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Nucleotide analogues with immunobiological properties: 9-[2-Hydroxy-3-(phosphonomethoxy)propyl]-adenine (HPMPA), -2,6-diaminopurine (HPMPDAP), and their N^6-substituted derivatives

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Abstract

Newly developed acyclic nucleoside phosphonates, derivatives of adenine and 2,6-diaminopurine bearing the 2-hydroxy-3-(phosphonomethoxy)propyl (HPMP) moiety at the N^9-side chain (i.e., HPMPA and HPMPDAP, respectively) were screened for in vitro immunobiological activity, using mouse resident peritoneal macrophages and splenocytes. Both HPMPA and HPMPDAP augmented the interferon-γ-triggered production of NO as well as expression of inducible nitric oxide synthase (iNOS) mRNA in macrophages. HPMPDAP activated secretion of tumor necrosis factor-α (TNF-α), chemokines "regulated-upon-activation, normal T cell expressed and secreted" (RANTES) and macrophage inflammatory protein-1α (MIP-1α), and marginally also secretion of interleukin-10 (IL-10) in both macrophages and splenocytes. The HPMPA, less prominently than HPMPDAP, elevated only secretion of RANTES and TNF-α. The compounds also activated secretion of TNF-α (HPMPDAP > HPMPA) in human peripheral blood mononuclear cells (PBMC). Distinct N^6-substituted derivatives, i.e., N^6-dimethyl-, N^6-cyclopropyl-, N^6-piperidin-1-yl-, N^6-(2-methoxyethyl)-, N^6-(2-hydroxyethyl)-, N^6-allyl- and N^6-2-(dimethylamino)ethyl-HPMPA/HPMPDAP as well as 6-thio and 6-hydroxy derivatives usually showed loss of the activity compared to the parent compounds. The immunomodulatory effects were found to be at least in part dependent on P_1 purinoreceptors, and mediated by transcriptional factor nuclear factor-κB.
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Keywords: Acyclic nucleotide analogues; HPMPA (9-[2-Hydroxy-3-(phosphonomethoxy)propyl]adenine); Immunostimulation; Nitric oxide; Cytokine

1. Introduction

Acyclic nucleoside phosphonates, notably derivatives of adenine, are being broadly used for treatment of virus diseases. The oral prodrug of 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA; adefovir) was approved by FDA for treatment of hepatitis B (Hepsera), and that of 9-(*R*)-[2-(phosphonomethoxy) propyl]adenine [(*R*)-PMPA; tenofovir] for treatment of acquired immunodeficiency syndrome (AIDS) (Viread). Another important representative of acyclic nucleoside phosphonates is 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (cidofovir) which was approved for treatment of cytomegalovirus retinitis in patients with AIDS (Rahhal et al., 1996). Several compounds of this distinct group of acyclic nucleoside phosphonates possess not only antiviral (Baba et al., 1987; De Clercq et al., 1986; Holý et al., 1990) but also antiparasitic (Botros et al., 2003; de Vries et al., 1991; Kaminsky et al., 1996) activities. In addition to the major mode of their antiviral action, i.e., the inhibition of virus-induced DNA polymerases (Kramata et al., 1996) or of reverse transcriptases (Crowe, 1999; Holý et al., 1990; Votruba et al., 1990), many of them are endowed with immunostimulatory potential. The anti-HIV effective (*R*)-PMPA activates secretion of several cytokines and augments the immune-triggered biosynthesis of virustatic molecule of nitric oxide (NO) (Zídek et al., 2001).

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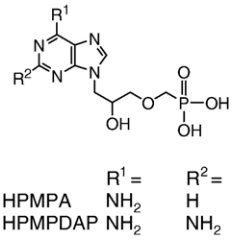
The immunobiological activities of acyclic phosphonates of heterocyclic bases such as adenine and 2,6-diaminopurine have been shown to be influenced by N^9-side chain moiety and N^6-substituents (Zídek et al., 2003). The present experiments were aimed at investigating possible immunobiological potential of closely related acyclic nucleoside phosphonates, namely of 9-[2-hydroxy-3-(phosphonomethoxy) propyl]adenine (HPMPA), its cognate 9-[2-hydroxy-3-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP), as well as their N^6-substituted derivatives. The compounds have originally been developed as potential antivirals. Therefore, we analyzed their effects on expression of several inducible factors implicated in antiviral immune defence such as chemokines, cytokines and NO. Special attention has been paid to β-chemokines "regulated-upon-activation, normal T cell expressed and secreted" (RANTES/CCL5) and macrophage inflammatory protein-1α (MIP-1α/CCL3), natural ligands of the chemokine receptor CCR5. The entry of HIV-1 into the cells of the immune system is primarily mediated by the CD4 receptor. However, chemokine receptors, called HIV entry co-receptors, are needed to ensure a productive infection (Deng et al., 1996; Dimitrov et al., 1999; Xiao et al., 1999). Blocking the appropriate β-chemokine receptors on both macrophages and lymphocytes is thus considered a prospective therapeutic approach against HIV (Arenzana-Seisdedos et al., 1996; Simmons et al., 1997; Ylisastigui et al., 1998). Another factor with prominent antiviral activity is NO (Karupiah and Harris, 1997) which inhibits replication of many viruses including Poxviridae, Herpetoviridae, Rhabdoviridae, Retroviridae, and Parvoviridae, e.g., hepatitis B virus (Guidotti et al., 2000), cytomegalovirus (Bodaghi et al., 1999), Epstein–Barr virus (Gao et al., 1999), vaccinia virus, ectromelia virus (Nathan and Hibbs, 1991), and HIV (Hori et al., 1999; Persichini et al., 1999). Biosynthesis of NO is tightly regulated by a number of cytokines. The major upregulatory signal for high-output NO production by immune-primed macrophages is provided by TNF-α (Ding et al., 1988; Drapier et al., 1988; Oswald et al., 1992). Numerous data show that IL-10 has an opposite function in this respect (Liew et al., 1991; Oswald et al., 1992). In addition, both cytokines, especially TNF-α (Wong et al., 1992), but also IL-10 (Mosmann, 1994; Nishio et al., 1999), possess antiviral activities.

