

CYTOTOXICITY OF PIVOXIL ESTERS OF ANTIVIRAL ACYCLIC NUCLEOSIDE PHOSPHONATES: ADEFOVIR DIPIVOXIL VERSUS ADEFOVIR

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Biological effectiveness of antiviral acyclic nucleoside phosphonate adefovir, 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) and its more lipophilic (bis)pivaloyloxymethyl ester prodrug adefovir dipivoxil (bis-POM-PMEA) were compared under in vitro conditions in mammalian cell systems. Proliferation of murine splenocytes was inhibited in a concentration-dependent manner, the bis-POM-PMEA being more effective than PMEA. In contrast to PMEA, bis-POM-PMEA inhibited production of nitric oxide (NO) in macrophages activated with interferon- γ (IFN- γ) and lipopolysaccharide (LPS). Viability of both splenocytes and macrophages remained uninfluenced by PMEA, whereas pronounced cytotoxic effects were exhibited by bis-POM-PMEA. The IC_{50} s reached the values of 15 μ M and 30 μ M in cultures of macrophages and splenocytes, respectively (assayed at the interval of 24 hrs). The effects could partly be mimicked by formaldehyde, a decomposition product of the pivoxil moiety of bis-POM-PMEA. The other possible product, pivalic acid, was ineffective in this respect. The present data are consistent with the view that pivoxil prodrug of PMEA, bis-POM-PMEA possesses enhanced but also broader spectrum of biological effects than the parent compound.

INTRODUCTION

Acyclic nucleoside phosphonate 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA; adefovir) is a recognized antiviral agent possessing both antiherpesvirus and antiretroviral activities including anti-HIV effects¹⁻⁴. The major disadvantage of this class of compounds under therapeutic usage is, however, rather poor intracellular delivery, intestinal permeation and limited oral bioavailability^{5,6}. Pharmaceutical formulations which would overcome these obstacles are therefore intensively explored. The negative charge of the PMEA molecule can be reduced through the introduction of the lipophilic (bis)pivaloyloxymethyl (pivoxil) moiety, giving rise to bis-POM-PMEA, i.e. adefovir dipivoxil. The prodrug shows considerably enhanced oral bioavailability in comparison with the parent structure of PMEA (ref.^{5,6}), and has been approved by FDA and EMEA for general treatment of hepatitis B (Hepsera[®]). Not only antiviral effectiveness⁷ but also more prominent antiarthritogenic activity of bis-POM-PMEA over PMEA⁸ have been reported. We have shown recently that PMEA suppresses proliferation of lymphocytes⁹ and in contrast to a number of other acyclic nucleoside phosphonates it does not augment production of immune-activated production of nitric oxide by macrophages¹⁰. The aim of this study was to investigate the influence of PMEA pivoxilation on these cell functions.

MATERIALS AND METHODS

Animals

Female mice of the inbred strain C57BL/6, 7-9 wks old, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in transparent plastic cages in groups of eight, and maintained in an Independent Environmental Air Flow Cabinet (ESI Flufrance, Wissous, France). Lighting was set on 0600 to 1800 h, temperature at 22 °C.

Compounds

9-[2-(Phosphonomethoxy)ethyl]adenine (PMEA; adefovir) was synthesized in-house (Inst. Organic Chemistry and Biochemistry) according to the procedure described

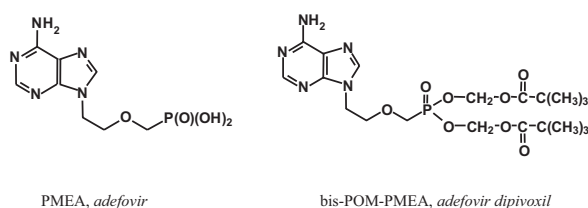


Fig. 1. Chemical structure of test acyclic nucleoside phosphonates.

elsewhere^{11,12}. The (bis)pivaloyloxymethyl ester prodrug adefovir dipivoxil (bis-POM-PMEA) was kindly donated by Gilead Sciences (Foster City, CA). Their structure is shown in Fig. 1. Stock solutions were prepared in incomplete phenol red-free RPMI-1640 culture medium containing NaHCO₃ (Sigma-Aldrich, Prague, CR). Working concentrations were prepared by diluting the stock solutions in complete medium RPMI-1640 (see below) and used fresh.

