Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 329-335

ACTIVITY OF ACYCLIC NUCLEOSIDE PHOSPHONATE ANALOGUES AGAINST HUMAN IMMUNODEFICIENCY VIRUS IN MONOCYTE/MACROPHAGES AND PERIPHERAL BLOOD LYMPHOCYTES

Jan Balzarini, Carlo-Federico Perno*, D. Schols and Erik De Clercq

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

**Dipartimento di Medicina Sperimentale e Scienze Biochimiche, II Universita degli Studi di Roma, 00173 Roma, Italy*

Received April 19, 1991

SUMMARY. A number of acyclic nucleoside phosphonate analogues, including 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and its 2,6-diaminopurine derivative PMEDAP, (*R,S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine [(*R,S*)-FPMPA] and its 2,6-diaminopurine derivative (*R,S*)-FPMPDAP were evaluated for their inhibitory effects on HIV-1 replication in two natural human cell systems, i.e. peripheral blood lymphocytes (PBL) and freshly prepared monocyte/macrophages (M/M). All compounds were potent inhibitors of HIV-1 replication in PBL [50% effective concentration (EC_{50}): 0.94-3.9 μM] and M/M (EC_{50}: 0.022-0.95 μM). In particular, (*R,S*)-FPMPA and (*R,S*)-FPMPDAP showed a greater antiviral selectivity than PMEA and PMEDAP due to the virtual lack of toxicity of the former compounds in these cell systems. Also, the antiviral selectivity of the acyclic nucleoside phosphonate analogues was much higher in M/M than in the human T-cell lines MT-4, ATH8 and CEM.

In 1986, a novel class of acyclic phosphonylmethoxyalkyl nucleoside derivatives was described with potent and selective activity against a broad spectrum of DNA viruses, i.e. herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus, cytomegalovirus, African swine fever virus, vaccinia virus and human adenoviruses (1). The prototype compound was (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA] (Fig. 1). 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) and 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP), which are structurally closely related to (*S*)-HPMPA (Fig. 1), show a marked anti-retrovirus activity *in vitro* and *in vivo*. Retroviruses that are sensitive to PMEA and PMEDAP include human immunodeficiency virus (HIV-1, HIV-2), simian immunodeficiency virus (SIV), simian AIDS-related virus (SRV), feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), Friend murine

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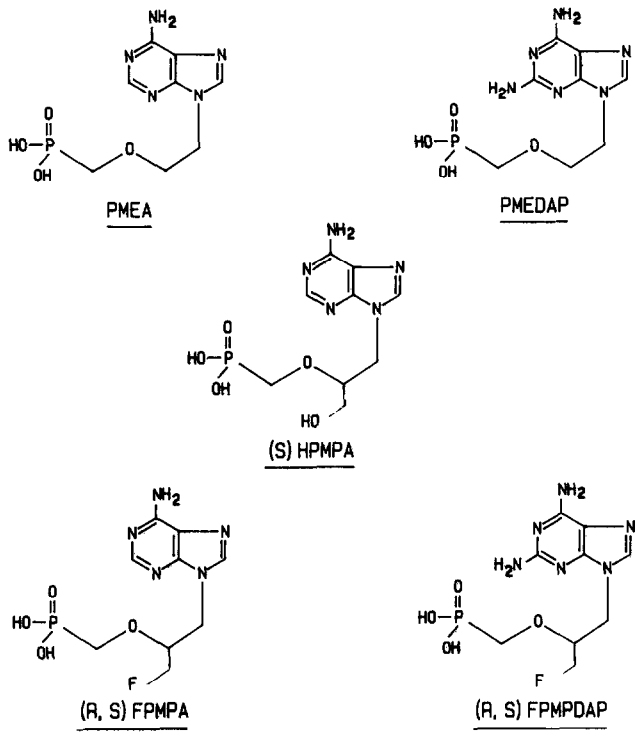

Fig. 1. Structural formulae of 9-(2-phosphonylmethoxyethyl)adenine (PMEA), 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP), (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], (*R,S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)adenine (FPMPA) and (*R,S*)-9-(3-fluoro-2-phosphonylmethoxypropyl)-2,6-diaminopurine (FPMPDAP).

leukemia virus, LP-BM5 retrovirus complex and Moloney murine sarcoma virus (MSV) (2-12).

