Pharmacokinetic and antiretroviral activity in mice of oral [P1,P2-bis[2-(adenin-9-yl)ethoxymethyl]phosphonate], a prodrug of 9-(2-phosphonylmethoxyethyl)adenine

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9-(2-Phosphonylmethoxyethyl)adenine (PMEA) is an antiviral drug with activity against herpes viruses, Epstein–Barr virus and retroviruses, including the human immunodeficiency virus. Unfortunately, oral PMEA administration, as required for long-term therapy, is hindered by its low bioavailability. In the present study, the synthesis, oral bioavailability and antiretroviral activity of a new prodrug of PMEA, consisting of two molecules of PMEA bound together by a P-O-P bond (Bis-PMEA), are reported. Pharmacokinetic experiments in mice showed that the oral bioavailabilities of PMEA following oral gavage of Bis-PMEA or PMEA (at a dose equivalent to 28 mg of PMEA/kg) were 50.8 and 13.5%, respectively. These results correlate with the antiviral efficacy of Bis-PMEA administered orally at a dose equivalent to 50 mg/kg of PMEA in C57 BL/6 mice infected with the retroviral complex LP-BM5. Oral treatment with Bis-PMEA proved to be more effective than oral treatment with PMEA given at equimolar doses. Moreover, oral Bis-PMEA was more effective than intraperitoneal PMEA (50 mg/kg) in reducing lymphoadenopathy, hypergammaglobulinaemia and lymph node proviral DNA content, overall in the first weeks post virus inoculation. Bis-PMEA thus appears to be an efficient oral prodrug of PMEA without significant toxicity, at least in this mouse model.

Introduction

9-(2-Phosphonylmethoxyethyl)adenine (PMEA, adefovir) is a prototype of the acyclic nucleoside phosphonate (ANP) analogues, which are broad-spectrum antiviral agents with potent and selective antiviral activity in vitro and in vivo.1 PMEA is able to inhibit both retroviruses (including human immunodeficiency virus, HIV-1) and herpes viruses (including herpes simplex virus type 1, HSV-1) both in vitro and in animal models.2–7 Thus, PMEA is of interest both as a potential antiretroviral drug for use in treating HIV-1 infections and also for the treatment of some of the opportunistic infections associated with AIDS. Since AIDS patients require a long-term treatment regimen, it would be convenient if drugs could be administered orally. Unfortunately, oral PMEA administration is hindered by its low cellular uptake due to the negative charge (at physiological pH) of the phosphonate group8 and hence needs to be injected, which is a major obstacle for long-term clinical applications. PMEA has been shown to have low oral bioavailability in mice,9 rats,10 cynomolgus monkey11 and humans.12 In order to mask the phosphonate charge and enhance the lipophilicity of the molecule, a large number of produgs of PMEA have been designed.

Among these, the bis (pivaloyloxymethyl) [bis(POM)] ester prodrug was selected as a potentially useful prodrug of PMEA,13 and clinical trials both with humans infected with HIV-1 and individuals infected with hepatitis B virus (HBV) were performed.14,15 Clinical trials with bis(POM)-PMEA (adefovir dipivoxil) in 442 patients infected with HIV have shown that the drug can add some extra anti-HIV effects to

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combination treatment, leading to an average decrease in viral load of just over 40% after 6 months. Furthermore, bis(POM)-PMEA works against several strains of HIV that are already resistant to AZT, d4T or ddI, and seems to have very little cross-resistance with other retroviral drugs. Unfortunately, in these studies more than a third of the people who took the highest dose (120 mg/day) for more than 6 months developed signs of serious kidney damage. For this reason, at present, the development of therapeutic strategies employing bis(POM)-PMEA against HIV is going on in Europe but not in the United States, where the development of the drug is instead continuing against HBV. In particular, for this disease, bis(POM)-PMEA is now in phase III clinical trials.

