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# Potent Inhibition of Human Immunodeficiency Virus and Herpes Simplex Virus Type 1 by 9-(2-Phosphonylmethoxyethyl)adenine in Primary Macrophages Is Determined by Drug Metabolism, Nucleotide Pools, and Cytokines

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## SUMMARY

The efficacy of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) against the replication of human immunodeficiency virus (HIV) and herpes simplex virus type 1 (HSV-1) and its cellular metabolism were investigated in human primary macrophages from seronegative donors. PMEA potently inhibited the replication of both HIV and HSV-1 in macrophages, with similar $EC_{50}$ values (0.025 and 0.032 $\mu$M, respectively), whereas the $EC_{50}$ values of PMEA in lymphocytic C8166 cells and fibroblastoid Vero cells were 150–200-fold higher (3.5 and 7.9 $\mu$M, respectively). Granulocyte/macrophage colony-stimulating factor and macrophage colony-stimulating factor, two cytokine enhancers of the replication of HIV (and HSV-1), decreased the activity of PMEA against both viruses, yet $EC_{50}$ values were still lower than in lymphocytes and fibroblasts. Thus, the selectivity index of PMEA in macrophages was >2 orders of magnitude higher than that in lymphocytes and fibroblasts and still > 1 log higher under conditions of enhancement of virus replication in macrophages. The intracellular levels of 2′-deoxyadenosine-5′-triphosphate, the natural competitor of PMEA-diphosphate at the level of viral DNA polymerase (either RNA or DNA dependent), were 5–12-fold lower in macrophages than in other cells. Furthermore, intracellular concentrations of PMEA-diphosphate (the active metabolite of PMEA) were unusually much higher in macrophages (with or without cytokines) than in lymphocytes and fibroblasts. Consequently, the ratio of PMEA-diphosphate to 2′-deoxyadenosine-5′-triphosphate in monocytes/macrophages was ~2 orders of magnitude higher in macrophages than in the other cells and correlated closely with the pronounced antiviral potency of PMEA. The dual potent activity of PMEA against HIV and HSV-1 stresses the importance of clinical trials to assess the role of this drug in the therapy of HIV-related disease.

PMEA, a prototype compound of the class of acyclic nucleoside phosphonates, is a potent inhibitor of both retroviruses and herpesviruses (1–3). Its antiviral effect is related to the direct inhibition of the DNA polymerase of the virus (either RNA or DNA dependent) and to DNA chain termination (4). Due to its efficacy, limited toxicity, and favorable pharmacokinetics, a prodrug of PMEA, bispivaloyloxymethyl-PMEA, is under evaluation in patients infected with HIV (5).

M/M are a main target of HIV in patients (6, 7); their infection is quite relevant in the progression of the disease and in the development of AIDS dementia complex (8, 9). M/M can also be easily infected by various herpesviruses, including HSV-1 and -2, herpes zoster virus, cytomegalovirus, human herpesvirus type 6, and pseudorabies (10–15). In addition, M/M can become a natural reservoir of latent HSV-1 in lungs, CNS, and other tissues. HSV-1/2 infection is a common feature in patients with HIV. There is clear evidence that HSV may activate or increase the replication of HIV and accelerate the progression of the disease. Furthermore, recent evidence shows that HIV and HSV-1 are able to mutually activate their replication during coinfection of tissue M/M of HIV-infected patients (16).

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**ABBREVIATIONS:** PMEA, 9-(2-phosphonylmethoxyethyl)adenine; HIV, human immunodeficiency virus; HSV-1, herpes simplex virus type 1; $CC_{50}$, 50% cytotoxic concentration; GM-CSF, granulocyte/macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; dATP, 2′-deoxyadenosine-5′-triphosphate; PMEApp, PMEA-diphosphate; CNS, central nervous system; M/M, monocytes/macrophages; $TCID_{50}$, 50% tissue culture infectious dose; NNRTI, non-nucleoside reverse transcriptase inhibitor; ddN, 2′,3′-dideoxynucleosides; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; AIDS, acquired immune deficiency syndrome.

In this context, PMEA may be of particular relevance in the therapy of infections sustained by HIV and/or HSV-1. Therefore, we investigated the effect of PMEA on HIV and HSV-1 replication in M/M and the factors potentially affecting its antiviral activity, such as the metabolism of PMEA and the intracellular levels of its endogenous counterpart, dATP.

## Materials and Methods

### Macrophages

Peripheral blood mononuclear cells were obtained from the blood of healthy seronegative donors by separation over Ficoll-Hypaque gradient. Pure macrophages were obtained following two different procedures, both described in detail previously (17). Briefly, adherent mature M/M were obtained by culturing mononuclear cells for 5 days in 48-well plastic plates (Costar, Cambridge, MA) in RPMI 1640 (GIBCO, Grand Island, NY) with the addition of 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 20% heat-inactivated, mycoplasma- and endotoxin-free fetal calf serum (the last from Hyclone, Logan, UT). Unless otherwise specified, this medium was used in all experiments. On the fifth day of culture, nonadherent cells were carefully removed by repeated washings. The great majority (>95%) of cells attached to the wells are differentiated M/M, as assessed by various methods (i.e., immunostaining with CD14, Giemsa and nonspecific esterase staining, and morphology).

