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Immunobiological activity of *N*-[2-(phosphonomethoxy)alkyl] derivatives of N^6-substituted adenines, and 2,6-diaminopurines

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Abstract

Acyclic nucleoside phosphonates are novel class of virostatics effective against replication of both DNA-viruses and retroviruses. We found recently, that in addition to the antimetabolic mode of action, some acyclic nucleoside phosphonates such as 9-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPA; *tenofovir*], which is used in treatment of human immunodeficiency virus (HIV) infection, possess immunostimulatory and immunomodulatory activities known to interfere with replication of viruses. The present experiments analyzed immunobiological effects of more than 70 novel derivatives of acyclic nucleoside phosphonates. They comprise substitutions at the N^6-amino function of adenine (A) or 2,6-diaminopurine (DAP) by monoalkyl, dialkyl, cycloalkyl, alkenyl, alkynyl or substituted alkyl group, and at the N^9-side chain represented by (*R*)- or (*S*)-enantiomeric 9-[2-(phosphonomethoxy)ethyl] (PME) and 9-[2-(phosphonomethoxy)propyl] (PMP) moieties. Their biological effects were investigated in vitro using mouse resident peritoneal macrophages. A number of the compounds under scrutiny, mainly the N^6-cycloalkyl derivatives of 9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine (PMEDAP) and (*R*)-enantiomeric 9-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPDAP] stimulate secretion of cytokines [tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10)] and chemokines [''regulated-upon-activation, normal T expressed and secreted'' (RANTES), macrophage inflammatory protein-1α (MIP-1α)]. Moreover, they substantially augment production of nitric oxide (NO) triggered by interferon-γ. The effects are produced in a dose-dependent fashion. The most potent derivatives, i.e. N^6-isobutyl-PMEDAP, N^6-cyclopentyl-PMEDAP, N^6-cyclooctyl-PMEDAP, N^6-dimethylaminoethyl-(*R*)-PMPDAP, N^6-cyclopropyl-(*R*)-PMPDAP, and N^6-cyclopentyl-(*R*)-PMPDAP are more effective than (*R*)-PMPA (*tenofovir*) itself. They exhibit immunostimulatory effects at concentrations as low as 1 to 5 μM. It is suggested that these compounds might be prospective candidates for antiviral therapeutic exploitation.

Keywords: Acyclic nucleoside phosphonate; Antiviral; Chemokine; Cytokine; Nitric oxide (NO)

1. Introduction

Acyclic nucleoside phosphonates are recognised class of novel compounds used in therapy of viral infections. They are effective against replication of both DNA-viruses and retroviruses, encompassing herpes simplex virus-1 and -2 (Yang and Datema, 1991), cytomegalovirus (Lalezari et al., 1995), varicella zoster virus (De Clercq, 1996), Epstein–Barr virus (Lin et al., 1991), human herpes virus types 6, 7 and 8 (Reymen et al., 1995), human papilloma virus (De Clercq, 1996), Moloney sarcoma virus (Balzarini et al., 1993), hepatitis B virus (Perrillo et al., 2000), visna virus (Thormar et al., 1995), Friend leukaemia virus (Naesens et al., 1994), and immunodeficiency viruses (Collier et al., 1993; Tsai et al., 1995). The oral prodrugs of the prototype compounds, i.e. 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA; *adefovir*), and 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPA; *tenofovir*] were approved by FDA for treatment of hepatitis B (Hepsera), and acquired immunodeficiency syndrome (AIDS) (Viread), respectively. 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 AIDS patients (Rahhal et al., 1996). However, it is active against all DNA viruses, including papilloma viruses (Bielamowicz et al., 2002; Calista, 2000) and poxviruses (De Clercq, 2002; Snoeck

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et al., 2002). The major mechanism of antiviral action of acyclic nucleoside phosphonates is 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).

Mounting evidence suggests that the early natural host defence against viral infections largely depends on nonspecific cell-mediated immune responses, orchestrated predominantly by natural killer (NK) cells and macrophages (Trinchieri, 1989). The role of immunocompetent cells in both innate and acquired immunity is mediated by a number of cytotoxic and immunomodulatory cytokines. The crucial role is obviously played by interferons. It is presumed that the direct interference of interferon-γ with viral replication is minimal and is rather attributed to its stimulatory effects on other effector mechanisms, such as double-stranded RNA activated protein kinase (PKR), 2′-5′ oligoadenylate synthetase (2-5A synthetase) (Esteban and Patino, 2000), dsRNA specific adenosine deaminase (dsRAD) (Boehm et al., 1997), indoleamine 2,3 dioxygenase (Bodaghi et al., 1999), and inducible nitric oxide synthase (iNOS) (Karupiah et al., 1993).

