Original Article

Influence of Acyclic Nucleoside Phosphonate Antivirals on Gene Expression of Chemokine Receptors CCR5 and CXCR4

(acyclic nucleoside phosphonate / HIV / CCR5 / CXCR4 / cytokine / RT-PCR)

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Abstract. Acyclic nucleoside phosphonates (ANPs) are potent antiviral agents effective against replication of DNA viruses and retroviruses including human immunodeficiency virus (HIV). Prototype compound 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine (tenofovir) is a principal component of drugs widely used in the treatment of HIV infection (Viread, Truvada). Besides their antimetabolic mode of action, ANPs possess immunomodulatory properties. A number of them have been previously found to stimulate secretion of cytokines and anti-HIV effective chemokines. In the present pilot experiments we analysed the *in vitro* effects of ANPs on the expression of chemokine receptors CCR5 and CXCR4 that are co-receptors of HIV-1 entry in cells. The impact of ANPs was investigated at the level of gene transcription of mRNA in mouse lymphocytes and macrophages using the RT-PCR method. The following compounds were included in the study: 9-*(R)*-[2-(phosphonomethoxy)propyl]adenine (tenofovir), N^6-cyclopropyl-(*R*)-9-[2-(phosphonomethoxy)-propyl]2,6-diaminopurine, N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy) propyl]2,6-diaminopurine, N^6-dimethylaminoethyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine, N^6-cyclopentyl-9-[2-(phosphonomethoxy) ethyl]2,6-diaminopurine, N^6-isobutyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine. Gene transcription of chemokine receptors CCR5 and CXCR4 was not affected after application of these acyclic nucleoside phosphonate antivirals.

Received May 12, 2014. Accepted December 4, 2014.

The work was supported by grant No. 305/03/1470 from the Grant Agency of the Czech Republic and No. 1M 6138896301 from the Centre for New Antivirals and Antineoplastics. It was performed as a part of Research Project of Charles University in Prague, PRVOUK P34.

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Abbreviations: AIDS – acquired immunodeficiency syndrome, ANP – acyclic nucleoside phosphonate, IL-10 – interleukin 10, HIV – human immunodeficiency virus, LPS – lipopolysaccharide, MCP – monocyte chemotactic protein, MIP-1α – macrophage inflammatory protein 1α, RT-PCR – reverse transcriptase-polymerase chain reaction, TNF-α – tumour necrosis factor α.

Introduction

Modern possibilities of acquired immunodeficiency syndrome (AIDS) pharmacotherapy are usually adequate for many patients suffering from HIV infection. However, in some situations new approaches in the field of human immunodeficiency virus (HIV) chemotherapy research may be appreciated, because the number of patients infected by HIV has been unfortunately increasing in some countries despite the effort targeted to prevent HIV infection and despite the progress in pharmacotherapy of AIDS (Giaquinto et al., 2008; De Clercq, 2013). It has been previously suggested that modern remedies against viral diseases should be able to enhance the immunological functions of patients (Holý, 2001; De Clercq, 2004).

Some of the representatives belonging to the class of antiviral drugs, named acyclic nucleoside phosphonates (ANPs), may possibly be considered to have such properties (Zídek et al., 2003; Potměšil et al., 2007). The major mechanism of their antiviral action is inhibition of virus-induced DNA polymerases (Kramata et al., 1996) or of reverse transcriptases (Holý et al., 1990). ANPs thus inhibit replication of both DNA viruses and retroviruses, including HIV. The oral prodrugs of the prototype compounds, i.e. 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPA; tenofovir] and 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA; adefovir) were approved by FDA and EMA for the treatment of AIDS (Viread) and hepatitis B (Hepsera), respectively (Potměšil et al., 2007).