In selected experiments, M/M were cultured with cytokines. In these cases, M/M were obtained by countercurrent centrifugal elutriation as described previously (18). M/M that were >95% pure were then cultured for 5 days in the presence or absence of 100 units/ml GM-CSF or 1000 units/ml M-CSF (both kindly donated by Dr. Clive Wood, Genetics Institute, Cambridge, MA) before infection. The characteristics, details, and advantages of these procedures are described in great detail elsewhere (17).

### Lymphocytes and Fibroblasts

A CD4-positive lymphocytic cell line, C8166, and an African green monkey fibroblastoid kidney cell line, VERO, were used for the comparative experiments of infection. These cell lines are highly sensitive to the cytopathic effect of HIV and HSV-1, respectively.

### Viruses

In the case of HIV-1 (referred to as HIV), two strains were used: a monocytotropic strain, HTLV($III_{BaL}$) (referred to as HIV-BaL), and a lymphocytotropic strain, HTLV($III_B$) (referred to as HIV-IIIB). HIV-BaL and HIV-IIIB were expanded and titrated in M/M and lymphocytes, respectively. For HSV-1, a dual tropic laboratory-adapted strain, named EB, was used in all experiments.

### Drugs

PMEA (>99% pure by HPLC analysis; kindly provided by Dr. N. Bishofsberger, Gilead Sciences, Foster City, CA) was dissolved in phosphate-buffered saline at a high concentration (1 mg/ml) and stored at 4° until used.

### Cell Infection and Virus Detection

**Infection with HIV.** M/M (with or without cytokines) were exposed to various concentrations of PMEA and 20 min later challenged with 300 $TCID_{50}$ of HIV-BaL. After a 2-hr incubation, M/M were carefully washed with warm medium to remove excess virus and then cultured in a humidified chamber with 5% $CO_2$ in the same medium containing the same concentrations of PMEA (and cytokines, when requested) as before. M/M were fed every 5–6 days with fresh medium. PMEA and cytokines were added at each change of medium to maintain the same concentrations. Virus production was assessed in the supernatants, usually 14 days after virus challenge, with a commercially available ELISA kit that could be used to detect HIV gag p24 (Abbott Labs, Pomezia, Italy).

In selected experiments, the PMEA-mediated inhibition of HIV production was also assessed by virus titration, using primary M/M as target cells (data not shown), as described previously (19, 20). The results obtained with this method were very similar with those achieved with the ELISA assay. Similarly, results obtained 21 days after virus challenge were superimposable with those achieved at day 14. Additional experiments were devoted to assess whether the antiviral activity of PMEA could be affected by varying the multiplicity of infection (i.e., $TCID_{50}$). As expected, the results (data not shown) consistently showed that the antiviral activity of PMEA linearly decreases with increase in the $TCID_{50}$ of HIV (or HSV-1) Thus, no increased interference by noninfectious particles was found, at least not with the $TCID_{50}$ values in the range of those used in our experiments.

Each experiment was run with primary M/M from a single blood donor, and minor variations have been found among different experiments. However, differences among triplicates (or quadruplicates) of the same experiment were <5% of the average values.

C8166 lymphocytic cell cultures were treated with PMEA and infected under the same experimental conditions, except that the lymphocytotropic strain HIV-IIIB was used. Virus production was assessed 5–6 days after virus challenge by visual inspection of the HIV-related cytopathic effect and by titration of the virus released in the supernatants.

**Infection with HSV.** Virus infection of M/M with HSV-EB (multiplicity of infection of 3 plaque-forming units/cell) was similar to that performed for HIV. Because M/M are sensitive to the cytopathic effect of HSV within 3–4 days from virus challenge, the assessment of virus production was performed in the supernatants of M/M by plaque formation assay (by using Vero cells as virus target) and by a commercially available ELISA kit able to recognize HSV antigens (Murex Diagnostics, Dartford, UK), starting at 24 hr after virus challenge. Data given refer to virus production 48 hr after virus challenge. Results at 24, 72, and 96 hr after virus challenge were superimposable with those obtained at 48 hr.

Subconfluent VERO cells were infected and treated with PMEA using the same procedure (including the same virus strain, HSV-1 EB, at the same amount of plaque-forming units/ml) performed for M/M. Also in this case, virus production was assessed by ELISA and by plaque formation assay at the same time points as for M/M.

The selectivity index of PMEA was established by the ratio of $CC_{50}$ to $EC_{50}$.