2. Materials and methods

2.1. Acyclic nucleoside phosphonates

Acyclic phosphonates of heterocyclic bases, i.e., adenine and 2,6-diaminopurine containing [2-hydroxy-3-(phosphonomethoxy)propyl] moiety at the N^9 position, were synthesized in-house (Institute of Organic Chemistry and Biochemistry) according to the procedures described elsewhere (Kreèmerová et al., 2004). Included in the study were two parent congener nucleotide analogues, which are not substituted at the N^6-amino group: 9-[2-hydroxy-3-(phosphonomethoxy)propyl]adenine (HPMPA), and 9-[2-hydroxy-3-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP) and their 6-substituted derivatives represented by a) dimethylamino, b) cyclopropylamino, c) piperidin-1-yl, d) (2-methoxyethyl)amino, e) (2-hydroxyethyl) amino, f) SH, g) allylamino, h) 2-(dimethylamino)ethylamino, and i) OH. The chemical structure of compounds is shown in Fig. 1.



	R^1 =	R^2 =
HPMPA	NH_2	H
HPMPDAP	NH_2	NH_2

Fig. 1. Chemical structure of HPMPA and HPMPDAP and their derivatives bearing the following substituents (R^1) in position 6: a) dimethylamino, b) cyclopropylamino, c) piperidin-1-yl, d) (2-methoxyethyl)amino, e) (2-hydroxyethyl)amino, f) SH, g) allylamino, h) 2-(dimethylamino)ethylamino, and i) OH.

Stock solutions of acyclic nucleoside phosphonates (5 mM) were prepared in incomplete $NaHCO_3$-containing, phenol red-free RPMI-1640 medium (Sigma-Aldrich, Praha, Czech Republic). They were sterile filtered using non-pyrogenic 0.22 μm filters (Costar, Cambridge, MA), used fresh or kept no longer than 8 weeks at −20 °C. Required working concentrations were prepared by diluting the stock solution in complete RPMI-1640 culture medium (described below). The pilot study was done using 100-μM drug concentration.

The chromogenic Limulus Amoebocyte Lysate assay (Kinetic-QCL; Cambrex Bio Science, Walkersville, MD) was used to check for possible contamination with lipopolysaccharide (LPS). The highest 100-μM concentrations of test compounds contained <10 pg/ml of LPS, an amount that is virtually ineffective to activate secretion of cytokines (Zídek et al., 2003).

2.2. Antagonists of adenosine receptors

The following nonspecific and specific antagonists of adenosine receptors (Sigma-Aldrich) have been used: nonspecific antagonists of adenosine $A_{1/2A/2B/3}$ receptors 9-chloro-2-(2-furanyl)-[1,2,4]triazolol[1,5-c]quinazolin-5-amine (CGS-15943), and 8-[4-[[[[2-aminoethyl]amino]carbonyl]methyl] oxy]phenyl]-1,3-dipropylxanthine (XAC); adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DCCPX); adenosine A_{2A} receptor antagonist 8-(3-chlorostyryl)caffeine (CSC); adenosine A_{2B} receptor antagonist benzo[g]pteridine-2,4(1*H*,3*H*)-dione (Alloxazine); adenosine A_3 receptor antagonist 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)dihydropyridine-3,5-dicarboxylate (MRS-1191).

Stock solutions of adenosine receptor antagonists were prepared in dimethylsulphoxide DMSO (Sigma-Aldrich). After appropriate diluting in culture medium, the antagonists were added to the cell cultures 20 min prior to administration of test acyclic nucleoside phosphonates. The effect of the appropriate solutions of DMSO had no effect on the responses studied.

2.3. Animals; isolation and cultivation of macrophages and lymphocytes

Female mice of the inbred strain C57BL/6, 8–10 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 on 6 to 18 h, temperature at 22 °C.

Animals, killed by cervical dislocation, were i.p. injected with 8 ml of sterile saline. Pooled peritoneal cells collected from mice (n=4–6 in individual experiments) were washed, resuspended in culture medium, and seeded into 96-well round-bottom microplates (Costar) in 100-μl volumes, 2×10^5 cells/well. Adherent cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5% CO_2, and then vigorously shaking the plate and washing the wells three times to remove non-adherent cells. Cultures were maintained at 37 °C, 5% CO_2 in humidified Heraeus incubator.

