# Inhibition of HIV-1 replication in macrophages by red blood cell-mediated delivery of a heterodinucleotide of azidothymidine and 9-(*R*)-2-(phosphono methoxypropyl)adenine

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**Monocyte-derived macrophages (M/M) are considered important *in vivo* reservoirs for different kinds of viruses, including HIV. Hence, therapeutic strategies are urgently needed to protect these cells from virus infection or to control viral replication. In this paper, we report the synthesis, target delivery and *in vitro* efficacy of a new heterodinucleotide (AZTpPMPA), able to inhibit HIV-1 production in human macrophages. AZTpPMPA consists of two established anti-HIV drugs [zidovudine (AZT) and tenofovir (PMPA)] chemically coupled together by a phosphate bridge. This drug is not able to prevent p24 production when administered for 18 h to M/M experimentally infected with HIV-1 Bal (inhibition 27%), but can almost completely suppress virus production when given encapsulated into autologous erythrocytes (inhibition of p24 production 97%). AZTpPMPA is slowly converted to PMPA, AZT monophosphate and AZT (36 h half-life at 37°C) by cell-resident enzymes. Thus AZTpPMPA should be considered a new prodrug of AZT and PMPA that is able to provide stechiometric amounts of both nucleoside analogues to macrophage cells and to overcome the low phosphorylating activity of M/M for AZT and the modest permeability of PMPA.**

**Keywords: erythrocytes, macrophages, HIV, azidothymidine (*R*)-PMPA**

## Introduction

Cells of the mononuclear phagocyte system play a decisive role as a reservoir of the HIV. Indeed, HIV selectively kills CD4 lymphocytes, but in macrophages it induces an infection characterized by a long-term production of virus particles with an important role in the pathogenesis of AIDS (Gartner *et al.,* 1986; Ho *et al.,* 1986; Meltzer *et al.,* 1990). The importance of macrophages as a reservoir of retroviruses was recently confirmed (Igarashi *et al.,* 2001). These authors reported that long-term infected macrophages sustain replication of the highly pathogenic simian immunodeficiency virus/HIV type 1 (SHIV) chimeric virus (SHIV-DH12R) in rhesus monkeys, even at the stage of CD4 depletion. Interestingly, the administration of 9-(*R*)-2-(phosphonomethoxy-propyl)adenine (PMPA, tenofovir), a potent reverse transcriptase inhibitor, blocked virus production during the early T-cell phase, but not during the later macrophage phase of the SHIV-DH12R infection. These results implicate tissue macrophage as an important reservoir of virus *in vivo*, which can be a major contributor to total body virus burden during the symptomatic phase of the human infection. So, to achieve a complete elimination of the virus, we need antiretroviral agents that can target HIV infections both in lymphocytes and in tissue macrophages.

The antiviral dideoxynucleoside analogue 3′-azido-3-deoxythymidine (AZT, zidovudine) is among the drugs of choice against HIV infection. To be pharmacologically active, AZT must be phosphorylated by cellular kinases (Mitsuya *et al.,* 1990) to produce its triphosphate form (AZT-TP), which is able to inhibit HIV reverse transcriptase. However, the first phosphorylation of the drug is a limiting step in the intracellular metabolism to active form (Furman *et al.,* 1986). AZT shows a substantial activity against HIV in macrophages, yet its efficacy is limited by the low levels of the enzymes responsible for the phosphorylation of nucleoside analogues (Perno *et al.,* 1988).

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**Figure 1.** Chemical structures of zidovudine and tenofovir

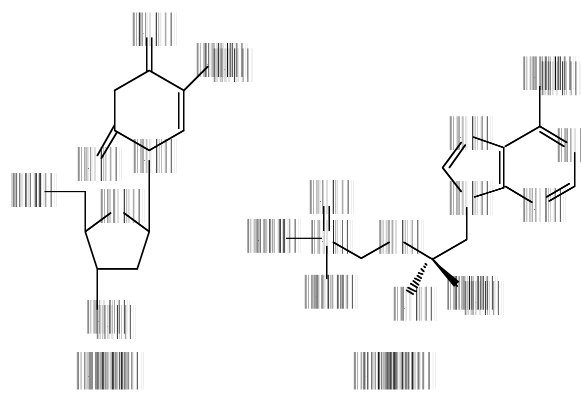

**Table 1.** Percentage of virus production by HIV-infected human macrophages treated with AZTpPMPA-loaded red blood cells of free drugs

| Treatments | p24 (%) |
|---|---|
| HIV-1 | 100 |
| Unloaded (500 RBC/macrophage) | 80 |
| AZTpPMPA-loaded RBCs (0.004 μmol/ml RBCs) | 42.5 |
| AZTpPMPA-loaded RBCs (0.25 μmol/ml RBCs) | 2.6 |
| AZTpPMPA 1.0 μM | 72.9 |
| AZT 1.0 μM | 90 |
| PMPA 1.0 μM | 73.2 |

RBC, red blood cells. All values are the means of quadruplicate cultures of a representative experiment, and show the p24 synthesized after 14 days of infection. P24 virus production from control, untreated macrophage cultures ranged 10000–30000 pg/ml in different experiments. RBCs were added at the ratio of 500 RBCs/machrophage. The free drugs were added only once for 18 h (as for RBCs).

