixed with 10% formaldehyde, stained with erythrosin-hematoxylin, and examined by light microscopy as described [13].

TABLE 1. Growth inhibition of rat choriocarcinoma RCHO cells upon incubation with PMEA at 20 or 50 μM during different exposure times

PMEA exposure time (hours)	PMEA conc. (μM)	Time point at which protein concentration was determined (hrs)*				
		6	24	48	72	96
6	0	100	100	100	100	100
	20	123 ± 11	109 ± 12	87 ± 17	63 ± 4	92 ± 6
	50	112 ± 22	106 ± 1	95 ± 0	57 ± 4	82 ± 6
24	20		120 ± 7	71 ± 6	45 ± 1	74 ± 1
	50		109 ± 13	75 ± 3	37 ± 4	47 ± 2
48	20			99 ± 8	49 ± 4	71 ± 8
	50			89 ± 11	37 ± 5	34 ± 13
72	20				57 ± 6	63 ± 5
	50				42 ± 4	31 ± 8
96	20					59 ± 9
	50					37 ± 11

* The results are expressed as percentages of untreated control cell cultures, measured at the same time point. Protein concentrations in untreated control cell cultures were 8.6, 9.8, 24, 46 and 51 μg/microplate well at 6, 24, 48, 72 and 96 hours, respectively. Extensive morphological changes, resulting from PMEA-induced differentiation of RCHO cells, did not allow accurate comparison of cell numbers of untreated (undifferentiated) RCHO cell cultures versus PMEA-treated RCHO cell cultures, showing varying degrees of giant cell formation depending on PMEA concentration and exposure time. Therefore, the total amount of cell material (total protein concentration) of the cell cultures was used as a parameter to estimate the growth inhibitory effect of PMEA on RCHO cells. The data represent the means ± SD of two independent experiments. The data of both experiments were calculated as the average values obtained from 5 identical microplate wells.

Cell Viability Assay

Viability of the drug-treated RCHO cell cultures was assessed by the trypan blue dye exclusion method. To each well, 100 μL of a 0.1% trypan blue solution in PBS was added and the microplates were incubated at 37° for 10 min. Thereafter, the stained cell cultures were examined microscopically.

Total Protein Concentration

Because extensive morphological and metabolic changes occur when cytotrophoblasts differentiate into giant cells, standard methods such as automated cell counting and MTT staining were not suitable to determine and compare cell numbers in untreated control cell cultures and in drug-treated, differentiated RCHO cell cultures. Moreover, selective loss of the less adhesive, proliferating cytotrophoblasts may occur during processing of the drug-treated RCHO cell cultures in standard cell proliferation assays. Attempts to assess DNA replication in drug-exposed RCHO cells by propidium iodide staining and flow cytometry were unsuccessful, since the highly differentiated giant cells are disrupted by passage through the nozzle. Thymidine incorporation, another standard procedure to monitor cell proliferation, is not convenient for studies on PMEA, which has been found to stimulate incorporation of radiolabeled thymidine into cellular TMP, TDP and TTP pools by 10- to 100-fold (unpublished results). Therefore, the protein concentration in drug-treated RCHO cell cultures (as a measure of the total amount of cell material) was used as a parameter to estimate the growth inhibitory effect of PMEA on RCHO cells. However, it should be kept in mind that the anti-proliferative activity of differentiation-inducing agents against RCHO cells may be underestimated by protein determination, because high protein synthesis may continue to occur in nonproliferating, differentiated giant cells. Therefore, several additional parameters were developed to better estimate and quantify the differentiation-inducing potential of PMEA (see below).

To determine the protein content of the drug-exposed RCHO cell cultures, 100 μL of water was added to each microplate well and the cells were lysed by repeated freezing (−70°) and thawing (37°). The cell lysates of identical wells were mixed together. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories) and expressed as percentages of untreated control cultures. These data were used for normalization of the results of the differentiation assays to equal amounts of cell material.

Alkaline Phosphatase Assay

The cells in the microplate wells were lysed by repeated freezing and thawing. Then, to each well was added 100 μL of substrate [2 mg/mL of disodium *p*-nitrophenyl phosphate (PNPP) (Sigma) in 50 mM Tris-HCl buffer, pH 9.5, containing 0.1% Tween-20]. After incubation at 37° for 90 min, the optical density was measured at 405 nm and at a reference wavelength of 620 nm. The data of each independent experiment represent the average values of six identical wells.

Detection of Progesterone in the Culture Supernatant

Unlike proliferating cytotrophoblasts, differentiated giant cells are able to synthesize progesterone, which is secreted into the extracellular medium. Progesterone levels in culture supernatants were determined by a radioimmunoassay using a monoclonal antibody (Laboratory for Experimental Medicine and Endocrinology, Leuven, Belgium).