Single-cell suspension of splenocytes was prepared by passing the fragmented pooled spleens of mice through a fine nylon sieve. Erythrocytes were removed by means of red blood cell lysing buffer (Sigma-Aldrich) containing 0.80% ammonium chloride in 0.01 M Tris–HCl, pH 7.5. After thorough washing (twice in phosphate-buffered saline, once in incomplete RPMI-1640 medium), the cells were re-suspended in complete RPMI-1640 medium and seeded in 96-well U-bottom cell culture plates (Costar). The number of cells was 5×10^5/well in final 100 μl. They were cultured in Heraeus incubator for 24 h (37 °C, 5% CO_2, 100% relative humidity).

Complete RPMI-1640 culture medium (Sigma-Aldrich) contained 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma).

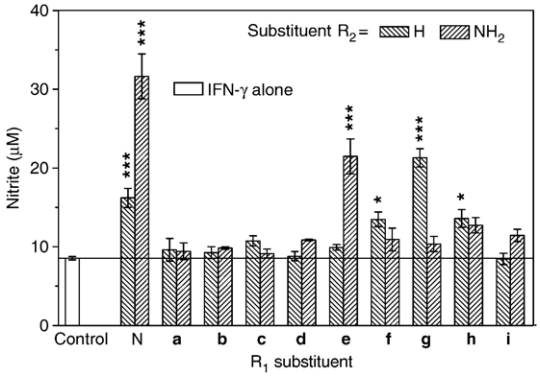


Fig. 2. Production of nitric oxide. Murine peritoneal macrophages were cultured (2×10^6/ml) for 24 h in the presence of test compounds (100 μM) and concomitantly applied interferon-γ (5000 pg/ml). The supernatant concentration of nitrite was determined using Griess reagent. Each bar is a mean±S.E.M. for duplicate culture wells. The results are representative of three identical experiments. N=non-substituted parent compounds. R^1 substituents of parent compounds: a, dimethylamino; b, cyclopropylamino; c, piperidin-1-yl; d, (2-methoxyethyl)amino; e, (2-hydroxyethyl)amino; f, SH; g, allylamino; h, 2-(dimethylamino)ethylamino; i, OH. ***, * statistically significant differences against the value of IFN-γ alone at $P<0.001$, and $P<0.05$, respectively.

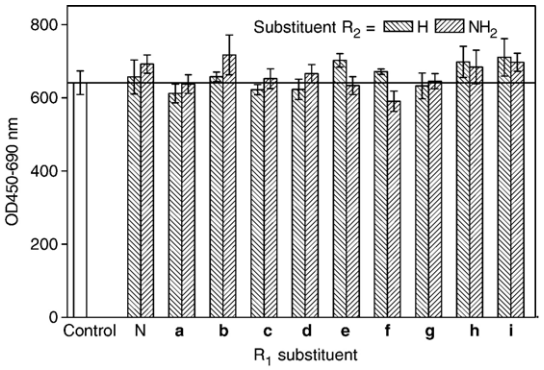


Fig. 3. Cleavage of tetrazolium salt (see Materials and methods for details) indicating viability of macrophages cultured 24 h in the presence of acyclic nucleoside phosphonates (100 μM). No significant differences were observed as compared to the viability of control cells. The bars are means±S.E.M.

All protocols were approved by the institutional ethics committee.

2.4. Isolation of human peripheral blood mononuclear cells (PBMC)

The sources of cells were buffy coats acquired from healthy donors (provided by the Institute of Hematology and Blood Transfusion, Prague, CZ). PBMC were separated by Ficoll–Paque gradient centrifugation (Amersham Biosciences) according to the manufacturer's instructions. They were cultured 22 h at a final density of 1.5×10^6 cells/ml in complete RPMI-1640 medium (250 μl per well).

2.5. Nitric oxide assay

Murine peritoneal macrophages were cultured 24 h in the presence of test compounds, applied either alone or in the presence of NO-priming immune stimuli, i.e., murine recombinant interferon-γ (IFN-γ, 5000 pg/ml; R&D Systems, Minneapolis, MN) or lipopolysaccharide (LPS from *E. coli* 0111:B4, 100 pg/ml; Sigma). The concentration of nitrites in supernatants of cells was taken as a measure of NO production (Marletta et al., 1988). It was detected in individual, cell-free samples (50 μl) incubated 5 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanilamide/0.1% naphtylendiamine/2.5% H_3PO_4). 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.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) for mouse inducible NO synthase (iNOS)

The expression of mouse iNOS was analyzed by a semiquantitative RT-PCR. Total RNA was isolated from 4×10^6 macrophages per sample using the RNeasy mini kit and RNAse free DNAse I set (Qiagen, Hilden, Germany). The yield and purity of RNA were quantitated by measuring the ratio of the optical density at 260 and 280 nm. 0.4 μg

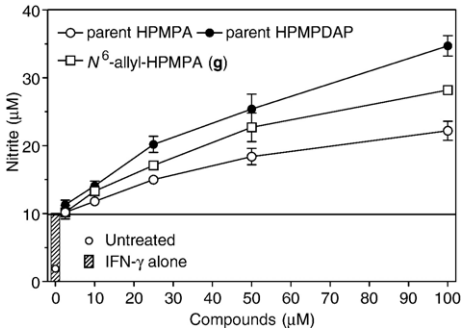


Fig. 4. Dose-dependent enhancement of NO production. Murine peritoneal macrophages were cultured (2×10^6/ml) for 24 h in the presence of increasing concentration of acyclic nucleoside phosphonates, together with interferon-γ (IFN-γ, 5000 pg/ml). The supernatant concentration of nitrite was determined using Griess reagent. Each point is a mean±S.E.M. for duplicate culture wells. The results are representative of two identical experiments.