Culture medium and other reagents

Complete RPMI-1640 medium contained 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 µg/ml gentamicin, and 5 × 10⁵ M 2-mercaptoethanol (all Sigma). Recombinant mouse interferon-γ (IFN-γ) was purchased from R&D Systems (Minneapolis, MN), lipopolysaccharide (LPS) of *S. typhimurium* origin from Difco Labs (Detroit, MI), the LDH cytotoxicity detection kit from Boehringer Mannheim (Germany), and methyl-³H-thymidine (specific activity 185 GBq/mmol) from Amersham (Little Chalfont, UK). Pivalic acid was obtained from Sigma.

Lymphocytes and lymphocyte proliferation assay

Single-cell suspension of splenocytes was prepared by passing fragmented pooled spleens of mice through a fine nylon sieve. Erythrocytes were removed using the red blood cells lysing buffer (Sigma) containing 0.83 % ammonium chloride in 0.01 M Tris-HCl, pH 7.5. After repeated thorough washing the cells were seeded in triplicate wells of 96-well U-bottom cell culture plates (Nunc, Roskilde, Denmark). The number of cells was 5 × 10⁵/well in final 100 µl. They were cultured for 72 h in the presence or absence of mitogens (ConA, 2.5 µg/ml or LPS, 5 µg/ml) in Heraeus incubator keeping 37 °C, 5 % CO₂, 100 % relative humidity.

PMEA and bis-POM-PMEA were added immediately after mitogens. Six hours prior to the collection of cells, they were pulsed with 0.5 µCi of methyl-³H-thymidine. Cells were harvested onto glass microfiber filters using Dynatech Multimash Harvester 2000. Thymidine incorporation into DNA (cpm) was determined via scintillation counting.

Macrophages and macrophage nitric oxide (NO) production

Mice, sacrificed by cervical dislocation, were injected intraperitoneally with 8 ml sterile saline. Collected and pooled lavage cells were washed, resuspended in culture medium and seeded into 96-well flat-bottom Nunc microplates in 100-µl volumes. Adherent peritoneal cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5 % CO₂, and then thrice vigorously shaking the plate and washing to remove non-adherent cells. Cultures were maintained with or without addition of PMEA and bis-POM-PMEA and in the absence or presence of IFN-γ (5000 pg/ml) plus LPS (1 µg/ml) in Heraeus incubator for 24 h. All experimental variants were set in triplicate.

The concentration of nitrites in supernatants was taken as a measure of NO production^{13,14}. It was detected in individual cell-free samples (50 µl) incubated for 10 min at 37 °C with an aliquot of a Griess reagent (1 % sulphanilamide/0.1 % naphthylendiamine/2.5 % H₃PO₄). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to µM nitrite.

Determination of cell viability

Cell death was analysed using the LDH assay. It is based on the determination of lactate dehydrogenase activity released from the cytosol of damaged cells into the supernatant. The cell supernatants were diluted 1 : 1 and mixed with an aliquot of the LDH kit. After 30-min incubation in the dark at ambient temperature, the reaction was stopped with 2 N HCl. Differences between absorbances at 492 and 690 nm were evaluated. The percentage cytotoxicity of test samples was related to the control samples and to the samples with 100 % dead cells evoked by 1 % Triton, according to the formula: [(exp. value - control value) / (Triton value - control value)] × 100. All control and experimental variants were run in triplicate.

Data analysis

Analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test, and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

RESULTS

Antiproliferative effects of PMEA and bis-POM-PMEA

The following values (means ± SEM) of incorporation of ³H-thymidine into DNA were found in controls (drugs absent): 1,700 ± 138 cpm for resident, mitogen-nonstimulated cells, 22,397 ± 1,059 cpm for LPS-stimulated, and 128,473 ± 1,690 cpm for ConA-stimulated cells. Proliferation of nonstimulated and mitogen-stimulated lymphocytes was differentially inhibited by PMEA and bis-POM-PMEA (Fig. 2). Expressed as a percentage of corresponding control values, the inhibitory effects run in parallel in individual experimental groups. The bis-POM-PMEA proved to be significantly more potent (P < 0.01) than PMEA in suppressing proliferation of all nonstimulated (IC₅₀s: 2.9 and 20.4 µM, respectively), LPS-stimulated (1.7 and 13.8 µM, respectively), and ConA-treated (0.44 and 1.02 µM, respectively) cells.