Some ANPs, such as tenofovir and several others, are endowed with immunomodulatory activities that may

be able to reduce replication of HIV and other viruses (Zídek et al., 2001). ANPs enhance production of virostatic molecule nitric oxide and stimulate secretion of cytokines tumour necrosis factor α (TNF-α), interleukin 10 (IL-10) and chemokines RANTES ("regulated upon activation, normal T cell expressed and secreted") and macrophage inflammatory protein 1α (MIP-1α) (Zídek et al.; 2001, 2003). Beta chemokines RANTES and MIP-1 are ligands of chemokine receptor CCR5, which is used as a co-receptor for HIV entry in cells, and these molecules can thus suppress HIV infection (Cocchi et al., 1995; Schols et al., 1997). Previously, we investigated the impact of ANPs on gene expression of mouse monocyte chemotactic proteins MCP-1, 2, 3, 5 that share similarities to human MCPs and we found increased expression of MCP-1 and MCP-3 (Potměšil et al., 2007). Some MCPs (MCP-3, for example) can also bind to chemokine receptors including HIV co-receptors (Blanpain et al., 1999), and thus MCP-3 could also block HIV entry into permissive cells (Schols et al., 1997; Modi et al., 2003).

The goal of the present experiments was to examine the possible interference of ANPs with gene expression of mRNA for chemokine receptors CCR5 and CXCR4. CCR5 antagonists and CXCR4 antagonists or dual inhibitors have already proceeded through successful preclinical and clinical development and some of them are used in clinical practice (Princen et al., 2004; De Clercq, 2013).

Material and Methods

Acyclic nucleoside phosphonates (ANPs) and other chemicals

All ANPs were synthesized in-house (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic v. v. i., Prague, Czech Republic) according to the procedures described earlier (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 9-[2-(phosphonomethoxy)ethyl] and 9-[2-(phosphonomethoxy)propyl] moieties. The following ANPs were included in RT-PCR experiments: (1) 9-*(R)*-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPA; tenofovir], (2) N^6-cyclopropyl-(*R*)-9-[2-(phosphonomethoxy)-propyl]2,6-diaminopurine [N^6-cyclopropyl-(*R*)-PMPDAP], (3) N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-cyclopentyl-(*R*)-PMPDAP], (4) N^6-dimethylaminoethyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-dimethylaminoethyl-(*R*)-PMPDAP], (5) N^6-cyclopentyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine [N^6-cyclopentyl-PMEDAP], (6) N^6-isobutyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine [N^6-isobutyl-PMEDAP]. Chemical structure of these ANPs is shown in Fig. 1.

Stock solutions of ANPs (5 mM) were prepared in incomplete, $NaHCO_3$-containing, phenol red-free