of total RNA was reverse transcribed to complementary DNA using random nonamers and recombinant Moloney murine leukemia virus reverse transcriptase in a total reaction volume of 20 μl in the presence of RNAse inhibitor. One fifth of resulting cDNA was then amplified by PCR. Primers for mouse iNOS were: sense 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, antisense 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′. The PCR reaction mix contained the forward and reverse primers (0.15 μM each), dNTPs (0.2 mM each), 10 mM Tris–HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase and 4 μl of cDNA in a total reaction volume of 50 μl. As a control, cDNA was also amplified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) commercially available primers (Clontech, Palo Alto, CA, USA). PCR chemicals were obtained from Top-Bio (Prague, Czech Republic). After initial denaturation (2 min, 94 °C), 28 amplification cycles were performed using a thermocycler Mastercycler gradient (Eppendorf, Hamburg, Germany). PCR was performed in a linear range. One cycle consisted of denaturation at 94 °C for 45 s, annealing at 61 °C for 45 s and extension at 72 °C for 45 s. After the last cycle, final extension at 72 °C for 7 min was carried out. The amplified DNA size was 450 bp for GAPDH and 496 bp for iNOS.

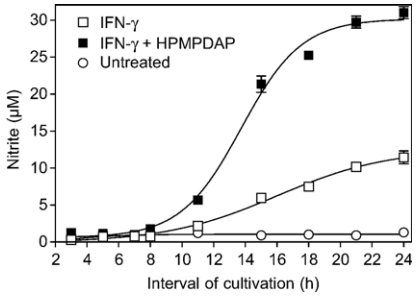


Fig. 5. Dynamics of NO production. Murine peritoneal macrophages were cultured (2×10^6/ml) for indicated time intervals in the presence of interferon-γ alone (IFN-γ, 5000 pg/ml) or altogether with acyclic nucleoside phosphonate HPMPDAP, i.e., 9-[2-hydroxy-3-(phosphonomethoxy)propyl]-diaminopurine (100 μM). The supernatant concentration of nitrite was determined using Griess reagent. Each point is a mean±S.E.M. for duplicate culture wells. The results are representative of two identical experiments.

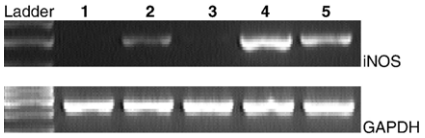


Fig. 6. Macrophage iNOS mRNA expression. Mouse macrophages (4×10^6 per well) were cultured for 3 h. Then the RNA was extracted and analyzed by RT-PCR. Lane 1: untreated cells. Lane 2: cells treated with IFN-γ, 5000 pg/ml. Lane 3: cells treated with HPMPDAP, i.e., 9-[2-hydroxy-3-(phosphonomethoxy) propyl]-diaminopurine, 100 μM. Lane 4: cells treated with IFN-γ plus HPMPDAP. Lane 5: cells treated with combination of IFN-γ, HPMPDAP and nonspecific adenosine A_1 receptor antagonist CPX (40 μM).

PCR products of predicted size were identified by electrophoresis on 1.5% agarose gel containing ethidium bromide. The gels were photographed in UV light.

2.7. Cytokine assays

Concentration of cytokines and chemokines (pg/ml) was assayed in supernatants of cells cultured for the interval of 5 h successive to addition of test acyclic nucleoside phosphonates. It was determined by enzyme-linked immunoabsorbent assay (ELISA) kits, following the manufacturer's instructions (R&D Systems, Minneapolis, MN). The effects of acyclic nucleoside phosphonates were compared with the effects of LPS applied at doses of 10^1 to 10^6 pg/ml.

2.8. Determination of cell viability

Viability of macrophages was determined using a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche Diagnostics, Mannheim, Germany). The cells were cultured as described above. After the 24-h culture, the WST-1 was added and the cells were kept in the incubator (37 °C) for additional 3 h. Optical density at 450–690 nm was evaluated.

2.9. Statistical analysis of data

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

3. Results

3.1. Augmented NO production

The parent compounds, i.e., HPMPA and HPMPDAP, upregulated the IFN-γ-triggered production of NO by murine macrophages (Fig. 2). The effect was more prominent with HPMPDAP than with HPMPA ($P<0.001$). Their derivatives substituted at the position 6 by dimethylamino (a), cyclopropylamino (b), piperidin-1-yl (c), (2-methoxyethyl) amino (d), and hydroxy (i) groups were devoid of the NO-enhancing potential. The effects of other substituents depended on the type of the heterocyclic base. Whereas

