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# A new acyclic heterodinucleotide active against Human Immunodeficiency Virus and Herpes Simplex Virus

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**Abstract**

The most common therapies against human herpes virus (HSV-1) and human immunodeficiency virus (HIV-1) infectivity are based on the administration of nucleoside analogues. Acyclovir (ACV) is the drug of choice against HSV-1 infection, while the acyclic nucleoside phosphonate analogue PMPA has shown marked anti-HIV activity in a phase I and II clinical studies. As monocyte-derived macrophages are assumed to be important as reservoirs of both HSV-1 and HIV-1 infection, new approaches able to inhibit replication of both viruses in macrophages should be welcome. ACVpPMPA, a new heterodinucleotide consisting of both an antiherpetic and an antiretroviral drug bound by a phosphate bridge, was synthesized and encapsulated into autologous erythrocytes modified to increase their phagocytosis by human macrophages. ACVpPMPA-loaded erythrocytes provided an effective in vitro protection against both HSV-1 and HIV-1 replication in human macrophages. 

*Keywords:* Acyclovir (ACV); Anti-HIV activity; Anti-HSV activity; Human macrophages; (*R*)PMPA

## 1. Introduction

Human herpes viruses (HSVs) are distributed worldwide and are among the most frequent viral infections in human immunodeficiency virus type 1 (HIV-1) immunocompromised patients. Hence, therapeutic strategies able to inhibit replication of

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both viruses are surely welcome (De Clercq, 1997). So far, the most common therapeutic strategies against Human herpes virus type 1 (HSV-1) and HIV-1 infectivity have been based on the administration of nucleoside analogues alone or in combination with other antiviral agents. However, these antiviral nucleosides have to be converted to their triphosphorylated derivatives by viral and/or cellular kinases (De Clercq, 1997). At the cellular level, the main problems involved in the use of such drugs are their limited phosphorylation in some cells (e.g. antiretroviral drugs in macrophages) and the cytotoxic side effects of the corresponding triphosphates. Acyclic nucleoside phosphonates (ANP), as mimics of 5′-monophosphate nucleosides, have proved to be effective inhibitors of different types of DNA viruses and retroviruses including HIV (De Clercq, 1997, 1998). These compounds enter the cells through an endocytosis-like process and are converted into diphosphorylated derivatives, which act as alternative substrate to the dNTP/chain terminator in the HIV reverse trancriptase (RT) reaction. Also the 3-hydroxy-2-(phosphonomethoxy)propyl (HPMP) derivatives, such as cidofovir (HPMPC), the 2-(phosphonomethoxy)propyl (PMP) derivatives, such as PMPA, and the 2-(phosphonomethoxy)ethyl (PME) derivatives, such as PMEA, in their diphosphorylated form (HPMCpp, PMPApp, and PMEApp) interact with the normal substrates for the viral DNA polymerases. PMPA [9-((*R*)-2-phosphonomethoxypropyl)adenine] is more a faithful chain terminator of the HIV-1 reverse transcriptase reaction than PMEA (De Clercq, 1997 and references cited therein). PMPA exhibits a slightly higher selectivity index in cell culture, which could be explained by the low affinity of the phosphorylated PMPApp form for host DNA polymerases. PMPApp proved to be a less potent inhibitor of each of the cellular polymerases, particularly DNA polymerase γ, than PMEApp. Both PMEApp and PMPApp were found to be substrates for the DNA polymerases α, β and γ, and to terminate DNA elongation after their incorporation into the primer. The lower cytotoxicity of PMPA, as compared to PMEA, could depend on its less efficient incorporation into DNA (Cihlar and Chen, 1997). PMPA does not afford activity against herpes viruses, but its activity is selective against a wide range of hepadnaviruses and retroviruses including the human immunodeficiency virus (De Clercq, 1997). In particular, PMPA is highly effective in the pre- or postexposure prophylaxis of simian immunodeficiency virus (SIV) infection in rhesus monkeys (Tsai et al., 1997). This acyclic nucleotide is currently undergoing phase III clinical trials for the treatment of HIV infection in AIDS patients. Acyclovir (ACV) is the drug of choice against HSV and varicella-zoster virus (VZV) infections in immunosuppressed patients.