Binding of the Monoclonal Antibodies 22H6 and 28G4 to RCHO Cells

The antibodies were prepared and purified in the Laboratory of Immunopathology, Rega Institute, Leuven, Belgium [13]. The mAb 22H6 reacts specifically with cytotrophoblasts but not with differentiated giant cells. In contrast, the 28G4 mAb reacts with both cell types. Therefore, the ratio of bound mAb 22H6 to bound mAb 28G4 decreases when cytotrophoblasts differentiate into giant cells. Unless specified, all the incubation steps were performed with 100 μL/well. After each step, the cell cultures were washed 3 to 5 times with PBS. Drug-treated and untreated control RCHO cell cultures were fixed with 10% formaldehyde in PBS for 10 min at room temperature. Aspecific protein binding sites were blocked with 10% normal goat serum (Sigma) in PBS at 37° for 30 min. Then, the cell cultures were incubated for 2 hr at 37° with the primary antibody (22H6 or 28G4), diluted to its optimal concentration in PBS containing 5% normal goat serum. Endogenous peroxidase activity was blocked by H_2O_2 (1.7 mL of H_2O_2 30% in 100 mL of methanol) for 30 min at 37°. Thereafter, the cells were incubated for 1 hr at room temperature with a 1/1000 dilution of biotinylated goat anti-mouse immunoglobulins (Mouse ExtrAvidin® Peroxidase Staining Kit, Sigma) in PBS containing 5% normal goat serum. After subsequent incubation for 30 min at room temperature with ExtrAvidin-Peroxidase (Sigma), diluted 1/500 in PBS containing 0.05% Tween-20, 200 μL of substrate was added (SIGMA FAST™ OPD Peroxidase Substrate Tablet Set, Sigma). The staining reaction was allowed to proceed for 30 min at room temperature in the dark. Then, the microplates were read at 450 nm on a microtiter plate reader (Multiskan MCC/340) and the average OD_{450} value of four identical wells was calculated. Background staining due to nonspecific binding of secondary antibody and ExtrAvidin-Peroxidase was corrected by subtraction of the OD_{450} value obtained when the procedure was performed in the absence of the primary antibody.

RESULTS

Effect of PMEA Exposure Time on Inhibition of RCHO Cell Proliferation (Table 1)

RCHO cells were exposed to PMEA at 20 or 50 μM for different time periods (i.e. 6, 24, 48, 72 or 96 hr). At each time point, the culture medium of multiplicate PMEA-treated RCHO cell cultures was aspirated. A PMEA-treated RCHO cell culture was used for immediate determination of cell growth and comparison with an untreated RCHO cell culture at the same time point. Identical PMEA-treated RCHO cell cultures were further incubated in drug-free culture medium and examined for cell growth at different subsequent time points (Table 1). The pronounced morphological and metabolic changes that occurred during PMEA-induced differentiation of cytotrophoblasts into giant cells, and the different adhesive properties of proliferating cytotrophoblasts and differentiated giant cells did not allow accurate comparison of cell numbers in nondifferentiated versus strongly differentiated RCHO cell cultures by standard procedures such as automated cell counting, MTT staining, thymidine incorporation or flow cytometry after propidium iodide staining. Therefore, protein measurement was found to be the only suitable method to estimate the inhibitory effect of the test compounds on cell growth (Table 1).

When examined immediately after a continuous 72-hr or 96-hr exposure to PMEA at 20 or 50 μM, the drug-treated RCHO cell cultures showed markedly pronounced inhibition of cell proliferation (Table 1). In contrast, no growth inhibition was observed immediately after exposure of RCHO cells to PMEA for 6, 24 or 48 hr (Table 1). However, when the latter PMEA-treated cells were further incubated in drug-free medium, a delayed inhibitory effect of PMEA on RCHO cell proliferation became apparent. At 72 hr, total protein concentrations of RCHO cell cultures that had been exposed to 20 μM PMEA for 6, 24, and 48 hr had declined to 63%, 45% and 49%, respectively, as compared to untreated control cell cultures at 72 hr (Table 1). Likewise, protein concentrations of 57%, 37% and 37% of control were noted at 72 hr for RCHO cell cultures exposed to 50 μM PMEA for 6, 24 and 48 hr, respectively (Table 1).

When further incubated, after removal of PMEA, in drug-free medium until 96 hr, the RCHO cell cultures treated with PMEA for 6, 24 and 48 hr resumed cell growth, except for the RCHO cells treated for 48 hr with 50 μM PMEA (Table 1). These data are consistent with the degrees of giant cell formation (as a result of cytotrophoblast cell differentiation) observed in the RCHO cell cultures at the time of PMEA removal (data not shown). PMEA-treated RCHO cell cultures consisting predominantly of differentiated, nonproliferating giant cells after PMEA exposure were not able to resume growth during subsequent incubation in drug-free medium. On the other hand, cell proliferation was restarted in those RCHO cell cultures that showed a low degree of differentiation when PMEA was removed from the cells. However, we have found that the irreversible differentiation afforded by a 48-hr exposure of RCHO cells to 50 μM PMEA can also be achieved by lower PMEA concentrations when treatment is prolonged [2].

