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Inhibition of Murine Macrophage Nitric Oxide Synthase Expression by a Pivoxil Prodrug of Antiviral Acyclic Nucleotide Analogue 9-(2-Phosphonomethoxyethyl)adenine

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ABSTRACT. The effect of the acyclic nucleotide analogue, 9-(2-phosphonomethoxyethyl)adenine (PMEA, *Adefovir*), and its (bis)pivaloyloxymethyl ester (bis-POM-PMEA, *Adefovir Dipivoxil*) on *in vitro* nitric oxide (NO) production by murine peritoneal macrophages was investigated. Bis-POM-PMEA inhibited in a concentration-dependent manner the formation of NO generated by interferon-γ and lipopolysaccharide, the IC_{50} being 15 μM. Suppressed transcription of mRNA for inducible NO synthase (EC 1.14.13.39) resulting in decreased synthesis of NO synthase protein was found. Parent compound PMEA was virtually ineffective. BIOCHEM PHARMACOL **54**;8:855–861, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. acyclic nucleotide analogues; 9-(2-phosphonomethoxyethyl)adenine (PMEA); pivoxil prodrug of PMEA; nitric oxide; inhibition of iNOS transcription

9-(2-Phosphonomethoxyethyl)adenine (PMEA,‖ *Adefovir*) is a prototype of the potent antiviral acyclic nucleotide analogues [1]. It possesses both anti-DNA viral and anti-retroviral activities, including human immunodeficiency viruses (HIV) [2–5], and has shown antiviral activity in phase I/II trials against HIV infections [6, 7]. The major disadvantage of PMEA and similar compounds is, however, rather poor intracellular delivery, limited intestinal absorption, and resulting low oral availability. These parameters were greatly improved by esterification with lipophilic pivaloyloxymethyl (POM) groups [i.e. bis-POM-PMEA, bis(pivaloyloxymethyl)ester of PMEA, *Adefovir Dipivoxil*] [8–10]. Therapeutic efficacy of PMEA has thus been considerably augmented [11, 12]. Recently, we found that bis-POM-PMEA was more effective in inhibiting *in vitro* lymphocyte proliferation compared with the parent compound (Zidek Z, Franková D, Holý A, unpublished data). The present experiments were aimed at comparing the effects of PMEA and its pivoxil prodrug bis-POM-PMEA on the immune-related enzymatic activity of macrophages, namely on the production of nitric oxide (NO).

Due to its antiviral, antibacterial, and anticancer effects, NO is considered to be an important defense mechanism in the nonspecific immune system of the organism [13, 14]. It is synthesized from L-arginine by nitric oxide synthases (NOS) (EC 1.14.13.39), which are present in many cell types. NO is formed via oxidation of terminal guanidino nitrogen atoms of L-arginine, yielding L-citrulline and the active radical. There are at least three isoenzymes that differ according to their cofactor dependence, genetic regulation, tissue and intracellular location, as well as physiological functions [15, 16]. Macrophages contain the type II NOS (inducible NOS; iNOS) which is transcriptionally activated by interferon-γ (IFN-γ) and lipopolysaccharides (LPS) [17].

Here, we present data that bis-POM-PMEA inhibits production of NO by IFN-γ/LPS-stimulated macrophages.

MATERIALS AND METHODS

Animals

Female mice of the inbred strain C57BL/6 NCrlBR, 7 to 9 weeks old, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in transparent plastic cages in groups of ten and maintained in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France). Lighting was set from 0600 to 1800 h, and the temperature was kept at 22 ± 1°.

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‖ Abbreviations: IFN-γ, interferon-gamma; LPS, lipopolysaccharide; NO, nitric oxide; NOS, NO synthase; PMEA, 9-(2-phosphonomethoxyethyl)adenine; bis-POM-PMEA, bis(pivaloyloxymethyl)ester of PMEA; RT-PCR, reverse transcriptase-polymerase chain reaction.