Adenine acyclic nucleoside phosphonates, such as phosphonomethoxy-ethyl (PMEA) or phosphonomethoxy-propyl (PMPA) have emerged as potent antiretroviral agents. These nucleotides, are considered to mimic the 5′-monophosphorylated form of the dideoxynucleosides (ddN) and to bypass the need for enzymatic 5′-phosphorylation (De Clercq *et al.,* 1997). In particular, (*R*)-PMPA is presently being evaluated clinically for treatment and prophylaxis of HIV infection (Deeks *et al.,* 1998). PMPA has been demonstrated to have marked anti-SIV activity in models of acute and chronic infection of rhesus macaques (Van Rompay *et al.,* 1996) and anti-HIV replication *in vitro* (Balzarini *et al.,* 1993).

Unlike other nucleoside analogues, such as AZT, whose phosphorylation is cell cycle-dependent, PMPA is efficiently phosphorylated in resting as well as cyclic peripheral blood lymphocytes (Robbins *et al.,* 1998). PMPA can also inhibit HIV-replication in different cell types that may target HIV, including primary human blood lymphocytes and macrophages (Perno *et al.,* 1992). Moreover, it was reported that (*R*)-PMPA demonstrated strong synergistic anti-HIV activity in combination with AZT *in vitro*, but minor synergistic inhibition of HIV replication in combination with other antiretroviral drugs such as didanosine (ddI) and nelfinavir (Mulato *et al.,* 1997).

Our purpose is to protect macrophages against HIV-1 infection by the simultaneous administration of AZT and PMPA, and try to overcome both the low ability of macrophages to phosphorylate AZT and the low cellular permeability of PMPA. We had previously developed a drug delivery system based on the use of autologous artificially aged red blood cells (RBCs) for the selective administration of antiviral drugs to macrophages (Magnani *et al.,* 1992). The idea of using erythrocytes as a drug delivery system has been succesfully explored (Green *et al.,* 1997). Erythrocytes are natural targets of the reticuloendothelial system, can be manipulated to increase their removal from circulation, influence the biodistribution of encapsulated drugs, decrease the drug toxicity to non-macrophage cells, and prolong drug action. This system was shown to be effective in the protection of macrophages in both the murine (Rossi *et al.,* 1993) and feline (Magnani *et al.,* 1994) models of AIDS. Furthermore, new antiviral prodrugs can be designed and synthesized, which, once in macrophages, are split into nucleoside analogues that are close precursors of the active phosphorylated drugs (Magnani *et al.,* 1996; Rossi *et al.,* 1998; Franchetti *et al.,* 2000). Based on these previous results, a new heterodinucleotide consisting of AZT and PMPA bound together by a phosphate bridge, giving a phosphate-phosphonate mixed anhydride AZTpPMPA [$P^1$ thymidine-3′-azido-2′,3′-dideoxy-β-D-riboside-5′-$P^2$-9-(*R*)-2-(phosphonomethoxy-propyl)adenine] was synthesized and encapsulated into autologous erythrocytes modified to increase their recognition and phagocytosis by human macrophages.

Macrophages are expected to split the dimeric nucleotide analogue with the formation of AZT-momophosphate (AZT-MP) and (*R*)-PMPA, which are then converted by cellular kinases into the corresponding active forms AZT-TP and PMPA-diphosphate (PMPA-DP), respectively. In this way, it is possible to overcome the low permeability of PMPA and the low ability of macrophages to phosphorylate AZT. We report here the chemical synthesis, characterization, macrophage-targetted delivery and antiretroviral activity of the heterodinucleotide AZTpPMPA.

## Materials and methods

### Chemistry

Solvents were dried and freshly distilled before use, according to literature procedures. Thin layer chromatography (TLC) was run on silica gel 60 $F_{254}$ plates (Merck, KgaA,

