Inhibition of HIV-1 replication in macrophages by a heterodinucleotide of lamivudine and tenofovir

Luigia Rossi1*, Palmarisa Franchetti2, Francesca Pierigé1, Loredana Cappellacci2, Sonja Serafini1, Emanuela Balestra3, Carlo-Federico Perno1, Mario Grifantini2, Raffaele Caliò3 and Mauro Magnani1

1Institute of Biochemistry ‘G. Fornaini’, University of Urbino ‘Carlo Bo’, 61029 Urbino, Italy; 2Department of Chemical Sciences, University of Camerino, 62032 Camerino, Italy; 3Department of Experimental Medicine, University of Rome ‘Tor Vergata’, 00133 Rome, Italy

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Objectives: (i) To generate a new heterodinucleotide (3TCpPMPA) comprising the drugs lamivudine and tenofovir which have been shown to act synergistically and (ii) to protect macrophages from ‘de novo’ HIV-1-infection through its administration.

Methods: 3TCpPMPA was obtained by coupling the morpholidate derivative of tenofovir with the mono n-tri-butylammonium salt of lamivudine 5’-monophosphate. Stability and metabolism were evaluated in vitro and in vivo in mice. 3TCpPMPA was encapsulated into autologous erythrocytes by a procedure of hypotonic dialysis, isotonic resealing and reannealing. 3TCpPMPA-loaded erythrocytes were modified to increase their phagocytosis by human macrophages. Macrophages were infected by HIV-1Ba-L and inhibition of HIV-1 replication was assessed by HIV p24gag quantification.

Results: Pharmacokinetic studies in mice revealed a rapid disappearance of the heterodinucleotide from circulation (t1/2 = 15 min) without any advantage compared with the administration of single drugs. Adding free 3TCpPMPA to macrophages (18 h), a 90% inhibition of viral replication up to 35 days post-treatment was achieved, while only a 60% inhibition was obtained by the combined treatment 3TC and (R)PMPA. When 3TCpPMPA was selectively targeted to the macrophage compartment by a single addition of loaded erythrocytes, the protection of macrophages from ‘de novo’ infection (99% protection 3 weeks post-treatment) was nearly complete.

Conclusions: Erythrocytes loaded with 3TCpPMPA and modified to increase their phagocytosis are able to protect macrophages from ‘de novo’ HIV-1 infection. 3TCpPMPA acts as an efficient antiviral pro-drug that, once inside macrophages, can be slowly converted into 3TCMP and (R)PMPA protecting these cells for a longer period of time.

Keywords: phagocytes, nucleoside analogues, HIV/AIDS, drug-delivery

Introduction

HIV-1, like other lentiviruses, has developed the ability to infect non-dividing cells including macrophages. Unlike T cells, HIV-1-infected macrophages appear to be resistant to the cytopathic effects of the virus1 and hence can persist in the tissues for a long period of time, supporting viral replication and contributing to the pathogenesis of the disease throughout the course of infection.2,3 Searching for factors that allow the monocyte-derived macrophage (M/M) cell lineage to survive HIV-1 infection, Garaci et al. have demonstrated that nerve growth factor (NGF), produced and released by the HIV-1-infected M/M, acts as an autocrine survival factor in such cells.4 Furthermore, the expression of phospho-STAT1 in infected cells prevents their death upon viral replication. Although the kinetics of virus release from cells of macrophage lineage are slower in comparison with CD4+ T cells, the lack of HIV-1-induced cytopathicity enables macrophages to continue to produce HIV-1
for a long period of time. Moreover, the capacity of M/M to migrate to organs makes them potential conveyors of HIV-1 infection, thus playing a pivotal role in the pathogenic progression of the disease. In fact, productively infected monocytes and macrophages can fuse with CD4+ T lymphocytes and transfer the virus to these cells and, in addition, are able to release cytokine factors that can mediate the activation of apoptosis in bystander cells such as CD4+ and CD8+ lymphocytes, neurons and astrocytes. Moreover, the expansion of CD16 monocyte-derived macrophages in the peripheral blood of HIV-infected patients and their subsequent recruitment into tissues, may contribute to establishment of viral reservoirs in resting T cells.

Despite the clinical improvement associated with the highly active combined, antiretroviral therapy (HAART), current antiretroviral drugs are not able to eradicate HIV-1 simply due to the persistence of the virus in cellular reservoirs (predominantly long-lived memory CD4+ T cells and cells of the macrophage lineage) and anatomical sanctuary sites (brain and possibly testes). Indeed, HIV-1 has been recovered from the peripheral blood monocytes of patients with maximal viral suppression while under HAART. Viral reservoirs established early during the infection remain unaffected by antiretroviral therapy for a long time and are able to replenish systemic infection following the failure or cessation of the treatment.