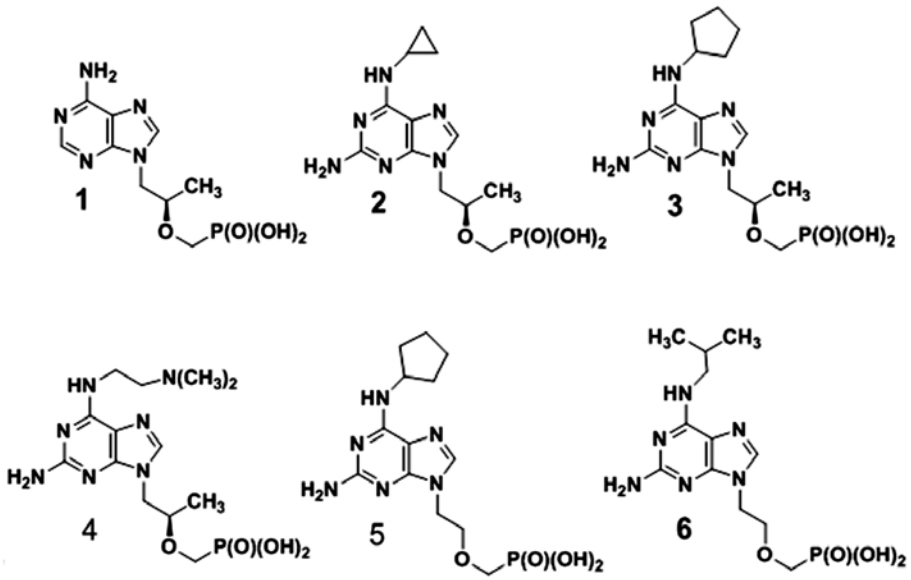


Fig. 1. Chemical structure of the tested acyclic nucleoside phosphonates
ANP 1 = 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine [(R)-PMPA; tenofovir],
ANP 2 = N^6-cyclopropyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-cyclopropyl-(*R*)-PMPDAP],
ANP 3 = N^6-cyclopentyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-cyclopentyl-(*R*)-PMPDAP],
ANP 4 = N^6-dimethylaminoethyl-(*R*)-9-[2-(phosphonomethoxy)propyl]2,6-diaminopurine [N^6-dimethylaminoethyl-(*R*)--PMPDAP],
ANP 5 = N^6-cyclopentyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine [N^6-cyclopentyl-PMEDAP],
ANP 6 = N^6-isobutyl-9-[2-(phosphonomethoxy)ethyl]2,6-diaminopurine [N^6-isobutyl-PMEDAP].

RPMI-1640 medium (Sigma-Aldrich, Prague, Czech Republic). They were sterile filtered using non-pyrogenic 0.22-μm filters (Costar, Cambridge, MA), used fresh or kept no longer than four weeks at -20 °C. The required concentration was prepared by diluting the stock 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 of ANPs with lipopolysaccharide (LPS). The highest final concentrations of all chemicals used contained < 10 pg/ml (i.e., approximately < 0.1 endotoxin units/ml), an amount that was previously found virtually biologically inactive (Zídek et al., 2003).

LPS from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich. It was applied as a reference agent in a final concentration of 1 μg/ml in lymphocyte and macrophage cell cultures.

Animals

Female mice of 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 Enviromental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France). Lighting was set at 6:00–18:00 h and temperature at 22 °C. All protocols were approved by the institutional ethics committee.

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 were washed, resuspended in complete RPMI-1640 medium (10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, 2×10^{-5} M 2-mercaptoethanol; all from Sigma-Aldrich) and seeded into 96-well round-bottom microplates (Costar) in 100-μl volumes, 2×10^5 cells per well. Adherent cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5% CO_2 in a humidified Heraeus incubator, and then vigorously shaking the plate and washing the wells three times to remove non-adherent cells. The number of macrophages used for RNA extraction was 4×10^6 cells per sample in final 2 ml volume in a 6-well culture plate (Costar). Macrophages were cultured for indicated time intervals in the presence or absence of test compounds.

Lymphocyte collection and culture

Single-cell suspension of lymphocytes was prepared by passing the fragmented pooled spleens through a fine nylon sieve. Erythrocytes were removed by means of red blood cell lysing buffer (Sigma-Aldrich) containing 0.8% ammonium chloride in 0.01 M Tris-HCl, pH 7.5. After thorough washing (twice in sterile saline), the cells were resuspended in culture medium (described above). The number of cells used for RNA extraction was 10×10^6 per sample in final 2 ml volume seeded in a 12-well culture plate (Costar). Lymphocytes were cultured in a humidified Heraeus incubator (37 °C, 5% CO_2) for indicated time intervals in the presence or absence of test compounds.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