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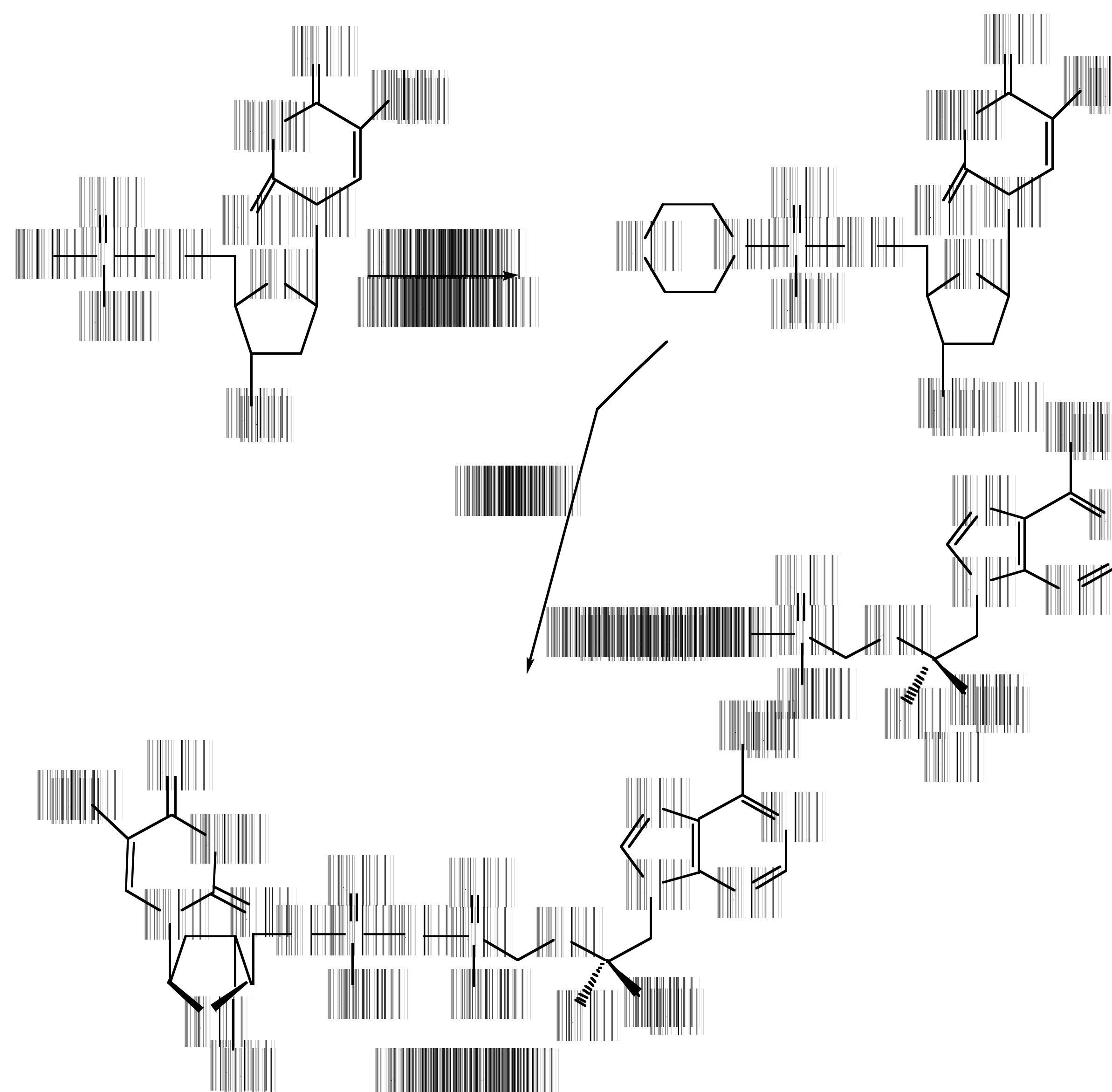
**Figure 2.** Synthesis of AZTpPMPA