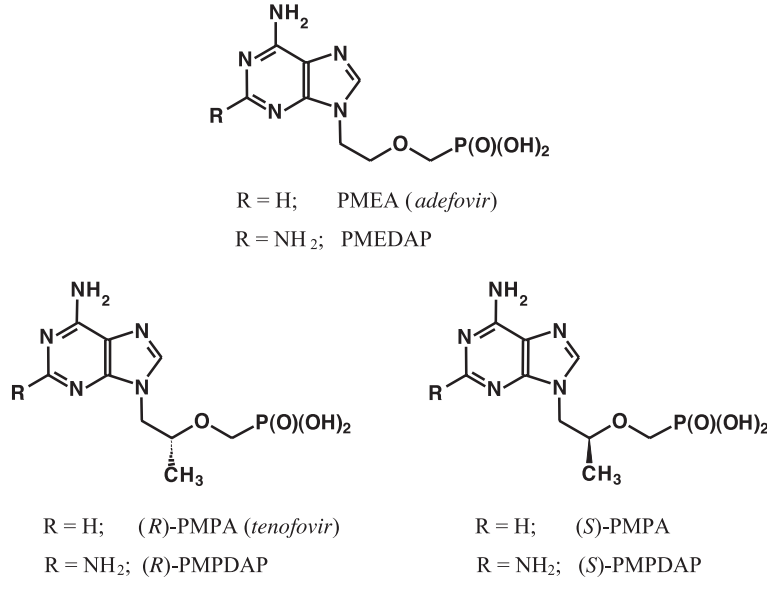
Conceivably, drugs which are used in therapy of viral infections should be devoid of negative influence upon the natural defence mechanisms. Contrariwise, they should rather support or restore them. We have found recently, that in addition to the antimetabolic mode of action, some acyclic nucleoside phosphonates, such as (*R*)-PMPA and several others, are endowed with immunostimulatory and immunomodulatory activities that may influence replication of viruses. Thus, (*R*)-PMPA stimulates secretion of various cytokines, including chemokines "regulated upon activation, normal T cell expressed and secreted" (RANTES) and macrophage inflammatory protein-1 (MIP-1α) (Zídek et al., 2001), ligands of receptors CCR5 and CXCR4, used as co-receptors for the entry of human immunodeficiency virus-1 (HIV-1) in cells (Berger et al., 1999). Besides, PMPA augments the interferon-γ-activated production of virustatic molecule of nitric oxide (NO) (Zídek et al., 1999). It may be presumed that these properties could contribute to the overall antiviral activity of the agents.

In search for new virustatic acyclic nucleoside phosphonates, we have synthesized a number of novel N^6-substituted derivatives of adenine and 2,6-diaminopurine. Their high activity against DNA viruses has been reported (Holý et al., 1995; Meerbach et al., 1998). The aim of the present study was to investigate their possible immunobiological activity.

2. Materials and methods

2.1. Compounds

All acyclic nucleoside phosphonates were synthesized in-house (Institute of Organic Chemistry and Biochemistry) according to the procedures described recently (Holý et al., 2001). They comprise alterations at the 6-amino group of the heterocyclic base, i.e. adenine (A) or 2,6-diaminopurine (DAP), and at the N^9-side chain represented by (*R*)- or (*S*)-enantiomeric 9-[2-(phosphonomethoxy)ethyl] (PME) and 9-[2-(phosphonomethoxy)propyl] (PMP) moieties. Their structure is shown in Fig. 1. The following parent, i.e. N^6-nonsubstituted acyclic nucleoside phosphonates have been included in the study: PMEA:9-[2-(phosphonome-



R = H; PMEA (*adefovir*)
R = NH_2; PMEDAP

R = H; (*R*)-PMPA (*tenofovir*)
R = NH_2; (*R*)-PMPDAP

R = H; (*S*)-PMPA
R = NH_2; (*S*)-PMPDAP

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

thoxy)ethyl]adenine; PMEDAP:9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine; (*R*)-PMPA and (*S*)-PMPA:9-[2-(phosphonomethoxy)propyl]adenine; (*R*)-PMPDAP and (*S*)-PMPDAP:9-[2-(phosphonomethoxy)propyl]2-6-diaminopurine. Chemical names of various mono- or dialkyl, cycloalkyl, alkenyl, or alkynyl N^6-substituents are listed in Table 1.

Stock solutions of acyclic nucleoside phosphonates (1 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 2 weeks at −20 °C. Required working concentrations were prepared by diluting the stock

Table 1
Production of NO by murine peritoneal macrophages cultured 24 h in the presence of interferon-γ (1000 pg/ml) and concomitantly applied acyclic nucleoside phosphonates (25 μM)