In addition, some adverse effects due to the pivaloyl moiety of the prodrug have been observed. To avoid the toxicity caused by the pivaloyl group, herein we report a novel approach based on the prodrug P1,P2-bis[2-(adenine-9H-yl)ethoxymethyl]phosphonate (Bis-PMEA), a compound consisting of two molecules of PMEA bound together by a P-O-P bond (Figure 1). We previously showed 16 that Bis-PMEA acts as an efficient antiviral prodrug able to protect macrophages against in vitro HIV or HSV infections following selective targeting by means of loaded erythrocytes. In this paper, the oral bioavailability of free Bis-PMEA in mice and its antiretroviral activity in a murine model of immunodeficiency were evaluated. The results obtained show that Bis-PMEA, administered by oral gavage, acts as a prodrug for a slow delivery of PMEA in circulation, giving a higher antiretroviral activity than oral PMEA without causing increased toxicity.

Materials and methods

Synthesis of Bis-PMEA and [14C]Bis-PMEA

The homodinucleotide Bis-PMEA was synthesized by coupling the morpholidate derivative of PMEA as N,N'-dicyclohexyl-4-morpholine-carboxamidinium salt with PMEA as mono n-tri-buty lammonium salt. In a similar way the radiolabelled dimer [14C]Bis-PMEA was obtained. PMEA n-tri-butylammonium salt was synthesized by treatment of nucleoside in pyridine/water (1:1, v/v) with n-tri-butylamine and dried in vacuo over phosphorus pentoxide. N,N'-Dicyclohexyl-4-morpholine-carboxamidinium 2-(adenine-9H-yl)-ethoxymethyl]morpholinophosphonate was synthesized by treatment of PMEA with morpholine and N,N'-dicyclohexylcarbodiimide (molar ratio 1/6.5) in hot aqueous tert-butyl alcohol (1:1, v/v) for 11 h as reported in Holy & Rosenberg,17 to obtain the compound as a white solid. 1H-NMR (300 MHz, D2O): δ 3.15, 3.30, 3.63 (3m, 8H, morpholino); 3.52 (d, J = 8.8 Hz, 2H, CH2P); 3.84 (t, J = 4.9 Hz, 2H, H-2'); 4.35 (t, J = 5.0 Hz, 2H, H-1'); 8.25 (2s, 2H, H-2, H-8). 31P-NMR (121 MHz, D2O): δ 18.0.

Coupling of the PMEA salt to PMEA morpholidate was carried out under anhydrous conditions (molar ratio 1/1) in N,N-dimethylformamide at 50°C for 40 h. The solvent was removed in vacuo and the oily residue was portioned between water and diethyl ether (1:2, v/v). The aqueous phase was evaporated to dryness and the residue was chromatographed on a silica gel column with PrOH/NH4OH/H2O (80:10:10, v/v/v). Evaporation of the appropriate fractions gave a solid residue that was solubilized in aqueous ammonia and washed with diethyl ether. The aqueous phase was evaporated to dryness and co-evaporated with anhydrous ethanol. The residue was washed with ethyl ether and acetone to obtain the bis(ammonium) Bis-PMEA as a white solid, which was dried over phosphorus pentoxide (50% yield). TLC (H2O/CH3CN, 80:20, v/v): Rf = 0.68. UV (H2O): λmax 258 nm (ε 23 500). MS (API-ESI) m/z = 527.0, 263.0 [M–H]+, 540.0 [M–2H + Na]+, consistent with the expected molecular structure. All measurements were performed in the negative ion mode, and scanning in the 300–800 mass range. 1H-NMR (300 MHz, D2O): δ 3.65 (t, J = 3.7 Hz, 4H, 2x CH2P); 3.81 (t, J = 4.5 Hz, 4H, H-2'); 4.18 (t, J = 4.5 Hz, 4H, H-1'); 7.95 (s, 2H, H-8); 7.98 (s, 2H, H-2'). 31P-NMR (121 MHz, D2O): δ 8.8. The chemical shift values are expressed in δ values (ppm) relative to tetramethylsilane and orthophosphoric acid as internal standards. The elemental analysis confirmed the structure of Bis-PMEA as a diammonium salt (Anal. Calc. for C16H28N12O7P2·(H2O): C 32.11, H 5.39, N 28.09. Found: C 32.43, H 5.48, N 27.89).