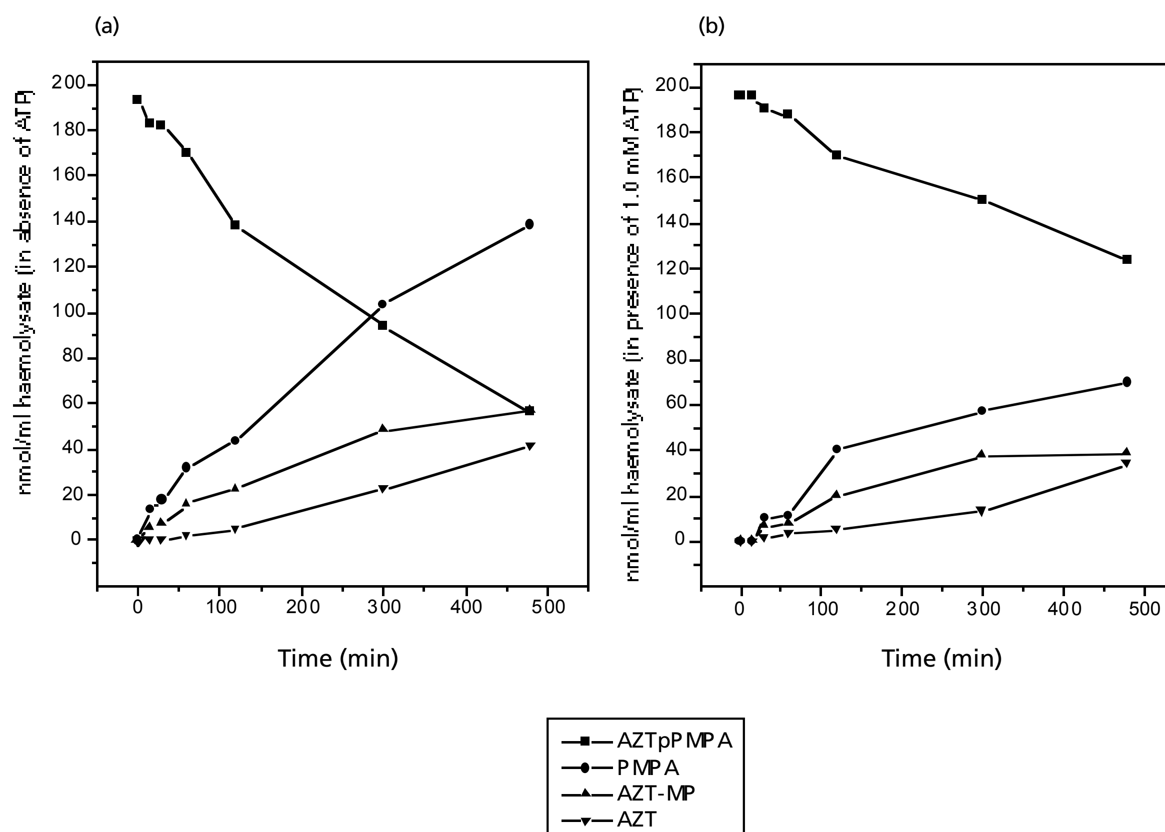


Darmstadt, Germany); silica gel 60 (230–400 mesh, Merck) was used for flash chromatography. Analitical high pressure liquid chromotography (HPLC) was run with a HP 1090 II series using a Beckman Ultrasphere C-18 (2mm×15cm) column (San Ramon, Calif., USA). Ion electrospray ionization (ESI) mass spectrum was obtained on a MSD HP 1100 series. All measurements were performed in the negative ion mode, scanning in the 300–800 mass range (API-ES). Nuclear magnetic resonance $^{1}$H-NMR (300 MHz) and $^{31}$P-NMR spectra (121 MHz) were recorded on a Varian VXR-300 spectrometer (Palo, Alto, Calif., USA) in $D_2O$. The chemical shift values are expressed in δ values (p.p.m.) relative to tetramethylsilane as an internal standard.

### N,N′-Dicyclohexyl-4-morpholine-carboxidinium-(thymidine-3′-azido-2′,3′-dideoxy-D-ribose)-5′-phosphoro-morpholidate (2)

AZT (300 mg, 1.12 mmol) was treated with $POCl_3$ in trimethylphosphate, as described by Yoshikawa *et al*. (1967). The reaction mixture was neutralized with 1N NaOH and the aqueous phase was washed with $CHCl_3$ (15 ml×3) and then evaporated to dryness. The residue was purified on DEAE-Sephadex A-25 (bicarbonate form) using $H_2O$ and

**Figure 3.** Stability of AZTpPMPA in human erythrocyte lysate




AZTpPMPA (0.2 mM) was incubated with the lysate at 37°C for 8 h in the absence (a) or presence (b) of 1.0 mM ATP. Aliquots of the incubation mixture were extracted with perchloric acid and analysed by HPLC (see materials and methods). The results shown are from one of three representative experiments that agree within 5% of the reported values.

a linear gradient of 0.01–0.2M $NH_4HCO_3$, and then on a Dowex 50w×8 cationic-exchange column ($H^+$ form) to obtain AZT 5′-monophosphate as a white solid, which was lyophilized (280 mg, 72% yield). AZT-MP (270 mg) was reacted with morpholine and 1,3-dicyclohexylcarbodiimide (DCCI) in t-BuOH/$H_2O$ (1/1, v/v) as described by Hostetler *et al*. (1990) to obtain the corresponding morpholidate. The crude compound was purified by flash chromatography, eluting with $CHCl_3$/MeOH (9:1 and then 6:4, v/v) to give the pure product as a foam (500 mg, 90%).

$^1$H-NMR ($D_2O$) δ2.40 (t, J 6.1 Hz, 2H, H2′), 2.92, 3.35, 3.55, 3.65 (4m, 8H, morpholino), 3.90 (m, 2H, H5′), 4.05 (m, 1H, H4′), 4.38 (q, J 5.4 Hz, 1H, H3′), 6.12 (t, J 6.6 Hz, 1H, H1′), 7.58 (s, 1H, H6); $^{31}$P-NMR ($D_2O$) δ 7.90 (s). MS (API/ES), m/z=415.3, as reported by Rossi *et al.* (1998).

### Bis(Ammonium) (P$^1$-thymidine-3′-azido-2′,3′-dideoxy-β-D-riboside-5′)-P$^2$-[9-(*R*)-2-(phosphono methoxypropyl)adenine] (1)

A solution of AZT-monophosphate morpholidate (480mg, 0.68 mmol) in dry pyridine (20 ml) was added to (*R*)-PMPA trioctylammonium salt (**3**) (338 mg, 0.34 mmol). The mixture was co-evaporated with dry pyridine (10 ml×3) and then solubilized in dry pyridine (20 ml). The solution was stirred under nitrogen atmosphere at room temperature for 72 h. Evaporation of the solvent gave a residue that was solubilized in $H_2O$ (10 ml) and treated with 1M NaOH to pH 8.0. The aqueous phase was washed with diethyl ether (10 ml×3), evaporated to dryness and purified by flash chromatography on silica gel eluting with $CHCl_3$/MeOH/$NH_4OH$/$H_2O$ (7:3:0.4:0.1, v/v/v/v). Evaporation *in vacuo* of the appropriate fraction gave the title compound as disodium salt (white solid, 92 mg, 43%). TLC (*i*PrOH/$NH_4OH$/$H_2O$, 8:1:1, v/v/v): R*f* 0.33. Analytical HPLC was run using $CH_3CN$/$H_2O$ (6:4, v/v) as eluents (F 1 ml/min; I 5 μl; Rt 7 min).