Effects of PMEA and bis-POM-PME on nitric oxide (NO) production

Production of NO, triggered primarily in mouse peritoneal macrophages by LPS (10 pg/ml) plus IFN-γ (5000 pg/ml), remained uninfluenced by PMEA (Fig. 3). PMEA was ineffective even in as high concentrations as 500 µM. On the other hand, effects of bis-POM-PMEA on NO production were strongly inhibitory. The dose of

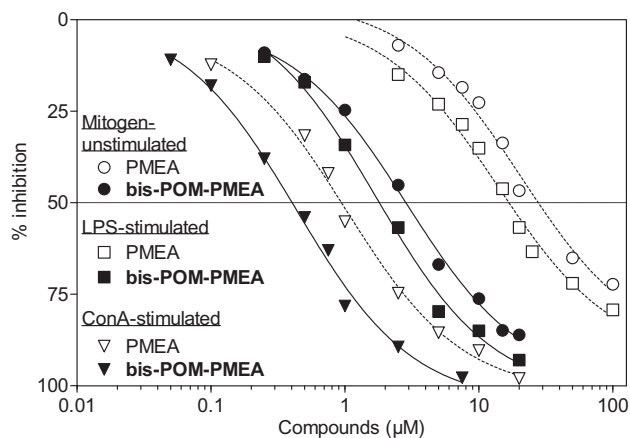


Fig. 2. Antiproliferative effects of PMEAs and bis-POM-PMEAs. Mouse lymphocytes were cultured for 72 h in the absence or presence of B-cell mitogen LPS and T-cell mitogen ConA. Six hours prior to the collection of cells, they were pulsed with 0.5 μCi of methyl- ^3H -thymidine. Its incorporation into DNA was determined by scintillation counting. Control cpm values are shown in Results.

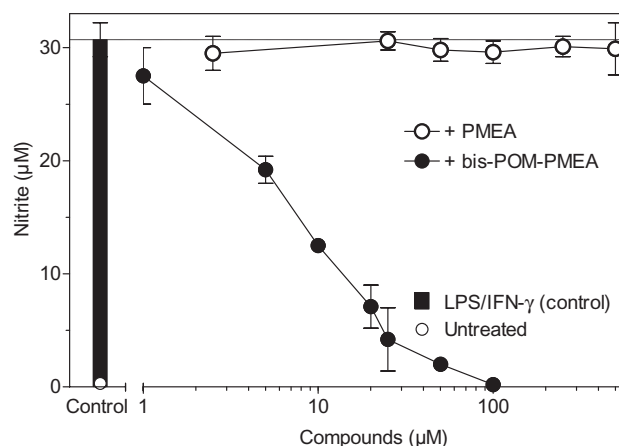


Fig. 3. Effects of PMEAs and bis-POM-PMEAs on production of NO by macrophages activated with LPS+IFN- γ . Nitrite concentration in supernatants of cells cultured for 24 h was determined by Griess reagent.

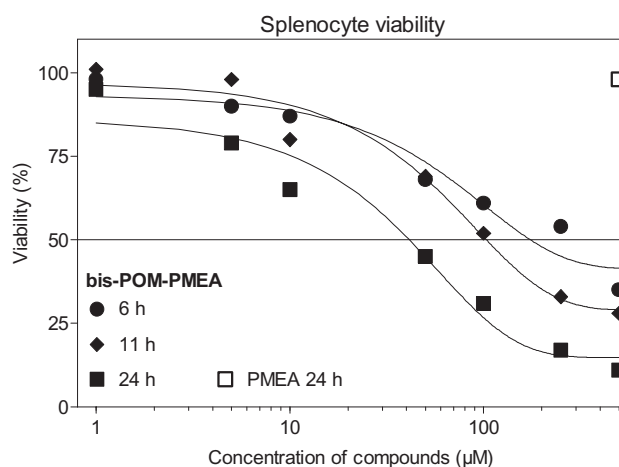
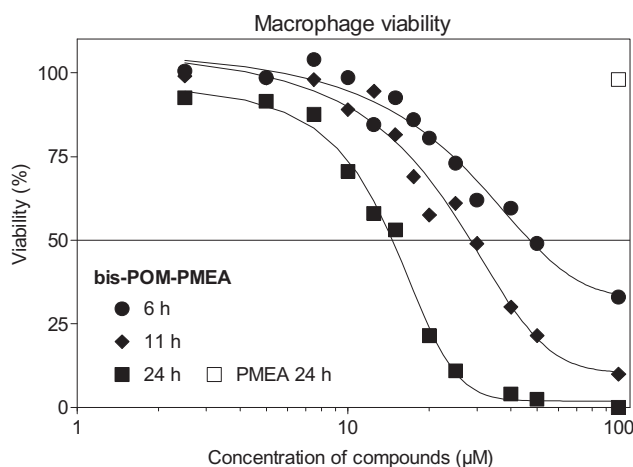