### Metabolism of PMEA

[2,8-$^3$H]PMEA (17 mCi/mol) was purchased from Moravek Biochemicals (Brea, CA). Approximately $5 \times 10^6$ cells (macrophages with/without cytokines, C8166, and VERO) were cultured for 24 hr in the presence of 0.4 μM (1 μCi/ml) [2,8-$^3$H]PMEA. Cells were then carefully detached (with the exception of C8166 cells, which grow in suspension), counted, and centrifuged at least three times in a large volume of cold phosphate-buffered saline to remove the excess of radiolabeled PMEA. Cell pellets were then dried, exposed to 400 μl of 60% methanol, and centrifuged for 10 min at 13,000 rpm, and the supernatant was injected in HPLC. Chromatography was performed on a Partisil-SAX-10-radial compression column.

**Assessment of dATP levels.** Separation of dATP was performed according to a modification of an HPLC method for the determination of several adenine nucleotide derivatives (21). Briefly, macrophages with/without cytokines, VERO, and C8166 were cultured in the same conditions as for infection. After detachment from plastic (requested for M/M and VERO), cells were counted and washed twice in cold saline. Pellets were carefully dried and deproteinized by 1.2 M ice-cold $HClO_4$. Acid cell extracts were centrifuged at 13,000 rpm for 10 min at 4° and subsequently neutralized by the addition of 5 M $K_2CO_3$. After centrifugation at 13,000 rpm for 3 min at 4°, samples were filtered through a 0.45-μm HV-Millipore filter and then loaded

(200 μl) for HPLC analysis onto the column (Alltima C-18; 250 mm × 4.6 mm, 5-μm particle size; Alltech Associates, Deerfield, IL). The column was equilibrated with a mobile phase (buffer A) containing 10 mM tetrabutylammonium hydroxide as the pairing reagent, 10 mM $KH_2PO_4$, and 0.25% methanol. The addition of HCl was necessary to adjust pH of buffer A to 7.00. A step gradient was obtained with a second buffer (B) containing 2.8 mM tetrabutylammonium hydroxide, 100 mM $KH_2PO_4$, and 30% methanol. HCl was added to adjust pH of buffer B to 5.50. Both buffers were prepared daily. Gradient was formed as follows: 22 min with 100% buffer A, 2 min with ≤58% buffer A, 11 min with ≤55% buffer A, and 20 min with ≤0% buffer A. The flow rate throughout chromatographic runs was 1.5 ml/min, and the temperature was maintained at 21° by thermostating the column with water-jacketed glassware.

## Results

Fig. 1 reports the comparative anti-HIV activity of PMEA in M/M and lymphocytes. PMEA treatment of M/M resulted in complete inhibition of HIV replication at concentrations of 0.3 μM and higher, with an $EC_{50}$ of 0.025 μM. In contrast, 92% inhibition of HIV replication was obtained in lymphocytes at PMEA concentrations of ≤8 μM, with $EC_{50}$ of 3.5 μM. This antiviral activity of PMEA is in agreement with previously reported inhibition values in peripheral blood lymphocytes (22) and very similar with that achieved in MT4 cell cultures, another $CD4^+$ lymphocytic cell line with characteristics similar to C8166 (data not shown). Thus, under our experimental conditions, PMEA proved to be ~140-fold more potent as an HIV inhibitor in M/M than in lymphocytes. To assess whether such greater antiviral activity of PMEA in M/M would be related to the virus strain and not to the cell type, we tested the activity of PMEA in M/M infected with the strain HIV-IIIB; as expected (18), M/M infection was very limited, with poor virus production overtime, yet the inhibition of virus replication induced by PMEA in these M/M ($EC_{50}$ = 0.035 μM) was quite similar to that induced in M/M infected with the monocytotropic strain HIV-BaL (data not shown).

The activity of PMEA against HSV-1 is reported in Fig. 2. Complete inhibition of HSV-1 replication in M/M was achieved at PMEA concentrations of ~1.6 μM, with an $EC_{50}$ of 0.032 μM. In agreement with data of the literature, the $EC_{50}$ of PMEA in VERO fibroblastoid cells was 7.9 μM, whereas complete inhibition could only be achieved at PMEA concentrations that exceeded 40 μM. The anti-HSV-1 activity of PMEA in M/M, based on $EC_{50}$ values, is ~240-fold higher than that found in fibroblastoid cells. Thus, the results show that PMEA (against either HIV or HSV-1) is ≥2 orders of magnitude more active in M/M than in replicating cells such as lymphocytes and fibroblasts and suggest that such enhanced antiviral effect is related to the characteristics of host cells (i.e., M/M) rather than to the type of virus.