$^1$H-NMR ($D_2O$) δ1.02 (d, J 6.4 Hz, 3H, $CH_3$), 1.60 (s, 3H, $CH_3$ thymine), 2.26 (m, 2H, H2′), 3.58 (dd, J 10.3, 13.1 Hz, 1H, $CH_2$P), 3.72 (dd, J 8.7, 13.3 Hz, 1H, $CH_2$P),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3.84 (m, 1H, OCH), 3.98 (m, 2H, H5a, H5b), 4.12 (dd, *J* 6.4, 14.9 Hz, 1H, H4′), 4.25 (d, J 3.0 Hz, 2H, $NCH_2$), 4.30 (m, 1H, H3′), 6.0 (t, J 6.7 Hz, 1H, H1′), 7.40 (s, 1H, H6 thymine), 8.15, 8.22 (2s, 2H, H2 and H8 adenine); $^{31}$P-NMR ($D_2O$) δ 9.03 (d, J 26.2 Hz, $CH_2P$), −10.9 (d, J 25.6Hz, O-P-O). MS (API/ES), m/z = 616.4.

## Biological assays

***HPLC-analysis of AZTpPMPA metabolites.*** Samples were extracted with perchloric acid as reported by Magnani *et al.* (1989). Neutralized extracts were then used for HPLC determinations. A 5 μM Res. Elut. 5 $C_{18}$ 90A column (150×4.60 mm ID; Varian, Harbor City, Calif., USA) protected by 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 $KH_2PO_4$ adjusted to pH 6.0 (buffer A), and buffer A containing 30% (v/v) acetonitrile (buffer B). All buffer solutions, as well as standards and samples solutions, were filtered through a 0.22-μm membrane filter (Millipore, Bedford, Mass., USA). 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 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 wavelenght was 260 nm. Analyses were performed at room temperature on a 50-μl sample, and quantitative measurements were obtained by injection of standards of known concentration. The detection limit was 10 μM. The retention times under the conditions used were 11.1 min for PMPA, 16.8 min for AZT, 12 min for AZT-MP and 29.5 min for AZTpPMPA.

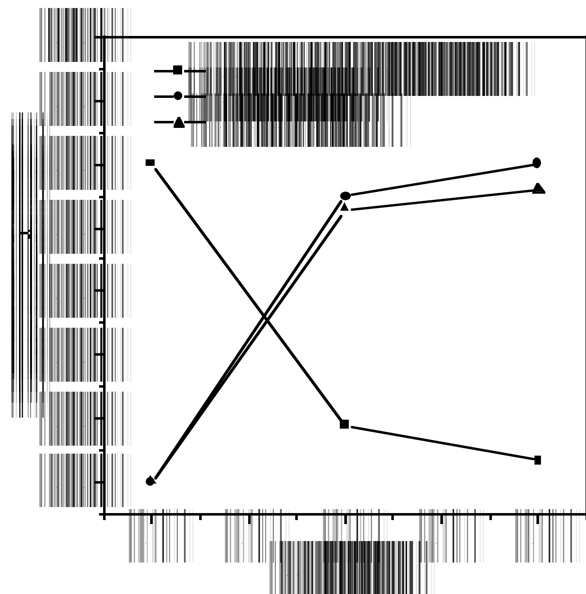
***AZTpPMPA metabolism in erythrocyte lysates.*** Human red blood cells lysates were obtained as described by Magnani *et al.* (1996). Haemoglobin concentration was 118 mg/ml haemolysate. AZTpPMPA was incubated in erythrocyte lysates 8 h at 37°C in the absence and presence of 1.0 mM ATP. At times 0, 0.25, 0.5, 1, 2, 5 and 8 h, 100-μl aliquots were removed and treated for HPLC determination.

***AZTpPMPA encapsulation in erythrocytes.*** AZTpPMPA was encapsulated in human erythrocytes by a procedure of hypotonic dialysis, isotonic resealing and reannealing as previously reported by Magnani *et al.* (1992) but with some modifications. Briefly, human erythrocytes were washed in 10 mM HEPES (pH 7.4) containing 154mM NaCl and 5 mM glucose (washing buffer) to remove leukocytes and platelets. RBCs were resuspended at 70% haematocrit in the same washing buffer and were dialysed for 75 min using a tube with a cut off of 12–14 kDa, against 50 vol of 10 mM $NaH_2PO_4$, 10 mM $NaHCO_3$ and 20 mM glucose (pH 7.4) containing 3 mM reduced glutathione and 2 mM ATP. The osmolarity of the buffer was about 60mmol, whereas the erythrocytes reached about 80mmol at the end of the dialysis time. All these procedures were performed at 4°C. After this step, two different amounts of AZTpPMPA (0.2 and 10 μmol) were added to each ml of dialysed erythrocytes, which were then incubated for 30min at room temperature under gentle mixing. Resealing of erythrocytes was obtained by adding 0.1 vol of 5 mM adenosine, 100 mM inosine, 2 mM ATP, 100 mM $MgCl_2$, 0.194 M NaCl, 1.606 M KCl and 35 mM $NaH_2PO_4$ (pH7.4) per volume of dialysed erythrocytes and incubating the resealed cells at 37°C for 25 min. Resealed cells were washed three times in the washing buffer and used as they were, or processed further, to increase their recognition by macrophages.

