Inhibition of murine AIDS by a heterodinucleotide of azidothymidine and 9-(R)-2-(phosphonomethoxypropyl)adenine

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Introduction

The acyclic nucleoside phosphonate 9-(R)-2-(phosphonomethoxypropyl)adenine (PMPA, tenofovir) is a highly potent and selective antiretroviral agent that has recently received US Food and Drug Administration (FDA) approval (as tenofovir disoproxil fumarate, Viread) for the treatment of HIV infection when taken in combination with other antiretroviral agents.

Unlike nucleoside analogues such as zidovudine (azidothymidine, AZT), PMPA possesses a metabolically stable phosphonoether moiety that does not require an initial phosphorylation by the nucleoside kinase to be activated. PMPA is phosphorylated by cellular enzymes to form the active metabolite PMPA diphosphate, which exhibits a long intracellular half-life in both resting and activated peripheral blood mononuclear cells, thus allowing once-daily dosing. This infrequent dosage regime adds a new dimension to its usefulness as an antiviral agent. In fact, not only does it assure greater convenience but also guarantees better compliance from the patients in adhering to their medicine.

Moreover, it was reported that PMPA demonstrated strong synergic anti-HIV activity in combination with AZT in vitro, but minor synergic inhibition of HIV replication in combination with other antiretroviral drugs such as didanosine (ddI) and nelfinavir. However, it is possible that during combination therapy, drug interactions may arise in vivo that would influence their antiviral activity. To investigate in vivo PMPA synergic antiretroviral activity in combination with AZT, experiments involving combination of PMPA and AZT were...
performed in a murine model of immunodeficiency (MAIDS), an AIDS-like disease caused by the LP-BM5 retroviral complex in susceptible C57BL/6 mice. However, since AZT has a low serum elimination half-life ($t_{1/2}$) of $\sim 1$ h, frequent daily administrations of this drug are needed to maintain therapeutically useful drug levels. Furthermore, the half-life is significantly different from that reported for PMPA.5 We showed previously that a new heterodinucleotide, consisting of tenofovir and zidovudine chemically coupled together by a phosphate bridge (AZTpPMPA), acts as an efficient antiviral prodrug able to protect macrophages against in vitro HIV infection (following selective targeting by means of loaded erythrocytes). Starting from these results, we first evaluated whether the single drug AZTpPMPA could act as a prodrug for a slow delivery of AZT and PMPA in circulation. Subsequently, the anti-retroviral activity of AZTpPMPA in the murine model of immunodeficiency was evaluated and compared with that obtained following both AZT plus PMPA and AZT or PMPA, administered as single drugs. The results obtained show that daily intraperitoneal AZTpPMPA administration is able to reduce MAIDS without causing toxicity, although to a minor extent compared with AZT plus PMPA, or treatment with PMPA alone. In addition, PMPA exhibits no synergic anti-retroviral activity in combination with AZT.

Materials and methods

Synthesis of AZTpPMPA

The heterodinucleotide AZTpPMPA was synthesized as described in a previous publication.6 PMPA was kindly provided by Norbert Bischofberger (Gilead Sciences, Foster City, CA, USA). AZT was obtained from Sigma, Milan, Italy.

Pharmacokinetic studies in mice

To investigate which route of administration in vivo is more convenient, two groups of ICR mice (Nossan, Milan, Italy) received intraperitoneal or oral gavage administration of AZTpPMPA, while another two groups received intraperitoneal or oral gavage administration of AZT plus PMPA. The intraperitoneal administration was chosen instead of the intravenous (iv) one since preliminary data showed that the pharmacokinetics of another phosphonate, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), administered iv or intraperitoneally provided essentially the same results. To evaluate the plasma concentration of AZT and PMPA after intraperitoneal AZTpPMPA or AZT plus PMPA administration, 7.5 µmol of AZTpPMPA (0.3 g/kg) or of AZT plus PMPA (0.12 and 0.13 g/kg, respectively) was intraperitoneally administered in PBS to each mouse (three mice for each time point were used). To evaluate the plasma concentration of AZT and PMPA after oral gavage administration of AZTpPMPA or of AZT and PMPA, the same amounts of drug were administered orally in distilled water. At different times (15 min, 1, 2, 4.5 and 6 h), 200 µL of blood was collected from CO2-anaesthetized mice into heparinized microhaematocrit capillary tubes by puncture of the retro-orbital sinus. Blood was immediately centrifuged at 900g for 10 min at 4°C and 70 µL plasma samples were extracted with perchloric acid: neutralized extracts were then used for the HPLC determinations of AZT, PMPA and AZTpPMPA.