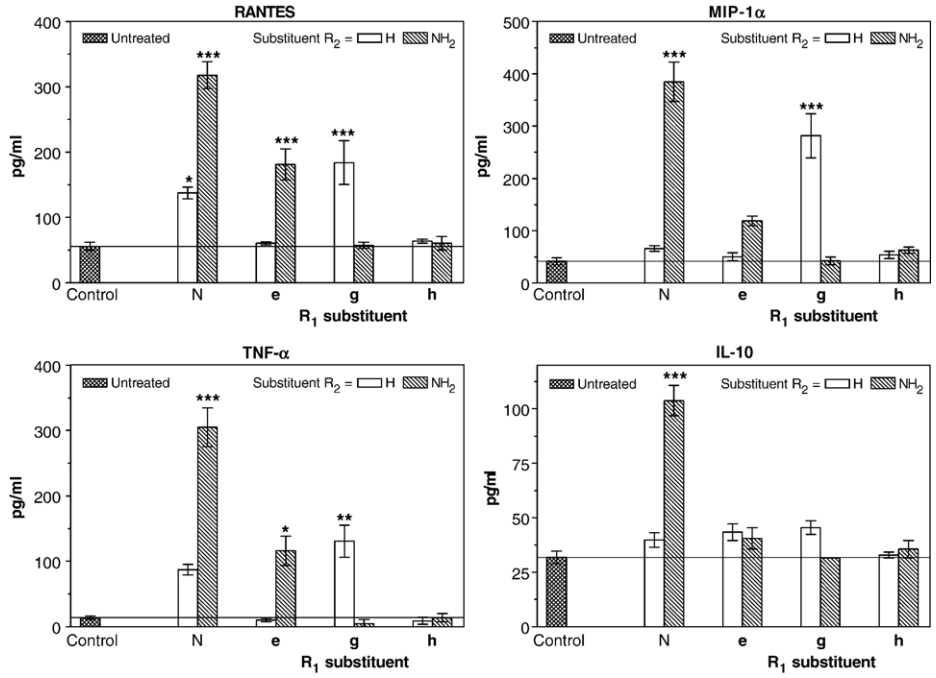


Fig. 7. Cytokine stimulatory activity of test compounds. Murine macrophages were cultured (2×10^6/ml) in the presence of acyclic nucleoside phosphonates solely (100 μM). Supernatant concentration of cytokines was evaluated using ELISA after the 5-h culture. Each bar is a mean ± S.E.M. for duplicate culture wells. The results are representative of two identical experiments. N = non-substituted parent compounds. R^1 = substituents in position 6 of parent compounds: e, (2-hydroxyethyl)amino; g, allylamino; h, 2-(dimethylamino)ethylamino. ***, * statistically significant differences against the value of untreated cells at $P<0.001$, and $P<0.05$, respectively.

the substitution by 6-(2-hydroxyethyl)amino group (e) led to the complete loss of biological activity of HPMPA, it only partially though statistically significantly ($P<0.001$) decreased the remarkable stimulatory potential of HPMPDAP. Derivatives with 6-SH (f), allylamino (g), and 2-(dimethylamino)ethylamino (h) substituents of HPMPDAP were completely ineffective to influence NO production. In contrast, they retained the stimulatory biological activity of parent HPMPA. The effect of N^6-allyl-HPMPA was even slightly higher ($P<0.05$) than that of the HPMPA itself.

All compounds were ineffective to stimulate NO production on their own. Similarly, they did not influence NO production activated primarily by LPS (data not shown).

3.2. Cell viability assay

No changes between control, i.e., untreated cells and those cultured 24 h in the presence of acyclic nucleoside phosphonates (100 μM), were observed in their ability to cleave the tetrazolium salt, irrespective of their ability/inability to augment NO formation (Fig. 3). We found previously that the final concentrations of adenosine receptor antagonists had no negative influence on cell viability (Kmoníèková et al., 2006).

3.3. Dynamics of NO production and expression of iNOS mRNA

The effects of active compounds were dose-dependent (Fig. 4). The differences against the effect of IFN-γ alone began to be apparent with the 10-μM concentration and turned out to be statistically significant with the concentration of 25 μM ($P<0.01$). The onset of enhanced NO biosynthesis was observed at the interval of approximately 11 h following addition of IFN-γ. Accumulation of nitrites in supernatants grew steadily towards the interval of 24 h, and it was substantially accelerated in the presence of the test acyclic nucleoside phosphonate HPMPDAP (Fig. 5).

Expression of iNOS mRNA, analyzed at the 3-h interval of culture, was undetectable in control macrophages and in those treated with HPMPDAP alone. It was increased by IFN-γ and further enhanced by co-treatment with HPMPDAP (Fig. 6).

3.4. Activated cytokine secretion

The compounds which augmented biosynthesis of NO were further screened for ability to stimulate secretion of cytokines by mouse peritoneal macrophages (Fig. 7). The most effective compound was non-substituted HPMPDAP. It stimulated secretion of all TNF-α, IL-10, RANTES, and MIP-1α ($P<0.001$, in all cases). The parent HPMPA stimulated RANTES ($P<0.05$) and marginally TNF-α. Out of the N^6-substituted HPMPA derivatives, it was solely the N^6-allyl substituent (g) that significantly elevated production of all TNF-α, RANTES and MIP-1α (not IL-10). The N^6-(2-hydroxyethyl) substituent (e) remained only effective in combination with HPMPDAP, notably in enhancing secretion of TNF-α and RANTES, but not secretion of IL-10 and MIP-1α.