***AZTpPMPA stability in human erythrocytes.*** The stability of AZTpPMPA in erythrocytes was evaluated by incubation of AZTpPMPA-loaded erythrocytes at 0.8% haematocrtit in RPMI 1640 medium containing 10% fetal calf serum (FCS). At different incubation times at 37°C in a 5% $CO_2$ atmosphere and under sterile conditions, 5-ml aliquots were processed to determine the concentration of AZTpPMPA and its metabolites. Briefly, AZTpPMPA-loaded RBCs were extracted with perchloric acid and analysed by HPLC as previously described, while media were submitted to solid-phase extraction using Isolute $C_{18}$ columns (International Sorben Technology, Mid-Glamorgan, UK) according to manufacturer instructions, before being analysed by HPLC. Efficiency of solid phase extraction was 90±5%.

***Targetting of AZTpPMPA-loaded erythrocytes to macrophages.*** Targetting of AZTpPMPA-loaded RBCs to macrophages was obtained, as described by Magnani *et al.* (1992), by promoting the clustering of band 3, the predominant transmembrane protein that functions as an anion transport system. The clustering of band 3 can be induced by some chemical agents, such as zinc and the peptide mellitin (Clague *et al.*, 1989), and can be made irreversible by addition of the cross-linking agent bis(sulfosuccinimidyl)suberate ($BS^3$) (Turrini *et al.*, 1991). Once clusters are formed, they are viewed by the immune system as non-self and consequently are opsonized by autologous antibodies. Loaded erythrocyte suspension (10% haematocrit) was incubated with 1.0 mM $ZnCl_2$ and treated with 1.0 mM $BS^3$ for 15 min at room temperature under gentle mixing. It was then washed once in washing buffer containing 10 mM ethanolamine (pH 7.4) and once in washing buffer containing 1% (w/v) bovine serum albumine (BSA). These cells were then incubated in autologous plasma for 60 min at 37°C at a haematocrit of 30% to induce IgG binding, washed once in washing buffer containing 2%

**Figure 4.** Metabolism of AZTpPMPA in intact erythrocytes



AZTpPMPA was encapsulated into human erythrocytes by a procedure of hypotonic dialysis and isotonic resealing to a final concentration of 0.25 mM. These cells were then incubated at 37°C following the conditions described in materials and methods. One of two similar and independent experiments is shown.

(w/v) BSA and once in washing buffer only. AZTpPMPA-loaded erythrocytes were then added to macrophages and their antiviral activity evaluated.

***Macrophages and virus.*** PBMCs were obtained from normal seronegative blood donors by separation over Ficoll–Hypaque gradient. Monocytes/macrophages (M/M) were separated as described by Perno *et al*. (1996). Briefly, adherent mature cells (about 100000/well) were obtained by culturing mononuclear cells for 5 days in 48-well plastic plates (Costar, Cambridge, Mass., USA) in 1 ml of RPMI 1640, with the addition of 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine and 20% heat-inactivated FCS. This medium was used in all experiments. On the fifth day of culture, non-adherent cells were carefully removed by repeated washings. Cells that attached to the wells were differentiated M/M as revealed by surface marker analysis. A monocytotropic strain of HIV-1, HTLV-III Bal (referred to as HIV-Bal), was used in experiments fitted up to evaluate the anti-HIV-activity. HIV-Bal was expanded and titrated in macrophages.

***Anti-HIV-1 activity assay.*** For the assay of antiretroviral activity on infected macrophages, RBCs loaded with AZT–PMPA were added at a ratio of 500 RBCs per macrophage. After 18 h of incubation, non-ingested RBCs were removed by extensive washing with culture medium. As a control, macrophage cultures were treated with unloaded RBCs, that is, cells RBCs submitted to the same procedure, including transient lysis and subsequent modification to increase macrophage recognition, but without of AZT–PMPA. M/M cultures receiving either AZT–PMPA-loaded or unloaded RBCs were then infected for 2 h with HIV-Bal [300 $TCID_{50}$ (50% tissue culture infective dose)/ml]. After incubation with the virus, cell cultures were extensively washed to remove any residual virus particles. Further controls were performed in each experiment: 1.0 μM AZTpPMPA, 1.0 μ M AZT and 1.0μM PMPA were given as free drugs for 18 h (as for RBCs), before virus challenge, and removed thereafter. Throughout the experiments, no treatment was repeated after infection. Virus production was assessed in the supernatants, 14 days after challenge, with an ELISA kit that could be used to detect HIV gag p24.