Antiretroviral therapies able to protect macrophages from HIV-1 infection are thus sorely needed. Antiretroviral drugs predominantly used in the treatment of HIV-1 infection include nucleoside and non-nucleoside reverse transcriptase inhibitors and protease inhibitors. Recently, enfuvirtide, a peptide that inhibits fusion of HIV-1 with the host cell membrane, has also been approved for use in the treatment of the disease. Most clinically relevant compounds belong to the family of nucleoside analogue inhibitors of reverse transcriptase, such as zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), lamivudine (3TC), stavudine (d4T) and abacavir (ABC) along with acyclic nucleoside phosphonates such as tenofovir [(R)PMPA]. Tenofovir is a highly potent and selective antiretroviral agent that has received US Food and Drug Administration (FDA) approval as tenofovir disoproxil fumarate (Viread) for the treatment of HIV-1 infection when taken in combination with other antiretroviral agents; it is also able to inhibit HIV-1 replication in macrophages. Moreover, an additive anti-HIV-1 activity of (R)PMPA when associated with lamivudine in in vitro studies on MT2 cells has been reported. Recently, clinical studies on 299 antiretroviral-naive patients performed during a period of 144 weeks, showed the efficacy and safety of tenofovir in combination with lamivudine and efavirenz. The combination of tenofovir and lamivudine has already been suggested in therapy guidelines for HAART-naive patients. In addition, it is noteworthy that the selection of M184V mutation by 3TC, which occurs in most cases of virological failure, seems to increase (R)PMPA activity. This is particularly true for dideoxynucleoside analogue phosphorylation. On the basis of our previous results, the heterodinucleotide 3TCpPMPA was synthesized and encapsulated into autologous erythrocytes modified to increase their recognition and their phagocytosis by human macrophages. Inside the macrophages, we expected this heterodinucleotide to be split, forming 3TC monophosphat (3TCMP) and (R)PMPA, which are then converted by cellular kinases into the corresponding active forms 3TCTP and PMPA-DP, respectively. In this way, it is possible to overcome the weak ability of macrophages to phosphorylate 3TC and the limited permeability of (R)PMPA. Herein, we report the chemical synthesis, characterization, pharmacokinetics, the macrophage-targeted delivery as well as the antiretroviral activity of the heterodinucleotide 3TCpPMPA.

Materials and methods

Cells and virus

Human peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy seronegative donors by Ficoll–Hypaque density gradient centrifugation. The PBMCs were resuspended in RPMI 1640 medium supplemented with 20% heat-inactivated (56 °C, 30 min) fetal calf serum, penicillin(100 U/mL), streptomycin(100 mg/mL) and l-glutamine (2 mM), then seeded into 48-well plates (1.8 x 10^7 cells/well). M/M were separated by adherence onto plastic. After 5 days, non-adherent cells were carefully removed by repeated gentle washings with warm medium, and adherent (>95% pure) M/M were cultured for an additional 3 days to mature and to form a monolayer. The number of cells present in each well was assessed by counting nuclei extracted from M/M with lysing buffer in a cell counting chamber under a phase contrast microscope, according to a previously published procedure. About 10^5 cells/well were present at the time of infection. Viability was assayed by the Trypan Blue exclusion method. A monocytotropic strain of HIV-1, named HIV-1Ba-L, was used in all experiments. The virus was expanded in M/M, collected, filtered and stored at -80 °C before use. The virus titre was assessed on M/M, calculated according to the Reed and Muench method and expressed as 50% tissue culture infective dose per mL (TCID50/mL).

Chemistry

Elemental analyses were determined on an EA 1108 CHNS-O (Fisons Instruments) analyser. Thin-layer chromatography (TLC)
was run on silica gel 60 F254 plates; silica gel 60 (70–230 mesh, Merck) for column chromatography was used. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian-Mercury Plus AS400 spectrometer using TMS and H2PO4 as internal standards for 1H-NMR and 31P-NMR, respectively. Chemical shifts are reported in parts per million (δ) as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). Mass spectroscopy was carried out on an HP-1100 MSD G19/46A instrument. All measurements were performed in a negative ion mode using atmospheric pressure electrospray ionization (API-ESI). (R)PMPA was kindly provided by Dr Norbert Bischofberger (Gilead Sciences Inc.) while 3TC was synthesized in our laboratories as reported.36

Ref: 3TCpPMPA, 16 min for 3TC, 5 min for 3TCMP and 14 min for 

Evaporation of the second fraction from the same column gave the pure heterodinucleotide IV as a white solid (55% yield). HPLC purity was more than 99%. 1H-NMR (400 MHz, D2O): δ 0.95 (d, J = 6.3 Hz, 3H, CH3), 2.90 (dd, J = 4.3, 12.1 Hz, 1H, H'2), 3.30 (dd, J = 5.5, 12.1 Hz, 1H, H'2), 3.64 (dd, J = 2.9, 9.5 Hz, 2H, CH3P), 3.82 (m, 1H, OCH), 3.97 (m, 1H, H5), 4.08 (m, 2H, H'S and N-CH2), 4.22 (dd, J = 3.3, 14.7 Hz, 1H, N-CH2), 5.20 (dd, J = 2.7, 4.7 Hz, 1H, H'4), 5.62 (d, J = 7.7 Hz, 1H, H5), 6.0 (t, J = 4.7 Hz, 1H, H1'), 7.60 (d, J = 7.7 Hz, 1H, H6), 8.0 (s, 1H, H2), 8.10 (s, 1H, H8). 31P-NMR (162 MHz, D2O): δ 8.35 (P1, d, J = 25.9 Hz), −11.64 (P2, d, J = 25.9 Hz). MS m/z: 577.0 [M−H]−, 288.0 [M−2H]2−. Anal. C17H22N8O9P2S.2NH4H2O; Calcd: C 36.61, H 5.46, N 28.46. Found: C 36.54, H 5.35, N 28.39.