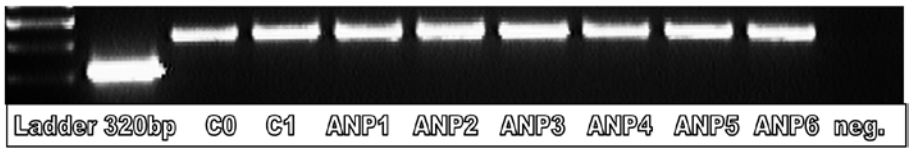
The expression of chemokine co-receptors CCR5, CXCR4 was analysed by semi-quantitative RT-PCR in linear range. Total RNA was isolated from macrophages or lymphocytes using the RNeasy mini kit and RNAse free DNAse I set (Qiagen, Hilden, Germany). The yield and purity of RNA was quantitated by measuring the ratio of the optical density at 260 and 280 nm. RNA integrity was checked by gel electrophoresis, and 0.4 μg of total RNA was then reverse transcribed to complementary DNA using random nonamers and recombinant Moloney murine leukaemia virus reverse transcriptase in a total reaction volume of 20 μl in the presence of RNAse inhibitor. Negative controls excluding reverse transcriptase were also set up. One fifth of the resulting cDNA was then amplified by PCR. PCR reactions were performed with commercially available specific primers and positive controls for mouse CCR5 and CXCR4 (R&D systems, Abingdon, UK). 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, Mountain View, CA). This housekeeping gene is frequently used on regular basis as a control because its expression is constitutive. RT-PCR chemicals were obtained from Top-Bio (Prague, Czech Republic). After initial denaturation (2 min, 94 °C), 27 amplification cycles were performed using a thermocycler Mastercycler gradient (Eppendorf, Germany). One cycle consisted of denaturation at 94 °C for 45 s, annealing at 57 °C (chemokine co-receptor primers) or 61 °C (GAPDH primers) 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, 433 bp for CCR5 and 415 bp for CXCR4. The amplified size of positive controls was 320 bp for CCR5 and 350 bp for CXCR4. PCR was performed in linear range. PCR products of predicted size were identified by electrophoresis in 1.5% agarose gel containing ethidium bromide. The gels were photographed in UV light.

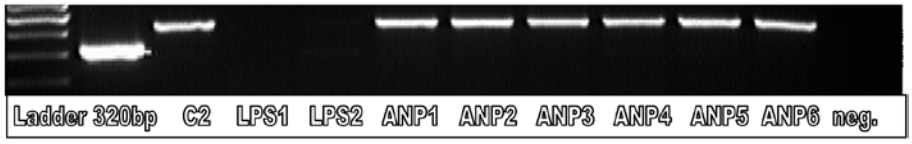
Results

CCR5 mRNA expression

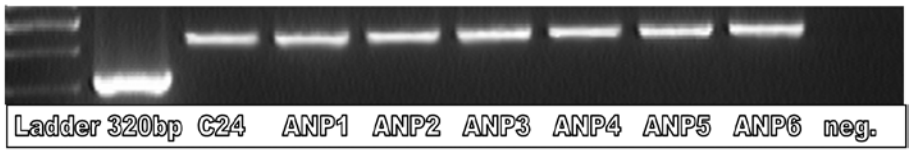
CCR5 mRNA was constitutively expressed in mouse lymphocytes. It was not affected by any of the six ANPs tested (100 μM) during 1-h, 2-h and 24-h cultivation pe-



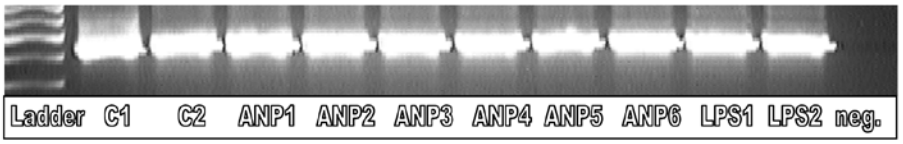
2a) cultivation of ANP 1-6 (100 μM) for 1 h