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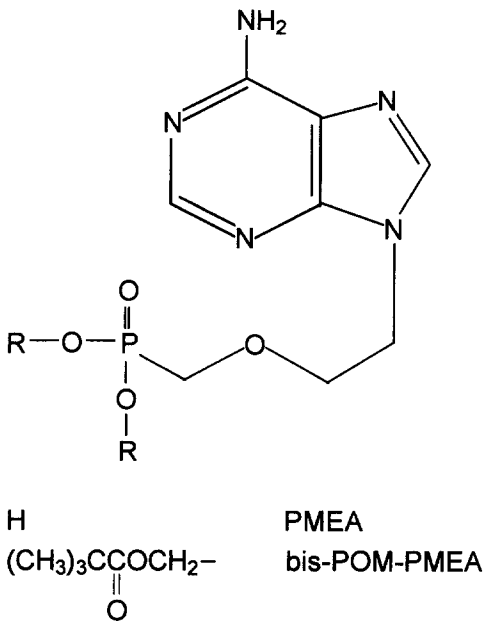

FIG. 1. Structural formula of test compounds.

Test Compounds

PMEA was synthesized according to the procedure described elsewhere [18] and used as a sodium salt. Bis-POM-PMEA was kindly donated by Gilead Sciences (Foster City, CA, USA). Their structure is shown in Fig. 1. Stock solutions (2 mg/mL) were prepared in incomplete phenol red-free RPMI-1640 culture medium containing $NaHCO_3$ (Sigma, St. Louis, MO, USA). They were sterile filtered using nonpyrogenic 0.22-μm filters (Costar, Cambridge, MA, USA) and used fresh. Required working concentrations were prepared by diluting the stock solution in complete RPMI-1640 (see below). Possible presence of endotoxin was tested using the chromogenic Limulus Amebocyte Lysate assay (QCL-1000, Bio-Whittaker, Walkersville, MD, USA). Test samples contained no more than 0.1 EU/mL.

Culture Medium and Other Reagents

Complete RPMI-1640 medium (phenol red-free) contained 10% heat-inactivated (56°, 30 min) fetal bovine serum (ICN Biomedicals, High Wycombe, UK), 2 mM L-glutamine, 50 μg/mL gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma). Recombinant mouse IFN-γ (specific activity, 1.1×10^7 units/mg), was purchased from Genzyme Corp. (Cambridge, MA, USA) and LPS of *Salmonella typhimurium* origin from Difco Labs (Detroit, MI, USA). Cytotoxicity Detection Kit (LDH) was purchased from Boehringer Mannheim (Germany).

Macrophage Isolation and Culture

Mice, sacrificed by cervical dislocation, were injected i.p. with 8 mL of sterile saline. Collected and pooled lavage cells were washed, resuspended in culture medium, and set into 24-well cell culture clusters (Costar, Cambridge, MA, USA) in 500-μL volumes (1×10^6 cells/well). Adherent peritoneal cells (macrophages) were isolated by incubating the cells for 2 h at 37°, 5% CO_2 and then thrice vigorously shaking the plate and washing to remove nonadherent cells. Cultures were maintained with or without addition of test compounds and in the absence or combined presence of LPS (5 μg/mL) + IFN-γ (50 units/mL) at 37°, 5% CO_2 in a humidified Heraeus incubator for 12 or 24 h. The 12-h cultivated cells were used for NOS enzyme assay and reverse transcriptase-polymerase chain reaction (RT-PCR).

Determination of Nitrites in Culture Supernatants

The concentration of nitrites in supernatants was taken as a measure of NO production [19, 20]. This was detected in individual cell-free samples (50 μL) incubated for 10 min at 37° with an aliquot of a Griess reagent (1% sulfanilamide, 0.1% naphtylethylendiamine, 2.5% H_3PO_4). The absorbance at 540 nm was recorded using a Uniskan II microplate reader (Labsystems, Helsinki, Finland). The background absorbance of the control medium sample was subtracted from the values of experimental samples.