Cells of macrophage lineage [monocyte/macrophages (M/M)] play a central role in the pathogenesis of infection by HIV and represent the main reservoir of the virus in the body. In addition, M/M can be targeted by other viruses, such as HSV-1, which are able to enhance the replication of HIV (Heng et al., 1994; Perno et al., 1997).

Magnani et al. (1992) have developed a drug targeting system that allows the selective administration of antiviral drugs to macrophages. This system has proved to be effective in the protection of macrophages both in the murine model of AIDS (Rossi et al., 1993) and feline AIDS (Magnani et al., 1994). Furthermore, it was shown that new antiviral prodrugs, once in the macrophage, can be split into phosphorylated (and thus activated) nucleoside analogues (Magnani et al., 1996).

Prompted by these considerations, we designed and synthesized ACVpPMPA, a new acyclic heterodinucleotide consisting of both an antiherpetic and an antiretroviral drug. In ACVpPMPA an acyclic nucleoside (acyclovir) and an acyclic nucleoside phosphonate (PMPA) were bound by a phosphate bridge, giving a mixed phosphate-phosphonate anhydride.

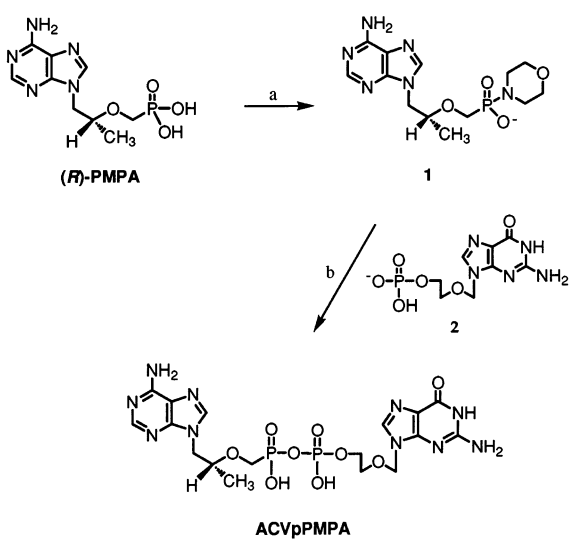
We propose ACVpPMPA as a single molecule active against both HSV and HIV replication. ACVpPMPA was incorporated into a carrier such as the red blood cells (RBCs) for targeted delivery thereby conferring protection on macrophages. These are expected to split the acyclic heterodinucleotide symmetrically with the formation of ACV

monophosphate and PMPA, or asymmetrically with the formation of ACV and PMPA monophosphate which could then be converted into the corresponding active diphosphate form by cellular kinases. In this way, it is possible to increase the uptake of phosphorylated drugs by cells of the M/M lineage and to overcome the low phosphorylating capabilities of macrophages. At the same time, ACVpPMPA acts as a prodrug that can be activated by endogenous enzymes to produce at low concentrations triphosphorylated nucleoside analogues that selectively inhibit the viral reverse transcriptase and/or viral DNA polymerase.

## 2. Materials and methods

### 2.1. Reagents

Acyclovir was purchased from Sigma Reagents. (*R*)PMPA was synthesized as reported by Holý et al. (1995a,b). PMEA was kindly provided by Gilead Sciences, Foster City, CA, USA.




Scheme 1. Reaction conditions: (a) morpholine, DCCI, *tert*-butanol: (b) pyridine.