2b) cultivation of ANP 1-6 (100 μM) for 2 h



2c) cultivation of ANP 1-6 (100 μM) 24 h



2d) expression of GAPDH

Fig. 2. Expression of CCR5 mRNA in mouse lymphocytes. The size of PCR product is 433 bp. The size of PCR product obtained by amplification of synthetic DNA supplied by the manufacturer of primers is 320 bp (positive control of PCR). As a ladder, "GeneRuler 100 bp DNA ladder" was used (see Material and Methods). Lymphocytes were cultivated for 1 h, 2 h and 24 h with different ANPs (**ANP 1–ANP 6**: **ANP 1** = tenofovir [(*R*)-PMPA], **ANP 2** = N^6-cyclopropyl-(*R*)-PMPDAP, **ANP 3** = N^6-cyclopentyl-(*R*)-PMPDAP, **ANP 4** = N^6-dimethylaminoethyl-(*R*)-PMPDAP, **ANP 5** = N^6-cyclopentyl-PMEDAP, **ANP 6** = N^6-isobutyl-PMEDAP; chemical structure and full names of compounds see Fig. 1). Final concentration of ANPs was 100 μM. Lipopolysaccharide (1 μg/ml) was added to cells for 1 h (**LPS 1**) and 2 h (**LPS 2**). Extraction of RNA and its analysis was then performed by RT-PCR. **C 0** = uninfluenced cells (not cultivated). **C 1**, **C 2**, **C 24** = uninfluenced cells, cultivated for 1 h, 2 h and 24 h. 320 bp = positive PCR control. Negative PCR control is marked as Neg. ANPs do not influence gene transcription of mRNA for CCR5 after cultivation periods of 1 h (**a**), 2 h (**b**) and 24 h (**c**). LPS inhibits gene transcription of mRNA for CCR5 after 1 h and 2 h. Example of expression of mRNA for constitutive gene *GAPDH* (size 450 bp) is shown in photograph **d)**.

riods (Fig. 2). LPS (1 μg/ml) inhibited transcription of CCR5 mRNA completely in 1-h and 2-h periods.

In mouse macrophages, no constitutive CCR5 mRNA expression was detected. Tenofovir, N^6-cyclopropyl-(*R*)-PMPDAP, N^6-isobutyl-PMEDAP (100 μM) and LPS (1 μg/ml) failed to induce transcription of CCR5 mRNA in 0.5-h, 1-h, 2-h and 24-h cultivation periods (data not shown).

CXCR4 mRNA expression

CXCR4 mRNA was constitutively expressed in mouse lymphocytes. Similar to CCR5, it was not affected by ANPs (100 μM) within 1-h, 2-h and 24-h cultivation periods (Fig. 3). LPS (1 μg/ml) slightly inhibited transcription of CXCR4 mRNA after 1 h of the cell treatment and markedly so after 2 h of cell cultivation.

In mouse macrophages, barely detectable CXCR4 mRNA expression was observed. Tenofovir, N^6-cyclopropyl-(*R*)-PMPDAP, N^6-isobutyl-PMEDAP (100 μM) failed to induce transcription of CXCR4 mRNA in 0.5-h, 1-h, 2-h and 24-h cultivation periods (data not shown).

Discussion

Cytokines, namely chemokines, play an important role in the host defence against infection, including HIV-1 infection. Moreover, they are closely implicated in a broad range of inflammatory and neoplastic diseases (Housmand and Zlotnik, 2003; Modi et al., 2003).

The involvement of chemokine receptors in the pathogenesis of infectious or neoplastic diseases has also been frequently highlighted by many researchers