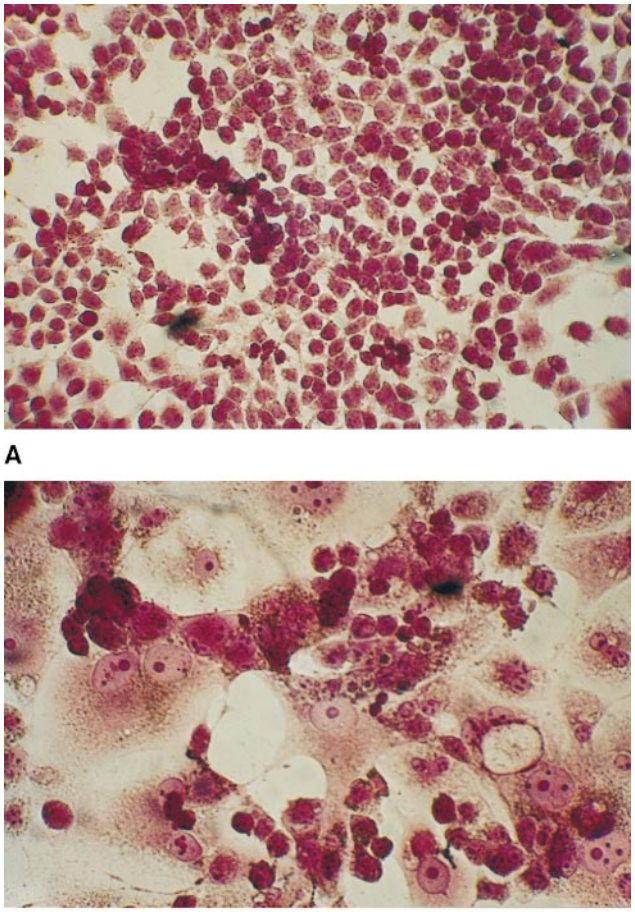

FIG. 2. Morphological changes accompanying differentiation of rat choriocarcinoma cytotrophoblast cells into giant cells. Panel A: untreated RCHO cell culture; panel B: RCHO cells after exposure to 20 μM PMEA for 96 hr.

Effects of PMEA, Methotrexate and PMPA on Viability, Proliferation and Differentiation-related Morphological Changes of RCHO Cells

RCHO cell cultures were exposed to PMEA at 2, 5, 10, 20 and 50 μM, to MTX at 0.004 and 0.02 μM, and to PMPA at 50 and 250 μM. After incubation for 96 hr, the viability of the drug-treated and untreated RCHO cell cultures was estimated by trypan blue dye staining and giant cell formation was examined microscopically. RCHO cell cultures that had been exposed to the highest concentrations of PMEA (20 and 50 μM) and MTX (0.02 μM) predominantly (>95%) consisted of fully differentiated giant cells (Fig. 2), the majority of which were blue-coloured and thus nonviable (data not shown). This is in agreement with the fact that giant cells no longer multiply but degenerate and die soon after they have been formed. The percentages of giant cells present in RCHO cell cultures exposed to the lower concentrations of PMEA (i.e. 2, 5 and 10 μM) and MTX (i.e. 0.004 μM), or to PMPA at 50 and 250 μM, were much lower (<40%) than observed at the highest drug concentrations (data not shown). These giant cells were blue-coloured as well, whereas the vast majority of nondifferentiated cytotrophoblasts in these cell cultures remained transparent (viable).

The giant cells resulting from spontaneous differentiation of cytotrophoblasts in untreated control RCHO cell cultures were also blue-stained, and generally represented less than 5% of the total cell population. The small numbers of undifferentiated, blue-coloured (nonviable) cytotrophoblasts (<5%) that were observed in RCHO cultures treated with the lower concentrations of PMEA and MTX, and also with PMPA, were most likely due to nutrient starvation after 4 days of incubation, because a comparable percentage of nonviable (blue-stained) trophoblast cells was also found in the untreated RCHO cell cultures.

In an independent set of experiments, the differentiation-related inhibition of cell proliferation by PMEA was compared with that of MTX and PMPA. As shown in Fig. 3A, the total amount of cell material was significantly ($P < 0.0002$) decreased in RCHO cell cultures treated with the highest concentrations of PMEA (i.e. 50 μM) and MTX (i.e. 0.02 μM) for 96 hr. Total protein concentrations in RCHO cell cultures exposed to PMEA at 20 and 50 μM were reduced to 84% and 60% of control, respectively, compared to 73% and 43% for MTX at 0.004 and 0.02 μM, respectively. In contrast, exposure of RCHO cells to PMPA at concentrations as high as 250 μM did not result in a significant decrease in the amount of cell material (Fig. 3A). The protein concentrations shown in Fig. 3A are consistently higher than those reported for the 96-hr time point in Table 1. This is obviously due to external factors such as different batches of fetal bovine serum used in the cell culture medium and different coating conditions of the microplates.