Figure 1. HPLC analyses of 3TCpPMPA and its metabolites

Samples were extracted with perchloric acid as described in Magnani et al.38 Preliminary experiments (not shown) have demonstrated that 3TCpPMPA is stable under these extraction conditions. Neutralized extracts were then used for HPLC determinations. A 5 μm Res. Elut. 5 C18 90A column (150 × 4.6 mm ID; Varian, Harbor City, CA, USA) protected with a guard column (Pelliguard LC-18, 20 × 4.6 mm ID, 40 μm particles) was used. The mobile phase consisted of two eluents: 25 mM KH2PO4, adjusted to pH 6.0 (buffer A) and buffer A containing 30% (v/v) acetonitrile adjusted to pH 6.0 (buffer B). All buffer solutions, as well as standards and sample solutions, were filtered through a 0.2 μm membrane filter (Millipore, Bedford, MA). The elution conditions were as follows: 5 min at 100% buffer A, up to 100% buffer B over 30 min and hold 10 min. The gradient was back to 100% buffer A over 3 min and the initial conditions restored in 2 min. The flow rate was 1 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 15 min for 3TCpPMPA, 16 min for 3TC, 5 min for 3TCMP and 14 min for (R)PMPA.

3TCpPMPA metabolism in culture medium

The ability of macrophage culture medium (complete RPMI 1640 medium containing 20% FCS) to split the heterodinucleotide into its metabolites was evaluated by incubating the medium for 24 h at 37°C in the presence of 0.3 mM 3TCpPMPA. At different incubation times: 0, 1, 2, 4, 8 and 24 h, 100 μL aliquots were removed and treated for HPLC determinations as described earlier. Values obtained were corrected for the recovery percentage (82% for heterodinucleotide; no correction for 3TC, 3TCMP and (R)PMPA was needed).

3TCpPMPA metabolism in murine plasma

Murine plasma was obtained from Swiss mice (Stefano Morini S.a.S., Reggio Emilia, Italy) by drawing blood from the retro-orbital sinus. To evaluate the presence in plasma of enzymes that can cleave the pyrophosphate bridge of 3TCpPMPA, 0.25 μmol of free drug/mL of murine plasma was incubated for 24 h at 37°C. At
different incubation times (0, 0.25, 0.5, 1, 2, 4, 8 and 24 h), 100 μL aliquots were collected and processed for HPLC analyses as reported above. Values obtained were corrected for the recovery percentage (80%).

**Pharmacokinetics of 3TCpPMPA in mice**

To determine the concentration of 3TCpPMPA and its metabolites in circulation, two groups of three Swiss mice (female, 2 months old, 20 g body weight) received intravenous (iv) or oral gavage administration of 5 mg (8.16 mol) of the heterodinucleotide in HEPES solution (10 mM HEPES, pH 7.4, containing 154 mM NaCl and 5 mM glucose) at a final volume of 300 and 400 μL, respectively, while another two groups (of three mice each) received iv or oral gavage administration of 3Tc plus (R)PMPA (8.16 μmol of each drug was administered as described earlier). At different times (0, 0.25, 0.5, 1, 2 and 4 h), 100 μL of blood was collected from CO2-anaesthetized mice into heparinized microhaematocrit capillary tubes by puncture of the retro-orbital sinus. Blood samples were immediately centrifuged for 5 min at 900 g at 4°C; plasma samples were extracted with perchloric acid, neutralized and used for the HPLC determinations as described earlier. Values obtained were corrected for the recovery percentage (80%). Animal studies were performed after approval from the Animal Ethics Committee of Urbino University (Italy).

**3TCpPMPA metabolism in erythrocyte lysates**

Human red blood cell lysates were obtained as described by Rossi et al. The haemoglobin concentration was 110 mg/mL of haemolysate. 3TCpPMPA was incubated in erythrocyte lysate at a final concentration of 0.3 mM for 24 h at 37°C, both in the absence and in the presence of 1.0 mM ATP. At times 0, 1, 2, 4, 8 and 24 h, 100 μL aliquots were removed and treated for HPLC determinations of the heterodinucleotide and its metabolites as reported above. Values obtained were corrected for the recovery percentage [54%, 80%, 72% and 76% for heterodinucleotide, (R)PMPA, 3TC and 3TCMP, respectively].