In the following set of experiments, we assessed whether the efficacy of PMEA in M/M is affected by GM-CSF and M-CSF, two cytokines able to potently enhance the replication of HIV (23, 24). For these experiments, elutriated M/M were used. Both cytokines enhanced the replication of HIV, GM-CSF to a greater extent than M-CSF (data not shown). In agreement with this phenomenon, a substantial decrease in the anti-HIV activity of PMEA was found. Indeed, as shown in Fig. 3, 1 μM PMEA induced complete inhibition of the replication of HIV, with an $EC_{50}$ value of 0.022 μM (quite similar to the $EC_{50}$ value of PMEA in 5-day adherent M/M; see above). However, concentrations of 1 μM PMEA were unable to induce complete inhibition of HIV replication in elutriated M/M treated with GM-CSF or M-CSF. The $EC_{50}$ values were also increased, being 0.52 and 0.06 μM in the presence of GM-CSF and M-CSF, respectively. Despite this cytokine-mediated increase in $EC_{50}$, the antiviral efficacy of PMEA in M/M was still markedly greater than that shown in lymphocytes (Table 1 and Fig. 1). Erythropoietin, a cytokine active against different bone marrow-derived cells, was unable to affect the anti-HIV efficacy of PMEA in M/M (Fig. 3) because it is unable to interfere with virus replication in these cells (24). This result supports the hypothesis that the

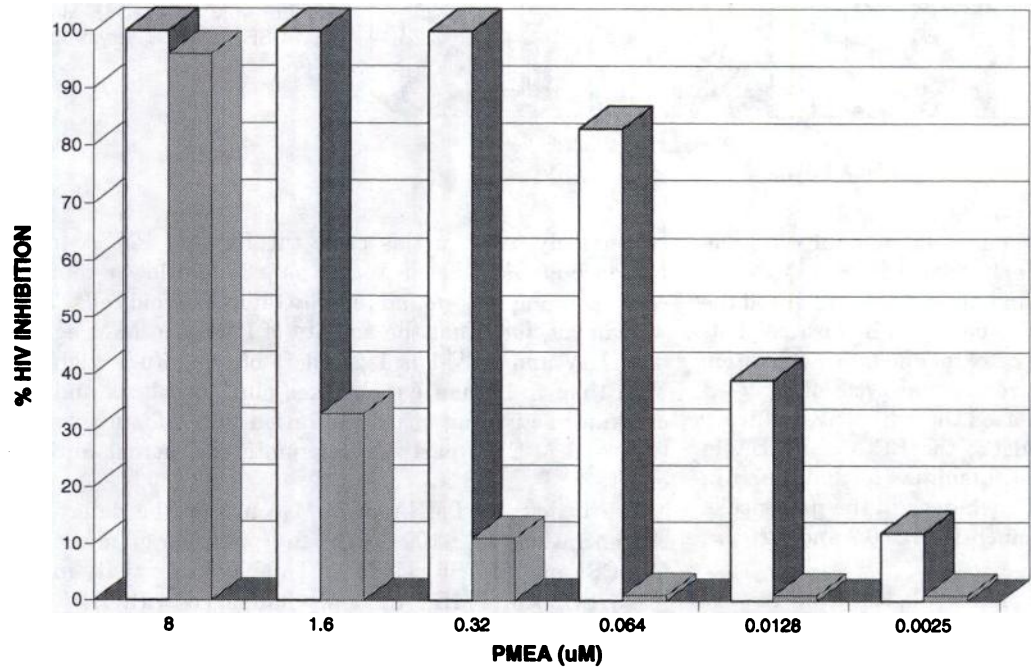


**Fig. 1.** Inhibition by PMEA of HIV replication in macrophages and lymphocytes. Results represent the average of five experiments, each run in triplicate (for M/M) or in duplicate (for C8166 lymphocytic cells). Experiments were performed as described in Materials and Methods. Virus production was assessed at day 14 (for M/M) and at day 5 (for C8166). PMEA-directed virus inhibition in M/M at day 14 was superimposable with that observed at day 21. □, 5-day adherent macrophages; ▨, C8166 lymphocytic cells.