Effects of PMEA, Methotrexate and PMPA on Alkaline Phosphatase Activity Induction in RCHO Cells

The increase in alkaline phosphatase activity, calculated for an equal amount of cell (protein) material as found in the untreated control cultures, was used as a biochemical parameter to evaluate the differentiation stage of drug-treated RCHO cell cultures. As shown in Fig. 3B, alkaline phosphatase activity gradually increased from 100% in untreated control cells to 131%, 182%, 269%, 360% and 491% in RCHO cells exposed to increasing PMEA concentrations. Interestingly, the elevation of alkaline phosphatase activity was less pronounced for 0.02 μM MTX (271%) than for 50 μM PMEA (491%), although at these concentrations the drugs gave a comparable inhibition of cell proliferation (Fig. 3A). At a concentration as high as 250 μM, PMPA did not afford a marked increase in alkaline phosphatase activity in RCHO cells (Fig. 3B).

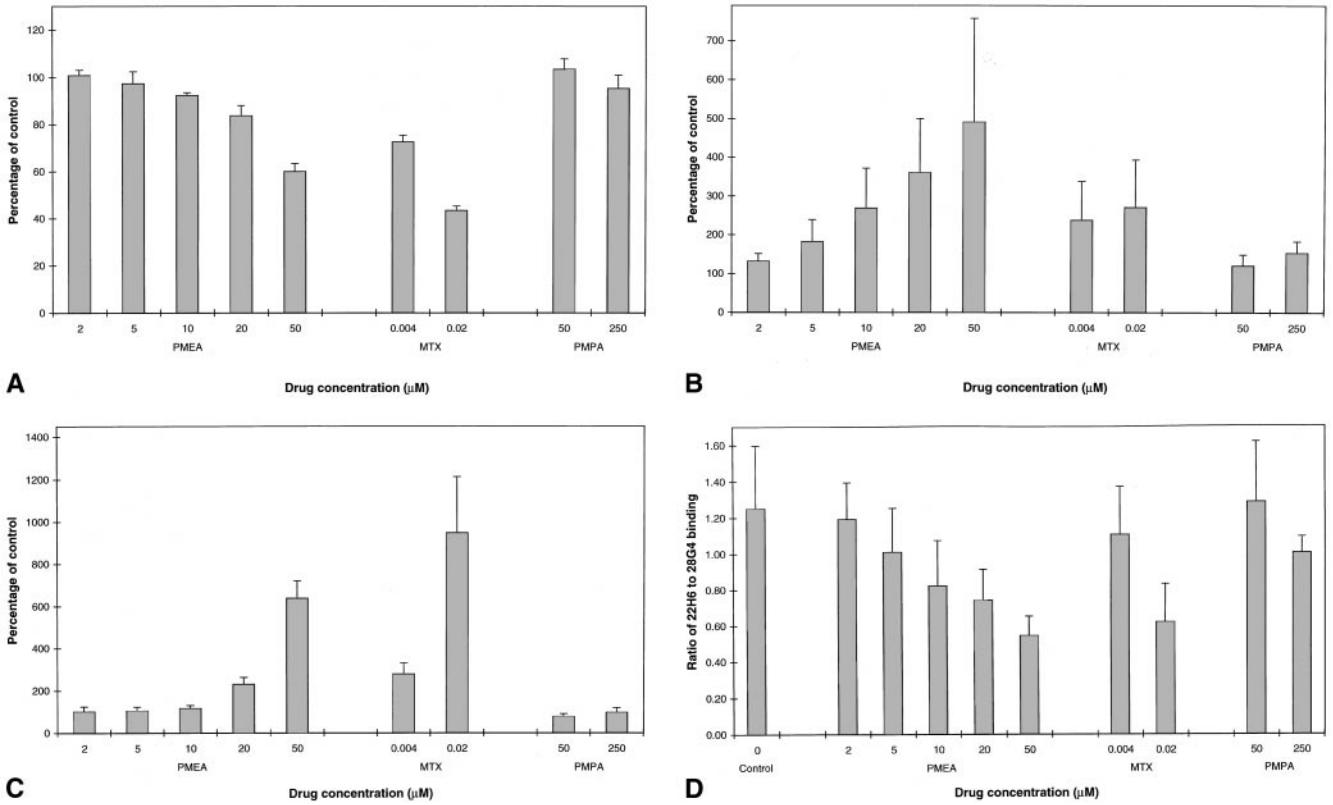

FIG. 3. (A) Total protein concentration of drug-treated RCHO cell cultures relative to untreated control cells. The total amount of cellular material, as measured by the BioRad Protein Assay, was used as a parameter to estimate inhibition of RCHO cell proliferation upon exposure to differentiation-inducing agents for 96 hr. Total protein amounts of drug-treated cell cultures are expressed as percentages of the value measured for untreated control cell cultures at 96 hr (i.e. 58 μg of protein per microplate well). The data represent the means ± SD of three independent experiments. Values for PMEA at 2 and 50 μM are significantly different ($P < 0.0002$). (B) Alkaline phosphatase activity as a marker of RCHO cell differentiation. Increase in alkaline phosphatase activity of drug-treated RCHO cells, compared to untreated control cells, is expressed as percent increase of the optical density at 405 nm after incubation of the cell cultures in the presence of a substrate of alkaline phosphatase. For each independent experiment, the results were calculated as the average values of six identical samples. The data were normalized to equal amounts of cell material using the results of the protein measurements. The data represent the means ± SD of three independent experiments. The values obtained for the different independent experiments varied considerably. However, the data within each experiment clearly displayed a similar concentration-response relationship. (C) Progesterone secretion by drug-treated RCHO cells relative to untreated control cells. Extracellular progesterone concentrations for drug-treated RCHO cell cultures are expressed as percentage of the background (untreated controls). The data were normalized to equal amounts of cell material using the results of the protein measurements. The data represent the means ± SD of three independent experiments. (D) Binding of specific monoclonal antibodies as a parameter to monitor the differentiation of cytotrophoblasts into giant cells. Unlike the cytotrophoblast-specific antibody 22H6, the mAb 28G4 recognizes both trophoblast cells and differentiated giant cells. Thus, the ratio of binding of both antibodies reflects the altered proportions of both cell types in drug-treated cell cultures, as compared to untreated control cell cultures. The data represent the means ± SD of three independent experiments and represent the ratio of the optical densities at 450 nm after an indirect enzyme immunoassay using 22H6 or 28G4 as primary antibody. For each independent experiment, the results were calculated as the average value of four identical samples and were normalized to equal amounts of cell material using the results of the protein measurements.