Fig. 4. Effect of bis-POM-PMEAs and PMEAs on viability of murine macrophages and lymphocytes. The LDH assay was performed at indicated time intervals subsequent to the addition of test compounds.

approximately 5 μM reduced the production of NO by about 50 %.

Cytocidal effects of bis-POM-PMEAs

Whereas even high doses (100–500 μM) of PMEAs did not influence viability of cells (Fig. 4), bis-POM-PMEAs proved to possess remarkable cytotoxic activity. The LC_{50} , determined at the interval of 24 h, reached the value of 15 μM for macrophages, and 30 μM for splenocytes. The effects were time-dependent, the significant inhibition of viability of both macrophages and lymphocytes being apparent as early as 5–6 h following the addition of bis-POM-PMEAs to cells (Fig. 4).

Comparison of biological effects of bis-POM-PMEAs with the effects of formaldehyde and pivalic acid

As compared to bis-POM-PMEAs, formaldehyde was less potent to inhibit production of NO by macrophages (Fig. 5). Pivalic acid was free of the NO-suppressive effect. Similarly, only formaldehyde, but not pivalic acid, showed moderate cytotoxic activity (Fig. 6).

DISCUSSION

Expression of biological potential of PMEAs, i.e. 9-[2-(phosphonomethoxy)ethyl]adenine (adefovir) is restricted by a relatively high anionic charge of this molecule, brought about by the phosphonate side chain, which

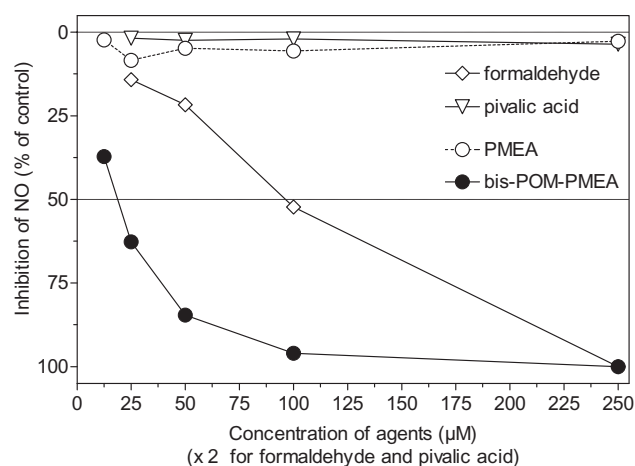


Fig. 5. Bis-POM-PMEA- and formaldehyde-induced suppression of NO production in macrophages stimulated with LPS+IFN- γ . Control value for the cells cultivated without the test compounds was $42 \pm 2.7 \mu\text{M}$ nitrite. Formation of NO was inspected after 24-h culture.

makes it otherwise resistant to metabolism. The pivoxil prodrug, i.e. the (bis)pivaloyloxymethyl ester of PME A, bis-POM-PMEA (adefovir dipivoxil), overcomes partially this obstacle and has been found to possess higher virostatic efficacy than parent PME A. Both compounds are transported across the membrane by endocytosis¹⁵. Once in the cell, bis-POM-PMEA is cleaved gradually to the parent drug⁶, and is subsequently phosphorylated to its mono- and diphosphate (analogues of dADP and dATP). These analogues are alternative substrates and inhibitors of DNA polymerases¹⁶ and act as chain terminators of the DNA synthesis de novo^{11,17}. The cleavage of bis-POM-PMEA and, in particular, the subsequent phosphorylation strongly depend on the cell type and on a variety of enzymes¹⁸⁻²⁰.