**3TCpPMPA encapsulation in erythrocytes**

Human erythrocytes were loaded with 3TCpPMPA by a procedure of hypotonic dialysis, isotonic resealing and reannealing as previously reported by Magnani et al. with some modifications. Briefly, human erythrocytes were washed in 10 mM HEPES (pH 7.4) containing 154 mM NaCl and 5 mM glucose (washing buffer) to remove leucocytes and platelets. RBCs were resuspended at 70% haematocrit in the same washing buffer and were dialysed for 70 min using a tube with a cut-off of 12–14 kDa, against 50 volumes of 10 mM NaH2PO4, 10 mM NaHCO3 and 20 mM glucose (pH 7.4) containing 3 mM reduced glutathione and 2 mM ATP. The osmolarity of the buffer was ~55 mOsm whereas the erythrocytes reached about 70 mOsm at the end of the dialysis time. All these procedures were performed at 4°C. After this step, 3TCpPMPA was added to each millilitre of dialysed erythrocytes (range 10–20 μmol/mL), which were then incubated for 30 min at room temperature under gentle mixing. Resealing of erythrocytes was obtained by adding 0.1 volume of 100 mM inosine, 20 mM ATP, 4 mM MgCl2, 190 mM NaCl, 1666 mM KCl and 33 mM NaH2PO4, 10 mM anhydrous glucose, 100 mM sodium pyruvate (pH 7.4) per volume of dialysed erythrocytes (PIGPA.C) and incubating the resealed cells at 37°C for 25 min. The resealed cells were washed three times in the washing buffer and used for the experiments described below as they were or processed further to increase their recognition by macrophages.

**3TCpPMPA stability in intact human erythrocytes**

The stability of 3TCpPMPA in loaded human erythrocytes was evaluated by incubation of loaded erythrocytes at 0.5% haematocrit in RPMI 1640 medium containing 10% FCS. At different incubation times (0, 1, 2, 3, 17 and 24 h) at 37°C in a 5% CO2 atmosphere and under sterile conditions, 5 mL aliquots were processed to determine the concentration of 3TCpPMPA and its metabolites. Briefly, RBC suspensions were centrifuged 10 min at 570g, the supernatants were removed and 3TCpPMPA-loaded RBCs were extracted with perchloric acid and analysed by HPLC as previously described.

**Targeting of 3TCpPMPA-loaded erythrocytes to macrophages**

3TCpPMPA-loaded erythrocytes were modified to increase their recognition by macrophages by a procedure described in Magnani et al. with some modifications. Briefly, suspensions of loaded erythrocytes (10% haematocrit) in 1.0 mM ZnCl2 were treated with 1.0 mM bis (sulphosuccinimidyl) suberate (BS) for 15 min at room temperature under gentle mixing and washed once in washing buffer containing 10 mM ethanolamine and once in washing buffer containing 1% (w/v) BSA. These cells were incubated in autologous plasma for 60 min at 37°C at a haematocrit of 30% and successively washed once in washing buffer containing 2% (w/v) BSA and once in washing buffer without any addition. During the procedure described here, 3TCpPMPA is fully retained in RBCs as revealed by HPLC analyses performed before and after RBC membrane modifications (data not shown). 3TCpPMPA-loaded erythrocytes were then added to macrophages and their antiviral activity was evaluated (see below).
Anti-HIV-1 activity assay

To evaluate the anti-HIV-1 activity of 3TC, (R)PMPA, 3TC plus (R)PMPA and 3TCpPMPA on human macrophages, all drugs were added to M/M overnight (18 h) at a concentration of 1.0 μM. Human macrophage cultures were then infected for 2 h with HIV-1 Ba-L (300 TCID 50/mL). After virus incubation, cell cultures were extensively washed to remove any residual virus particles. All cell cultures were maintained up to 35 days at 37°C and 5% CO 2 and medium was changed weekly. No drug was added after infection throughout the experiments. At different time points (range 2–7 weeks), virus replication was assessed by measuring HIV p24 antigen in the M/M supernatant with a commercially available enzyme-linked immunosorbent (ELISA) kit that can detect the virus (Bio-Rad, France).

Similarly, the anti-HIV-1 activity of the heterodinucleotide-loaded RBCs was evaluated. In detail, RBCs loaded with 3TCpPMPA (0.8 μmol/mL of RBCs) were added to M/M cultures at a ratio of 100 RBCs per macrophage for 18 h of incubation before the infection. After this time, non-ingested RBCs were removed by extensive washing with culture medium. As a control, macrophage cultures were treated with ‘unloaded’ (UL) RBCs, i.e. RBCs submitted to the same procedure including transient lysis and subsequent modification to increase macrophage recognition, but without addition of 3TCpPMPA. Human macrophage cultures receiving either 3TCpPMPA-loaded or unloaded RBCs were then infected with HIV-1 Ba-L, as described earlier and the same experimental protocol was followed.