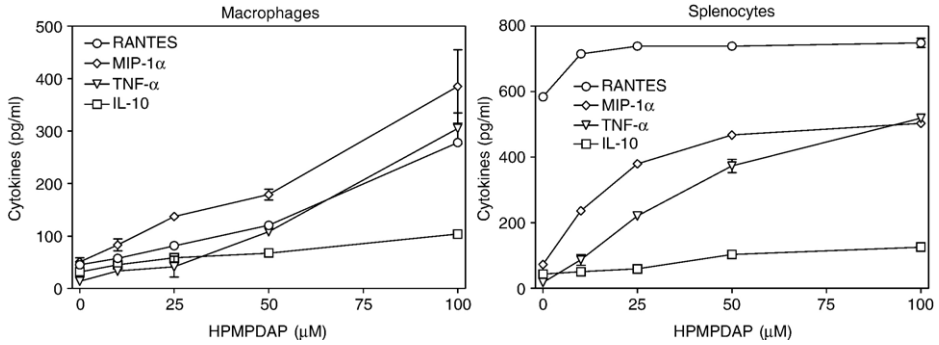


Fig. 8. Dose-dependent activation of cytokine secretion by murine peritoneal macrophages and splenocytes. The cells were cultured for 5 h in the presence of varying concentrations of HPMPDAP, i.e., 9-[2-hydroxy-3-(phosphonomethoxy)propyl]-diaminopurine. Supernatant concentration of cytokines was evaluated using ELISA. Each point is a mean±S.E.M. for duplicate culture wells. The results are representative of two identical experiments.

The cytokine stimulatory effects of HPMPDAP were dose-dependently expressed in both macrophages and lymphocytes (Fig. 8). The dose for a common immunostimulatory agent LPS which was needed to induce effects equipotent to the effects of 100-μM HPMPDAP was approximately 1–10 ng/ml (Fig. 9).

HPMPDAP dose-dependently activated secretion of TNF-α also in human PBMC. The effects of HPMPA were much less apparent (Fig. 10).

3.5. Dependence of immunobiological effects on transcriptional factor NF-κB

Addition of pyrrolidine dithiocarbamate (PDTC; Sigma), an inhibitor of transcriptional factor nuclear factor-κB (NF-κB) (Schreck et al., 1992), led to a dose-dependent suppression of NO production stimulated by IFN-γ alone (5000 pg/ml) as well as by combination of IFN-γ plus acyclic nucleoside phosphonate HPMPDAP (100 μM) (Fig. 11). The IC_{50} for IFN-γ-induced NO was 20.6 μM (11.6–26.4; 95% limits of confidence), and 19.0 (17.6–20.5) μM for IFN-γ/HPMPDAP-induced NO production. Complete inhibition of NO biosynthesis was reached at the 40-μM concentration of PDTC.

Similarly, secretion of TNF-α was dose-dependently inhibited by PDTC (Fig. 11), the estimate of IC_{50} being 15.8 μM (8.9–28.1 μM, 95% limits of confidence).

3.6. Dependence of immunobiological effects on adenosine receptors

The NO-enhancing activity of acyclic nucleoside phosphonate HPMPDAP was inhibited by a specific antagonist of A_1 adenosine receptor CPX, and by nonspecific antagonists of A_{1-3} adenosine receptors CGS-15934 and XAC (Fig. 12). The inhibitory potential of these antagonists was very similar: the IC_{50}s (95% limits of confidence in parentheses) reached the values of 8.8 (5.5–14.2) μM, 8.6 (5.6–13.2) μM and 13.7 (7.7–24.3) μM, respectively. Remarkably less pronounced inhibition was also observed with adenosine A_{2A} receptor antagonist CSC; significantly decreased NO production ($P<0.01$) was found with 20 μM concentration and remained about the same (approximately 20%) despite of an increasing dosing. Both specific adenosine A_{2B} and A_3 receptor antagonists alloxazine and MRS-1191, respectively, were ineffective to influence the NO response.

The same profile of NO inhibition by adenosine receptor antagonists was found for parent HPMPA (data not shown).

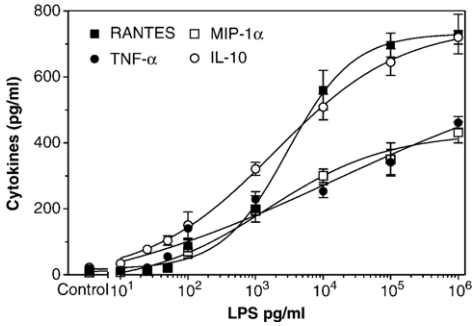


Fig. 9. Secretion of cytokines by murine macrophages cultured (2×10^6/ml) for 5 h in the presence of varying concentrations of lipopolysaccharide (LPS). Supernatant concentration of cytokines was determined using ELISA. Each bar is a mean±S.E.M. for two independent experiments done in duplicate culture wells.

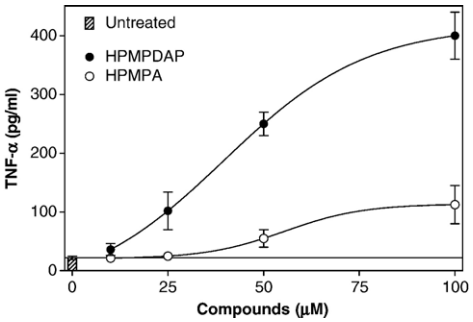


Fig. 10. Secretion of TNF-α by human PBMC (1×10^6/ml) cultured for 5 h in the presence of varying concentrations of acyclic nucleoside phosphonates HPMPA and HPMPDAP. Supernatant concentration of the cytokine was determined using ELISA. Each point is a mean±S.E.M. for duplicate culture wells. The results are representative of two identical experiments.