NOS Enzyme Assay

Macrophage monolayers were washed three times with PBS (Sigma), scraped into PBS, and centrifuged at 600 × *g* for 10 min at 4°. The cells were resuspended in 500 μL of 40 mM TRIZMA buffer (pH 8) containing 5 μg/mL pepstatin A, 1 μg/mL leupeptin, 5 μg/mL aprotinin, and 100 μM phenylmethylsulfonyl fluoride (all Sigma). The cells were disrupted by sonicator (1 × 10 s) on ice. Enzyme activity of iNOS was measured as described [21–23]. Twenty μL of macrophage lysate were incubated for 3 h at 37° in 80 μL of 20 mM TRIZMA (pH 8) containing 2 mM NADPH (Boehringer Manheim, Germany), 4 μM tetrahydrobiopterin (Calbiochem, La Jolla, CA, USA), 4 μM FAD, 3 mM dithiothreitol, 1 mM L-arginine (all Sigma). Final assay pH was 7.9. After 3 h of cultivation, 10 μL of 1 units/mL nitrate reductase (from *Aspergillus sp.*; Boehringer) was added. Samples were incubated for 20 min at 37° in the dark. Reaction was stopped (5 min, 37°) by adding 10 units/mL lactate dehydrogenase (from rabbit muscle; Boehringer) and 10 mM sodium pyruvate (Boehringer) in a final volume of 120 μL. Using a nitrate standard curve, overall nitrite accumulation was then determined by Griess reagent as described above. Recovery of nitrate was >95%, and the assay results were not significantly affected by the presence of proteins. The activity of NO synthase was referred to as nanomoles of nitrites produced per mg of protein/h. Samples were run in triplicate.

Protein Determination

Protein was determined according to Bradford [24] modified for 96-well plates [25], using the dye reagent from Bio-Rad

Laboratoires (Richmond, CA, USA) and BSA (Sigma) as protein standard.

RT-PCR Assay

For the RT-PCR assay, RNA from 12-h cultured macrophages was extracted and reverse transcribed into cDNA with M-MLV reverse transcriptase using an RNA Blue PCR kit (EXBIO, Prague, CR) according to the manufacturer's instructions. cDNA was amplified by PCR techniques using iNOS-specific primers designed from iNOS sequence of mouse macrophage: 5'-CCCTTCCGAAGTT TCTGGCAGCAGC-3' and 5'-GGCTGTCAGAGCCTC GTGGCTTTGG-3' (Clontech Laboratories, Palo Alto, CA, USA). As a control, cDNA of mouse β-actin was also amplified using β-actin-specific primers 5'-GTGGGCCGCTCTAG GCACCAA-3' and 5'-CTCTTTGATGTCACGCACGA TTTC-3' (Clontech). PCR products were analyzed on 1.5% agarose gels, stained with ethidium bromide, and their amount estimated by densitometry using gel documentation and analysis system with Gel Base/Gel Blot Pro software (UVP Life Sciences Cambridge, U.K.).

Determination of Cell Viability

Colorimetric assay was applied for the quantification of cell death and cell lysis. Cytotoxicity Detection Kit (LDH, Boehringer Mannheim) was used for this purpose. It is based on the determination of lactate dehydrogenase activity released from the cytosol of damaged cells into the supernatant. Briefly, the cell supernatants were diluted 1:1 and mixed with an aliquot of the LDH kit. After a 30-min incubation period in the dark at ambient temperature, the reaction was stopped with 2N HCl. Differences between absorbances at 492 and 690 nm were evaluated. The percentage of cytotoxicity of test samples was related to the control (compounds absent) samples and to the samples with 100% dead cells artificially caused by the presence of 1% Triton, according to the following formula: % cytotoxicity = [(exp. value − control value)/(Triton value − control value)] × 100. All control and experimental variants were run in triplicate. In parallel, cell viability was checked by the methylene blue (Sigma) trapping [25] or mitochondrial respiration (Cell Proliferation Reagent WST-1; Boehringer) assays. Results of all tests were virtually identical.