Recently, we showed that (*R,S*)-9-(3-fluoro-2-phosphonylmethoxypropyl) derivatives of adenine [(*R,S*)-FPMPA] and 2,6-diaminopurine [(*R,S*)-FPMPDAP] (Fig. 1) has selective activity against retroviruses (13). These novel compounds are less toxic than the corresponding 2-phosphonylmethoxyethyl derivatives, both *in vitro* and *in vivo* (13). The *in vitro* anti-retrovirus data obtained with the phosphonate derivatives were obtained in the human T-cell lines MT-4, CEM, Molt/4, HUT-78, H9 and ATH8. In this paper, we describe the anti-HIV-1 activity of the representative phosphonate derivatives [i.e. PMEA, PMEDAP, (*S*)-HPMPA, (*R,S*)-FPMPA and (*R,S*)-FPMPDAP] in natural human cell systems [i.e. freshly prepared monocyte/macrophages (M/M) and PHA-stimulated peripheral blood lymphocytes (PBL)]. We found that in PBL cells (*R,S*)-FPMPA and (*R,S*)-FPMPDAP are more selective anti-HIV-1 agents than PMEA and PMEDAP. Their anti-HIV-1 activity is more pronounced in M/M than in PBL or MT-4 cells. Our observations point to the potential of the acyclic nucleoside phosphonate analogues, in

particular (*R*,*S*)-FPMPA and (*R*,*S*)-FPMPDAP, as candidate drugs for the treatment of retrovirus infections, including AIDS.

MATERIALS AND METHODS

Compounds. The synthesis of (*S*)-HPMPA, PMEA and PMEDAP has been published previously (14-16). The synthesis of (*R*,*S*)-FPMPA and (*R*,*S*)-FPMPDAP will be reported elsewhere.

Preparation, infection by HIV-1 and exposure of human monocyte/macrophages (M/M) to the test compounds *in vitro*. The M/M assays were essentially as described previously (17,18). Briefly, peripheral blood mononuclear cells (PBMC) were obtained from healthy, HIV-negative human volunteers upon separation by the Ficoll/Hypaque technique. More than 95% pure monocyte/macrophage (M/M) preparations were obtained by adherence of the cell suspension to plastic wells (10^6 cells/well) of a 48-well microtiter plate (Costar, Cambridge, Mass.) after the cells were cultured for 5 days. Non-adherent cells were removed by extensive washing with a phosphate-buffered saline (PBS) solution. Purity of the M/M preparation was ascertained according to non-specific esterase activity determination and Giemsa staining. M/M were then exposed to the test compounds at 100, 20, 4, 0.8, 0.16, 0.032 and 0.0016 μM for 20 min prior to challenge with the monocytotropic HIV-1 (Ba-L) strain (19) at 300 $CCID_{50}$/well. Virus excess was removed at 2 days after virus challenge, and the M/M cell cultures were continuously kept in RPMI-1640 medium supplemented with 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 Units/ml penicillin and 50 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY). At 15 days after virus challenge, viral p24 antigen production was evaluated by an ELISA assay (Abbott, Illinois). Also, syncytium formation was evaluated by viral inspection of the M/M culture under an inverted microscope. Drug toxicity was assessed by trypan blue exclusion.