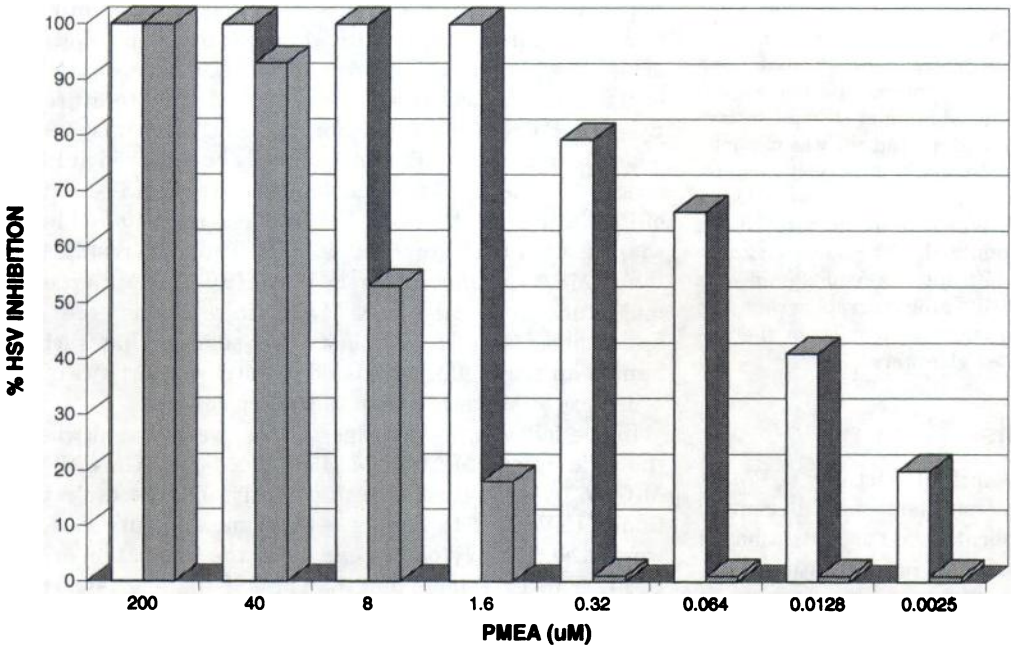


**Fig. 2.** Inhibition of HSV-1 replication in macrophages and fibroblasts by PMEA. Results represent the average of six experiments, each run in triplicate (for M/M) or in duplicate (for Vero fibroblastoid cells). Virus production was assessed at day 2 for both cell types. Percentage of virus inhibition obtained at this time point was superimposable on that achieved at days 1, 3, and 4. □, macrophages; ▨, fibroblasts.

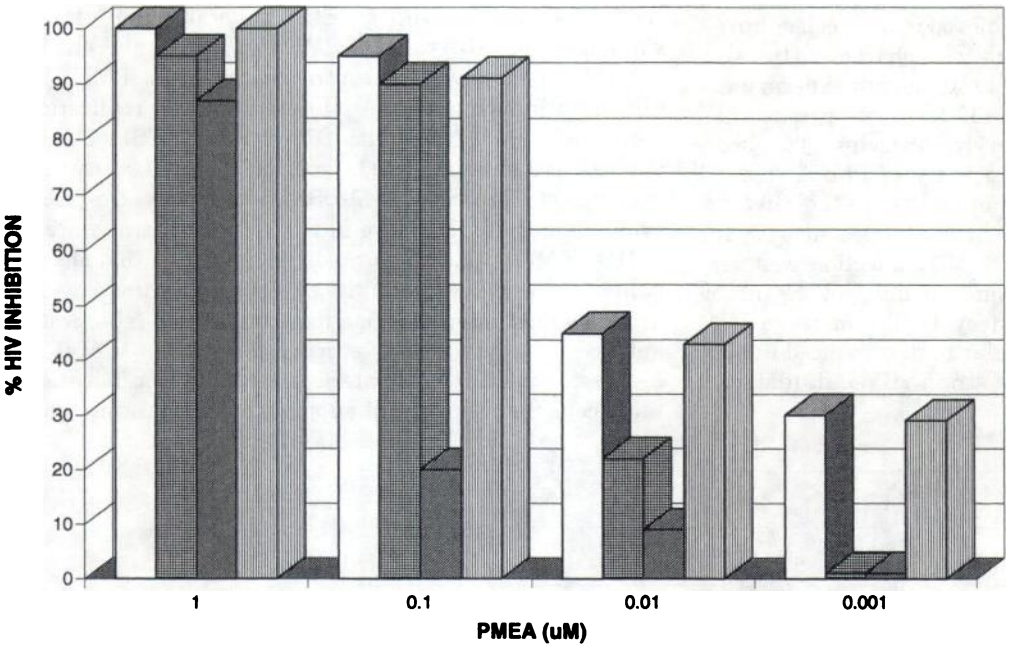


**Fig. 3.** Anti-HIV activity of PMEA in M/M exposed to various cytokines. Results represent the average of four experiments, all run at least in triplicate. Virus production was assessed at day 14 after virus challenge by ELISA HIV-gag p24 assay. Virus production in M/M not treated with PMEA was as follows: control M/M, 24,000 pg/ml; M-CSF-treated M/M, 57,000 pg/ml; GM-CSF-treated M/M, 73,000 pg/ml; M/M treated with erythropoietin (*Epo*), 27,500 pg/ml. □, control M/M; ■, M-CSF M/M; ▨, GM-CSF M/M; ■, erythropoietin M/M.

decreased efficacy of PMEA is in part related to the cytokine-related enhancement of virus replication.