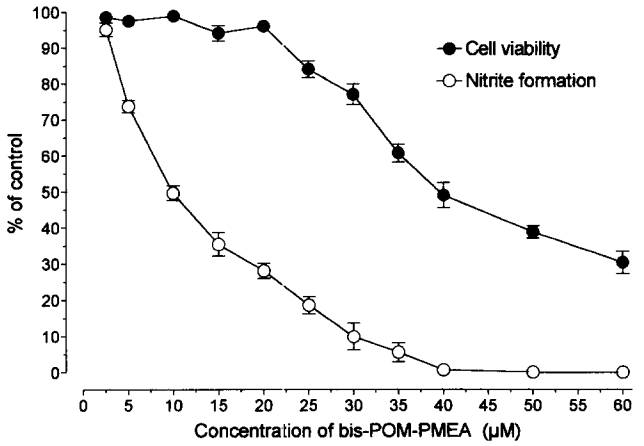

FIG. 2. Dose-dependent inhibitory effect of bis-POM-PMEA on *in vitro* IFN-γ/LPS-generated formation of nitrites by mouse peritoneal macrophages and their viability. The IFN-γ/LPS cocktail (50 units/mL and 5 μg/mL, respectively) and bis-POM-PMEA were administered simultaneously. Assays were performed after a 24-h cultivation period. Each point is the mean ± SEM of triplicate combinations. Nitrite concentration for IFN-γ/LPS control (in the absence of bis-POM-PMEA) was 66.3 ± 0.3 μM, while that for IFN-γ/LPS-untreated cells was 2.3 ± 1.4 μM. The figure is representative of two independent experiments.

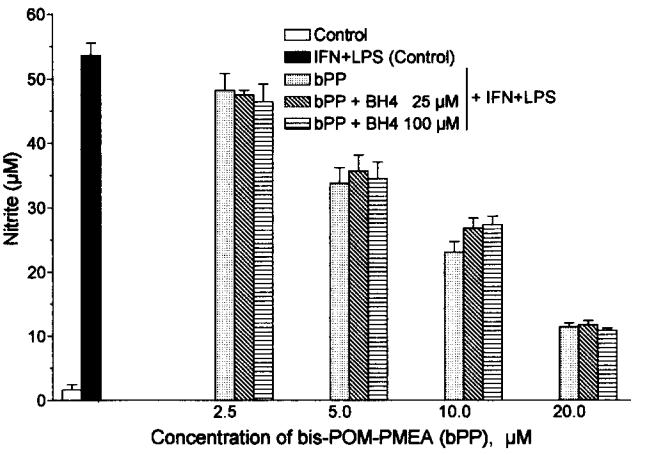

FIG. 3. Production of nitrites by mouse peritoneal macrophages *in vitro* stimulated with the IFN-γ/LPS cocktail (50 units/mL and 5 μg/mL, respectively). The cells were cultured for 24 h in the presence of bis-POM-PMEA (25 μM) ± tetrahydrobiopterin (BH4). No effect of BH4 on bis-POM-PMEA-triggered suppression of nitrite formation was observed. Data are expressed as means ± SEM of quadruplicate settings.

Statistical Analysis

Statistical significances were evaluated by means of ANOVA using the Prism program (GraphPad Software, San Diego, CA, USA).

RESULTS

In vitro production of NO by mouse peritoneal macrophages was generated by the IFN-γ/LPS cocktail (50 units/mL and 5 μg/mL, respectively). It was suppressed in a concentration-dependent manner by bis-POM-PMEA (Fig. 2), the IC_{50} being 15 μM. The same effect was found when LPS and IFN-γ were used separately (data not shown). No changes in cell viability were observed up to the concentration of 20 μM bis-POM-PMEA (Fig. 2). All further experiments were performed with the concentration of 25 μM, which, without any prominent cytotoxicity, caused at least 80% inhibition of nitrite formation.