Effects of PMEA, Methotrexate and PMPA on the Production and Secretion of Progesterone by RCHO Cells

In addition to alkaline phosphatase activity, secretion of progesterone (solely synthesized by highly differentiated giant cells) into the extracellular culture medium was used as a second biochemical parameter to monitor RCHO cell differentiation. The results are displayed in Fig. 3C. PMEA and MTX markedly stimulated progesterone production in RCHO cells. For RCHO cell cultures incubated in the presence of 10 μM PMEA, the extracellular progesterone concentration (0.5 μg/100 mL of culture medium) was not significantly higher than the background level of untreated control cell cultures (0.4 μg/100 mL of culture medium). However, at 20 μM, PMEA clearly stimulated the production and secretion of progesterone (0.9 μg/100 mL) and at 50 μM, a 6.5-fold increase in the extracellular concentration of progesterone (2.6 μg/100 mL), compared to the untreated control, was found (Fig. 3C). For RCHO cells exposed to 0.02 μM MTX, a 9.5-fold increase in extracellular progesterone concentration was found, as compared to the untreated control. Again, PMPA did not stimulate progesterone production at 50 or 250 μM (Fig. 3C).

Effects of PMEA, Methotrexate and PMPA on the Expression of a Cytotrophoblast-specific Surface Antigen on RCHO Cells

Differentiation of RCHO cell cultures implies a shift in the composition of the cell population from predominantly trophoblast cells toward mainly giant cells. This change in the relative proportions of both cell types is reflected by the altered binding of specific monoclonal antibodies. Both giant cells and cytotrophoblasts give a positive reaction with the monoclonal antibody 28G4, whereas the mAb 22H6 exclusively recognizes a cytotrophoblast-specific surface antigen. Therefore, the ratio of bound 22H6 to bound 28G4 reflects the proportion of nondifferentiated cytotrophoblasts in the RCHO cell line. After exposure of the cells to the test compounds, an antibody binding assay was performed with both 22H6 and 28G4 as primary antibody. The resulting OD_{450} values were corrected to reflect equal amounts of cell (protein) material, and the ratio of the OD_{450} values obtained for 22H6 binding versus 28G4 binding was calculated. Figure 3D shows the gradual decrease of this ratio with increasing PMEA concentrations. For untreated RCHO cells, a ratio of 1.25 was found. When the cells were exposed to increasing PMEA concentrations (2, 5, 10, 20 and 50 μM), the ratios were 1.19, 1.01, 0.82, 0.74 and 0.54, respectively. After treatment with MTX, the RCHO cells showed antibody binding ratios of 1.11 and 0.62 for 0.004 and 0.02 μM MTX, respectively. PMPA had virtually no effect on the 22H6/28G4 mAb binding ratio (Fig. 3D).

Effect of the Combined Administration of PMEA and MTX on RCHO Cell Differentiation

RCHO cells were simultaneously exposed to PMEA and MTX for 96 hr. The compounds were combined at concentrations affording minor degrees of RCHO cell differentiation when administered separately to the cells (i.e. 2.5, 5 or 10 μM for PMEA and 0.001, 0.002, 0.004 or 0.008 μM for MTX). Based on giant cell formation and alkaline phosphatase activity, the combination experiment did not reveal any marked synergistic action of PMEA and MTX on differentiation induction (data not shown).