The radiolabelled dimer [14C]Bis-PMEA was obtained as above, by coupling the mono n-tri-butylammonium *PMEA [adenine-8-14C] salt to PMEA morpholidate. The *PMEA [adenine-8-14C] n-tri-butylammonium salt was synthesized from a mixture of 16.5 mg of *PMEA [adenine-8-14C] and 24.75 mg of PMEA in pyridine/water (1:1, v/v), with n-tri-butylamine (molar ratio 1/1). [14C]Bis-PMEA was characterized as described above. PMEA and *PMEA [adenine-8-14C] were kindly provided by Dr Norbert Bischofberger, Gilead Sciences, Foster City, CA, USA.

HPLC determination of Bis-PMEA and PMEA in plasma

The metabolism of Bis-PMEA in plasma was evaluated as follows: 0.14 mM Bis-PMEA was incubated in murine plasma at 37°C and at different times of incubation (15 min, 30 min, 1 h,
Effects of clarithromycin on biofilm

Pharmacokinetic studies in mice

To determine the concentration of Bis-PMEA and PMEA in circulation after oral Bis-PMEA administration, 45 mg of Bis-PMEA in 350 µL of distilled water was administered by oral gavage to four Institute of Cancer Research (ICR) mice. At different times (1, 2, 5 and 8 h), 500 µL of blood was collected from CO2-anaesthetized mice (one mouse/time point) into heparinized Pasteurs by puncture of the retro-orbital sinus. Blood was immediately centrifuged at 900 g for 10 min at 4°C and 250 µL plasma samples were extracted with perchloric acid; neutralized extracts were then concentrated with a Speed Vac concentrator (Savant Instruments, Hicksville, NY, USA) to a final volume of 100 µL and used for the HPLC determinations of Bis-PMEA and PMEA, as described above.

Pharmacokinetic studies in mice were performed as follows: female ICR mice (weight 22 ± 1 g) received either [14C]PMEA by intravenous (iv) bolus injection (via the retro-orbital sinus) or oral gavage, or [14C]Bis-PMEA by oral gavage. Both compounds were given at equimolar doses of PMEA, i.e. 28 mg/kg for [14C]PMEA (with a specific activity of 11 257 cpm/nmol) and 28 mg/kg for [14C]Bis-PMEA (with a specific activity of 22 977 cpm/nmol). The [14C]PMEA used for iv administration was suspended in physiological saline solution [5 mM glucose, 5 mM KH2PO4/NaH2PO4 and 0.9% (w/v) NaCl, pH 7.4], whereas [14C]PMEA and [14C]Bis-PMEA used for oral gavage administration were suspended in distilled water and 2 M sodium hydroxide was added to obtain a final pH of 7.0. For each drug administration (iv) the concentration (AUCpo,0-t last) equals the AUC for [14C]PMEA following iv injection of [14C]PMEA.

Virus and animals

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.18 Five-week-old female C57BL/6 mice (Nossan, Milan, Italy) 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. ICR mice (Nossan) were used to assess the pharmacokinetic studies. Mice were housed at 22 ± 1°C with a 12 h light/dark cycle, 60 ± 5% humidity, and 12 air changes per hour.

Drug administration

After viral infection (7 h after the second viral injection), mice were treated with PMEA by mouth, Bis-PMEA by mouth and PMEA intraperitoneally, 50 mg/kg for each drug tested, five consecutive days weekly for a period of 4 or 9 weeks, as already reported for other treatment regimens investigated in this animal model.19,20 PMEA and Bis-PMEA dosages were adjusted every 2 weeks on the basis of body weight.