### 2.2. Synthesis

The heterodinucleotide ACVpPMPA was obtained by coupling the morpholidate derivative of 9-((*R*)-2-phosphonomethoxypropyl)adenine (1) with the mono *n*-tri-butylammonium salt of ACV monophosphate (ACV-MP, 2) as reported in Scheme 1. Morpholidate 1 was obtained as *N*,*N*′-dicyclohexyl-4-morpholine-carboxamidinium salt by treatment of PMPA with morpholine in the presence of *N*,*N*′-dicyclohexyl-carbodiimide (DCCI) as activating reagent (molar ratio 1/6), in hot aqueous *tert*-butyl alcohol for 11 h. ACV-MP was synthesized as described by Yoshikawa et al. (1967), except that after the reaction the mixture was poured into diethyl ether, and the resulting precipitate was then separated by centrifugation. The product was then dissolved in water and the aqueous solution was adjusted to pH 7.5 with a 1 M NaOH solution; the solvent was removed under reduced pressure and dried in vacuo. The final product (100% yield) was identified by mass spectrometric analysis using an atmospheric pressure ionization electrospray ionization (API-ESI) source, which confirmed the expected molecular mass of 305.5 m/z. The tributylammonium salt of ACV-MP was then obtained as described by van Wijk et al. (1992). Reaction of morpholidate 1 with ACV-MP (2) (molar ratio 1/1) in anhydrous pyridine for 2 days at room temperature gave crude ACVpPMPA. The obtained reaction mixture was evaporated to dryness and purified by chromatography on silica gel column using 2-propanol/$NH_4OH$/$H_2O$ (75:20:5, v/v/v) as eluents. The appropriate fraction was evaporated in vacuo to give a solid residue that was solubilized in aqueous ammonia (30%) and washed several times with diethyl ether. The aqueous fraction was concentrated in vacuo to give ACVpPMPA as diammonium salt. The final product (52% yield) was analyzed by mass spectrometry as described above, and showed a mass value of 574.1 m/z, consistent with the expected molecular structure. All measurements were performed in the negative ion mode, and scanning in the 300–800 mass range. The elemental analysis confirmed the structure of ACVpPMPA as diammonium salt (Anal. Calcd. for $C_{17}H_{24}N_{10}O_9P_2$ $2NH_3$: C 33.56, H 4.97, N 27.62. Found: C 33.38, H 4.71, N 27.40).

Finally, the structure of ACVpPMPA was also confirmed by nuclear magnetic resonance $^1$H-NMR spectrum in $D_2O$ recorded at 300 MHz with a Varian VXR-300 spectrometer. The chemical shift values are expressed in $\delta$ values (parts per million) relative to tetramethylsilane as an internal standard. The $^1$H-NMR spectrum showed the presence of two singlets at $\delta$ 7.82 (1H, H-2 of adenine) and at $\delta$ 8.13 (2H, H-8 of adenine and guanine). Moreover, the $^{31}$P-NMR spectrum in $D_2O$, recorded by the above apparatus at 121 MHz, revealed the presence of two singlets at $\delta$ 9.02 ($CH_2P$) and $\delta$ −10.59 (O–P–O).

### 2.3. HPLC analysis of ACVpPMPA metabolites

Samples were extracted with perchloric acid (Magnani et al., 1989); neutralized extracts were then used for HPLC determinations essentially as described by Rossi et al. (1998) with some modifications: the flow rate was 0.9 ml/min and the detection wavelength was 254 nm. The retention times under the conditions used were 8.5 min for ACV, 11.5 min for ACV-MP and 15.4 min for PMPA.

### 2.4. ACVpPMPA metabolism in erythrocyte lysate

Erythrocyte lysate was obtained as described by Rossi et al. (1998). The hemolysate (123 mg of hemoglobin/ml) was incubated at 37°C in the presence of 0.08 mM ACVpPMPA, in absence or in presence of 1.0 mM ATP. At 0, 0.5, 1.0, 2.0, 4.0, and 7.0 h, 100 μl aliquots were removed and processed for HPLC determination.

### 2.5. ACVpPMPA encapsulation in RBCs

Human erythrocytes were loaded with ACVpPMPA by a procedure of hypotonic dialysis, isotonic resealing, and reannealing, essentially as reported by Magnani et al. (1992) with some modifications: RBCs were dialyzed for 70 min and at the end of the dialysis time 10 μmol of ACVpPMPA were added to each milliliter of erythrocytes. The mixture was then incubated 30 min at room temperature before adding 0.1 vol of the compound solution per volume of dialyzed erythrocytes. Cells were washed twice in 10 mM HEPES (pH 7.4) containing 154 mM NaCl and 5 mM glucose (buffer A) and used for the experiments described below.