N^6-substituent	PMEA	PMEDAP	(*R*)-PMPA	(*S*)-PMPA	(*R*)-PMPDAP	(*S*)-PMPDAP
Control: Effect of interferon-γ alone: 8.3 ± 0.9 μM						
nonsubstituted	8.2 ± 0.2	6.9 ± 0.3	*26.9 ± 1.7*	12.7 ± 0.3	10.8 ± 0.8	8.0 ± 0.8
methyl		8.5 ± 0.5				
dimethyl	8.9 ± 0.7	8.3 ± 0.3		7.9 ± 0.5	8.0 ± 1.0	6.9 ± 0.9
2-hydroxyethyl	8.4 ± 0.6					
bis(2-hydroxyethyl)		8.2 ± 1.8				
2-methoxyethyl		8.2 ± 0.6				
bis(2-methoxyethyl)		8.2 ± 1.4				
diethyl	9.6 ± 0.4					
ethyl,methyl		9.2 ± 0.6				
2-dimethylaminoethyl		9.5 ± 0.5		9.2 ± 1.2	*41.9 ± 0.9*	
trifluorethyl		8.3 ± 1.3			7.6 ± 0.8	
propyl		8.2 ± 0.6				
isopropyl	7.0 ± 0.4			7.8 ± 0.8		
2-hydroxypropyl	8.2 ± 0.6	8.7 ± 0.5				
3-hydroxypropyl		8.2 ± 1.6				
3-aminopropyl		8.6 ± 0.2				
butyl		9.7 ± 0.9			*16.4 ± 1.0*	
isobutyl		*45.3 ± 1.7*	8.1 ± 0.1	8.1 ± 0.5	11.2 ± 0.6	
2-butyl		11.3 ± 0.7				
1-buten-3-yl		9.5 ± 0.9				
2-butenyl		8.4 ± 0.8				
allyl	7.1 ± 0.5	9.1 ± 0.5		7.8 ± 1.0		8.6 ± 0.6
bis(allyl)		11.2 ± 0.4				
homoallyl		7.2 ± 0.8				
2-methylallyl		8.9 ± 0.7				
cyclopropyl	8.8 ± 1.0	8.8 ± 2.0	14.4 ± 0.6	7.1 ± 2.1	*51.2 ± 1.4*	7.9 ± 0.7
di-cyclopropyl		7.8 ± 1.2				
cyclopropylmethyl		*21.4 ± 0.4*				
1-cyclopropyl-1-ethyl		8.3 ± 0.5				
cyclobutyl		8.1 ± 1.5				
cyclopentyl		*30.4 ± 1.2*			*50.0 ± 2.0*	9.2 ± 0.4
cyclohexyl	7.8 (0.8)	9.2 ± 0.2				
cyclohexylmethyl		*24.6 ± 2.0*				
cycloheptyl		*23.8 ± 1.2*				
cyclooctyl		*53.7 ± 1.9*				
phenylethyl		11.0 ± 1.2				
benzyl					10.9 ± 0.7	
pyridylmethyl		7.9 ± 0.3				
pyrrolidino	7.8 ± 1.2	*26.0 ± 3.8*	7.0 ± 0.2	7.6 ± 0.8	8.0 ± 1.0	8.0 ± 0.4
pyrazino		8.8 ± 0.6				
homopyrazino		10.5 ± 1.1				
piperidino	*26.8 ± 1.0*	7.8 ± 0.2			10.8 ± 1.2	
propargyl		*24.3 ± 1.7*				
morfolino		7.9 ± 0.9			9.9 ± 1.3	
8-aza					8.6 ± 0.4	
8-bromo		7.3 ± 0.3				

Data are means ± S.E.M. of supernatant nitrite concentrations (μM) assayed in triplicate culture wells. Substantially enhanced NO production, as compared to the effect of interferon-γ alone, is indicated in bold italics (grey boxes). Similar co-stimulatory effects of the compounds were confirmed in one to three other independent experiments. Note: When applied alone, acyclic nucleoside phosphonates did not stimulate NO production.

solution in complete RPMI-1640 culture medium (described below).

The chromogenic Limulus Amoebocyte Lysate assay (Kinetic-QCL; BioWhittaker, Walkersville, MD) was used to check for possible contamination with lipopolysaccharide. The test samples of acyclic nucleoside phosphonates contained <15 pg/ml lipopolysaccharide at the concentration of 25 μM. The effects of acyclic nucleoside phosphonates were always compared with the effects of lipopolysaccharide (*Escherichia coli* 055:B5, BioWhittaker), applied at doses 2–100 pg/ml, which was employed as positive reference control.

2.2. Animals

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 eight, and maintained in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France). Lighting was set on 0600 to 1800 h, temperature at 22 °C.

2.3. Isolation and cultivation of macrophages

Animals, killed by cervical dislocation, were i.p. injected with 8 ml of sterile saline. Pooled peritoneal cells collected from mice ($n=5–11$ 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 for 24 h (NO assay) or 5 h (cytokine assays).

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

2.4. Nitric oxide (NO) assay

The concentration of nitrites in cell supernatants was taken as a measure of NO production (Marletta et al., 1988). It was detected in individual, cell-free samples (50 μl) incubated 10 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.

2.5. Cytokine assays; immunochemicals

Concentration of cytokines and chemokines in cell supernatants was determined using enzyme-linked immunoabsorbent assay (ELISA) kits, following the manufacturer's instructions (R&D Systems, Minneapolis, MN). Recombinant mouse interferon-γ (lot No. CFP031051) was purchased from R&D Systems. Lipopolysaccharide from *E. coli* 055:B5 (product No. 7306, lot No. 8L3420) was from BioWhittaker.