Supplementation of cultures with tetrahydrobiopterin

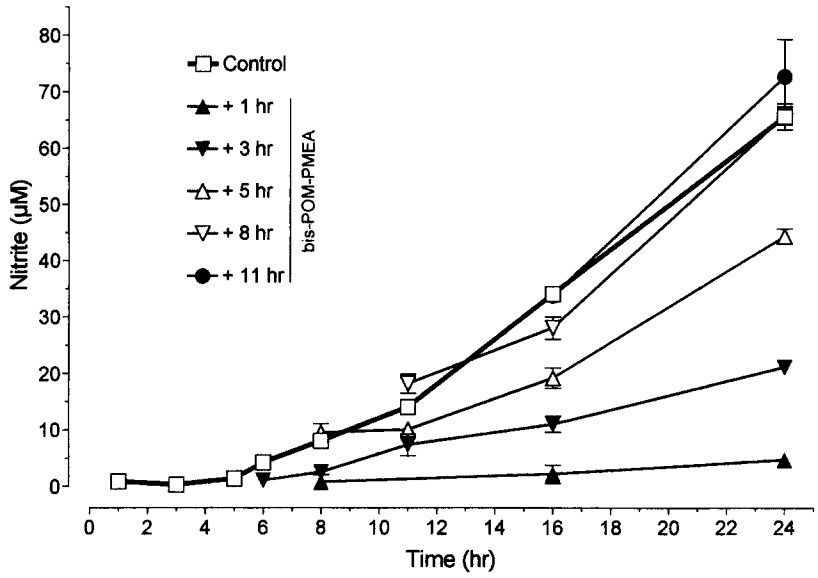

FIG. 4. Inhibitory effect of bis-POM-PMEA is dependent on its timing. Bis-POM-PMEA (25 μM) was added at indicated time intervals after administration of the IFN-γ/LPS cocktail (50 units/mL and 5 μg/mL, respectively) to mouse peritoneal macrophages. Concentration of nitrites in supernatants was determined at various points during the 24-h cultivation period. The data are the means ± SEM for triplicate setups.

(25 μM or 100 μM) did not reverse the NO-inhibitory effect of bis-POM-PMEA (Fig. 3).

In the above experiment, both IFN-γ/LPS and bis-POM-PMEA were added simultaneously. Delayed administration (1, 3, 5, 8, or 11 h) of bis-POM-PMEA resulted in gradually decreasing nitrite formation (Fig. 4). When added as late as 8 h following IFN-γ/LPS, bis-POM-PMEA was totally ineffective.

The IFN-γ/LPS cocktail was able to enhance accumulation of nitrites if present in cell cultures for 2 h only and then washed out. The efficacy of the washing procedure was checked by pulsing the cells with IFN-γ/LPS for a duration of 10 s; this treatment had no effect on nitrite production. The amount of nitrite formed during the consecutive 24 h was indistinguishable from that produced in the permanent (24 h) presence of IFN-γ/LPS (54.8 ± 1.2 μM and 58.8 ± 1.6 μM, respectively; means ± SEM). A small but statistically significant NO-suppressive effect of bis-POM-PMEA added immediately after IFN-γ/LPS removal was observed (25.7%, $p < 0.01$). The same results were obtained after the 5-h or 8-h IFN-γ/LPS preincubation (data not shown).

As demonstrated in Fig. 5A, formation of nitrites plus nitrates in lysates obtained from cells cultured 12 h in presence of bis-POM-PMEA (plus IFN-γ/LPS) was decreased in a concentration-dependent manner. The effect was in parallel with changes in concentration of nitrites accumulating in supernatants of these cells (Fig. 5B). Concomitant RT-PCR analysis showed considerably suppressed synthesis of iNOS mRNA, starting with a 2.5 μM concentration of bis-POM-PMEA (Fig. 6). No significant changes in cell viability were observed in this experiment (data not shown).

The parent compound PMEA, given mutually with IFN-γ/LPS, exhibited no inhibitory influence at the concentration of 1 mM (13.8%, $p > 0.5$). Higher concentrations tested, i.e. 2, 3, or 4 mM, were only moderately and uniformly inhibitory (35.8%, 36.3%, and 34.6%, respectively, $p < 0.01$) and had no cytocidal effects (data not shown).

DISCUSSION

The present data document that bis-POM-PMEA (*Adefovir Dipivoxil*), a bis(pivaloyloxymethyl)ester prodrug of the antiviral acyclic nucleoside phosphonate PMEA (*Adefovir*) [8, 9], interferes with *in vitro* production of nitrites in macrophages stimulated with IFN-γ and LPS. Biotransformation of L-arginine to NO and subsequently to nitrites is inhibited in a concentration-dependent manner by bis-POM-PMEA, the IC_{50} being 15 μM. The inhibition is only partially influenced by the cytolytic effects of this compound, since increased cell death occurs when approximately 80% inhibition of nitrite formation has already been achieved.