Inhibitory Effect of PMEA on Choriocarcinoma Tumor Growth in an In Vivo *Rat Model*

Syngeneic WKA/H rats were inoculated under the kidney capsule with 10^4 RCHO cells. The RCHO-inoculated animals were treated by daily i.p. injections of PMEA at doses of 0, 25, 50, 100 or 250 mg/kg/day for 10 consecutive days, starting on the day before tumor cell grafting. Immediately after the end of the treatment, the rats were sacrificed and autopsied. We found a clear dose-response relationship between the administered PMEA dose and the antitumor effect of the compound. At the highest (subtoxic) dose (i.e. 250 mg/kg/day), PMEA completely suppressed the development of choriocarcinoma tumors in the RCHO-inoculated kidney. The antitumor effect of PMEA gradually decreased with lower drug doses, but tumor growth still proceeded markedly slower in rats treated with PMEA at 25 mg/kg/day (data not shown). Unlike PMEA, PMPA at a dose as high as 200 mg/kg/day completely failed to inhibit choriocarcinoma tumor outgrowth in the RCHO-inoculated kidneys (data not shown).

DISCUSSION

PMEA is the prototype of a new class of compounds (the acyclic nucleoside phosphonate derivatives) and is endowed with a potent antiviral activity against retroviruses, herpesviruses and hepatitis B virus in cell culture, animal models and HIV- and hepatitis B virus-infected individuals [1, 3–7]. Previously, we have reported that PMEA induces tumor cell differentiation *in vitro* in several cell lines, including human erythroleukemia K562 and promyelocytic HL-60 cells [2]. This paper describes the remarkable differentiation-inducing potential of PMEA in the *in vitro* RCHO model.

The marked concentration-dependent induction of RCHO cell differentiation by PMEA could be demonstrated by morphological changes in the cell cultures, induction of alkaline phosphatase activity and production and secretion of progesterone by the differentiated giant cells, and decreased binding of a cytotrophoblast-specific surface antigen antibody. The differentiation-inducing potential of PMEA *in vitro* was compared to that of methotrexate, an analogue of folic acid [17] with strong antimitotic activity, which is widely used as a chemotherapeutic agent for the treatment of post-gestational choriocarcinoma in humans [18, 19]. Due to differentiation-related morphological changes, it is difficult to determine exact IC_{50} values (50% inhibitory concentrations for cell proliferation) for PMEA and MTX in the RCHO cell line. However, the IC_{50} values of PMEA and MTX are 27 μM and 0.011 μM, respectively, in the differentiation-susceptible human erythroleukemia K562 cell line. Based on these data, our current results on the effects of PMEA and MTX on total protein concentration and expression of differentiation markers in drug-treated RCHO cell cultures clearly indicate that PMEA and MTX afford a comparable degree of RCHO cell differentiation at concentrations showing a similar growth inhibitory effect. It should also be noted that PMEA and MTX are structurally unrelated molecules with an entirely different metabolism and different antimetabolic effects. In this respect, it cannot be excluded that choriocarcinoma cells that have become refractory to the inhibitory effects of MTX (i.e. by mutations at the level of uptake and/or metabolism of the drug and/or by altered expression of target enzymes) remain fully sensitive to PMEA and vice versa. Furthermore, the side-effects that may arise during long-term treatment with MTX will probably be strikingly different from those associated with

PMEA treatment. However, combined administration of PMEA and MTX to RCHO cells did not result in a synergistic differentiation-inducing effect. This may suggest that the different anti-metabolic effects afforded by PMEA and MTX finally trigger a common cascade leading to the differentiated phenotype. Indeed, both compounds eventually lead to an S-phase block, which may possibly be the primary event that activates a programme of cell differentiation. In the case of PMEA, S-phase progression is inhibited as a result of inhibition of DNA polymerase α by PMEApp (Hatse S and Balzarini J, unpublished results) MTX inhibits purine *de novo* synthesis and thymidylate formation, resulting in an imbalance of the 2′deoxyribonucleotide pools and, consequently, inhibition of S-phase progression [17].

It has been excluded by the trypan blue cell viability assays that the pronounced growth inhibitory effect of PMEA in RCHO cell cultures simply results from selective elimination of the proliferating cytotrophoblast cells by a nonspecific cytotoxic effect of PMEA. In agreement with this are our recent findings with a PMEA-resistant variant of the human erythroleukemia K562 cell line, suggesting that the differentiation-inducing effects of PMEA and other differentiation-inducing agents can be clearly distinguished from their cytostatic effects [20]. Together with the observation that PMPA, a structurally closely related analogue of PMEA, proved unable to induce differentiation of RCHO cells (and to afford an antitumor effect in choriocarcinoma-bearing rats), the results from the viability assays point to the high specificity of the differentiation-inducing capability of PMEA. Indeed, structurally unrelated differentiation-inducing agents such as resveratrol [21], thymidine and ara-C proved inhibitory to RCHO cell proliferation at concentrations that did not significantly induce RCHO cell differentiation. In addition, the differentiation-inducing properties of PMEA seem to be cell type-specific, as illustrated by the inability of the compound to induce tumor cell differentiation in rat pheochromocytoma PC12 cells, rat glioma C6 cells (Anciaux K, unpublished results) and human alveolar lung carcinoma A549 cells (Hozumi M, unpublished results). The signal that triggers choriocarcinoma cytotrophoblast cells to initiate a programme of differentiation toward nonproliferating, hormonally active giant cells upon exposure to PMEA is currently under investigation.