2.6. Analysis of data

Analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test, and graphical presentation

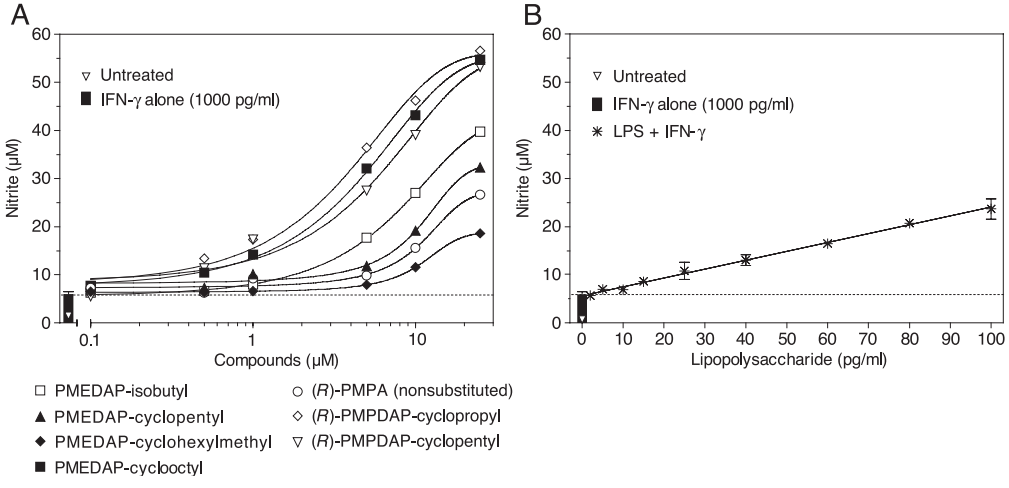


Fig. 2. Dose-dependent effects of selected acyclic nucleoside phosphonates on production of NO by murine peritoneal macrophages cultured (2×10^6/ml) 24 h in the presence of interferon-γ (IFN-γ; 1000 pg/ml) (A). The effects of test compounds were compared to the synergistic NO-augmenting influence of interferon-γ given together with varying concentrations of lipopolysaccharide (B). Each point is a mean ± S.E.M. for triplicate culture wells. The results are representative of two identical experiments.

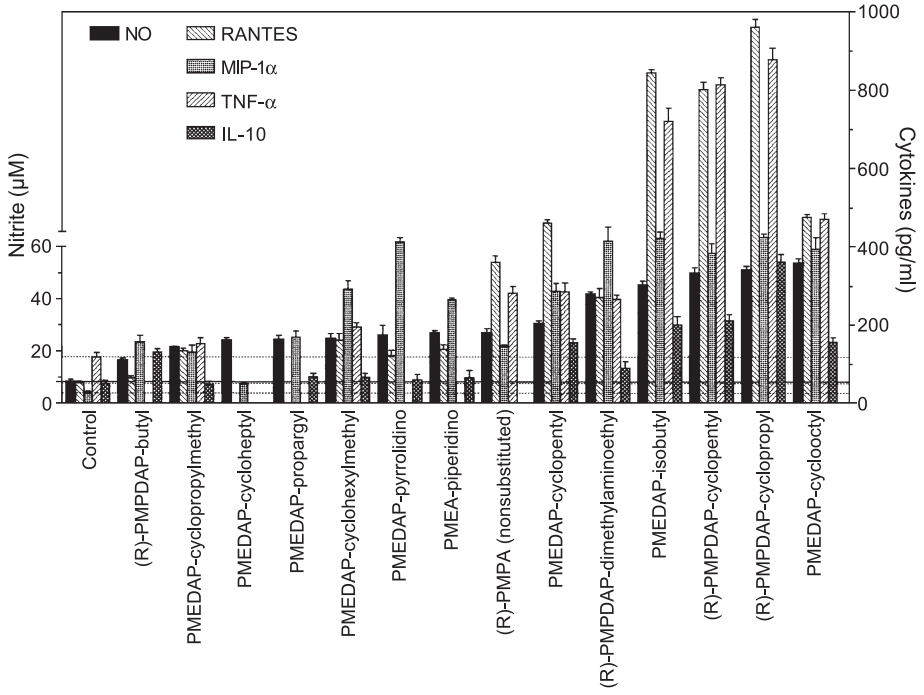


Fig. 3. Concentration of cytokines (pg/ml) secreted by murine peritoneal macrophages. The cells were cultured 5 h at the density of 2×10^6/ml (in duplicate) in presence of acyclic nucleoside phosphonates, applied at concentration of 25 μM. For comparative reasons, the corresponding data from Table 1, showing production of NO, have been included. The bars are means ± S.E.M. Note: The NO data were generated in the presence of interferon-γ and the cytokine data in its absence.

of data were done using the Prism program (GraphPad Software, San Diego, CA).