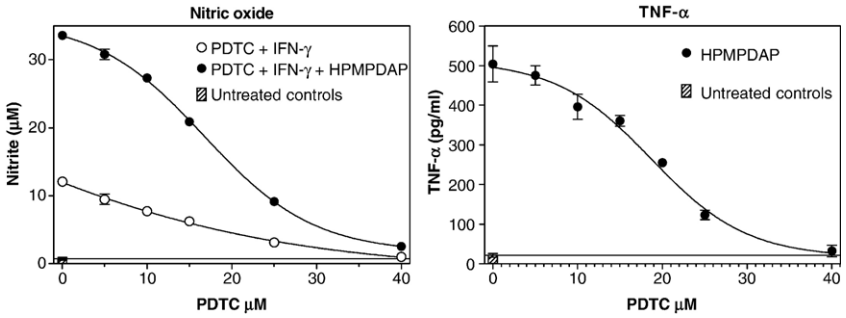


Fig. 11. Inhibitory effects of the inhibitor of NF-κB, pyrrolidine dithiocarbamate (PDTC), on expression of immunostimulatory activity of acyclic nucleoside phosphonate HPMPDAP, i.e., 9-[2-hydroxy-3-(phosphonomethoxy)propyl]-diaminopurine (100 μM). Murine peritoneal macrophages (2×10^6/ml) were cultured either for 24 h (NO assay) or 5 h (TNF-α assay) in the presence of test compounds. PDTC was added 10 min before HPMPDAP. The points represent means ± S.E.M.

The NO-stimulatory effect of IFN-γ alone remained unaltered by adenosine receptor antagonists (data not shown).

Also the expression of iNOS mRNA induced by co-treatment of macrophages with HPMPDAP was suppressed by adenosine A_1 receptor antagonist CPX (Fig. 6).

4. Discussion

Our previous experiments demonstrated immunobiological potential of acyclic phosphonates of adenine and 2,6-diaminopurine containing 2-(phosphonomethoxy)propyl moiety (PMPA and PMPDAP, respectively) at the N^9-side chain (Zídek et al., 2003). The present data extend this knowledge in showing that also acyclic nucleoside phosphonates with hydroxylated PMP side chain, i.e., with N^9-[2-hydroxy-3-(phosphonomethoxy) propyl] (HPMP) moiety, possess immunostimulatory activity. Notably, production of NO and secretion of cytokines TNF-α, IL-10, and of β-chemokines RANTES/CCL5 and MIP-1α/CCL3 were investigated. Both parent acyclic nucleoside phosphonates, i.e., HPMPA and HPMPDAP, dose-dependently augmented the IFN-γ-triggered production of NO. In principle, the time dynamics of NO production after combination of IFN-γ/HPMPDAP was very similar to that observed after IFN-γ

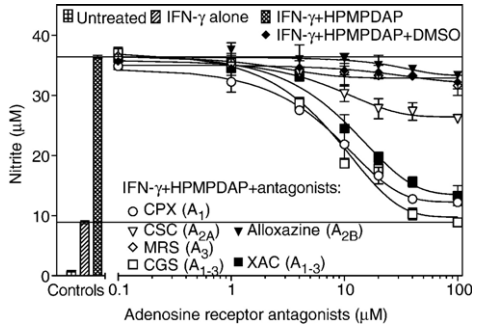


Fig. 12. Influence of purinoreceptor antagonists on production of NO by murine peritoneal macrophages (2×10^6/ml) stimulated primarily with IFN-γ (5000 pg/ml) and co-stimulated with acyclic nucleoside phosphonate HPMPDAP, i.e., 9-[2-hydroxy-3-(phosphonomethoxy)propyl]-diaminopurine (100 μM). The adenosine receptor antagonists were applied 15 min before HPMPDAP. The cells were cultured 24 h in triplicate wells. The supernatant concentration of nitrite was determined using Griess reagent. Production of NO stimulated by IFN-γ alone was not influenced (not depicted). The values are means ± S.E.M. The results are representative of two identical experiments.

alone, but it was greatly accelerated. The RT-PCR study suggests that the underlying mechanism for this effect is a substantially enhanced expression of iNOS mRNA. HPMPA and HPMPDAP also activated, without the aid of IFN-γ or any other cytokine co-activator, secretion of TNF-α and RANTES. The effects of HPMPDAP were more prominent than the effects of HPMPA. In addition, HPMPDAP (not HPMPA) was an activator of MIP-1α and to a lesser extent of IL-10. In this respect, the effects of HPMPA are similar to the immunostimulatory effects of (*R*)-PMPA (Zídek et al., 2001). On the other hand, while both (*R*)- and (*S*)-PMPDAP were found to be biologically virtually inactive (Zídek et al., 2003), HPMPDAP in this study was remarkably effective to stimulate NO and cytokine production. Significant cytokine stimulatory activity also was expressed by N^6-allyl-HPMPA, which is more potent compared to the parent HPMPA. Other analogues, including HPMPA itself, were less effective. Similar to the N^6-(2-hydroxyethyl)-HPMPDAP, HPMPA only significantly enhanced secretion of RANTES and TNF-α, but not of MIP-1α and IL-10. The immunostimulatory effectiveness of HPMPDAP may be considered less pronounced than is the effectiveness of some other types of acyclic nucleoside phosphonates (Zídek et al., 2003). However, the magnitude of the effects of HPMPDAP (100 μM) on cytokine expression (except IL-10) was equal or more pronounced than that of the effects of recognized immunostimulator LPS at the concentration of 1–10 ng/ml. Needless to say that all test acyclic nucleoside phosphonates proved to be devoid of LPS contamination.