Not only is the inhibitory effect on nitrite formation dependent on the concentration of bis-POM-PMEA, it is also dependent on its timing with respect to the stimulus provided by IFN-γ/LPS. It is most pronounced when bis-POM-PMEA is given simultaneously with IFN-γ/LPS. Delayed addition of bis-POM-PMEA results in a gradually decreasing extent of inhibition; when given 8 h post-IFN-γ/LPS, the inhibitory potential of bis-POM-PMEA is not manifested at all. These data suggest that bis-POM-PMEA mainly interferes with very early events in the complex mechanism of L-arginine-dependent NO formation. With respect to the fact that it exerts only a marginal or no inhibitory effect on nitrite production already triggered by preincubation of macrophages with IFN-γ/LPS, we consider that the most important target for the inhibitory action is the transcription of iNOS. Indeed, we have demonstrated that the drug inhibits induction of iNOS protein in macrophages activated with IFN-γ and LPS. The results of RT-PCR analysis support the view that this effect clearly depends on reduced expression of iNOS mRNA.

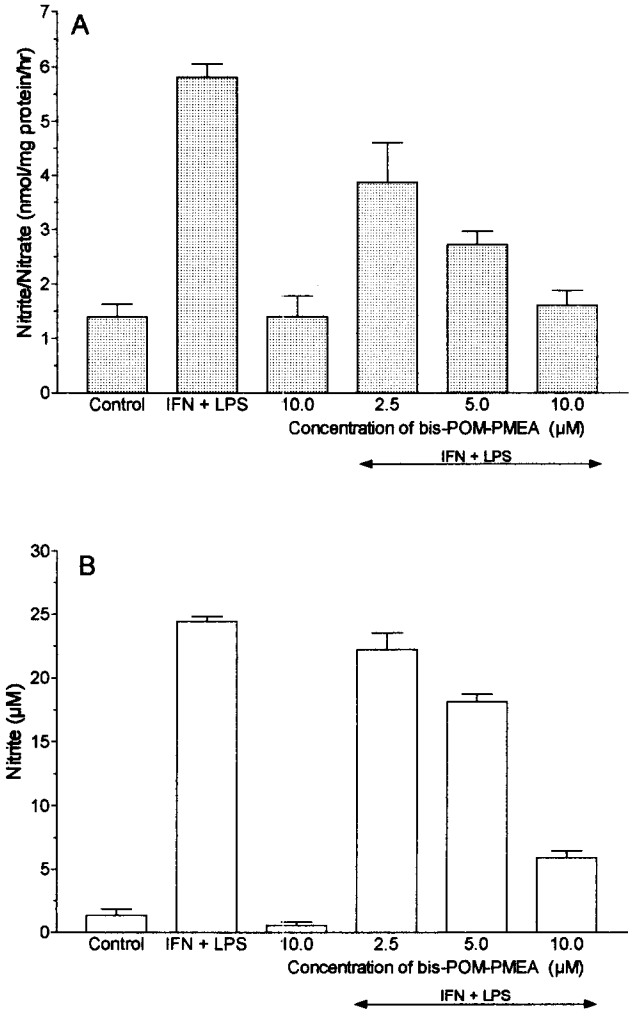

FIG. 5. The concentration-dependent effect of bis-POM-PMEA on formation of nitrites/nitrates by cell lysates containing the iNOS protein (A) and accumulation of nitrites in culture supernatants (B). Mouse peritoneal macrophages were cultured for 12 h in the presence of the IFN-γ/LPS cocktail (50 units/mL and 5 μg/mL, respectively), with or without bis-POM-PMEA. All assays, run in triplicate, were performed using the same cell cultures. The data are the means ± SEM representing two independent experiments.