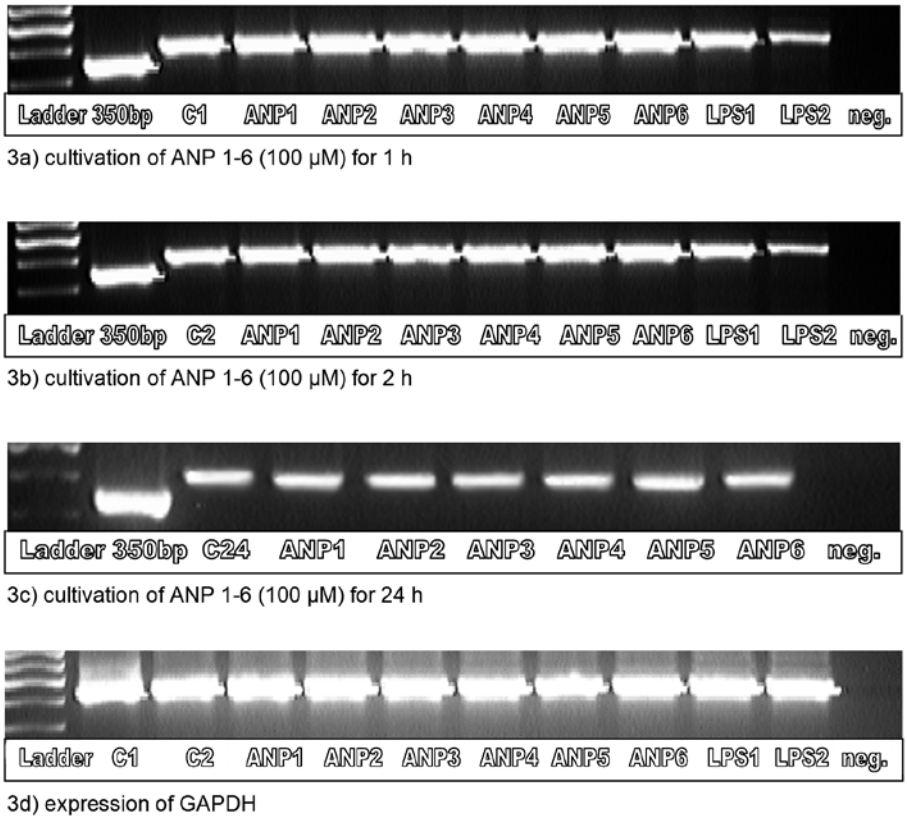


Fig. 3. Expression of CXCR4 mRNA in mouse lymphocytes. The size of PCR product is 415 bp. The size of PCR product that was obtained by amplification of synthetic DNA supplied by the manufacturer of specific primers was 350 bp. "GeneRuler 100 bp DNA ladder" was used as a ladder (see Material and Methods). Lymphocytes were cultivated for 1 h, 2 h and 24 h with different ANPs (**ANP 1–ANP 6**: **ANP 1** = tenofovir [(*R*)-PMPA], **ANP 2** = N^6-cyclopropyl-(*R*)-PMPDAP, **ANP 3** = N^6-cyclopentyl-(*R*)-PMPDAP, **ANP 4** = N^6-dimethylaminoethyl-(*R*)-PMPDAP, **ANP 5** = N^6-cyclopentyl-PMEDAP, **ANP 6** = N^6-isobutyl-PMEDAP; chemical structure and full names of the tested compounds see Fig. 1 in Material and Methods). Final concentration of ANPs was 100 μM. Lipopolysaccharide (1 μg/ml) was added to cells for 1 h (**LPS 1**) and 2 h (**LPS 2**). Extraction of RNA and analysis of RNA was then performed using RT-PCR. **C 1**, **C 2**, **C 24** = uninfluenced cells, cultivated for 1 h, 2 h and 24 h. 350 bp = positive PCR control. Negative PCR control is indicated as Neg. ANPs do not influence transcription of mRNA for CXCR4 after cultivation periods 1 h (**a**), 2 h (**b**) a 24 h (**c**). LPS inhibits transcription of mRNA for CXCR4 after 1 h and also after 2 h. Example of expression of mRNA for constitutive gene *GAPDH* (the size of PCR product is 450 bp) is shown in photograph **d)**.

(Michael et al., 1997; Juarez et al., 2001). The chemokine receptors that are most relevant to progression of HIV-1 infection are CCR5 and CXCR4. They serve as co-receptors for HIV-1 entry into permissive cells. Utilization of chemokine receptors as co-receptors appears to be a common strategy among lentiviruses (Berger et al., 1999; Moore et al., 2004). CCR5 plays a critical role in transmission of HIV-1 infection and progression to AIDS (Dean et al., 1996; Paxton et al., 1998). Individuals with low CCR5 expression, caused by the Δ32 deletion, are less susceptible to HIV-1 infection (Dean et al., 1996; Michael et al., 1997). In addition, CCR5-deficient humans have not shown increased susceptibility to the disease (Mueller and Strange, 2004). Expression of CCR5 is not critical for T cell-mediated antiviral immunity, and this molecule constitutes a logical and safe target for anti-HIV therapies (Nansen et al., 2002).