HPLC determinations

Samples were extracted with perchloric acid as reported by Magnani et al.;7 neutralized extracts were then used for HPLC determinations. A 5 µm Res. Elut. 5 C18 90A column (150 × 4.60 mm I.D.; Varian, Harbor City, CA, USA) protected by a guard column (Pelliguard LC-18, 20 × 4.6 mm I.D., 40 µm particles) was used. The mobile phase consisted of two eluents: 25 mM KH2PO4 adjusted to pH 6.0 (buffer A) and buffer B containing 30% (v/v) acetonitrile (buffer B). All buffer solutions, as well as standards and sample solutions, were filtered through a 0.22 µm membrane filter (Millipore, Bedford, MA, USA). The elution conditions were as follows: 5 min at 100% buffer A, up to 100% buffer B over 30 min and hold for 10 min. The gradient was returned to 100% buffer A over 3 min and the initial conditions restored in 2 min. The flow rate was 0.9 mL/min and the detection wavelength was 260 nm. Analyses were performed at room temperature and quantitative measurements were obtained by injection of standards of known concentration. The retention times under the conditions used were 14.9 min for AZT-MP (where MP stands for monophosphate), 15.2 min for PMPA, 17.5 min for AZT and 24.9 min for AZTpPMPA.

Metabolism of AZTpPMPA in plasma

To evaluate the presence in plasma of enzymes able to cleave the phosphate bridge of AZTpPMPA, 200 µM AZTpPMPA was incubated in murine plasma for 8 h at 37°C under sterile conditions. At different times of incubation (0, 15 min, 30 min, 1, 2, 5 and 8 h), 100 µL aliquots were extracted with perchloric acid and used for HPLC determinations as reported above.

Virus, animals and drugs administration

The LP-BM5 viral mixture was kindly provided by Robert Yetter (Veterans Administration Hospital, Baltimore, MD, USA) and was maintained in a persistently infected SC-1 cell line as previously described.8 Five-week-old female C57BL/6 mice (Nossan) were infected by means of two intraperitoneal injections, one 24 h after the other, of 0.125 mL of the virus stock containing 1 U of reverse transcriptase. Mice were housed at 22 ± 1°C with a 12-h
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light/dark cycle, 60 ± 5% humidity and 12 air changes per hour. The study was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. After viral infection (7 h after the second viral injection), mice were intraperitoneally treated with equimolar doses of drugs (2.8 µmol/mouse) as follows: AZT (45 mg/kg), PMPA (48 mg/kg), AZT plus PMPA (45 and 48 mg/kg, respectively) or AZTpPMPA (111 mg/kg), five times per week for a period of 10 weeks. Drug dosages were adjusted every 2 weeks on the basis of body weight.

Serum immunoglobulin determination

At 10 weeks post-infection, blood was drawn from the retroorbital sinus for use in determining serum IgG levels via an enzyme-linked immunosorbent assay (ELISA) technique. Briefly, polystyrene microtitre plates (Dynex Technologies, Inc., Chantilly, VA, USA) were coated with goat anti-mouse IgG (Sigma, St Louis, MO, USA) diluted 1:100 in 0.135 M NaCl and incubated for 24 h at 37°C. The plates were washed four times with 0.1% Tween 20 in 10 mM NaH₂PO₄, 154 mM NaCl, pH 7.0 (TPBS) and blocked with 5% bovine serum albumin (BSA) in TPBS for 1 h at 37°C. After four washings in TPBS, serial dilutions of murine serum in 50 mM sodium borate, pH 8.5, were added and incubated for 1 h at 37°C. After four washings in TPBS, 100 µL of goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad, Richmond, CA, USA), diluted 1:1000 in PBS, was added. After incubation for 1 h at 37°C, serum IgG levels were determined using a colour development solution containing 2.2 mM o-phenylenediamine. Absorbance was measured at 492 nm on a Model 2550 enzyme immunoassay (EIA) reader (Bio-Rad). Absolute serum IgG concentrations were obtained using known concentrations of standard mouse IgG.