Serum immunoglobulin determination

At different times post virus inoculation (4 and 9 weeks), blood was drawn from the mouse’s tail vein for use in determining serum IgG levels via an enzyme-linked immunosorbent assay (ELISA) technique, as described elsewhere.21

Semi-quantitative PCR analysis of LP-BM5 proviral DNA

Total cellular DNA was isolated from lymph nodes as previously described.20 A semi-quantitative PCR method was used to analyse BM5d proviral DNA in lymph nodes. The oligonucleotide primers and the procedure used were those described in Fraternale et al.22

Evaluation of mtDNA content

Total cellular DNA was isolated from lymph nodes of infected and infected-treated mice 9 weeks post virus inoculation as described by Rossi et al.23 The extracted DNAs were carefully quantified with an agarose–ethidium bromide staining gel using the Gel Doc 1000 System. For each sample, 2 µg of DNA was digested with BamH1 restriction enzyme, analysed on a 0.8% (w/v) agarose gel and transferred to a nylon membrane. A specific 1237 bp mtDNA probe was used in hybridization experiments to detect mtDNA content. The intensities of the radiolabelled bands were quantified in a GS-250 molecular imager (Bio-Rad).
Flow cytometric analysis

The fluorescent monoclonal antibodies used for fluorescence-activated cell sorting (FACS) analysis were purchased from PharMingen, Becton Dickinson Co. (Milan, Italy). Blood samples were stained with fluorescent monoclonal antibody and analysed by FACS (Becton Dickinson, San Jose, CA, USA) as described in the study by Palamara et al. 19

Lymphocyte proliferative index

[3H]Thymidine incorporation on lymphocytes stimulated by either phytohaemagglutinin (PHA) for T cells or lipopolysaccharide for B cells was determined as described by Brandi et al. 24

Histological examinations

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

Cell cultures

U937 human monoblastoid cells and L1210 mouse leukaemia cells were grown in RPMI 1640 medium (PBI International, Milan, Italy) supplemented with 10% heat-inactivated fetal calf serum (PBI International), 2 mM L-glutamine (Sigma), penicillin and streptomycin, 100 U/mL and 100 mg/L (Bio-Whittaker, Verviers, Belgium), respectively, in 5% CO2/95% air at 37°C. U937 and L1210 cells were seeded in 96 wells at a density of 1 × 104 cells/well and incubated at 37°C, 5% CO2/95% air. PMEA toxicity was evaluated by determining the number of viable cells with the Trypan Blue dye exclusion test after exposing U937 and L1210 cells to different concentrations (0–4 µM) of PMEA for 8 days.

Statistical analysis

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

Other determinations

Haematological parameters were evaluated using ABX Micros ‘OT’ (ABX, Parc Euromedicine, Montpellier, France); creatinine was evaluated in plasma samples with a test-combination kit (Boehringer-Mannheim).

Results

Bis-PMEA metabolism in murine plasma

The ability of murine plasma enzymes to convert Bis-PMEA into PMEA was evaluated. As shown in Figure 2, plasma possesses enzymes able to cleave the P-O-P bond of Bis-PMEA with the stoichiometric production of PMEA (t1/2 ~60 min).

Pharmacokinetic studies in mice

The chromatographic analysis of plasma samples obtained from mice receiving oral Bis-PMEA administration (45 mg/kg) revealed the presence of PMEA and the total absence of Bis-PMEA in circulation (data not shown). Unfortunately, the HPLC method used did not allow us to determine PMEA concentration accurately below 1 µM and thus time points after 8 h could not be evaluated. In addition, since only PMEA was found in circulation at all time points examined following Bis-PMEA administration, the chromatographic separation of these two compounds was not required for pharmacokinetic studies. For these reasons, radiolabelled compounds ([14C]PMEA and [14C]Bis-PMEA) were used and pharmacokinetic profiles obtained directly by evaluating [14C]PMEA radioactivity in plasma samples.