Results

Anti-HIV-1 activity of 3TC and (R)PMPA in infected human macrophages

At first, the ability of 3TC and (R)PMPA to protect macrophages against ‘de novo’ HIV-1 infection, both as single drugs and in combination, was evaluated. Drugs were administered at a concentration of 1.0 μM for 18 h before infection and virus production was evaluated over a period of 35 days. The results obtained are reported in Table 1, columns 1, 2 and 3, and show, as expected, that the combination 3TC plus (R)PMPA works better than the separate drugs. However, only a 60% virus replication inhibition was registered 35 days post-treatment. To test if different kinetics of drug release could improve the performance of the combination, the heterodinucleotide 3TCpPMPA was synthesized as a pro-drug to allow a slow and controlled delivery of lamivudine and tenofovir to macrophages. When the heterodinucleotide was incubated in culture medium in the presence of 20% FCS, a time-dependent decrease in its concentration (t 1/2 = 2 h) was observed and a slow and linear release of 3TCMP, 3TC and (R)PMPA was obtained (Figure 2). In Table 1, column 4, the anti-HIV-1 activity of 3TCpPMPA administered 18 h prior to infection is reported. More than 90% inhibition of viral replication 35 days post-treatment was obtained. Starting from these encouraging results and with the objective to carry out in vivo studies, the metabolism of 3TCpPMPA in murine plasma was evaluated.

Table 1. Percentage of virus production in HIV-1-infected human macrophages

<table>
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<tr>
<th>Time (days)</th>
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3TCpPMPA, [P-4’-{4-amino-1’-{2(R,5S)-2-[(phosphonoxy)methyl]-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone}-P₂-{9-[(R)-2-phosphonomethoxy]propyl]} adenine]; (R)PMPA, 9-[(R)-2-phosphonomethoxy]propyl]adenine (tenofovir); 3TC, (−)-2’-deoxy-3’-thiacytidine (lamivudine). Macrophages were treated with 3TC or (R)PMPA, 3TC plus (R)PMPA and with 3TCpPMPA for 18 h before infection. Values are expressed as percentage of p24 production compared with untreated infected macrophages. All values are the mean (±SD) of sextuplicate cultures of a representative of three experiments and show the percentage of inhibition in p24 produced until 35 days after infection. One hundred percent virus production corresponds to 19 000 ± 950 pg of p24 antigen/mL at day 14, 27 000 ± 2250 pg of p24 antigen/mL at day 21, 24 000 ± 1500 pg of p24 antigen/mL at day 28 and 24 500 ± 1750 pg of p24 antigen/mL at day 35.

Metabolism of 3TCpPMPA in murine plasma

The ability of murine plasma to convert 3TCpPMPA into 3TC and (R)PMPA was evaluated. As shown in Figure 3, murine plasma possesses enzymes that can cleave the phosphate bridge of 3TCpPMPA with the stoichiometric production of (R)PMPA and 3TC (the latter is quickly obtained starting from 3TCMP). The results obtained show that 3TCpPMPA is stable enough in murine plasma (t 1/2 = 14 h); the half-lives for the appearance of (R)PMPA and 3TC were 5.5 and 6.5 h, respectively. When 3TCpPMPA was incubated in human plasma, similar results to those obtained with murine plasma were observed (data not shown). These good stability results suggested the possibility of using 3TCpPMPA as a pro-drug for a slow delivery of 3TC and (R)PMPA into circulation.

Pharmacokinetic studies of 3TCpPMPA in mice

To evaluate whether or not 3TCpPMPA could be administered as a pro-drug for the slow delivery of its metabolites and to chose the better administration route, 8.16 μmol of the heterodinucleotide was administered to mice both intravenously and by oral gavage. The amount of administered drugs was calculated as expected, that the combination 3TC plus (R)PMPA works better than the separate drugs. However, only a 60% virus replication inhibition was registered 35 days post-combined-treatment. To test if different kinetics of drug release could improve the performance of the combination, the heterodinucleotide 3TCpPMPA was synthesized as a pro-drug to allow a slow and controlled delivery of lamivudine and tenofovir to macrophages. When the heterodinucleotide was incubated in culture medium in the presence of 20% FCS, a time-dependent decrease in its concentration (t 1/2 = 2 h) was observed and a slow and linear release of 3TCMP, 3TC and (R)PMPA was obtained (Figure 2). In Table 1, column 4, the anti-HIV-1 activity of 3TCpPMPA administered 18 h prior to infection is reported. More than 90% inhibition of viral replication 35 days post-treatment was obtained. Starting from these encouraging results and with the objective to carry out in vivo studies, the metabolism of 3TCpPMPA in murine plasma was evaluated.

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Anti-HIV-1 activity of 3TCpPMPA

3TCpPMPA shows a rapid and biphasic decline (Figure 4). 3TCpPMPA quickly disappears from circulation ($t_{1/2} = 15$ min) until reaching 0.2% of its initial concentration (time 0) 4 h post-injection; the disappearance of the dimer coincides with the presence of the two separate drugs. In the inset of the figure, the pharmacokinetic profiles of 3TC and (R)PMPA, when administered as a combination of the two separate drugs, are shown. To define the advantages which might be obtained by administering 3TC and (R)PMPA as a single molecule rather than as a 3TC plus (R)PMPA combination, values for the area under the curve in the range 0–4 h (AUC$_{0-4}$) were determined. The results obtained are reported in Table 2 and show that no advantage is obtained from heterodinucleotide administration; in fact, 3TCpPMPA injection yielded lower plasma levels of drugs 3TC and (R)PMPA (~30–40%) compared with the levels reached when the drugs were administered separately. When the oral drug administration was evaluated, no data were obtained except for the presence of low plasma levels of 3TC following 3TC drug administration was evaluated, no data were obtained except (three mice per time point, three experiments). 3TCpPMPA, P1-[4-amino-1-[(2R,5S)-2-[(phosphonooxy)methyl]-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone]-P2-[9-[(R)- (2-phosphonooxy)propyl]adenine; (R)PMPA, 9-[(R)-2-phosphonomethoxy]propyl]adenine (tenofovir); 3TC, (−)2′-deoxy-3′-thiacytidine (lamivudine); 3TCpMP, (−)2′-deoxy-3′-thiacytidine-5′-phosphate (lamivudine 5′-monophosphate).