CXCR4 is a less attractive therapeutic target in preventing HIV-1 infection or other diseases because this chemokine receptor is necessary for optimal immune defence, haematopoiesis and embryonal development (Juarez et al., 2001). On the other hand, CXCR4 is the most widely expressed chemokine receptor in many tumours, and therefore it may represent an important target receptor for preventing metastasis (Housmand and Zlotnik, 2003).

Development of antiretrovirals that would be able to down-regulate expression of chemokine co-receptors in addition to their effect on reverse transcriptase may be a possible challenge for pharmacological research. Thus, the aim of our pilot study was to investigate whether tenofovir and other ANPs could influence CCR5 and CXCR4 mRNA transcription in mouse lymphocytes and macrophages. The effects of these ANPs that have been previously found to enhance production of cytokines TNF-α and IL-10 were analysed (Zídek et al., 2001, 2003). These cytokines have been shown to influence, both positively and negatively, the expression of chemokine receptors, as described in the literature. TNF-α induces expression of CCR5 and CXCR4 in human astro-

cytes (Croitoru-Lamoury et al., 2003) or CXCR4 in monocytes (Biswas et al., 2001) and on the other hand, TNF-α down-regulates CCR5 expression on human lymphocytes (Hornung et al., 2000). Similarly, differential effects of IL-10 on the expression of CCR5 and CXCR4 receptors have been reported in dependence on cell types. Thus, CCR5 mRNA was rapidly up-regulated by exposure of murine dendritic cells to IL-10 (Takayama et al., 2001). In contrast, IL-10 suppressed CCR5 expression in human lymphocytes (Patterson et al., 1999). CXCR4 mRNA transcription was increased by IL-10 in macrophages (Ancuta et al., 2001) but down-regulated in lymphocytes (Jinquan et al., 2000). MCP-1 itself had no effect on CCR5 expression (Sica et al., 1997).

We determined the levels of mRNA for CCR5 and CXCR4 up to 24 h of incubating the cells in the presence of ANPs, i.e. at the intervals of both absence (1 h), or presence (2 h and more) of endogenous TNF-α and IL-10 in supernatants of the treated cells. Our data show that ANPs have no effect on CCR5 and CXCR4 mRNA transcription in both macrophages with no or negligible constitutive expression of chemokine co-receptors, and in lymphocytes with marked constitutive expression of chemokine co-receptors. The lack of the capacity of ANPs to influence CCR5 and CXCR4 mRNA transcription may thus be considered in our opinion as a desirable feature of these antiretroviral agents, although inhibition of CCR5 mRNA expression would be of course more welcome. In the case of CXCR4, it has been summarized previously by Juarez et al. (2001) that this receptor is necessary for processes such as normal haematopoiesis, so that blocking the *CXCR4* gene expression by antivirals would not be as advantageous as inhibition of the *CCR5* gene expression. In our experiments, LPS inhibited transcription of CCR5 and CXCR4 mRNA, in accordance with literature data (Sica et al., 1997; Verani et al., 2002). LPS was used as a reference agent in our experiments because this component of G^- bacterial cell wall has been reported to negatively interfere with the expression of chemokine co-receptors (Sica et al., 1997) and because in some experiments LPS also inhibited spreading of HIV infection (Verani et al., 2002).

Our preliminary study demonstrates that ANPs, despite stimulating cytokine and/or chemokine production, do not interfere with mRNA transcription for chemokine receptors CCR5 and CXCR4 that are usually used as co-receptors for HIV-1 entry in cells. Therapeutic exploitation of ANPs for generation of antivirals with these properties can be, in our opinion, substantiated. On the other hand, discovery of a chemical compound that could block reverse transcriptase and at once antagonize a chemokine HIV entry co-receptor would be more advantageous, and this drug could also be interesting from the pharmaco-economic point of view.

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