## Results

### Chemistry

The heterodimer AZTpPMPA was prepared by coupling AZT 5′-monophosphate morpholidate with bis-trioctylammonium PMPA salt (Figure 2). AZT-monophosphate morpholidate (**2**) was obtained by the purified corresponding monophosphate as described by Hostetler *et al.* (1990), and purified by flash chromatography. (*R*)-PMPA trioctylammonium salt (**3**), obtained by treatment of PMPA in dry MeOH with trioctylamine, was coupled to **2** (molar ratio 1:0.5) under anhydrous conditions in pyridine at room temperature for 72 h. After evaporation of the reaction mixture, the residue in $H_2O$/NaOH was washed with diethyl ether and then was purified by flash chromatography to give the desired pure heterodimer **1** as disodium salt. Unreacted monophosphate **2** and PMPA were detected in the reaction mixture by nuclear magnetic resonance $^{1}$H- and $^{31}$P-NMR analysis. A small amount of the homodimer bis-PMPA was also obtained and characterized by $^{31}$P-NMR and mass spectrometry (data not shown). The structure of the heterodimer **1** was determined by HPLC/mass spectrometry which gave the expected m/z ratio of 616.4. This structure was also confirmed by $^{1}$H-, and $^{31}$P-NMR spectra in $D_2O$.

### Metabolism of AZTpPMPA in erythrocyte lysates

AZTpPMPA was incubated in erythrocyte lysates 8 h at 37°C in the absence and in presence of 1.0 mM ATP (Figure 3). The time courses show a faster cleavage of the heterodimer in PMPA and AZT-MP (then dephosphorylated to AZT) in the absence than in the presence of 1.0 mM ATP. Thus, after 8 h at 37°C, 62% of AZTpPMPA was still present in the incubation mixture supplemented

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with ATP, while without the addition of ATP, residual AZTpPMPA was 30% at the same time. This result is in agreement with the previous observation that an ADP-ribose pyrophosphatase is present in human erythrocytes (Zocchi *et al.* 1993), acts on a number of $P^1$–$P^2$ dinucleotides and is inhibited by physiological ATP concentration (Magnani *et al.*, 1996; Rossi *et al.,* 1998, 2001). This condition resembles the *in vivo* situation, where red blood cell ATP is 1.5 mM.

### Metabolism of AZTpPMPA in intact erythrocytes

AZTpPMPA was encapsulated into human erythrocytes to a final concentration of 0.25 μmol/ml RBC. This value was obtained by adding 2.5 μmol of AZTpPMPA to 250 μl of dialysed RBCs at the end of the dialysis time. AZTpPMPA-loaded RBCs, at 0.8% haematocrit, were incubated 4 days at 37°C in RPMI 1640 medium. On days 0, 2 and 4 of incubation, the concentration of AZTpPMPA in erythrocytes and the presence of its metabolites in culture medium were evaluated. As shown in Figure 4, the decrease in AZTpPMPA in RBCs (down to 10% of the starting level after 4 days of incubation), was paralleled by the stoichiometric production of AZT and PMPA in the medium culture.

### Anti-HIV activity of AZTpPMPA-loaded RBCs

Human monocyte-derived macrophages were cultured for 10 days and then treated with AZTpPMPA-loaded or unloaded RBCs in culture medium for 18 h before infection with a macrophage-tropic HIV-Bal strain. Two different concentrations of AZTpPMPA inside RBCs were evaluated (0.004 and 0.25 μmol/ml RBCs). These concentrations were obtained by adding, to each ml of dialysed erythrocytes, 0.2 and 10 μmol of AZTpPMPA, respectively. The highest concentration used corresponds to a mean drug concentration (in the medium culture well) of approximately 1 μM. Since we have previously demonstrated that each macrophage, under these experimental conditions, phagocytoses one erythrocyte, we have calculated that the mean drug concentration in the macrophages corresponds to 0.04 and 2.5 pmol×$10^5$ macrophages, respectively. Because a differentiated macrophage has a mean volume in the 8–60 pl range, 2.5 pmol×$10^5$ macrophages corresponds to a macrophage AZTpPMPA concentration in the region of 0.4–3.1 μM range. This mean concentration is comparable to the 1μM assays of free drugs.

The antiviral activity of AZTpPMPA-loaded RBCs was determined 14 days after infection by evaluating p24 production (Table 1). The results show that by administering AZTpPMPA-loaded RBCs (0.25 μmol/ml RBC), 97% inhibition of HIV replication was obtained. However, almost 60% inhibition in p24 production was observed by the addition of RBCs loaded with 0.004 mM AZTpPMPA only. Interestingly, about 20% inhibition of HIV replication was also obtained in macrophages treated with unloaded RBCs. As controls, macrophages were exposed to 1.0 μM AZTpPMPA, 1.0 μM AZT and 1.0μM PMPA, given as free drugs only once for 18 h (as for RBCs). With PMPA and AZTpPMPA addition, a low inhibition in p24 production (27%) was observed, while no effect at all was achieved by treatment with AZT. It is worth noting that AZTpPMPA is completely hydrolysed by serum enzymes to AZT and PMPA after 4 h incubation (not shown). Thus, addition of AZTpPMPA can be considered equivalent to the addition of 1 μM AZT and 1 μM PMPA.