### 2.6. ACVpPMPA metabolism in intact erythrocytes

ACVpPMPA-loaded RBCs were resuspended at a 0.7% hematocrit in RPMI 1640 medium containing 10% fetal calf serum (FCS) and incubated at 37°C, 5% $CO_2$ under sterile condition. At different incubation times, 5 ml aliquots were processed for the determination of ACVpPMPA and its metabolites by extraction with perchloric acid and analysis by HPLC, as described above. ACVpPMPA metabolites in the incubation medium were extracted by solid-phase extraction using Isolute TM $C_{18}$ columns (International Sorben Technology, Mid-Glamorgan, UK) according to the manufacturer's instructions and analyzed by HPLC, as described above.

### 2.7. Targeting of drug-loaded erythrocytes

ACVpPMPA-loaded RBCs were modified to increase their recognition by macrophages by a procedure described in Magnani et al. (1992) and Perno et al. (1997). Briefly, loaded erythrocyte suspensions (10% hematocrit) in 1.0 mM $ZnCl_2$ were treated with 1.0 mM bis(sulfosuccinimidyl)suberate ($BS^3$) for 15 min at room temperature and washed once in buffer A containing 10 mM ethanolamine, and once in buffer A containing 1% (w/v) bovine serum albumin. Erythrocytes were then incubated at 37°C in autologous plasma for 60 min at a hematocrit of 40% and after this time washed once in buffer A containing 2% (w/v) bovine serum albumin and once in buffer A. Finally, opsonized ACVpPMPA-loaded RBCs were resuspended in buffer A at a hematocrit of 20% and used for the targeting to macrophages.

### 2.8. Cells and viruses

Peripheral blood mononuclear cells (PBMCs) were obtained from normal seronegative blood

Table 1
Percent virus inhibition (p24) in HIV-infected human macrophages treated with drug-loaded RBCs or free drugs[a]

| Treatments | % HIV inhibition | | | |
|---|---|---|---|---|
| | Days | | | |
| | 14 | 20 | 27 | 34 |
| None | 0 | 0 | 0 | 0 |
| Unloaded RBCs (1:100) | – | 31 | 51 | 50 |
| Unloaded RBCs (1:500) | – | 28 | 48 | 46 |
| ACVpPMPA-loaded RBCs (1:100) | >99 | >99 | >99 | >99 |
| ACVpPMPA-loaded RBCs (1:500) | >99 | >99 | >99 | >99 |
| ACVpPMPA 1.0 μM (overnight) | 0 | 0 | 0 | 0 |
| PMPA 1.0 μM (overnight) | 14 | 13 | 13 | 14 |
| PMEA 1.0 μM (overnight) | 2 | 18 | 2 | 3 |
| ACVpPMPA 1.0 μM (maintained) | 78 | 64 | 68 | 79 |

[a] Drugs were added to human macrophages 18 h before infection and maintained overnight until infection, except for 1.0 μM of ACVpPMPA whose efficacy was evaluated also when maintained throughout the entire experiment. When 1.0 μM PMEA and 1.0 μM PMPA were maintained throughout the entire experiment, an almost total protection was obtained. All values are the means of quadruplicate cultures in which variability never exceeded 5%. One-hundred-percent virus production corresponds to 10 000, 22 000, 30 000, and 35 000 pg of p24/ml 14, 20, 27, and 34 days after infection, respectively.

donors by separation on Histopaque solution (Sigma, St. Louis, MO). Monocytes were separated as described by Rossi et al. (1998) or Perno et al. (1996). After removal of nonadhering cells by repeated washings, monocytes were scraped and collected in a phosphate saline solution. Cell viability was greater than 90% as determined by the trypan blue dye exclusion test. The cells were suspended in RPMI 1640 medium supplemented with 15–20% heat-inactivated FCS and 1% antibiotics (complete medium), and adjusted to a final concentration of $5 \times 10^5$ cells/ml. After culture, the monocytes had matured into macrophages, as revealed by surface marker analysis, and had formed a monolayer. A monocytotropic strain of HIV-1, HTLV-$III_{Ba\text{-}L}$ and a laboratory-adapted strain of HSV-1, Mc Entyre (the latter kindly provided by M. Barbi, Institute of Virology, University of Milan, Italy), were used in experiments of infection with HIV-1 and HSV-1, respectively. Both viruses grew and easily replicated in macrophages (Perno et al., 1996).