Competitive PCR analysis of BM5d proviral DNA

Total cellular DNA was isolated from lymph nodes as previously described. A competitive PCR assay was used to analyse BM5d proviral DNA content as described in Casabianca et al. Histological and other examinations

Samples of lymph node, liver, kidney and spleen tissues from three animals in each treatment group were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned and stained with haematoxylin–eosin. Haematological para-

Figure 1. Concentrations of AZT and PMPA in the plasma of mice after intraperitoneal (a) or oral gavage (b) administration of AZTpPMPA and after intraperitoneal (c) or oral gavage (d) administration of AZT plus PMPA. All compounds were given at a dose equivalent to 7.5 µmol/mouse for each drug, which corresponds to 0.3, 0.12 and 0.13 g/kg for AZTpPMPA, AZT and PMPA, respectively. Data are the average values for three independent experiments (three mice per time point in each experiment).
meters were evaluated using ABX Micros 'OT' (ABX, Parc Euromedicine, Montpellier, France).

### Statistical analysis

Statistical analysis of data was performed with the parametric test (t-test) of the Microcal Origin program (Microcal Software, Inc., Northampton, MA, USA) with a *P* value of <0.05 used to determine significance.

### Results

#### Pharmacokinetics in mice

To evaluate the route of administration able to give the highest and/or maintained levels of drugs in circulation, AZT plus PMPA and AZTpPMPA were administered both intraperitoneally and orally. As shown in Figure 1, the best results were obtained following intraperitoneal administration. In particular, PMPA plasma concentrations were 0.3 and 0.5 mM (at time point 15 min) after the intraperitoneal administration of AZTpPMPA and AZT plus PMPA, respectively. Very low levels of PMPA were found following oral gavage administration, probably because of its low bioavailability. On oral gavage AZTpPMPA administration, PMPA plasma ranged from 3 to 17 µM, whereas on oral gavage AZT plus PMPA treatment, PMPA concentrations ranged from 2 to 7 µM. The plasma concentration of AZT following both intraperitoneal and oral gavage administration of drugs was similar. Moreover, no more drugs were found in circulation at time point 4.5 h post-intraperitoneal AZT plus PMPA administration, whereas PMPA was still detectable 6 h after intraperitoneal heterodinucleotide administration. The half-lives (*t*<sub>1/2</sub>) of AZT and PMPA when administered intraperitoneally as prodrug, were compared with values obtained when the drugs themselves were administered. Data were calculated starting from drug concentrations at time 15 min, and for both PMPA and AZT slightly higher *t*<sub>1/2</sub> values were observed when administered as prodrug (55 and 205 min for PMPA and AZT, respectively) than when administered as free drugs (35 and 85 min for PMPA and AZT, respectively). Values for the area under the curve, calculated in the range 0.25–6 h (AUC<sub>0.25–6</sub>) (Table 1) confirmed the advantage of intraperitoneal administration compared with oral gavage. Thus, prompted by these data, we chose to administer the drugs intraperitoneally.

#### AZTpPMPA stability in murine plasma

The ability of murine plasma to convert AZTpPMPA into AZT and PMPA was evaluated. As shown in Figure 2, plasma possesses enzymes able to cleave the phosphate bridge of AZTpPMPA (*t*<sub>1/2</sub> 30 min) with the stoichiometric production of PMPA and AZT-MP, which is then converted into AZT. The half-lives for the appearance of PMPA and AZT were 42 and 102 min, respectively. Therefore, AZTpPMPA can act as a prodrug for the release of both AZT and PMPA. Similar results were obtained in human plasma.