Preparation, infection by HIV-1 and exposure of human peripheral blood lymphocytes (PBL) to the test compounds *in vitro*. Peripheral blood lymphocytes (PBL) from 2 different normal HIV-negative human volunteers were obtained by the Ficoll/Hypaque technique, washed twice with phosphate-buffered saline (PBS) and cultured with phytohemagglutinin (PHA, 72 μg/ml, Wellcome Diagnostics, Erembodegem, Belgium) for 3 days at 37°C in a CO_2-controlled incubator. PHA-stimulated PBL cells were washed twice with PBS and then infected with a concentrated HIV-1 ($HTLV\text{-}III_B$) stock solution. After 60 min incubation at 37°C, non-adsorbed virus was removed by 5 intensive and successive washing steps with fresh culture medium, and the HIV-1-infected and mock-infected PBL cells were then suspended at 5×10^5 cells/ml in RPMI 1640 medium supplemented with 15% heat-inactivated foetal calf serum and 5% recombinant interleukin-2 (Boehringer Mannheim, Mannheim, Germany) and cultured in the presence of varying concentrations (50, 10, 2, 0.4 and 0.08 μM) of the test compounds in 24-well culture plates (Costar) (1 ml per well). Fresh medium without test compound was added at 4 and 8 days after infection. HIV-1 p24 core antigen was quantified in the cell culture supernatants at 12 days after infection by an antigen-capture assay using a sandwich ELISA technique (Dupont, Brussels, Belgium). Cytotoxicity was determined by the trypan blue exclusion method (at day 12).

RESULTS

The acyclic nucleoside phosphonate analogues PMEA, PMEDAP, (*S*)-HPMPA, (*R*,*S*)-FPMPA and (*R*,*S*)-FPMPDAP were evaluated for their anti-

Table 1. Inhibitory effects of acyclic nucleoside phosphonate analogues on HIV-1 (Ba-L) replication in human monocyte/macrophages *in vitro*

Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)	Selectivity (CC_{50}/EC_{50})
PMEA	0.022	> 20	> 909
PMEDAP	0.076	> 20	> 263
(*S*)-HPMPA	3.2	20	6
(*R,S*)-FPMPA	0.95	> 100	> 105
(*R,S*)-FPMPDAP	0.20	> 100	> 500

[a] 50% Effective concentration or compound concentration that inhibits by 50% HIV-1 p24 antigen release in the supernatant of HIV-1(Ba-L)-infected monocyte/macrophages at day 21 after infection. Data are the mean of 2 independent experiments. Control cultures contained 210,000 and 259,000 ng p24 per ml at day 21 after the infection (two separate experiments).

[b] 50% Cytotoxic concentration, or compound concentration that reduces the viability of monocyte/macrophages by 50% on day 21 of the experiment.

HIV-1 activity in freshly prepared human monocyte/macrophages (M/M). Anti-HIV-1 activity was estimated from the reduction in p24 antigen production. The release of p24 antigen in the supernatant closely correlated with the appearance of syncytia. PMEA and PMEDAP showed a marked anti-HIV activity in M/M (Table 1). Their 50% effective concentrations (EC_{50}) were 0.022 and 0.076 μM, respectively. (*R,S*)-FPMPA and (*R,S*)-FPMPDAP were about 5- to 40-fold less effective; yet, their EC_{50} was still below 1 μM (Table 1). (*S*)-HPMPA was the least effective, its EC_{50} being 3.2 μM, which is only 6-fold lower than its 50% cytotoxic concentration (CC_{50}: 20 μM). In contrast, PMEA and PMEDAP showed no toxicity at a concentration of 20 μM at 21 days after exposure of the M/M cells to the compounds. Also, (*R,S*)-FPMPA and (*R,S*)-FPMPDAP proved non-toxic at a concentration of 100 μM. Consequently, antiviral selectivity indices (CC_{50}/EC_{50} ratios) are higher than 100 or 900 for all compounds except for (*S*)-HPMPA.