### 2.9. Assays of anti-HIV-1 activity

For the assays of antiretroviral activity on infected macrophages, RBCs loaded with 1.0 mM ACVpPMPA were added at a ratio of 100 or 500 RBCs per macrophage (Table 1). After 18 h of incubation, noningested RBCs were removed by extensive washing with culture medium. As a control, macrophages culture were treated with unloaded RBCs, i.e. RBCs submitted to the same procedure including transient lysis and successive modifications to increase macrophage recognition, but without addition of ACVpPMPA (see below). Human macrophage cultures receiving either ACVpPMPA loaded or unloaded RBCs were then infected for 2 h with HIV-$1_{Ba\text{-}L}$ [300 $CCID_{50}$ (50% cell 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) 1.0 μM ACVpPMPA given overnight (as for RBCs) or maintained throughout the entire experiment; (2) 1.0 μM PMEA given overnight (as for RBCs); (3) 1.0 μM PMPA given overnight (as for RBCs), and (4) unloaded RBCs prepared and cultured with macrophages exactly as the loaded ones. Cell cultures were then maintained at 37°C and 5% $CO_2$ for 5 weeks. Fresh medium was replaced every 5–6 days and virus production was assessed in the supernatants, with a commercially available enzyme linked immuno-

sorbent assay (ELISA) kit able to detect HIV $p24^{gag}$.

### 2.10. Assays for anti-HSV-1 activity

For the assays of antiviral activity on infected macrophages, unloaded RBCs and ACVpPMPA-loaded RBCs were added overnight before infection at a ratio of 50 RBCs per macrophage. Noningested erythrocytes were removed by extensive washing with culture medium. Human macrophage cultures receiving ACVpPMPA-loaded RBCs and unloaded RBCs were then infected for 2 h with HSV-1 [3 pfu (plaque-forming units)/cell]. After incubation with the virus, cell cultures were extensively washed, and fresh medium was added. As a control, 15 μM ACVpPMPA was added for the same time as the RBCs. The inhibitory effect of the compounds on the replication of HSV-1 was evaluated 48 h after infection by determining the infectious virus in the supernatant by plaque assay in Vero cells (Table 2).

In all the antiviral assays, loaded and unloaded erythrocytes were used within 24 h of preparation.

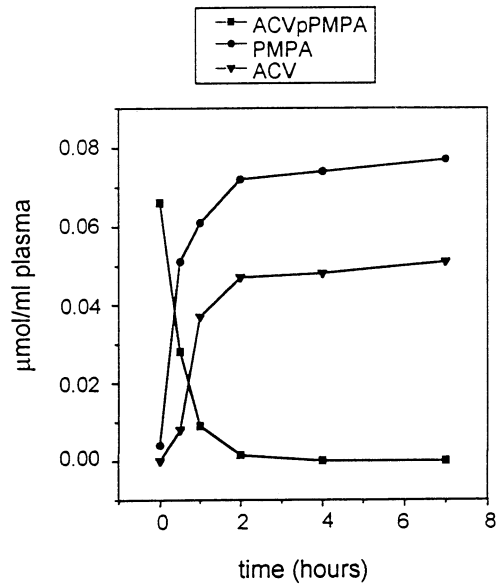



Fig. 1. Stability of ACVpPMPA in plasma. ACVpPMPA (0.08 μmol/ml plasma) was incubated for 7 h at 37°C. Perchloric acid extracts were prepared at different incubation times and analyzed by HPLC, as described in Section 2.

## 3. Results

### 3.1. Metabolism of ACVpPMPA

To evaluate the metabolism of ACVpPMPA in human plasma, 0.08 μmol of free drug/ml of plasma was incubated for 7 h at 37°C. At different incubation times (0, 0.5, 1, 2, 4, and 7 h), aliquots were collected and processed for HPLC analyses. The results obtained (Fig. 1) showed that ACVpPMPA was almost completely converted to ACV and PMPA already after 1 h of incubation.