![Figure 2](https://academic.oup.com/jac/article-abstract/50/5/639/754326)
Inhibition of murine AIDS by AZTpPMPA

The heterodinucleotide AZTpPMPA was examined for its ability to inhibit MAIDS development in LP-BM5-infected C57BL/6 mice compared with the free drugs AZT and PMPA alone or in combination. In C57BL/6 mice, LP-BM5 retrovirus complex causes a pathology similar to human AIDS, with marked hypergammaglobulinaemia, lymphoadenopathy and splenomegaly. Mice infected with LP-BM5 developed MAIDS within 4 weeks after infection. In this study, six groups of mice (eight mice for each group) were used: control mice (uninfected and untreated), infected mice (infected and untreated) and four other groups that were infected and treated with AZTpPMPA, AZT plus PMPA, PMPA or AZT, respectively. The drugs were administered intraperitoneally at equimolar dosages (2.8 μmol/mouse), which correspond to 45 mg/kg body weight for AZT, 48 mg/kg for PMPA and 111 mg/kg for AZTpPMPA. Each drug was administered post-infection, daily, 5 days per week, for a period of 10 weeks. After 10 weeks of infection, mice of all experimental groups were sacrificed and several parameters characterizing the progression of the disease were evaluated. The degree of hypergammaglobulinaemia is shown in Figure 3a: the administration of the heterodinucleotide AZTpPMPA causes a 40% reduction in IgG level, whereas a 64% decrease was observed in infected mice that received intraperitoneal AZT plus PMPA; the same result (62% reduction) was obtained with PMPA alone. A lower reduction (28%) was observed in infected mice treated with AZT.

The degree of lymphoadenopathy and splenomegaly is shown in Figure 3b. The results obtained after 10 weeks of treatment showed a marked reduction in lymph node weights...
following the administration of AZTpPMPA, or AZT plus PMPA or PMPA (88%, 91% and 91%, respectively), whereas a reduction of only 36% was observed with AZT. AZTpPMPA, AZT plus PMPA or PMPA were also effective in reducing splenomegaly (64%, 67% and 68%, respectively), but AZT only caused a 22% reduction in spleen weight.

The amount of BM5d proviral DNA in lymph nodes of infected mice was also evaluated (Figure 4). The results obtained showed a 49% reduction of proviral DNA content in the lymph nodes of infected mice receiving AZTpPMPA; upon AZT plus PMPA or PMPA treatment, higher reductions (75% and 70%, respectively) were observed. AZT was able to give an inhibition of the proviral DNA content of only 26%. Thus, AZTpPMPA is much better than AZT but similar to or even less efficient than PMPA, in reducing the progression of MAIDS evaluated following the typical signs of the disease and proviral DNA content in lymph nodes.

We also evaluated the possible haematological toxicity of these treatment regimens (Table 2). At 10 weeks post-infection, all treatment groups showed near normal control haematological parameters. In particular, red and white cell counts, haematocrit percentage and haemoglobin content were found to be higher (and then quite similar to controls) in all treated groups of mice with respect to the infected-untreated animals.

**Histological examinations**

Typical signs of MAIDS include changes in the architecture and cellularity of spleen and lymph nodes and portal lymphoid infiltration in liver. The administration of AZT was able to restore both normal cellularity in lymph nodes (repopulation of T and B cells) and architecture in spleen. Similar results were obtained upon both PMPA administration and combination therapy. In addition, the administration of AZT alone was able to partially reduce infiltrates in liver, while by administering PMPA or AZTpPMPA liver sections were similar to controls (Figure 5). Finally, no kidney damage was ever observed (not shown).

**Discussion**

In this study, the antiretroviral efficacy of the heterodinucleotide AZTpPMPA in a murine model of AIDS was evaluated. Furthermore, AZTpPMPA efficacy has also been compared with that of free AZT and PMPA, separately or simultaneously administered.