From the wash-out experiments (Table 1), we learned that a 48-hr exposure of RCHO cells to 50 μM PMEA results in an irreversible growth-inhibitory and differentiation-inducing effect. Interestingly, we could also demonstrate in our *in vivo* rat model that choriocarcinoma tumor growth is irreversibly suppressed by a 10-day treatment with PMEA at subtoxic doses [22]. The wash-out experiments also suggest that continuous exposure of the RCHO cells to PMEA for 2–3 days is required to achieve marked growth-inhibitory (and differentiation-inducing) activity. Apparently, intracellular accumulation of PMEA to a certain extent is essential for optimal activity of the compound. These data are in accordance with our earlier findings on PMEA-induced differentiation in the human erythroleukemia K562 cell line [23].

PMEA is phosphorylated intracellularly to its mono- and diphosphate forms, designated PMEAp and PMEApp, respectively [24]. PMEApp is responsible for the antiviral activity of the compound through inhibition of viral DNA polymerases and reverse transcriptase. Whether tumor cell differentiation is caused by PMEA itself, or by one of its metabolites, is still unclear. However, the delayed, long-term inhibitory effect of PMEA that we observed after a short exposure of RCHO cells to PMEA points to PMEApp as the growth-inhibitory and differentiation-inducing entity. Indeed, the long intracellular half-life (approximately 18 hr) [24] of PMEApp may account for the observed long-lasting effect of PMEA, whether antitumoral or antiviral [25]. This assumption is also supported by our earlier findings on K562 cells, in which the differentiationinducing effect of PMEA reached its maximum level 5 days after drug removal, and was still pronounced after 11 days of K562 cell incubation in drug-free medium [23].

PMEA also effectively suppresses tumor growth in an *in vivo* RCHO model in a dose-dependent manner [22]. Moreover, PMPA, which failed to induce differentiation in RCHO cell cultures, was also ineffective in inhibiting choriocarcinoma tumor growth *in vivo* [22]. The question as to whether or not the induction of RCHO cell differentiation plays a major role in the antitumor effect of PMEA against RCHO *in vivo* is now under investigation.

Induction of tumor cell differentiation implies withdrawal of the proliferating cells from the cell cycle and, consequently, growth arrest. Hence, differentiation therapy may offer an attractive strategy for cancer treatment. Yet, among the agents that are known to be endowed with differentiation-inducing properties, only very few have entered clinical trials at present. PMEA, in its oral prodrug form bis(POM)-PMEA, is currently subject of clinical trials for the treatment of HIV and hepatitis B virus infections [1, 7]. Because patients with AIDS frequently develop several types of malignancies, including Kaposi's sarcoma and lymphomas, the differentiation-inducing effect of PMEA on these and several other differentiation-susceptible tumors should be further explored. In this regard, it is important to note that the PMEA concentrations affording marked differentiation in our *in vitro* tumor cell systems can be readily attained *in vivo*. Indeed, phase I clinical trials with PMEA have revealed that drug plasma levels of 10 μg/mL (35 μM) can be achieved in patients without severe toxic side-effects [26].

In conclusion, PMEA emerged as an entirely novel and promising differentiation-inducing agent which proved highly specific in its tumor cell-differentiating activity. These properties of PMEA warrant further investigation on the compound to fully explore its potential as a differentiation-inducing agent in cancer chemotherapy.