When ACVpPMPA was incubated in erythrocyte lysate in absence of ATP, it was converted to ACV-MP and PMPA (Fig. 2); ACV-MP was rapidly converted to ACV. However, in presence of 1 mM ATP, a significant inhibition of this conversion was obtained; in this condition 65% of ACVpPMPA was still present after 7 h of incubation at 37°C.

To evaluate the metabolism of ACVpPMPA in intact erythrocytes, human erythrocytes were loaded with ACVpPMPA to a final concentration of 0.58 mM. The encapsulation of the heterodimer, by the procedure of hypotonic dialysis, isotonic resealing and reannealing, did not result in any appreciable alterations in erythrocyte mor-

Table 2
Inhibition of HSV-1 replication by ACVpPMPA-loaded RBCs or free drugs[a]

| Treatments | % HSV inhibition |
|---|---|
| None | 0 |
| Unloaded | 46 |
| ACVpPMPA-loaded RBCs | 69* |
| ACVpPMPA 15 μM (overnight) | 36 |

[a] Human macrophages were cultured for 10 days before treatment with the drugs. Cells were infected for 2 h with 3 pfu/cell of HSV-1. The inhibitory effect of the compounds was evaluated 48 h after infection by a plaque assay in Vero cells. The values are the mean of three experiments, each performed in duplicate. Standard errors were less than 10%.

* $P$ value $< 0.05$ versus unloaded ($T$ test). The titer of HSV-1 production in the supernatants of infected untreated macrophages ranged from 0.5 to $1.0 \times 10^6$ pfu/ml.

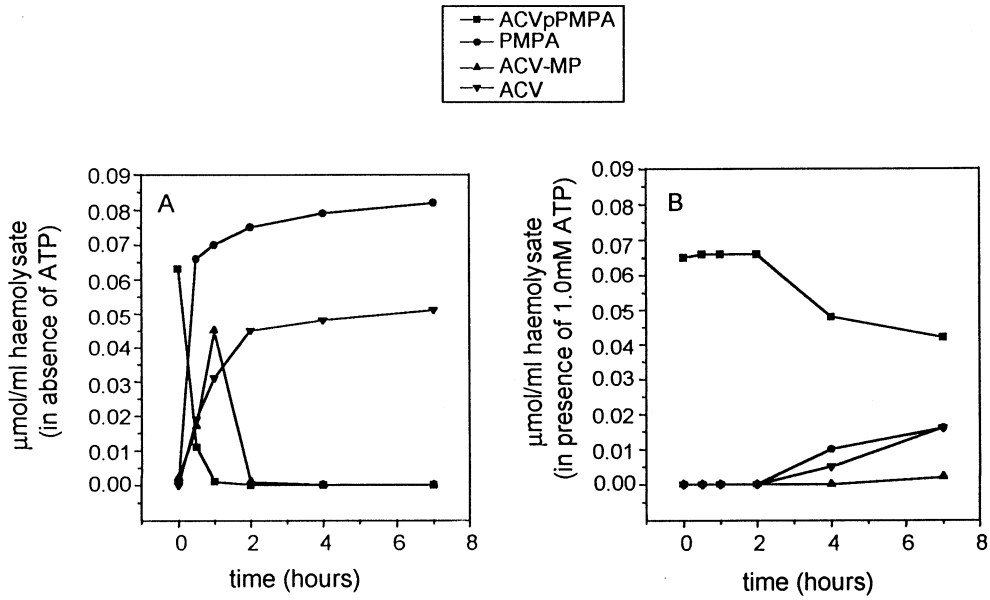



Fig. 2. Stability of ACVpPMPA in human erythrocyte lysates. ACVpPMPA (0.08 μmol/ml) was incubated with the lysates at 37°C for 7 h in the absence (A) or presence (B) of 1 mM ATP. Aliquots of the incubation mixture were extracted with perchloric acid and analyzed by HPLC, as described in Section 2.

phology and metabolism (not shown). ACVpPMPA-loaded RBCs were then incubated up to 10 days at 37°C in RPMI 1640 medium under sterile conditions. At different times of incubation, the presence of ACVpPMPA and its metabolites was evaluated (Fig. 3). The results obtained show that the intracellular ACVpPMPA decreases down to about 2% of the starting level after 10 days of incubation. The decrease of the heterodimer in the RBCs was paralleled by the stoichiometric production of ACV and PMPA released in the culture medium. As suggested by the results on ACVpPMPA stability in erythrocyte lysates in the presence of 1 mM ATP (see above), the heterodimer is sufficiently stable in RBCs to allow the use of ACVpPMPA in erythrocytes as a drug targeting system.