The acyclic nucleoside phosphonate analogues were also evaluated for their inhibitory effect on HIV-1 replication in human peripheral blood lymphocytes (PBL). The EC_{50} values recorded with PMEA, PMEDAP, (*R,S*)-FPMPA and (*R,S*)-FPMPDAP in PBL cells (Table 2) were higher than those obtained in M/M cells (Table 1). For (*S*)-HPMPA no anti-HIV activity was recorded in PBL cells. A striking difference was noted between PMEA and PMEDAP on the one hand, and (*R,S*)-FPMPA and (*R,S*)-FPMPDAP on the other hand, with regard to their toxicity for PBL cells. While PMEA and PMEDAP were cytotoxic at a CC_{50} of 50 and 12 μM, respectively, neither (*R,S*)-FPMPA nor (*R,S*)-FPMPDAP showed toxi-

Table 2. Anti-HIV-1 activity of acyclic nucleoside phosphonate analogues in human peripheral blood lymphocytes (PBL) *in vitro*

Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)	Selectivity (CC_{50}/EC_{50})
PMEA	3.7[c]	50	14
PMEDAP	0.94	12	13
(*S*)-HPMPA	> 20	50	< 2.5
(*R,S*)-FPMPA	3.9	> 500	> 128
(*R,S*)-FPMPDAP	1.85	> 500	> 270

[a]50% Effective concentration, or concentration required to inhibit by 50% HIV-1 p24 antigen release in the supernatant of HIV-1 (HTLV-III_B)-infected PBL cells at day 12 after infection.
[b]50% Cytotoxic concentration, or concentration required to reduce PBL viability by 50% on day 12 of the experiment.
[c]Data represent mean values of at least 2 to 3 independent experiments.

city even at a concentration as high as 500 μM. As a consequence, the anti-HIV-1 selectivity indices of (*R,S*)-FPMPA and (*R,S*)-FPMPDAP were 10- to 20-fold higher than those of PMEA and PMEDAP (Table 2).

DISCUSSION

There is an urgent need for novel and more potent and/or selective anti-retrovirus agents than those that are presently used in the treatment of HIV infections in humans. A series of acyclic nucleoside phosphonate analogues (i.e. PMEA, PMEDAP) have been identified as potent anti-retrovirus inhibitors *in vitro* and *in vivo* (2-12). However, these compounds have now been examined in two natural human cell systems, i.e. peripheral blood lymphocytes (PBL) and freshly prepared monocyte/macrophages (M/M). In PBL cells, PMEA and PMEDAP were equally effective as in the MT-4 cell line. However, they were about 100-fold more inhibitory to HIV-1 replication in monocyte/macrophages than in PBL or MT-4 cells. These observations together with the previously reported activity of these compounds against MSV, FLV, FIV, SIV and other retroviruses *in vitro* and *in vivo* point to their potential as candidate drugs for the treatment of HIV infections in humans.

The novel 3-fluoro-2-phosphonylmethoxypropyl derivatives (*R,S*)-FPMPA and (*R,S*)-FPMPDAP that were recently shown to exhibit greater anti-retrovirus selectivity than PMEA and PMEDAP *in vitro* and *in vivo* (13), also proved highly selective in inhibiting HIV replication in M/M and PBL cells. Thus, (*R,S*)-FPMPA and (*R,S*)-FPMPDAP also represent promising candidate drugs for AIDS chemotherapy. Also, the virtual

lack of toxicity of these compounds for human bone marrow cells and their lack of toxicity in newborn mice at relatively high doses (13) further attest to their potential therapeutic usefulness.

In conclusion, the acyclic nucleoside phosphonate analogues PMEA, PMEDAP, (*R,S*)-FPMPA and (*R,S*)-FPMPDAP exhibit potent and selective anti-HIV-1 activity in human peripheral blood lymphocytes and freshly prepared monocyte/macrophages. They are more selective in these natural human cell systems than previously reported in the well-established human T-cell lines MT-4, ATH8 and CEM. Moreover, the FPMP derivatives are more selective than their PME counterparts, owing to a markedly lower toxicity for the host cells.

ACKNOWLEDGMENTS

We thank Ann Absillis for her skillful technical assistance and Christiane Callebaut for her dedicated editorial help. This research was supported in part by the AIDS Basic Research Programme of the European Community and by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Projects 3.0026.91 and 3.0097.87), the Belgian Geconcerteerde Onderzoeksacties (project 90/94-2) and the Janssen Research Foundation.

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