Metabolism of 3TCpPMPA in erythrocyte lysates and intact erythrocytes

To evaluate whether or not 3TCpPMPA is stable enough in RBCs for use as an efficient delivery system to reach the macrophage compartment, the metabolism of 3TCpPMPA in

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</tr>
<tr>
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<td>599 ± 42</td>
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3TCpPMPA, P1-[4-amino-1-[(2R,5S)-2-[(phosphonooxy)methyl]-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone]-P2-[9-[(R)-(2-phosphonomethoxy)propyl]adenine]; (R)PMPA, 9-[(R)-(2-phosphonomethoxy)propyl]adenine (tenofovir); 3TC, (−)2′-deoxy-3′-thiacytidine (lamivudine). 3TCpPMPA and 3TC plus (R)PMPA were administered at equimolar dose of 8.16 μmol/mouse. Data are the average values ± SD for three independent experiments (three mice per time point in each experiment). Time points were 0, 0.25, 0.5, 1, 2 and 4 h.

Table 2. AUC determination upon intravenous administration of 3TCpPMPA or 3TC plus (R)PMPA

Figure 2. Stability of 3TCpPMPA in culture medium. 3TCpPMPA (0.3 mM) was incubated in culture medium for 24 h at 37°C. Percoll acid extracts were prepared at different incubation times and analysed by HPLC, as described in the Materials and methods section. All values are the mean (± SD) of three different experiments. 3TCpPMPA: P1-[4-amino-1-[(2R,5S)-2-[(phosphonooxy)methyl]-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone]-P2-[9-[(R)-(2-phosphonooxy)propyl]adenine]; (R)PMPA: 9-[(R)-2-phosphonomethoxy]propyl]adenine (tenofovir); 3TC, (−)2′-deoxy-3′-thiacytidine (lamivudine); 3TCpMP, (−)2′-deoxy-3′-thiacytidine-5′-phosphate (lamivudine 5′-monophosphate).

Figure 3. Stability of 3TCpPMPA in murine plasma. 3TCpPMPA (0.25 mM) was incubated in murine plasma for 24 h at 37°C. Percoll acid extracts were prepared at different incubation times and analysed by HPLC, as described in the Materials and methods section. All values are the mean (± SD) of three different experiments. 3TCpPMPA, P1-[4-amino-1-[(2R,5S)-2-[(phosphonooxy)methyl]-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone]-P2-[9-[(R)-(2-phosphonooxy)propyl]adenine]; (R)PMPA, 9-[(R)-2-phosphonomethoxy]propyl]adenine (tenofovir); 3TC, (−)2′-deoxy-3′-thiacytidine (lamivudine).

Figure 4. Concentrations of 3TCpPMPA, 3TC and (R)PMPA in the plasma of mice after iv bolus injections of heterodinucleotide. In the inset: concentration of 3TC and (R)PMPA in the plasma of mice after intravenous injection of 3TC plus (R)PMPA. All compounds were administered at a dose equivalent to 8.16 μmol/mouse, which corresponds to 0.25, 0.095 and 0.115 mg/kg of 3TCpPMPA, 3TC and (R)PMPA, respectively (three mice per time point, three experiments). 3TCpPMPA, P1-[4-amino-1-[(2R,5S)-2-[(phosphonooxy)methyl]-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone]-P2-[9-[(R)-(2-phosphonooxy)propyl]adenine]; (R)PMPA, 9-[(R)-2-phosphonomethoxy]propyl]adenine (tenofovir); 3TC, (−)2′-deoxy-3′-thiacytidine (lamivudine).
erythrocyte lysates and in intact erythrocytes was evaluated. The results obtained show that erythrocyte lysates possess enzymes that can form the related metabolites starting from the heterodinucleotide. The time courses show a faster cleavage of 3TCpPMPA into (R)PMPA and 3TCMP (which is then dephosphorylated to 3TC) in the absence of (Figure 5a) rather than in the presence (Figure 5b) of 1.0 mM ATP. In fact, a significant inhibition of this conversion was obtained from incubation mixture supplemented with ATP. In this condition, 37% of 3TCpPMPA was still present after 8 h while, without the addition of ATP, residual 3TCpPMPA was only 14% at the same time, showing that the pro-drug is sufficiently stable for the indicated times when physiological ATP concentrations are present. This result is in agreement with the previous observation that an ADP-ribose pyrophosphatase is present in human erythrocytes, that it acts on a number of P1-P2-dinucleotides and is inhibited by physiological ATP concentration.39–41 To study the stability of the heterodinucleotide in intact erythrocytes, human erythrocytes were loaded to a final concentration of 0.3 µmol/mL of packed RBCs. The encapsulation of the heterodinucleotide, through the procedure of hypotonic dialysis, isotonic resealing and reannealing, did not result in any appreciable alteration in erythrocyte morphology and metabolism (not shown). 3TCpPMPA-loaded erythrocytes were then incubated for up to 24 h at 37°C in RPMI 1640 medium. At different times of incubation (0, 1, 2, 3, 17 and 24 h), the concentration of 3TCpPMPA in erythrocytes was evaluated. As shown in Figure 6, a slow decrease in the intracellular presence of 3TCpPMPA was observed, reaching concentrations as low as 74% of the starting level after 24 h of incubation. As strongly suggested by these results, the heterodinucleotide is stable enough in RBCs to allow their use as a drug targeting system.