3. Results

3.1. Nitric oxide (NO) production

When applied alone at 25 μM concentration, none of the acyclic nucleoside phosphonates stimulated production of NO by macrophages (data not shown). Similarly, no NO was formed after combined treatment with lipopolysaccharide. The amount of lipopolysaccharide used, i.e. 50 pg/ml, albeit virtually ineffective on its own (nitrite concentration in cell supernatants was 1.3 ± 0.9 μM, compared to 0.4 ± 0.8 μM in untreated cells), was sufficient to augment NO biosynthesis triggered by interferon-γ 250 or 1000 pg/ml (nitrite concentration was 15.7 ± 0.1 and 25.6 ± 0.4 μM, respectively). A number of derivatives of acyclic nucleoside phosphonates did enhance production of NO when administered together with interferon-γ, however. Interferon-γ alone (1000 pg/ml) increased the concentration of nitrites from virtually zero in untreated cells to 8.3 μM. It was two- to seven-fold enhanced in supernatants of cells cultured

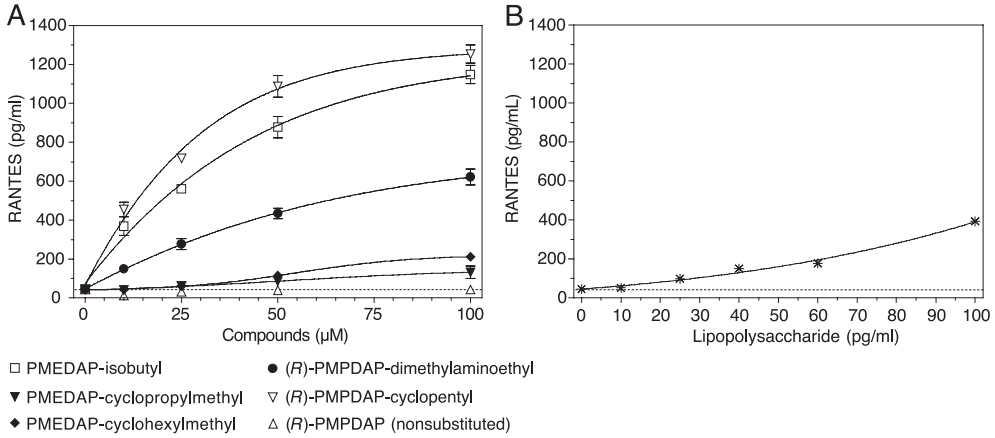


Fig. 4. Concentration of RANTES in supernatants of murine peritoneal macrophages cultured (2×10^6/ml) 5 h in the presence of varying concentrations of acyclic nucleoside phosphonates (A) or lipopolysaccharide (B). Each point is a mean ± S.E.M. for duplicate culture wells.

simultaneously with certain acyclic nucleoside phosphonate derivatives (Table 1). The most effective were N^6-cyclooctyl-PMEDAP, N^6-cyclopropyl-(*R*)-PMPDAP, and N^6-cyclopentyl-(*R*)-PMPDAP, the NO-stimulatory potential thereof being statistically highly significant ($P<0.01$) at as low concentration as 1 μM (Fig. 2A). Immunomodulatory activity of several other acyclic nucleoside phosphonates could be detected at 5–10 μM concentration (Fig. 2A). The effects were more pronounced or approximately equal to the synergistic effects of interferon-γ plus relatively high dose (100 pg/ml) of the most potent immunostimulant, lipopolysaccharide (Fig. 2B).

3.2. Cytokine secretion

Chemokines and cytokines were determined in supernatants of macrophages cultured for 5 h in the presence of acyclic nucleoside phosphonates alone, applied at concentration of 25 μM. Acyclic nucleoside phosphonates that were found to augment production of NO also activated macrophages for secretion of RANTES, MIP-1α, and TNF-α; the least influenced remained interleukin-10 (Fig. 3). The compounds which were ineffective to augment the interferon-γ-triggered NO production did not influence cytokine secretion neither (data not shown). Similar to NO, none of the (*S*)-PMPA and (*S*)-PMPDAP derivatives affected cytokine expression. The highest cytokine-stimulatory potential was found to be possessed by N^6-isobutyl-PMEDAP, N^6-cyclopropyl-(*R*)-PMPDAP, and N^6-cyclopentyl-(*R*)-PMPDAP that greatly elevated secretion of all cytokines under study. Both RANTES and MIP-1α were produced in a dose-dependent manner, the lowest effective dose being approximately 10 μM (Figs. 4A and 5A, respectively). The effects of the most efficacious N^6-substituted (*R*)-PMPA derivatives were more prominent compared to the effects of (*R*)-PMPA (*tenofovir*) (Fig. 5A). They also exhibited more pronounced stimulatory effects than relatively high concentrations (100 pg/ml) of lipopolysaccharide (Figs. 4B and 5B, respectively), which was used as a positive reference control.

4. Discussion

In the present study, a total of 77 novel N^6-substituted derivatives of acyclic nucleoside phosphonates differing by substitution of the amino group at C-6 of the heterocyclic base, i.e. adenine (A) and 2,6-diaminopurine (DAP), and at the N^9-side chain moieties, i.e. 9-[2-(phosphonomethoxy)ethyl] (PME; i.e. PMEA, and PMEDAP) and (*R*)- or (*S*)-enantiomers of 9-[2-(phosphonomethoxy)propyl] (PMP; i.e. (*R*)-PMPA, (*S*)-PMPA, (*R*)-PMPDAP, and (*S*)-PMPDAP) were screened for their immunobiological properties. Parent purine acyclic nucleoside phosphonates are primarily exploited for clinical treatment of viral infections, including HIV and hepatitis B virus (HBV). Therefore, we investigated the effects of test compounds on expression of soluble factors that play crucial roles in the host control of virus replication and/or of cell penetration, such as NO, cytokines and chemokines. The tests have been done under in vitro conditions using mouse resident peritoneal macrophages. This cell type is known to be the main source of high-output NO production (Förstermann and Kleinert, 1995), and prominent producer of a number of other cytotoxic molecules. Moreover, they play an important role in initiation and pathogenesis of HIV infection (Fauci, 1988).