We have found that the both drugs inhibit proliferation of murine splenocytes, irrespective of the kind of mitogen stimulation, or without any immune stimulation. The bis-POM-PMEA is several-fold more effective than parent PME A. As far as the intracellular concentration has been shown to be a crucial factor determining biological efficacy of PME A^{21,22}, it could be presumed that the enhanced effectiveness of bis-POM-PMEA results from the different abilities of PME A versus bis-POM-PMEA to permeate across cell membranes.

In sharp contrast to a quantitative character of these differences, the influence on production of NO is a novel property and is bound solely to bis-POM-PMEA. Even enormously increased concentrations of PME A (up to $500 \mu\text{M}$) were absolutely devoid of any positive and negative interference with biosynthesis NO. However, the introduction of pivoxil moiety in the molecule of bis-POM-PMEA led to the occurrence of inhibitory effects on NO production

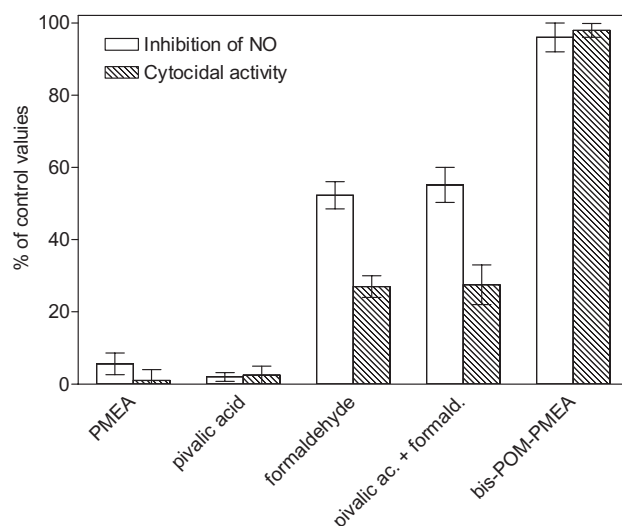


Fig. 6. Comparison of the NO-inhibitory and cytotoxic effects of PME A ($100 \mu\text{M}$), bis-POM-PMEA ($100 \mu\text{M}$), and its decomposition products formaldehyde ($200 \mu\text{M}$) and pivalic acid ($200 \mu\text{M}$). The both effects are statistically significantly correlated (Pearson $r = 0.93$, $P < 0.05$).

This finding prompted us to investigate possible cytotoxic potential of bis-POM-PMEA, as well as a profile of biological effects of decomposition products of the pivoxil moiety, i.e. formaldehyde and pivalic acid. Indeed, bis-POM-PMEA was found to remarkably decrease viability of both lymphocytes and macrophages. Similarly, in agreement with other reports²³, formaldehyde (but not pivalic acid) decreased the cell viability. Furthermore, it inhibited production of NO by macrophages. It may be presumed that enhanced antiproliferative effects of bis-POM-PMEA are due to improved transmembrane permeability, but at least in part they are influenced by formaldehyde-induced dying the cells. Inhibition of NO production seems to be however entirely dependent on formation of formaldehyde, and not on enhanced intracellular concentration of PME A following administration of the prodrug. It should be noted that formaldehyde (as well as pivalic acid) was applied at concentrations that could theoretically arise if all pivoxil moieties of bis-POM-PMEA molecule are transformed to formaldehyde and pivalic acid. Despite of this, all inhibitory effects of bis-POM-PMEA were more pronounced than those of formaldehyde. Plausible explanation for the discrepancy might be a difference in intracellular accumulation of formaldehyde reached after its direct administration into the cell cultures, or alternatively delivered indirectly by bis-POM-PMEA in the form of pivoxil ancestry. It cannot be excluded that intracellular concentrations of formaldehyde are higher after treatment of cells with bis-POM-PMEA than after addition of exogenous formaldehyde. This question remains to be investigated.

Apart from the inhibitory effects on NO formation and cell viability, formaldehyde has been shown to possess a number of other unwanted effects. It has been sug-

gested to contribute to allergic respiratory disease²⁴, to be a risk factor for initiation of endothelial injury leading to atherosclerosis²⁵, and for neurogenic inflammation²⁶. Inasmuch as some drugs including antibiotics²⁷⁻²⁹ and anticancer agents^{30,31} are pharmaceutically formulated as pivoxil prodrugs, careful evaluation of their possibly formaldehyde-related side effects and therapeutic implications warrant special consideration.

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