Interestingly, both GM-CSF and M-CSF also enhanced the replication of HSV-1 in M/M compared with controls. This effect seems to be related to the cytokine-mediated activation of more than one step of the replicative cycle of HSV-1.[1] GM-CSF and M-CSF also decreased the anti-HSV-1 efficacy of PMEA in M/M (Fig. 4). Indeed, the $EC_{50}$ of PMEA in control elutriated M/M is 0.04 μM (similar to that found in 5-day adherent M/M, see Fig. 2), whereas in the presence of GM-CSF or M-CSF, the $EC_{50}$ is increased to 0.3 and 0.25 μM, respectively. Also in this case, the $EC_{50}$ of PMEA in the presence of GM-CSF or M-CSF is ≥25-fold lower than the corresponding $EC_{50}$ found in VERO fibroblastoid cells. Thus, we can conclude that the activity of PMEA in M/M against both HIV and HSV-1 is 1–2 orders of magnitude higher in M/M than in human lymphocytes and fibroblasts and that cytokines activating virus replication in M/M, such as GM-CSF and M-CSF, partially attenuate this potent antiviral effect.

The toxicity of PMEA in M/M could not be detected at concentrations of ≤100 μM in the presence or absence of GM-CSF and M-CSF (Table 1). Thus, the selectivity indices ($CC_{50}/EC_{50}$) of PMEA in M/M challenged with HIV were >4000, >192, and >1666 for control, GM-CSF-treated, and M-CSF-treated M/M, respectively. In M/M challenged with

[1] C. F. Perno, S. Gessani, S. Aquaro, L. Conti, E. Balestra, S. Panti, A. Cenci, F. Serra, N. Villani, C. D. Pesce, and R. Caliò. Activity of GM-CSF and M-CFS upon replication of HIV and other DNA- and RNA-viruses in primary macrophages. Manuscript in preparation.

TABLE 1

**Toxicity and selectivity index of PMEA in macrophages, lymphocytes, and fibroblasts**

Noninfected cells were cultured under the same conditions as for virus-infected cells. Toxicity was evaluated by the trypan blue dye exclusion method at the same time point at which virus production was assessed in infected cell cultures.

| | $EC_{50}$ | $CC_{50}$ | Selectivity index[a] |
|---|---|---|---|
| Cells infected by HIV | | $\mu M$ | |
| 5-Day adherent M/M | 0.025 | >100 | >4000 |
| Elutriated M/M | 0.022 | >100 | >4545 |
| Elutriated M/M + GM-CSF | 0.52 | >100 | >192 |
| Elutriated M/M + M-CSF | 0.06 | >100 | >1666 |
| Lymphocytic C8166 cells | 3.5 | 40 | 11 |
| Cells infected by HSV-1 | | | |
| 5-Day adherent M/M | 0.032 | >100 | >3125 |
| Elutriated M/M | 0.040 | >100 | >2500 |
| Elutriated M/M + GM-CSF | 0.3 | >100 | >333 |
| Elutriated M/M + M-CSF | 0.25 | >100 | >400 |
| Vero fibroblastoid cells | 7.9 | 120 | 15.2 |

[a] Ratio of $CC_{50}$ to $EC_{50}$.

HSV-1, the selectivity indices were >2500, >333, and >400 for control, GM-CSF-treated, and M-CSF-treated M/M, respectively. $CC_{50}$ values of PMEA in C8166 lymphocytes and VERO fibroblasts were 40 and 120 $\mu M$, respectively, with selectivity indices of 11 and 15, respectively. Thus, the selectivity index of PMEA against HIV and HSV-1 in M/M is ~2 logs higher than that found in human lymphocytes and fibroblasts. In the presence of GM-CSF and M-CSF, the selectivity index in M/M was decreased, although still ≥15 higher than that of replicating cells.

To determine the underlying mechanism(s) able to determine the antiviral effect of PMEA in M/M, we assessed both the intracellular levels of dATP (the natural endogenous competitor of PMEApp at the level of HIV reverse transcriptase and HSV-1/DNA polymerase) and the intracellular concentrations of PMEA and its metabolites. The levels of dATP were substantially lower in M/M than in replicating cells (Table 2). Indeed, dATP in 5-day adherent M/M is ~10- and ~25-fold lower than in lymphocytes and fibroblasts, respectively. Table 3 shows the levels of dATP in elutriated M/M treated with cytokines. Both GM-CSF and M-CSF increase ~2-fold the dATP levels in M/M compared with elutriated controls (not treated with cytokines). This is not surprising because GM-CSF and M-CSF activate DNA metabolism in M/M and may induce a few cycles of cell replication. However, even under these enhancing conditions, dATP is 2–5-fold lower in M/M treated with GM-CSF/M-CSF than in lymphocytes and fibroblasts, respectively.

When PMEApp was examined, we found levels in M/M 6–12-fold higher than those found in lymphocytes and fibroblasts (Table 2). The treatment of elutriated M/M with GM-CSF or M-CSF does not significantly modulate the rate of phosphorylation of PMEA because the concentrations of PMEApp are approximately the same as those found in control elutriated M/M (Table 3).