Nitric oxide is one of the most important effector molecules in a repertoire of nonspecific immune defence mechanisms, having antiparasitic, antibacterial, and antiviral properties (Akarid et al., 1995). It is considered to mediate inhibitory effects of interferon-γ and/or other cytokines on replication of a great many number of viruses, including poxviridae, herpetoviridae, rhabdoviridae, retroviridae, and parvoviridae, e.g. hepatitis B virus (Guidotti et al., 2000),

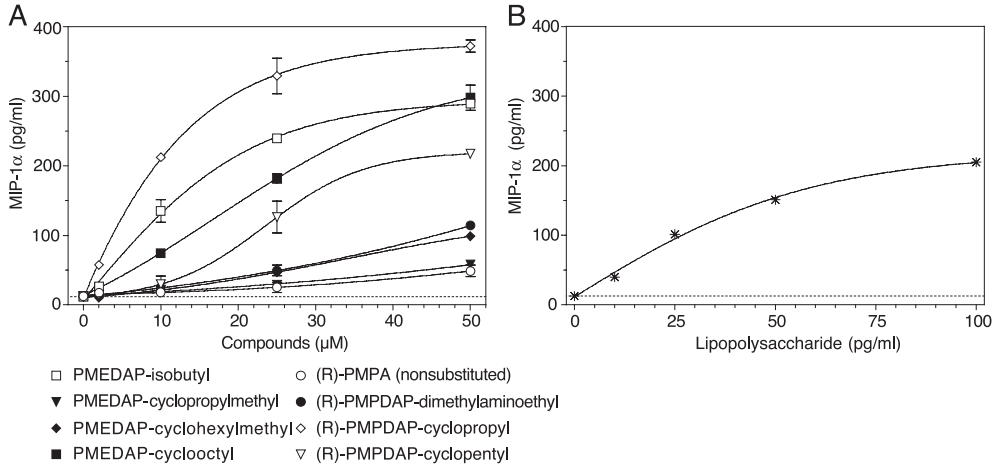


Fig. 5. Concentration of MIP-1α in supernatants of murine peritoneal macrophages cultured (2×10^6/ml) 5 h in the presence of varying concentrations of acyclic nucleoside phosphonates (A) or lipopolysaccharide (B). Each point is a mean ± S.E.M. for duplicate culture wells.

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).

While the test compounds were ineffective to stimulate NO when applied either alone or in combination with lipopolysaccharide, 14 of 83 acyclic nucleoside phosphonates included in the study have been found to up-regulate production of NO triggered by interferon-γ. Interferon-γ alone is known to be the most important stimulus for activation of the inducible NO synthase (iNOS) (Stuehr and Marletta, 1987). Its inner activity may be synergistically enhanced by lipopolysaccharide, and by a number of cytokines. All test derivatives of acyclic nucleoside phosphonates that proved to augment the interferon-γ-induced NO production, also were found to stimulate secretion of cytokines such as TNF-α and interleukin-10, and chemokines RANTES and MIP-1α. TNF-α belongs among those cytokines which are generally recognized for their NO up-regulatory functions (Ding et al., 1988). The effects of interleukin-10 are ambiguous: both NO-stimulatory (Chesrown et al., 1994) and NO-inhibitory effects (Bogdan et al., 1991) have been reported. Our previous experiments showed that production of NO by mouse peritoneal macrophages stimulated with interferon-γ plus (*R*)-PMPA could be suppressed by both anti-TNF-α and anti-interleukin-10 antibodies (Zídek et al., 1997). The role of chemokines is much less known in this respect, though some reports suggest that RANTES, MIP-1α, and MIP-1β (Villalta et al., 1998) augment formation of NO. The acyclic nucleoside phosphonates themselves do not activate secretion of interferon-γ (data not shown). Conceivably, cultivation of macrophages with the drugs alone or in combination with lipopolysaccharide does not influence the constitutive NO production. Despite of the ultimate dependence on the presence of interferon-γ, the NO-enhancing effect may be of practical importance, since the early phases of infections caused by viruses such as cytomegalovirus (Orange et al., 1995), vaccinia virus (Rolph et al., 1996), influenza virus (Sarawar and Doherty, 1994), and HIV (Fauci, 1996) are accompanied by enhanced levels of interferon-γ.