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References

1. Naesens L, Snoeck R, Andrei G, Balzarini J, Neyts J and De Clercq E, HPMPC (cidofovir), PMEA (adefovir) and related acyclic nucleoside phosphonate analogs: a review of their pharmacology and clinical potential in the treatment of viral infections. *Antiviral Chem Chemother* **8:** 1–23, 1997.
2. Balzarini J, Verstuyf A, Hatse S, Goebels J, Sobis H, Vandeputte M and De Clercq E, The human immunodeficiency virus (HIV) inhibitor 9-(2-phosphonylmethoxyethyl)adenine (PMEA) is a strong inducer of differentiation of several tumor cell lines. *Int J Cancer* **61:** 130–137, 1995.
3. De Clercq E, Holy A, Rosenberg I, Sakuma T, Balzarini J and Maudgal PC, A novel selective broad-spectrum anti-DNA virus agent. *Nature* **323:** 464–467, 1986.
4. Heijtink RA, De Wilde GA, Kruining J, Berk L, Balzarini J, De Clercq E, Holy A and Schalm SW, Inhibitory effect of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) on human and duck hepatitis B virus infection. *Antiviral Res* **21:** 141–153, 1993.
5. Balzarini J, Naesens L, Slachmuylders J, Niphuis H, Rosenberg I, Holy A, Schellekens H and De Clercq E, 9-(2-phosphonylmethoxyethyl)adenine (PMEA) effectively inhibits retrovirus replication *in vitro* and simian immunodeficiency virus infection in rhesus monkeys. *AIDS* **5:** 21–28, 1991.
6. Naesens L, Balzarini J and De Clercq E, Therapeutic potential of PMEA as an antiviral drug. *Rev Med Virol* **4:** 147–159, 1994.
7. Barditch-Crovo P, Toole J, Hendrix CW, Cundy KC, Ebeling D, Jaffe HS and Lietman PS, Anti-human immunodeficiency virus (HIV) activity, safety, and pharmacokinetics of adefovir dipivoxil (9-[2-(bis-pivaloyloxymethyl)phosphonylmethoxyethyl]adenine) in HIV-infected patients. *J Inf Dis* **176:** 406–413, 1997.
8. Teshima S, Shimosato Y, Koide T, Kuroki M, Kikuchi Y and Aizawa M, Transplantable choriocarcinoma of rats induced by fetectomy and its biologic activities. *Jap J Cancer Res* **74:** 205–212, 1983.
9. Verstuyf A, Sobis H, Goebels J, Fonteyn E, Cassiman JJ and Vandeputte M, Establishment and characterization of a continuous *in vitro* line from a rat choriocarcinoma. *Int J Cancer* **45:** 752–756, 1990.
10. Botte V, Tramontana S and Chieffi G, Histochemical distribution of some hydroxysteroid dehydrogenase in the placenta, fetal membranes and uterine mucosa of the mouse. *J Endocrinol* **40:** 189–194, 1968.
11. Campbell WJ, Larsen D, Deb S, Kwok SCM and Soares MJ, Expression of alkaline phosphatase in differentiated rat labyrinthine trophoblast tissue. *Placenta* **12:** 227–237, 1991.
12. Hunt JS and Soares MJ, Expression of histocompatibility antigens, transferrin receptors, intermediate filaments, and alkaline phosphatase by *in vitro* cultured rat placental cells and rat placental cells *in situ*. *Placenta* **9:** 159–171, 1988.
13. Verstuyf A, Fonteyn E, Sobis H and Vandeputte M, A rat cytotrophoblast antigen defined by a monoclonal antibody. *Am J Reprod Immunol* **28:** 6–11, 1992.
14. Tsai C-C, Follis KE, Sabo A, Beck TW, Grant RF, Bischofberger N, Benveniste RE and Black R, Prevention of SIV infection in macaques by (*R*)-9-(2-phosphonylmethoxypropyl) adenine. *Science* **270:** 1197–1199, 1995.
15. Holy A and Rosenberg I, Synthesis of 9-(2-phosphonylmethoxyethyl)adenine and related compounds. *Collect Czech Chem Commun* **52:** 2801–2809, 1987.
16. Verstuyf A, Goebels J, Sobis H and Vandeputte M, Influence of different growth factors on a rat choriocarcinoma cell line. *Tumor Biol* **14:** 46–54, 1993.
17. Burres NS and Cass CE, Inhibition of methotrexate-induced differentiation of cultured human choriocarcinoma (BeWo) cells by thymidine. *Cancer Res* **47:** 5059–5064, 1987.
18. Friedman SJ and Skehan P, Morphological differentiation of human choriocarcinoma cells induced by methotrexate. *Cancer Res* **39:** 1960–1967, 1979.
19. Pommier Y and Lokiec F, Le méthotrexate. *Chimiothérapie anticancereuse* **31:** 3083–3089, 1981.
20. Hatse S, De Clercq E and Balzarini J, 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. *Mol Pharmacol* **50:** 1231–1242, 1996.
21. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC and Pezzuto JM, Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275:** 218–220, 1997.
22. Hatse S, Naesens L, Degrève B, Segers C, Vandeputte M, Waer M, De Clercq E and Balzarini J, Potent antitumor activity of the acyclic nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine (PMEA) in choriocarcinoma-bearing rats. *Int J Cancer*, **76:** 595–600, 1998.
23. Hatse S, Balzarini J and De Clercq E, Induction of erythroid differentiation of human leukemia K562 cells by the acyclic nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine (PMEA). *Nucleosides & Nucleotides* **14:** 649–652, 1995.
24. Balzarini J, Hao Z, Herdewijn P, Johns DG and De Clercq E, Intracellular metabolism and mechanism of anti-retrovirus action of 9-(2-phosphonylmethoxyethyl)adenine, a potent anti-human immunodeficiency virus compound. *Proc Natl Acad Sci USA* **88:** 1499–1503, 1991.
25. Balzarini J, Naesens L and De Clercq E, Anti-retrovirus activity of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) *in vivo* increases when it is less frequently administered. *Int J Cancer* **46:** 337–340, 1990.
26. Cundy KC, Barditch-Crovo P, Walker RE, Collier AC, Ebeling D, Toole J and Jaffe HS, Clinical pharmacokinetics of adefovir in human immunodeficiency virus type 1-infected patients. *Antimicrob Agents Chemother* **39:** 2401–2405, 1995.