PMPA, as its oral prodrug form bis(isopropyloxycarbonyloxymethyl)-PMPA, is the first nucleotide analogue reverse transcriptase inhibitor approved for the treatment of HIV. As a nucleotide, it remains in cells for longer periods of time than many other antiretroviral drugs, allowing for once-daily dosage. However, it must be taken in combination with other antiretroviral agents, as stated by the US FDA. AZT is the most popular compound that is able to reduce the morbidity

**Table 2. Haematological parameters in mice at 10 weeks post-infection**

<table>
<thead>
<tr>
<th>Mice</th>
<th>RBC (cells x 10^3/µL)</th>
<th>Ht (%)</th>
<th>Hb (g/dL)</th>
<th>MCH (pg/cell)</th>
<th>MCHC (g/dL)</th>
<th>WBC (cells x 10^3/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.3 ± 0.6</td>
<td>38.8 ± 1.3</td>
<td>11.3 ± 0.5</td>
<td>13.5 ± 0.2</td>
<td>29.2 ± 0.3</td>
<td>8.4 ± 1.6</td>
</tr>
<tr>
<td>Infected (I)</td>
<td>5.7 ± 1.2</td>
<td>28 ± 5.6</td>
<td>8.4 ± 1.8</td>
<td>14.3 ± 0.4</td>
<td>29.8 ± 1.0</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td>I + AZTpPMPA</td>
<td>6.8 ± 0.5</td>
<td>34.6 ± 2.5</td>
<td>10.1 ± 0.5</td>
<td>14.1 ± 0.4</td>
<td>29.3 ± 0.9</td>
<td>5.9 ± 2.4</td>
</tr>
<tr>
<td>I + AZT + PMPA</td>
<td>7.2 ± 0.6</td>
<td>34.3 ± 2.8</td>
<td>10.3 ± 0.9</td>
<td>14.1 ± 0.4</td>
<td>29.9 ± 0.7</td>
<td>5.9 ± 3.0</td>
</tr>
<tr>
<td>I + PMPA</td>
<td>7.3 ± 0.8</td>
<td>35.5 ± 2.6</td>
<td>11.2 ± 1.4</td>
<td>13.8 ± 0.6</td>
<td>29.7 ± 0.9</td>
<td>6.9 ± 2.8</td>
</tr>
<tr>
<td>I + AZT</td>
<td>6.6 ± 0.6</td>
<td>32.5 ± 2.7</td>
<td>9.4 ± 0.8</td>
<td>14.1 ± 0.3</td>
<td>28.8 ± 0.4</td>
<td>7.7 ± 2.5</td>
</tr>
</tbody>
</table>

All values are means ± s.d. of eight animals.

RBC, red blood cells; Ht, haematocrit; Hb, haemoglobin; MCH, mean cellular haemoglobin; MCHC, mean cellular haemoglobin concentration; WBC, white blood cells.
Inhibition of murine AIDS by AZT-pPMPA

and mortality associated with severe HIV infection, particularly in combination with other drugs. In addition, AZT has demonstrated in vitro strong synergic anti-HIV activity in combination with PMPA; however, since it has a low serum elimination half-life (1 h), frequent daily AZT administrations are needed. Since we have previously shown that the heterodinucleotide AZT-pPMPA was able in vitro to inhibit HIV replication in macrophages, we thought that it could act as a prodrug able to give therapeutic plasma levels of AZT and PMPA. The goals of our work were: (a) to evaluate the ability of the heterodinucleotide AZT-pPMPA to release AZT and PMPA in circulation; (b) to evaluate in vivo the antiretroviral efficacy of AZT-pPMPA; and (c) to evaluate in vivo the synergic antiretroviral activity of AZT and PMPA.