TNF-α is a recognized antiviral agent in many viral infections. Its effects are usually more expressed in a synergistic manner with interferon-γ. For example, it thus suppresses replication of cytomegalovirus (Orange and Biron, 1996), herpes simplex virus (Feduchi et al., 1989), varicela zoster virus (Ito et al., 1991), and adenovirus (Mayer et al., 1992). It is also effective against HIV (Cotter et al., 2001) although there are some opposite observations (Poli et al., 1990). The anti-HIV activity may depend on TNF-α-induced delay and decrease of expression of chemokine receptors CCR5 and CXCR4 (Hornung et al., 2000). TNF-α also has antibacterial properties that are not necessarily NO-dependent as documented in the case of *Listeria monocytogenes* (Leenen et al., 1994) or *Mycobacterium bovis* infection (Bekker et al., 2001). On the other hand, interleukin-10 may indirectly, through mechanisms such as down-regulation of interleukin-12 production, impair the antiviral defence mechanisms of T cells and NK cells (Mosmann, 1994). Yet, the role of interleukin-10 in infection immunity is not as much clear since its inhibitory effects upon replication of encephalomyocarditis virus were observed when applied on days 0 or 1 after inoculation, but was ineffective later (Nishio et al., 1999). Also, interleukin-10 suppressed *B. streptococcus* sepsis, but only if applied 20 or 4 h before inoculation, not later (Finkel et al., 1992). Moreover, numerous reports document inhibitory effects of interleukin-10 on HIV replication, e.g. (Akridge et al., 1994), although it enhanced interleukin-1β-, and granulocyte-macrophage colony stimulating factor (GM-CSF)-induced HIV-1 expression in U1 promonocytic cells (Angel et al., 1995), and also in T-cells (Rabbi et al., 1998). The discrepancies may be caused by differential effects of interleukin-10 on expression of CCR5 receptor in dependence on cell types. It has been found uninfluenced in T cells (Wang et al., 1999), suppressed in $CD4^+$ lymphocytes (Patterson et al., 1999), whilst stimulated in astrocytic, microglial and neuronal cell lines (Speth et al., 2000), human placental macrophages (Torres et al., 2001) and monocytes (Sozzani et al., 1998).

The present data demonstrate that acyclic nucleoside phosphonates are potent stimulators of chemokines RANTES and MIP-1α secretion. They are natural ligands for the chemokine receptors CCR5 and CXCR4 which also are used as co-receptors for the entry of HIV-1 into the cells of the immune system, and in cooperation with CD4 receptor they ensure a productive infection (Xiao et al., 1999). Since blocking the appropriate β-chemokine receptors on both macrophages and lymphocytes is presently considered as a therapeutic approach against HIV (Ylisastigui et al., 1998), this is an important finding from the point of view of HIV infection treatment.

Both immunostimulatory and immunomodulatory effects, i.e. cytokine secretion and up-regulation of NO production, respectively, were expressed in a dose-dependent fashion. The most potent acyclic nucleoside phosphonates are effective at concentrations as low as 1–5 μM. Even the 25-μM concentration of the compounds, used for the primary screening of the effects, is relevant to the clinical practice, because it is very close to concentrations of various acyclic nucleoside phosphonates reached after their intravenous administration to humans or macaques (cf. Zídek et al., 2001).

Although the data do not allow to draw definite conclusion regarding the structure–activity relationships, it seems that the immunobiological potential is absent in (*S*)-enantiomers of both PMPA and PMPDAP series. There was solely one moderately active compound within the PMEA derivatives, i.e. N^6-piperidino-PMEA, and the only one within the (*R*)-PMPA series, namely the N^6-nonsubstituted (*R*)-PMPA per se. The highest frequency of immunomodulatory acyclic nucleoside phosphonates is obviously bound

to the 2,6-diaminopurine series, where 8 of 41 PMEDAP derivatives stimulated secretion of cytokines and co-stimulated NO production. Similarly, there were 4 active N^6-derivatives in a series of 13 N^6-substituted (*R*)-PMPDAP compounds. Interestingly, the N^6-nonsubstituted PMEDAP and (*R*)-PMPDAP were devoid of immunostimulatory effects. The influence of N^6-substitution may be regarded specific in dependence on the N^9-side chain moiety of acyclic nucleoside phosphonates. Thus, only (*R*)-PMPDAP- but neither PMEDAP- nor (*S*)-PMPA-derivatives acquired immunostimulatory/immunomodulatory properties after introduction of N^6-(2-dimethylaminoethyl) group. The same regards N^6-butyl, and N^6-cyclopropyl substituents. On the other hand, the introduction of N^6-isobutyl or N^6-pyrrolidino substituents stimulated the immunobiological activity in compounds derived from PMEDAP, but not from adenine analogs or PMP derivatives. Only the N^6-cyclopentyl substitution was effective in both PMEDAP and (*R*)-PMPDAP (but not in (*S*)-enantiomer). Substitution by N^6-piperidino grouping showed immunomodulatory effects solely in PMEA series, whereas no enhancement of activity was detected in PMEDAP or (*R*)-PMPDAP derivative upon such a substitution. It is therefore difficult to suggest any general rules that would allow to design novel acyclic nucleoside phosphonates with immunomodulatory properties on basis of N^6-substituents. It seems however, that the N^6-cycloalkyl and N^6-cycloalkylmethyl derivatives are the most promising candidates in this respect. Many of them, such as N^6-cyclopropyl-(*R*)-PMPDAP, N^6-cyclopropylmethyl-PMEDAP, N^6-cyclopentyl-PMEDAP, N^6-cyclopentyl-(*R*)-PMPDAP, N^6-cyclohexylmethyl-PMEDAP, N^6-cycloheptyl-PMEDAP, and N^6-cyclooctyl-PMEDAP possess significant immunomodulatory properties.