Two other NO-inhibitory derivatives of nucleic acid components have been described in the literature. 2,4-Diamino-6-hydroxypyrimidine acts through inhibition of GTP cyclohydroxylase I and thus suppresses biosynthesis of tetrahydrobiopterin [25]. 2-Methylthio-ATP, a partial agonist of the P_{2Y} purinoceptor, inhibits NO production at the level of macrophage NOS gene expression [26]. No data are as yet available on the possible affinity of bis-POM-PMEA or other adenine-based acyclic nucleotide analogues to the purinoceptor system; however, our findings strongly argue against its interaction with biosynthesis of tetrahydrobiopterin, which is regarded as a key cofactor for NO production at the posttranscriptional level [27].

Due to the high anionic charge of the phosphonate moiety, the delivery of PMEA into cells, even at relatively high extracellular concentrations, is rather low [28, 29].

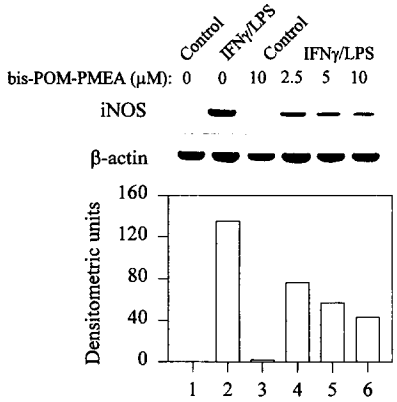

FIG. 6. Inhibitory effect of bis-POM-PMEA on iNOS mRNA induction. Mouse peritoneal macrophages were cultured for 12 h with or without the IFN-γ/LPS cocktail (50 units/mL and 5 μg/mL, respectively) and in the absence or presence of various concentrations of bis-POM-PMEA. Total RNA was isolated, and the levels of iNOS and β-actin mRNA were assessed by RT-PCR as described under "Materials and Methods." Ethidium bromide-stained DNA fragments generated by RT-PCR are shown in the upper part. Results of densitometric analysis of gels are presented as a histogram. The relative level of iNOS mRNA expression was determined after normalization to the respective β-actin signal to account for variability in the amount of RNA that had been extracted from cells. Lane designations are identical for the gels and histogram: 1, untreated controls; 2, IFN-γ/LPS-stimulated controls; 3, controls cultured with bis-POM-PMEA (10 μM/L) solely; and 4–6 cells stimulated with IFN-γ/LPS plus differing concentrations of bis-POM-PMEA (2.5 μM to 10 μM).

This obstacle is circumvented by the lipophilic properties of bis-POM-PMEA. After its uptake, bis-POM-PMEA is rapidly cleaved to PMEA [10, 30] and transformed into the diphosphate, which is considered to be a prerequisite for PMEA antiviral activity [31–33]. We found that the parent compound of bis-POM-PMEA, i.e. PMEA, was virtually ineffective in inhibiting NO (and resulting nitrite) production even at a concentration as high as 1 mM. The NO-inhibitory effect of bis-POM-PMEA seems thus unlikely to arise from enhanced intracellular concentration of PMEA *per se*. Our recent findings* suggest that the pivoxil moiety (or its decomposition products) of the PMEA prodrug is most probably responsible for inhibition of IFN-γ/LPS-generated macrophage NO production. Although biological relevance of this effect under *in vivo* conditions remains to be firmly established, the hitherto gathered data from phase I/II clinical trials with PMEA and bis-POM-PMEA [6–11, 34] indicate that the NO-inhibitory potential of bis-POM-PMEA has no serious bearing on the favorable antiretroviral (anti-AIDS) therapeutic efficacy of PMEA. The *in vitro* concentration of PMEA required to achieve 50% inhibition of HIV in monocytes/macrophages is lower by several orders of magnitude [35,

* Zídek Z and Holý A, Differences in biological effects of antiviral acyclic nucleotide analog 9-(2-phosphonomethoxyethyl)adenine (PMEA) and its pivoxil prodrug bis-POM-PMEA: cell proliferation and nitric oxide production. Submitted for publication.

36] than that of bis-POM-PMEA necessary for 50% inhibition of NO production.

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