### *3.2. Antiviral activity of ACVpPMPA-loaded RBCs*

To evaluate the anti-HIV activity of ACVpPMPA-loaded RBCs, human macrophages were treated with ACVpPMPA (approx. 1.0 mM) inside RBCs or unloaded RBCs. The production of p24 was determined at different days post infection (Table 1). As control, the free drugs 1.0 μM ACVpPMPA, 1.0 μM PMPA and 1.0 μM PMEA were used. The results show that ACVpPMPA-loaded RBCs were able to completely protect macrophages against HIV-1 infection.

To evaluate the anti-HSV-1 activity of ACVpPMPA-loaded RBCs, human macrophages were treated with ACVpPMPA-loaded RBCs (approx. 1.0 mM inside RBCs). After extensive washing, macrophages were infected for 2 h with HSV-1 (3 pfu/cell). As a control, 15 μM ACVpPMPA added for the same time as RBCs was used. The inhibitory effect of the compound was evaluated 48 h after infection by a plaque assay in Vero cells (Table 2). The results obtained show that ACVpPMPA-loaded RBCs were able to inhibit HSV-1 replication by almost 70%, compared to $< 50\%$ inhibition induced by unloaded RBCs. Addition of the free drug in the medium reduced HSV-1 production by 36%.

It is worth noting that the administration of unloaded RBC was able to inhibit the replication of HIV-1 and HSV-1 by about 30–50%. Similar results were previously reported and discussed by Piedimonte et al. (1992) and Rossi et al. (1998). The inhibitory effects of unloaded RBC depend on the experimental condition used (including donor variability, viral load, RBC/macrophages ratio, etc.) and were also observed with latex beads. This antiviral status may be due to an activation of macrophage functions and/or the production of certain cytokines (Borghi et al., 1995). In any case, the inhibition observed by administering drug-loaded RBC was always significantly higher than that observed with unloaded RBC administration (Table 1 and Table 2).

When 1.0 mM ACVpPMPA-loaded RBCs was added to control (non-infected) macrophages, no cytopathic effect was observed (data not shown).

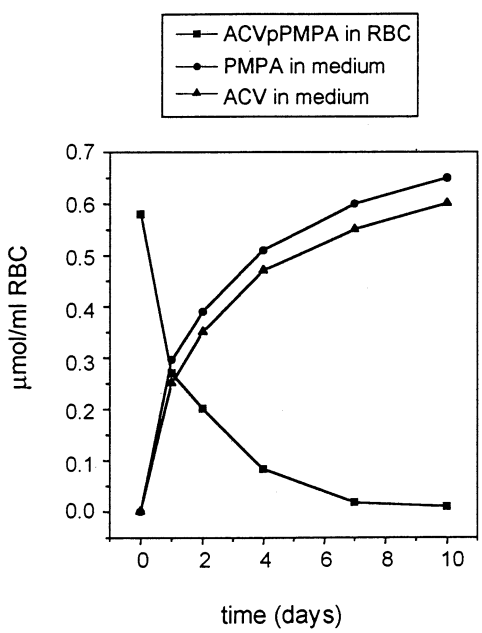



Fig. 3. Metabolism of ACVpPMPA in intact erythrocytes. ACVpPMPA was incapsulated in human erythrocytes by a procedure of hypotonic dialysis and isotonic resealing to a final concentration of 0.58 mM. These cells were then incubated at 37°C following the conditions described in Section 2.