Thus, we could demonstrate that M/M have not only markedly decreased dATP levels but also substantially increased intracellular concentrations of the active metabolite of PMEA (PMEApp). The combined effect of these two nucleotide levels is that the ratio of PMEApp/dATP is >100-fold higher in M/M than in lymphocytes and fibroblasts (Tables 2 and 3). These ratios were somewhat decreased (~2.5-fold) in the presence of GM-CSF or M-CSF (and this can in part explain the loss of activity of PMEA in cytokine-exposed M/M) but are still ≥50-fold higher than those recorded in lymphocytes and fibroblasts. The increase in the PMEApp/dATP ratio in M/M versus this ratio in lymphocytes and fibroblasts significantly correlates with the more pronounced antiviral activity of PMEA in M/M compared with that found in lymphocytes and fibroblasts. Regarding the importance of this ratio, we obtained the data with two NNRTIs, R82913 and TSAO-$m^3T$, leading compounds of the classes of TIBO and TSAO, respectively. NNRTIs do not require phosphorylation to be active and do not show any competition with 2′-deoxynucleoside-triphosphates at the level of reverse transcriptase. Indeed, R82913 and TSAO-$m^3T$ showed a similar range of activity in M/M and lymphocytes, with $EC_{50}$ values of 0.05 and 0.03 $\mu M$ (for R82913), and 0.08 and 0.045 (for TSAO-$m^3T$), respectively (Table 4). This further supports the importance of both dATP and its ratio with PMEApp in the regulation of the activity of PMEA.

## Discussion

PMEA is one of the most promising compounds belonging to the structural class of acyclic nucleoside phosphonates, currently studied (in a prodrug formulation) in HIV-infected patients (5). One of the most interesting features of PMEA is its ability to inhibit both retroviruses (including HIV) and herpesviruses (including HSV-1) not only *in vitro* but also *in vivo* in animal models (25–28). This dual antiviral efficacy is of clinical relevance because HSV-1 is well recognized as an important cofactor in the progression of AIDS (29). HSV-1 (even if replication defective) is able to activate HIV replication by various mechanisms (i.e., *trans*-activation of the HIV long-terminal repeat, activation of the nuclear factor κB system, and so on) (30, 31). In addition, tissue M/M of some HIV-infected patients have been found coinfected by HIV and HSV-1 and able to produce atypical virus progenies with characteristics of both virus types (16). Inhibition of HSV-1 may thus be therapeutically relevant in HIV-infected patients. Indeed, *in vitro* evidence shows that acyclovir (an antiherpetic drug commonly used in the therapy of HSV infection) is able to inhibit the replication of HIV in lymphocytic cells harboring the HSV-1/thymidine kinase gene (32). Furthermore, a recent analysis of clinical data suggests a potential advantage of the addition of acyclovir to zidovudine in terms of rate of progression to AIDS and survival time (33). It should be mentioned that acyclovir has been reported to act synergistically with zidovudine against HIV-1 replication in ATH8 cells (34), a phenomenon that may be abscribed to a potential inhibition of HIV-1 reverse transcriptase by the triphosphate derivative of acyclovir (35).

The results that we present show that PMEA inhibits the replication of both HIV and HSV-1 in M/M at concentrations far below those active in lymphocytes. From the data, we cannot exclude in principle that the greater antiviral effect in M/M is related to a somewhat slower replicative pace of HIV in these cells (compared with lymphocytes). However, the following points should be considered. First, as we report, NNRTIs TSAO-$m^3T$ and R82913 show similar antiviral activity in M/M and lymphocytes. Second, most inhibitors of binding of HIV on target cells and of late stages of virus replication are less effective in M/M than in lymphocytes (the opposite of what occurs with PMEA and the other nucleoside