Figure 3 shows the time course of PMEA concentrations in plasma following the iv administration of [14C]PMEA and the oral administration of [14C]PMEA and [14C]Bis-PMEA to ICR mice at 28 mg/kg.

The drug concentration–time curve after the iv bolus injection of [14C]PMEA showed a rapid and biphasic decline. In contrast, oral gavage of [14C]PMEA or [14C]Bis-PMEA resulted in rather low, yet sustained, concentrations of PMEA in
plasma that were still detectable up to 2 days after administration. Moreover, in mice receiving [14C]Bis-PMEA by mouth, [14C]PMEA reached higher concentrations in plasma than after oral administration of [14C]PMEA (maximum plasma PMEA concentrations, 0.89 and 0.316 mg/L following oral administration of [14C]Bis-PMEA and [14C]PMEA, respectively). The oral bioavailability of PMEA, defined as the ratio of the AUC for PMEA following oral administration of PMEA or of its prodrug Bis-PMEA to the AUC for iv PMEA, was 13.5% and 50.8% for oral PMEA and oral Bis-PMEA, respectively (Table 1).

**Inhibition of murine AIDS (MAIDS) development in vivo**

Infection of C57BL/6 mice with the LP-BM5 retrovirus complex causes a disease with a pathology that resembles that of human AIDS;25 as a result, this animal model has been used extensively for preclinical studies of antiviral drugs.24,26 In the present study, LP-BM5-infected mice were treated with the antiviral agent PMEA, or its prodrug Bis-PMEA, by oral gavage administration. Drugs were administered at equimolar doses of PMEA at a concentration of 50 mg/kg. The experiment was performed as follows: one group of mice was infected but not treated, one group was infected and treated by mouth with PMEA, and another group was infected and treated by mouth with the prodrug Bis-PMEA. Both drugs were administered 5 days a week for 4 or 9 weeks. As controls, uninfected/untreated mice, and infected/intraperitoneally PMEA-treated mice at a concentration of 50 mg/kg, were used. Mice infected with LP-BM5 developed MAIDS within 4 weeks after infection. At 4 and 9 weeks post-infection, mice (three and seven, respectively) were sacrificed and several parameters characterizing the progression of the disease were evaluated. Table 2 reports the degree of lymphadenopathy and splenomegaly. PMEA was found to be able to slightly reduce lymph node weight both after 4 weeks (10%) and after 9 weeks of treatment (11%), whereas a higher reduction was obtained following Bis-PMEA administration (36% and 27% after 4 or 9 weeks of treatment, respectively). However, these lymph node weight reductions were not statistically significant. PMEA and Bis-PMEA administration were not effective in reducing splenomegaly; moreover, an increase in the spleen weights of PMEA-treated mice as compared with infected-untreated ones was observed. The degree of hypergammaglobulinaemia is shown in Figure 4. The results obtained after 4 weeks of treatment showed a 40% reduction in hypergammaglobulinaemia following Bis-PMEA administration as compared with infected-untreated mice, whereas a lower inhibition (20%) was observed with PMEA; however, when IgG levels were determined after 9 weeks, only a 10–15% reduction was observed with both treatments.

The amount of BM5d proviral DNA in lymph nodes of infected mice receiving oral PMEA or Bis-PMEA was also evaluated. The results obtained at the 4th week post virus inoculation (Figure 5) showed an almost complete elimination of proviral DNA content in the lymph nodes of mice receiving Bis-PMEA and a marked (>50%) reduction in mice receiving PMEA. When the proviral DNA content was evaluated 9 weeks post virus inoculation, a reduction was always observed (33 ± 9% and 32 ± 8% of reduction with PMEA and Bis-PMEA, respectively).