Possible immunostimulatory effects of acyclic nucleoside phosphonates were also ascertained in human cell in vitro system. HPMPDAP was found to activate secretion of TNF-α in PBMC. Similar to the influence on mouse macrophages and lymphocytes, HPMPA was less effective than HPMPDAP to enhance TNF-α expression.

The immunobiological potential of (*R*)-enantiomers (interestingly, not (*S*)-enantiomers) of both PMPA and PMPDAP analogues can be either suppressed or enhanced by distinct substituents at the N^6-site of the heterocyclic base (Zídek et al., 2001). In general, the same principle applies for HPMPA and HPMPDAP derivatives. Introduction of dimethylamino, cyclopropylamino, piperidin-1-yl, (2-methoxyethyl)amino, SH, and OH groups at the position 6 of these compounds completely abrogated or substantially attenuated the NO-

enhancing capability of both HPMPA and HPMPDAP. The lack of NO-modulatory influence of the (2-hydroxyethyl) amino substituent was related to HPMPA structure, whereas that of allylamino and 2-(dimethylamino)ethylamino groups was related to HPMPDAP derivatives solely. The effects of N^6-allyl-HPMPA and N^6-[2-(dimethylamino)ethyl]-HPMPA were virtually indistinguishable from the effects of parent HPMPA. None of the test acyclic nucleoside phosphonates inhibited the intrinsic NO-stimulatory activity of IFN-γ, suggesting thus that they are not cytotoxic. The lack of possible cytocidal activity was further affirmed by the finding of no interference with the cleavage of tetrazolium salt.

Ability of acyclic nucleoside phosphonates to stimulate secretion of cytokines on their own suggests that they are activators of certain transcription factors undermining expression of immunostimulatory effects. We have addressed possible participation of one of them, NF-κB, which is believed to be a key element for the induction of iNOS in response to various stimuli (Kleinert et al., 1996; Xie et al., 1994). It also plays a central role in activation of TNF gene expression (Shakhov et al., 1990; Scheinman et al., 1995) while its involvement in expression of chemokines may depend on the type of immune stimuli (Ciesielski et al., 2002). In contrast to TNF-α gene, NF-κB does not seem to play a central role in the activation of IL-10 gene (Marchant et al., 1996). In our experiments, production of NO and secretion of TNF-α were inhibited by the inhibitor of NF-κB activation, PDTC. It can be therefore presumed that the immune-active acyclic nucleoside phosphonates are activators of NF-κB signaling pathway. The fact that NF-κB is necessary but at least in some cases not sufficient for transcriptional induction of TNF-α (Albrecht et al., 1995) and iNOS (Flodström et al., 1996) brings about a question whether and what other signalling pathways could be utilized by acyclic nucleoside phosphonates. This problem remains to be elucidated.

We found that the ability of HPMPDAP to augment the IFN-γ-triggered production of NO can be substantially suppressed by antagonist of adenosine A_1 receptors and less prominently by antagonist of adenosine A_{2A} receptors. The underlying mechanism for their inhibitory mode of action obviously is a negative interference with expression of iNOS mRNA that was detected as early as 3 h of macrophage cultivation. A plausible explanation for this effect is the inhibition of acyclic nucleoside phosphonates-induced secretion of NO-upregulatory cytokines, e.g., TNF-α, which was observed in our recent experiments (Kmoníèková et al., 2006). TNF-α is considered to be the major cytokine synergizing with IFN-γ for NO production (Bogdan et al., 1994). It may be supposed, therefore, that at least some of the interactions of N^9-[2-hydroxy-3-(phosphonomethoxy)propyl] derivatives of adenine and 2,6-diaminopurine with immune functions of macrophages depend on P_1 purinoreceptor system. The same receptor system dependence has been confirmed for PMPA and PMPDAP, i.e., N^9-[2-(phosphonomethoxy)propyl] derivatives of adenine and 2,6-diaminopurine to enhance NO production (Zídek et al., 2004).

The present data introduce a novel class of immunomodulatory agents, i.e., acyclic phosphonates of adenine and 2,6-diaminopurine bearing the [2-hydroxy-3-(phosphonomethoxy) propyl] moiety at the N^9 position. The strongest activity is expressed by parent HPMPDAP. This compound is also effective in human PBMC, at least to stimulate the TNF-α secretion. From the mechanistic point of view, the effects resemble those possessed by a related family of acyclic nucleoside phosphonates with the [2-(phosphonomethoxy) propyl] N^9-side chain moiety.

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