Anti-HIV activity of 3TCpPMPA-loaded RBCs in infected human macrophages

To assess the anti-HIV-1 activity of 3TCpPMPA-loaded RBCs, human macrophages were treated with 3TCpPMPA-loaded (~0.8 µmol/mL of RBCs) or unloaded RBCs in culture medium for 18 h before infection with a macrophage-tropic HIV$_{\text{Ba-L}}$ strain. Since we have previously demonstrated33 that each macrophage under these experimental conditions phagocytoses an average of one erythrocyte, we have calculated that the mean drug concentration in macrophages is ~64 pmol x 10$^6$ macrophages. Since a differentiated macrophage has a mean volume in the 8–10 µL range, a concentration of 64 pmol x 10$^6$ macrophages corresponds to a macrophage 3TCpPMPA concentration falling in the 6–8 µM range. The antiviral activity was determined up to 5 weeks post-infection by evaluating p24 production (Figure 7). The data obtained show that a single pre-infection treatment with dimer-loaded RBCs is very effective in inhibiting HIV-1 production to an almost undetectable level 14 days post-infection. Even more, a high long-term inhibition was observed, with an HIV reduction of 80%, compared with control, 35 days post-infection. Therefore, heterodinucleotideloaded RBCs are shown to be an effective drug-targeting system for the selective delivery of the heterodinucleotide, demonstrating the possible protection of a refractory cell compartment such as that of macrophages against ‘de novo’ HIV-1 infection.

Discussion

Several lines of evidence show that macrophages play a pivotal role in HIV-1 persistence and pathogenesis. Recently, the
Anti-HIV-1 activity of 3TCpPMPA

**Figure 7.** Inhibition of HIV-1 replication in human macrophages by 3TCpPMPA-loaded RBCs. Monocyte-derived macrophages (M/M) were cultured for 10 days and then treated for 18 h with both unloaded (UL) and 3TCpPMPA-loaded RBCs (0.8 μmol/mL) at a ratio of 100 RBCs per macrophage. Non-ingested RBCs were then removed and macrophages were cultured for 10 days and then treated for 18 h with both unloaded (UL) and 3TCpPMPA-loaded RBCs (0.8 μmol/mL) at a ratio of 100 RBCs per macrophage. Non-ingested RBCs were then removed and macrophages were infected for 2 h with HIV-1ΔNL4-3 (300 TCID50/mL). Results are expressed as percentages compared with infected untreated macrophages. All values are the mean (± SD) of sextuplicate cultures of three experiments and show the percentage of inhibition in p24 produced until 35 days after infection. One hundred percent virus production corresponds to 24 630 ± 2102 pg of p24 antigen/mL (value is the mean ± SD of virus production at 14, 21, 28 and 35 days post-infection). 3TCpPMPA, P2′-[(4-amino-1-[(2R,5S)-2-[(phospho-nooxy)methyl]-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone]-P2′-[(R)-(2-phosphonomethoxy)propyl]adenine].

A combination of lamivudine and tenofovir has been suggested for HAART-naïve patients, and a study to evaluate its efficacy in macrophages has previously been carried out. In this paper, we show that it is possible to protect macrophages against 'de novo' HIV infection by the treatment with a single pro-drug consisting of lamivudine and tenofovir bound together by a phosphate bridge (3TCpPMPA). To our knowledge, this is the first study in which the combination 3TC and (R)PMPA has been evaluated in primary monocyte/macrophage cells. Our results show that both lamivudine and tenofovir, when separately added for 18 h at 1.0 μM concentration (a value in their EC50 range) are partially able to protect macrophages against HIV infection, reaching 50% inhibition of viral replication at 3 weeks post-treatment, on average. In contrast, when lamivudine and tenofovir are simultaneously added, a 95% inhibition is reached at the same time point. This finding confirms, in macrophages as well, the efficacy of this combination therapy previously observed in MT-2 cells,42 and in other drug combination studies of tenofovir (www.viread.com). In addition, the combination therapy proved to be more efficient than separate treatment and was maintained for longer observation times (5 weeks post-treatment) although no more than a 60% viral replication inhibition was reached at the latest observation time. With the aim to increase lamivudine/tenofovir-combination performance, we reasoned that a pro-drug able to slowly release the two drugs, could improve their uptake and antiretroviral efficacy. To this end, we synthesized a heterodinucleotide of lamivudine and tenofovir bound together by a phosphate bridge (3TCpPMPA).