As control, 10 infected mice were treated intraperitoneally with PMEA (50 mg/kg). The results obtained are summarized

<table>
<thead>
<tr>
<th>Compound and route of administration</th>
<th>Dose (mg/kg)</th>
<th>AUC(_{0-t}) (µg·h/mL)</th>
<th>Bioavailability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMEA by mouth</td>
<td>28</td>
<td>3.0 ± 1.3</td>
<td>13.5 ± 3.5</td>
</tr>
<tr>
<td>Bis-PMEA by mouth</td>
<td>28</td>
<td>11.28 ± 2.6</td>
<td>50.8 ± 3.1</td>
</tr>
<tr>
<td>PMEA iv</td>
<td>28</td>
<td>22.2 ± 4.2</td>
<td>–</td>
</tr>
</tbody>
</table>

Data represent the means for three individual experiments.
in Table 3 and show that intraperitoneal PMEA has a low efficacy at the beginning of treatments (4th week) whereas it becomes more effective than by mouth PMEA or Bis-PMEA in slowing the development of the disease after 9 weeks of treatment. However, as concerns the evaluation of the content of BM5d proviral DNA in lymph nodes, a lower efficacy was observed with respect to oral gavage treatments. Only hypergammaglobulinaemia data showed statistical significance.

The responsiveness of T- and B-cell spleen lymphocytes to mitogenic stimuli was also evaluated. At 9 weeks post virus inoculation, spleen lymphocytes from infected mice had almost completely lost their ability to respond to mitogenic stimuli. Neither PMEA nor Bis-PMEA was able to restore the ability of both T and B cells to proliferate in vitro. In addition, a decrease in B cells was observed in treated mice as compared with infected-untreated ones; in particular, mice receiving PMEA or Bis-PMEA by mouth showed a further 35% decrease in the ability of B cells to proliferate in vitro, whereas a 48% decrease was observed when PMEA was administered intraperitoneally. Based on these results, we also analysed the percentage of CD19+ lymphocytes in the spleen by flow cytometry (Figure 6). The results obtained revealed a 35% reduction in the infected mice receiving PMEA by mouth as compared with infected-untreated ones, whereas with Bis-PMEA administration only a slight decrease (<10%) was observed. In addition, a further decrease in the percentage of CD19+ cells (50%) was observed when PMEA was administered intraperitoneally. Thus, Bis-PMEA does not cause a reduction in the number of spleen lymphocytes in infected mice as observed by PMEA administration. This can be due to a reduced Bis-PMEA toxicity or to a higher antiviral activity. The results available do not permit a clear conclusion.

**PMEA toxicity**

PMEA was not approved by the Food and Drug Administration (FDA) as an anti-HIV-1 drug essentially because of its renal toxicity. To evaluate renal toxicity in mice following PMEA administration for 9 weeks post-infection, the plasma concentration of creatinine was determined. As shown in Table 4, LP-BM5 infection caused a decrease (30%) in creatinine level; however, by administering PMEA by mouth or intraperitoneally, or Bis-PMEA by mouth, no additional reduction was observed. In a separate experiment the effects of PMEA and Bis-PMEA on plasma creatinine concentration in uninfected control mice were determined and found to be unmodified (data not shown). In addition, no differences were observed in histological examination of kidney tissue following drug treatments (data not shown).

### Table 2. Effect of oral PMEA or BIS-PMEA administrations on lymph node and spleen weights in LP-BM5-infected C57BL/6 mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Lymph node weight (g)</th>
<th>Spleen weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4th week</td>
<td>9th week</td>
</tr>
<tr>
<td>Controls</td>
<td>0.017 ± 0.005</td>
<td>0.038 ± 0.0086</td>
</tr>
<tr>
<td>Infected</td>
<td>0.103 ± 0.04</td>
<td>1.018 ± 0.337</td>
</tr>
<tr>
<td>Infected + PMEA</td>
<td>0.093 ± 0.009</td>
<td>0.908 ± 0.347</td>
</tr>
<tr>
<td>Infected + BIS-PMEA</td>
<td>0.066 ± 0.03</td>
<td>0.740 ± 0.265</td>
</tr>
</tbody>
</table>