## Discussion

The non-cytopathic infection of macrophages by HIV-1 makes these long-lived cells a major virus reservoir with an important role in the pathogenesis of AIDS (Gartner *et al.,* 1986; Ho *et al.,* 1986; Meltzer *et al.,* 1990). In this article, we have shown that it is possible to protect macrophages against '*de novo*' HIV infection by the administration of a single prodrug consisting of two nucleoside analogues (azidothimidine, AZT and (*R*)- PMPA) bound by a phosphate bridge (AZTpPMPA). AZT is the most popular compound that is able to reduce the morbidity and mortality associated with severe HIV infection, particularly in combination with other drugs (Clague *et al.,* 1989; Perno *et al.,* 1992). Unfortunately, well differentiated macrophages possess a substantially lower ability to phosphorylate AZT than T cells (Perno *et al.,* 1988). Unlike AZT, the dAMP analogue PMPA possesses a phosphonoether moiety that mimics the 5′-monophosphorylated form of the ddNs, thus bypassing the need for enzymatic 5′-phosphorylation (De Clercq, 1997). However, PMPA is characterized by a low cellular permeability. To overcome these limitations, and because AZT has demonstrated strong synergistic anti-HIV activity in combination with PMPA (Mulato *et al.,* 1997), we thought to target a single molecule, that has anti-HIV activity after intracellular cleavage into AZT-MP and PMPA (AZTpPMPA), selectively to macrophages. The selective targetting of this prodrug to macrophages was obtained by exploiting their natural property of erythrophagocytosis following the artificial aging of AZTpPMPA-loaded erythrocytes. The prodrug AZTpPMPA should be stable enough in the carrier RBCs to be metabolically converted to the nucleoside triphosphate analogues (AZT-TP and PMPA-DP) within infected macrophages. Our results show that human erythrocytes possess a dinucleotide pyrophosphatase able to cleave the pyrophosphate bridge of AZTpPMPA with subsequent production of AZT-MP and PMPA.

However, its activity seems to be rather low, probably

because of its susceptibility to ATP inhibition, as shown by the reduced AZTpPMPA 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 been already described (Zocchi *et al.* 1993). When AZTpPMPA was encapsulated into RBCs, 50% of the compound was still present inside cells after 36 h of incubation (erythrophagocytosis is allowed for 18 h), suggesting that the heterodimer is stable enough in RBCs to allow their use as a drug delivery system. Once delivered to macrophages, AZTpPMPA degradation occurs, which yields pharmacologically active metabolites, as demonstrated by the reported antiviral activity. The mechanism hypothesized involves first the cleavage of AZTpPMPA into AZT-MP and PMPA, which are then phosphorylated to AZT-TP and PMPA-DP, respectively by cellular kinases.

Protection of macrophages against '*de novo*' HIV infection is almost complete (97%) upon the administration of erythrocytes loaded with the highest AZTpPMPA concentration tested (0.25 mM). When AZTpPMPA, or AZT and PMPA, were added to macrophages as free drugs, for the same time as when administered encapsulated into RBCs (18 h), only a low inhibition in p24 production was obtained. In detail, while 1.0 μM AZT was almost ineffective, confirming the low ability of macrophages to phosphorylate AZT, 27% of inhibition was observed both by 1.0μM PMPA and 1.0 μM AZTpPMPA. Since AZTpPMPA does not cross the cellular membranes, its antiviral activity might be due to the hydrolysis of AZTpPMPA in AZT and PMPA by means of serum enzymes present in the RPMI complete culture medium. Another point of interest is the finding of a limited effect of unloaded RBCs on the replication of HIV in macrophages. This antiviral status induced by RBCs in macrophages may be due to an activation of macrophage functions and/or the production of certain cytokines (Piedimonte *et al.*, 1993) that still needs to be characterized. This effect has been shown and discussed in previous papers (Rossi *et al.*, 1998; Franchetti *et al.*, 2000).

In conclusion, the results reported in this paper show that AZT and PMPA can be chemically coupled into a new heterodinucleotide that is able to perform as a prodrug for the stechiometric release of both moieties. When selectively delivered to M/M, AZTpPMPA is very effective in inhibiting HIV-1 production to almost undetectable levels, suggesting that these cells can be efficiently protected if proper drugs and proper drug delivery systems are used. The administration of AZTpPMPA-loaded RBC may complement the existing therapies that aim to protect all cell compartments that actively participate in the maintenance of virus production in the infected host.

Interestingly, the same drug targetting system could be used for the delivery of other heterodimers, possibly including both a reverse transcriptase inhibitor and a protease inhibitor. If this is feasible, we will be able to protect non-infected macrophages from new infection, and infected macrophages from viral replication.

## Acknowledgments

This work was supported by grants from AIDS Project of the Italian National Institute of Health, and from CNR Target Project on Biotechnology. We thank Dr N Bischofberger (Gilead Sciences, Foster City, Calif., USA) for providing (*R*)-PMPA, and Fabbio Marcuccilli for the relevant technical support.

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Received 4 April 2001 ; accepted 1 June 2001