## 4. Discussion

The development of new therapeutic systems against HIV and HSV infections, based on nucleoside analogues and designed to achieve both antiretroviral and antiherpes activity in specific cell types like monocytes/macrophages, requires a combination of different features. (i) Pharmacologically active triphosphorylated nucleoside analogues can display unacceptable cell toxicity due to inhibition of enzyme targets in the host cell other than viral enzymes. Therefore PMPApp and ACV-TP and similar compounds cannot be safely encapsulated as such into RBCs for targeting to infected cells. (ii) Accordingly, triphosphorylated nucleoside analogues should be formed in the desired cell type at low concentrations such as to inhibit selectively viral reverse transcriptase and/or viral DNA polymerase; therefore, metabolically suitable prodrugs that can be activated by endogenous enzymes are necessary. (iii) Targeting of such prodrugs to macrophages, where their bioconversion is required, can be greatly favored by exploiting the natural property of erythrophagocytosis. (iv) The selected prodrugs should be stable enough in the carrier RBCs to be metabolically converted to the nucleoside triphosphate analogues only within infected macrophages. Furthermore, to favor compliance, single drugs with multiple activities should be preferred instead of multiple drugs active against each pathogen.

All the above requirements seem to be fulfilled by ACVpPMPA loaded into RBCs. Although a dinucleotide pyrophosphorylase in human red cells is present, its activity is rather low, probably because of its inhibition by ATP, as shown in Fig. 2. On the other hand, this heterodinucleotide, while being stable in the loaded red cells, disappears in the macrophages within time intervals that exceed those required to complete erythrophagocytosis (data not shown). This property is consistent with metabolism of ACVpPMPA largely occurring in macrophages. That ACVpPMPA behaves as a prodrug in macrophages is demonstrated by the anti-HIV and anti-HSV-1 activity of this compound in HIV-$1_{Ba-L}$- and HSV-1-infected macrophages.

It is worth noting that protection of macrophages from HIV-1 infection is complete upon drug-loaded RBCs phagocytosis and persists up to the end of the experiment (34 days) at both RBC/macrophage ratios investigated (Table 1). The same drug given free in solution and maintained throughout the entire experiment reduced p24 production from 70 to 80%. This limited activity is likely due to extracellular conversion of the ACVpPMPA dimer to PMPA, which then enters the macrophages. In fact, ACVpPMPA diffusion through the cell membrane is unlikely, as shown by the retention of the drug when it is encapsulated into RBCs (i.e. the ACVpPMPA dimer has never been found intact in the RBC culture medium during the stability studies reported above). PMPA or PMEA maintained in culture for only 18 h were of very limited efficacy.

The results show that the antiviral effect of the dimer is substantially different in M/M infected by HIV, as compared to M/M infected by HSV-1. These apparently surprising results, can be explained by the different virus lifecycles. Indeed, HIV-RT works only in one step at the beginning of virus replication cycle. After proviral integration, RT is no longer necessary for virus production. On the contrary, HSV-DNA polymerase works during the whole HSV lifecycle, since production of new viral DNA genome is continuously required. Thus, it is conceivable that the effect of the dimer on HIV-RT can be amplified by the limited rate of activity of this enzyme in infected M/M (O'Brien et al., 1994), while, by contrast, the effect of the same dimer can be reduced by the continuous activity of the HSV-DNA polymerase. Further studies are required to achieve final proof of this issue.

Another point of interest is the finding of a consistent effect of unloaded RBC upon the replication of both HIV-1 and HSV-1 in macrophages (ranging from 30 to 50% depending on the experimental condition used) as already discussed (see Section 3). However, administration of ACVpPMPA-loaded RBC completely inhibited HIV-1 replication in macrophages.

In conclusion, ACVpPMPA is a new heterodimer with adequate stability in RBCs and protective activity in human macrophages against HIV-l and HSV infections. This dimer maintains the high antiretroviral activity of PMPA and at the same time compensates for the lack of anti-HSV activity of PMPA. The approach used further suggests that nucleoside analogues, if present only for limited periods of time (i.e. 18 h in our experiment), are of limited efficacy in protecting the macrophages against HIV-l and HSV infections. Thus, careful pharmacokinetic studies are needed to define the optimal drug dosage able to maintain in vivo therapeutic concentrations for longer periods of time. When encapsulated into the appropriate carriers, these nucleoside/nucleotide analogues are able, upon single pulsed administration, to provide improved protection.

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