PMEA and Bis-PMEA were given at equimolar doses of PMEA at a concentration of 50 mg/kg. Drugs were administered by oral gavage 5 days per week for a period of 4 or 9 weeks. Values are the means ± s.d. of three animals determined 4 weeks post virus inoculation and seven animals at 9 weeks post virus inoculation.
The haematological parameters (Table 4) were similar in infected-untreated and by mouth PMEA-treated mice. Slightly lower values for red and white cell counts, haematocrit percentage and haemoglobin content were observed following Bis-PMEA treatment. Moreover, when PMEA was administered intraperitoneally, a more marked decrease in these parameters was obtained.

The content of mitochondrial DNA in lymph nodes was also evaluated and no decrease following PMEA (intraperitoneally or by mouth) or Bis-PMEA administration was revealed (data not shown).

Table 3. Effect of intraperitoneal PMEA administration on signs of LP-BM5 infection in C57BL/6 mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Percentage reduction versus infected-untreated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4th week</td>
</tr>
<tr>
<td>Lymphoadenopathy</td>
<td>0</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>0</td>
</tr>
<tr>
<td>Hypergammaglobulinaemia</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>BM5d proviral DNA in lymph nodes</td>
<td>44 ± 21</td>
</tr>
</tbody>
</table>

PMEA was administered intraperitoneally at a concentration of 50 mg/kg, 5 days per week for a period of 4 or 9 weeks. Values are the means of three animals, 4 weeks post virus inoculation and of seven animals, 9 weeks post virus inoculation.

The chronic nature of several viral infections, including HIV-1 and hepatitis, requires long-term treatments with antiviral drugs, as is the case with commercially available anti-HIV agents such as zidovudine, zalcitabine, didanosine, lamivudine and stavudine. For long-term treatments, the compounds should preferentially be given orally, but PMEA oral bio-

![Figure 5](https://academic.oup.com/jac/article-abstract/50/3/365/735940/15March2019)

**Figure 5.** Determination of BM5d proviral DNA in lymph nodes. Semi-quantitative PCR detection of BM5d proviral DNA in infected and infected-treated C57BL/6 mice 4 weeks post-infection. A 141 bp fragment of the BM5d gag gene and 203 bp of the G6PD gene as internal controls (data not shown) were amplified with specific primers. Quantification of DNA bands was obtained using GEL DOC 1000 (Bio-Rad), po, by mouth. Percentage of inhibition refers to the infected mice. aP = 0.0708 (versus infected); bP = 0.00234 (versus infected).

The haematological parameters (Table 4) were similar in infected-untreated and by mouth PMEA-treated mice. Slightly lower values for red and white cell counts, haematocrit percentage and haemoglobin content were observed following Bis-PMEA treatment. Moreover, when PMEA was administered intraperitoneally, a more marked decrease in these parameters was obtained.

The content of mitochondrial DNA in lymph nodes was also evaluated and no decrease following PMEA (intraperitoneally or by mouth) or Bis-PMEA administration was revealed (data not shown).

Finally, PMEA toxicity was evaluated in two different cell lines: U937 human monoblastoid cells and L1210 murine leukaemia cells. The results obtained showed that PMEA had the same toxicity profile in both human and murine cells, inhibiting cell proliferation in a dose-dependent manner (IC$_{50} = 3.88 ± 0.78$ µM, L1210; IC$_{50} = 4.3 ± 0.14$ µM, U937).