A single overnight (18 h) treatment of macrophages with the heterodinucleotide was able to amply protect cells from HIV infection for a long period of time, showing more than a 90% inhibition of viral replication 5 weeks post-treatment. Since 3TCpPMPA dimer does not cross the cellular membranes (data not shown), this anti-HIV activity was due to the single drugs [3TCMP, 3TC, (R)PMPA] formed by hydrolysis of the heterodinucleotides by the serum enzymes present in the RPMI complete culture medium, as shown. However, as the only difference between 3TC plus (R)PMPA or 3TCpPMPA administration lies in the different kinetics of appearance of the single drug in culture medium, our results strongly suggest the advantage of using the dimer form as a pro-drug. In addition, it is worth noting that serum enzymes split the dimer in (R)PMPA and 3TCMP which is very slowly dephosphorylated to 3TC. Starting from these encouraging results, pharmacokinetic studies of 3TCpPMPA in mice were performed. Unfortunately, following oral gavage administration, neither 3TCpPMPA nor its metabolites could be detected in systemic circulation. This is probably the consequence of a slow hydrolysis of the phosphate bridge during transit across the intestinal epithelium and the lack of absorption of the intact combined drug. Moreover, when lamivudine plus tenofovir were administered orally, only a low plasma level of 3TC could be detected, in agreement with reports on the limited oral bioavailability of (R)PMPA.43,44 In contrast, following iv injection, both 3TCpPMPA and its metabolites were found in circulation. However, 3TCpPMPA vanished too quickly (t1/2 = 15 min) to act as a pro-drug for the slow delivery of lamivudine and tenofovir in circulation. Promoted by these results, we decided to target the heterodinucleotide selectively to M/M through erythrocytes to protect macrophages against 'de novo' HIV-1 infection.

Thus, 3TCpPMPA was encapsulated into autologous RBCs modified to increase their recognition and their phagocytosis by human macrophages. The pro-drug should be stable enough in the carrier RBCs to be metabolically converted into 3TCTP and PMPADP within macrophages. Although our results show that dinucleotide pyrophosphatase is present in human cells, its activity seems to be rather limited, probably because of its susceptibility to ATP inhibition as observed by the reduced 3TCpPMPA degradation in RBC haemolysate supplemented with 1 mM ATP. The presence of a dinucleotide pyrophosphatase in human erythrocytes and its susceptibility to ATP inhibition has already been described45 and confirmed in our previous works.35,45,46 When 3TCpPMPA was encapsulated into RBCs, more than 70% of the compound was still present inside cells after 24 h of incubation (erythropagocytosis was allowed for 18 h prior to infection), suggesting that the heterodinucleotide is stable enough in RBCs to allow their use as a drug delivery system. Once delivered to macrophages, 3TCpPMPA degradation occurs yielding pharmacologically active metabolites, as demonstrated by the reported anti-HIV-1 activity. The mechanism hypothesized first involves the cleavage of 3TCpPMPA into 3TCMP and (R)PMPA which are then phosphorylated by cellular kinases to 3TCTP and PMPADP, respectively. The protection of macrophages against 'de novo' HIV infection is almost complete (99%) all through the 14 days post-infection and is still remarkable (~80%) even 5 weeks post-treatment. A limited effect of unloaded RBCs on HIV-1 replication has also been found. As previously observed and discussed,37,46 this effect may be due to an activation of macrophage functions and/or the production of certain cytokines.47 Moreover, our data show that slightly higher levels of protection against HIV-1 infection can be observed by treating macrophages with 3TCpPMPA as free drug rather than through RBCs, at the longest times post-treatment (4 and 5 weeks). This result could be due to the fact...
that not all macrophages have phagocytosed drug-loaded RBCs; in fact, we have previously demonstrated\textsuperscript{13} that under our experimental conditions, each macrophage phagocytoses one erythrocyte on average and thus we cannot exclude the presence of some unprotected cells. Moreover, the treatment with 3TCpPMPA encapsulated in RBCs may be particularly advantageous, considering that in M/M the development of resistance is far lower and slower than in lymphocytes.\textsuperscript{45} Another advantage rising from the heterodimer is the possibility of administering lamivudine and tenofovir in equimolar doses; this is of particular interest since lamivudine and tenofovir have EC\textsubscript{50} values in similar ranges.

In summary, the data reported in this paper prove that a new heterodinucleotide consisting of lamivudine and tenofovir bound together, once encapsulated into autologous erythrocytes modified to increase their recognition and phagocytosis, is able to protect macrophages from ‘de novo’ HIV-1 infection.

Very recent studies\textsuperscript{42} have demonstrated that the combination of tenofovir with emtricitabine (FTC, a cytosine analogue with a similar activity and resistance profile to 3TC) shows synergistic anti-HIV activity \textit{in vitro} superior to the combination 3TC plus (R)PMPA. This suggests the possibility of applying our strategy to other new heterodinucleotides to further improve macrophage protection against HIV-1 infection.

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Transparency declarations

We have no affiliations with the pharmaceutical industry or any related commercial concerns.

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