Numerous compounds of these series have been scrutinized for antiproliferative effects in an in vitro model of murine lymphocytes (Holý et al., 1996). Independently on

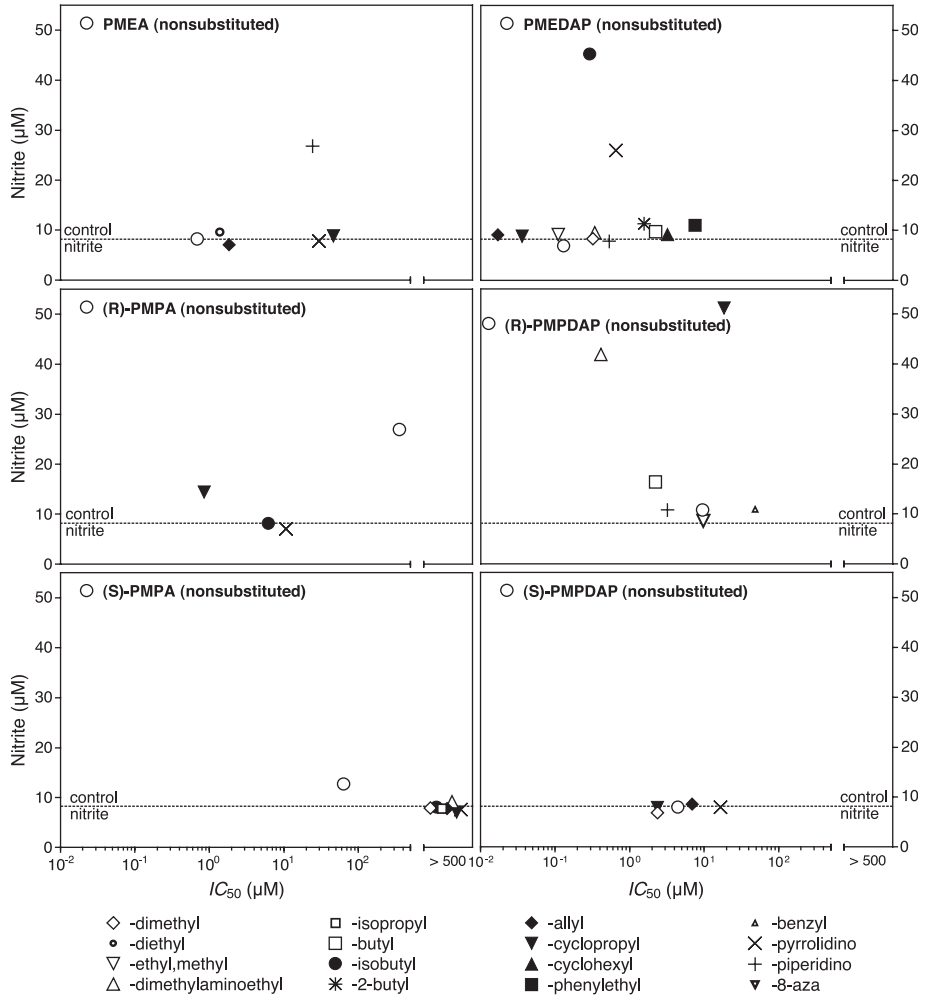


Fig. 6. Relationship between the immunomodulatory and cytostatic effects of acyclic nucleoside phosphonates. The immunomodulatory potential of compounds is represented by their co-stimulatory influence on interferon-γ-triggered production of NO by macrophages (data from Table 1). The quantitative measure of cytostatic activity is considered the interference of acyclic nucleoside phosphonates with proliferation of murine splenocytes (data from Holý et al., 1996, and unpublished results). This was estimated by the medial inhibitory concentrations (IC_{50}s). The figure documents no tight relationship between the two effects.

expression of cytostatic activity, determined by the medial inhibitory concentrations (IC_{50}), the compounds do or do not possess immunomodulatory properties (Fig. 6). For example, both N^6-dimethylaminoethyl-(*R*)-PMPDAP and N^6-cyclopropyl-(*R*)-PMPDAP stimulate production of cytokines and enhance NO production, while their IC_{50}s differ substantially: 0.41 and 18.5 μM, respectively. Several acyclic nucleoside phosphonates with relatively high cytostatic potential characterized by the IC_{50}s lying within the range of 0.1–1.0 μM, e.g. N^6-isobutyl-PMEDAP and N^6-pyrrolidino-PMEDAP possess immunomodulatory activity, while many others occurring within the same IC_{50} range, do not. The data demonstrate that the cytostatic and immunomodulatory potentials of acyclic nucleoside phosphonates are completely dissociated.

In summary, a number of N^6-substituted derivatives of acyclic nucleoside phosphonates possess immunostimulatory and immunomodulatory activities that may significantly influence replication of viruses. Several of them are more potent than a prototype compound (*R*)-PMPA (*tenofovir*) which is used clinically in anti-HIV therapy. It is suggested that these novel acyclic nucleoside phosphonates might become candidates for antiviral therapy.

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