**Discussion**

The chronic nature of several viral infections, including HIV-1 and hepatitis, requires long-term treatments with antiviral drugs, as is the case with commercially available anti-HIV agents such as zidovudine, zalcitabine, didanosine, lamivudine and stavudine. For long-term treatments, the compounds should preferentially be given orally, but PMEA oral bio-
### Table 4. Haematological parameters and creatinine levels in plasma in mice at 9 weeks post-infection

<table>
<thead>
<tr>
<th></th>
<th>Red blood cells</th>
<th>White blood cells</th>
<th>Creatinine in plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cells x 10^3/µL)</td>
<td>(cells x 10^3/µL)</td>
<td>mg creatinine/100 mL</td>
</tr>
<tr>
<td>Control</td>
<td>9.3±0.6</td>
<td>41.6±1.8</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Infected</td>
<td>7.9±0.9</td>
<td>36.6±3.5</td>
<td>3.5±0.9</td>
</tr>
<tr>
<td>Infected + PMEA by mouth</td>
<td>7.3±1.0</td>
<td>34.3±3.4</td>
<td>11.1±0.9</td>
</tr>
<tr>
<td>Infected + Bis-PMEA by mouth</td>
<td>7.3±1.2</td>
<td>34.5±2.2</td>
<td>26.8±0.4</td>
</tr>
<tr>
<td>Infected + PMEA intraperitoneal</td>
<td>6.7±1.2</td>
<td>7.3±1.2</td>
<td>2.4±0.3</td>
</tr>
</tbody>
</table>

Hb: haemoglobin; MCH: mean cell haemoglobin; MCHC: mean cellular haemoglobin concentration; Lymph.: lymphocytes; Gran.: granulocytes.

PMEA and Bis-PMEA were given at equimolar doses of PMEA at a concentration of 50 mg/kg. Drugs were administered by mouth or intraperitoneally 5 days per week for a period of 9 weeks. All values are means ± S.D. of seven animals.

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be due to a short treatment regimen in our murine model. A reduction in the response of B-splenocytes to mitogenic stimuli was observed with both PMEA and Bis-PMEA treatments compared with the response in infected-untreated mice. In addition, the CD19+ level was almost the same between infected and Bis-PMEA treated mice, whereas with PMMEA administration (by mouth and overall intraperitoneally) a marked reduction in the percentage of these lymphocytes as compared with the percentage in infected-untreated mice was reported. Thus, altogether, Bis-PMEA administered by mouth is more effective and less toxic than PMMEA by mouth.

In conclusion, the results obtained upon oral treatment with PMMEA and its prodrug Bis-PMEA were compared with those obtained by intraperitoneal PMMEA administration. It had already been shown that intraperitoneal PMMEA is not able to prevent MAIDS but does slow down the progression of the disease.35 In our experiments, the administration of PMMEA (50 mg/kg) by intraperitoneal injection was less effective than oral Bis-PMEA in reducing lymphoadenopathy, hypergammaglobulinaemia and lymph node proviral DNA content, at least in the first weeks post-infection. Moreover, following intraperitoneal PMMEA administration some signs of drug toxicity appeared (Figure 6 and Table 4). In addition, in vitro studies in two different cellular lines (murine L1210 and human U937) revealed the same toxicity profiles between murine and human cells, suggesting that mice represent a good animal model with which to evaluate the efficacy of different PMMEA delivery systems.

Overall, our results show that Bis-PMEA administered by oral gavage can act as a prodrug for a better delivery of PMMEA in circulation than that obtained with oral gavage PMMEA, assuring higher antiviral efficacy without causing significant toxicity. Moreover, our data suggest that charged molecules other than PMMEA [for example the most efficient antiretroviral drug 9-(2-phosphonylmethoxypropyl)adenine (PMPA)] can most likely be coupled by a P-O-P bond to increase their bioavailability, as shown in this paper for Bis-PMEA. It provides support to the idea that a new class of phosphonate prodrugs with good oral bioavailability, improved pharmacokinetics and reduced toxicity can be generated from the Bis-PMEA model.

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References