The results obtained show that AZT-pPMPA is cleaved into AZT and PMPA following both oral gavage and intraperitoneal administration. AZT-pPMPA does not reach the systemic circulation intact when orally or intraperitoneally administered to mice, in fact only AZT and PMPA were found in plasma samples taken at different times post-heterodinucleotide administration. This is probably the consequence of hydrolysis of the phosphate bridge of the heterodinucleotide during transit across the intestinal epithelium or after absorption in the portal blood (plasma enzymes are able to convert AZT-pPMPA into AZT and PMPA; Figure 2). In agreement with reports on PMPA limited oral bioavailability, a low level of PMPA in circulation was found when the heterodinucleotide was administered orally. In contrast, by intraperitoneal injection, higher plasma levels of PMPA were found and, overall, PMPA was still present at therapeutic levels, at least 6 h post-heterodinucleotide administration (Figure 1). The bioavailability of AZT when
administered orally is in accordance with what has been reported by other authors. They carried out pharmacokinetic studies of AZT in mice comparing concentrations in the 15–60 mg/kg range; we obtained proportional AUC values with a dose of 120 mg/kg. In addition, the calculation of AUC_{0.25–6} for AZT and PMPA following the two different routes of AZT-PMPA or AZT plus PMPA administration, confirmed intraperitoneal delivery as the best administration route for in vivo antiviral studies.

The antiretroviral efficacy of the intraperitoneal AZT-PMPA administration was then evaluated in C57BL/6 mice infected with the retroviral complex LP-BM5, which causes a severe immunodeficiency, i.e. MAIDS. We have chosen the MAIDS model because it shows many similarities to human AIDS, including abnormal T- and B-lymphocyte functions, polyclonal B-cell proliferation, lymphoid adenopathy, splenomegaly, hypergammaglobulinaemia and enhanced susceptibility to infections, and thus is a convenient pre-clinical animal model for initial studies. AZT-PMPA in MAIDS was able to markedly reduce lymphoadenopathy, splenomegaly and lymph node BM5 proviral DNA content (88%, 64% and 49%, respectively) and partially (40%) hypergammaglobulinaemia.

Moreover, the tissue histological examinations have shown that upon AZT-PMPA administration, liver and spleen were as controls and no sign of drug toxicity was found. In addition, an increase in red blood cell number, haematocrit, haemoglobin concentration and white cell count was observed compared with infected-untreated mice. As expected from pharmacokinetic studies, the antiviral efficacy of AZT-PMPA is similar to that obtained upon intraperitoneal administration of AZT in combination with PMPA, at least concerning lymphoadenopathy and splenomegaly reduction and histological examinations. However, upon treatment with AZT plus PMPA, a higher reduction of hypergammaglobulinaemia (64%) and, overall, of the proviral DNA content in lymph nodes (75%) was achieved. These results are probably due to the initial higher plasma levels of AZT and PMPA observed upon AZT plus PMPA administration rather than following the heterodinucleotide treatment.

Finally, we have also evaluated the possible synergic anti-retroviral activity of AZT in combination with PMPA. The results obtained show that AZT alone was only partly able to reduce disease progression (20–40% reduction in most of the parameters evaluated), whereas PMPA alone gave better results than AZT, confirming what was observed by other authors. However, with PMPA alone, results similar to those of the combined therapy (AZT plus PMPA) were obtained, excluding possible synergic effects between AZT and PMPA. Based on this evidence, we have not tested further concentrations of both drugs and we cannot find evidence for possible additional advantages in administering the combination of PMPA with AZT in MAIDS.

In conclusion, the results reported in this study show that the new heterodinucleotide AZTpPMPA, consisting of AZT and PMPA chemically coupled by a phosphate bridge, is able to perform as a prodrug for slow release of both drugs in circulation. When intraperitoneally administered to LP-BM5-infected mice, AZTpPMPA was able to reduce most of the signs of the disease. However, this drug does not appear to be more efficient than AZT plus PMPA or even PMPA alone, at least in mice. Thus, in view of the reported synergy of AZT and PMPA in vitro in HIV-1-infected cells, further studies are now needed to evaluate AZTpPMPA in a different pre-clinical animal model. The favourable pharmacokinetics, absence of toxicity and significant antiviral activity of this new heterodinucleotide suggest a possible alternative to the use of new combination therapies based on single molecules consisting of different antiviral moieties each with different intracellular pharmacokinetics.

Acknowledgements

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