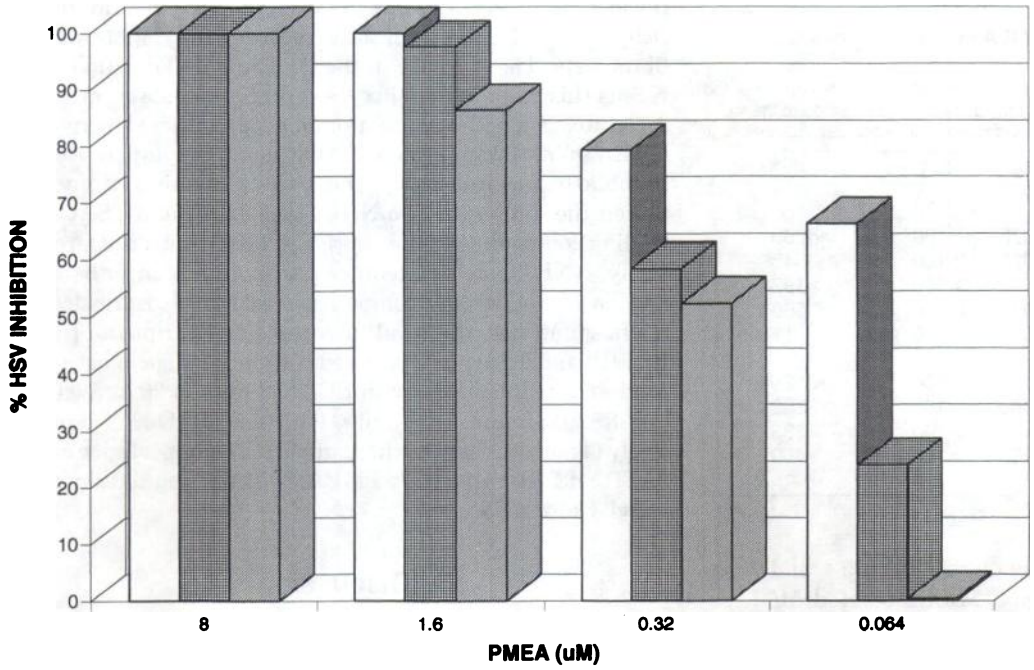


**Fig. 4.** Anti-HSV-1 activity of PMEA in M/M exposed to various cytokines. Data represent the average of six experiments, all run at least in triplicate. Virus production was assessed at day 2 after virus challenge by virus titration (plaque-forming units/well) and ELISA assay. Both methods gave comparable results. Also, similar results, in terms of PMEA-mediated inhibition of virus replication, were obtained at days 1, 3, and 4. □, control M/M; ■, M-CSF M/M; ▨, GM-CSF M/M.

TABLE 2

**Intracellular levels of PMEApp and dATP in various cell types**

Cells were exposed to 0.4 μM [2,8-$^3$H]PMEA (first column) or not exposed to labeled PMEA (second column) for 24 hr and then harvested, counted, lysed, and prepared for analysis by HPLC.

| Cell type | PMEApp | dATP | PMEApp/dATP |
|---|---|---|---|
| | *pmol/10$^9$ cells* | | |
| Macrophages[a] | 47 | 50 | 0.94 |
| Lymphocytes | 3.8 | 484 | 0.008 |
| Fibroblasts | 7.9 | 1280 | 0.006 |

[a] 5-Day adherent macrophages.

TABLE 3

**Intracellular levels of PMEApp and dATP in macrophages exposed to GM-CSF or M-CSF**

Cells were exposed to 0.4 μM [2,8-$^3$H]PMEA (first column) or not exposed to labeled PMEA (second column) for 24 hr and then harvested, counted, lysed, and prepared for analysis by HPLC.

| Cell type | PMEApp | dATP | PMEApp/dATP |
|---|---|---|---|
| | *pmol/10$^9$ cells* | | |
| Macrophages[a] | 91 | 120 | 0.75 |
| Macrophages + GM-CSF[a] | 89 | 260 | 0.34 |
| Macrophages + M-CSF[a] | 72 | 220 | 0.32 |

[a] Elutriated macrophages.

TABLE 4

**Antiviral activity of NNRTIs in lymphocytes and macrophages infected by HIV-1**

Noninfected cells were cultured under the same conditions as for virus-infected cells. Toxicity was evaluated by the trypan blue dye exclusion method at the same time point at which virus production was assessed in infected cell cultures.

| Compound | $EC_{50}$ | | $CC_{50}$ | |
|---|---|---|---|---|
| | C8166 HIV-IIIB | M/M HIV-BaL | C8166 | M/M |
| R-82913 | 0.03 | 0.05 | >10 | >10 |
| TSAO-m$^3$T | 0.045 | 0.08 | >10 | >10 |

analogue inhibitors of reverse transcriptase) (19, 36). Third, Bagnarelli *et al.*[2] recently found that the difference in the number of RNA copies produced by M/M (infected with HIV-BaL) and lymphocytes (infected by HIV-IIIB) is within 1 log, whereas PMEA is >2 logs more active in M/M. Thus, overall data suggest that there is no straightforward correlation between the replicative cycle of a virus (in lymphocytes and macrophages) and the comparative activity of antiviral drugs in these cells.

In the case of PMEA, it is conceivable that its increased antiviral activity in M/M is mainly related to the unique characteristics of M/M for the following reasons. (i) Both HIV and HSV-1 are equally inhibited by PMEA in M/M. (ii) Inhibitory activity of PMEA against both viruses correlates well with the increased levels of PMEApp in M/M and with a markedly higher ratio of PMEApp to dATP in M/M than that found in lymphocytes and fibroblasts. (iii) Cytokines enhanced the replication of both HIV and HSV-1 in M/M (but not in other cells) and concomitantly decreased the efficacy of PMEA against each virus. Nevertheless, even under these conditions, the activity of PMEA against HIV and HSV-1 in M/M is substantially higher than that found in replicating cells. This latter phenomenon is therapeutically relevant because M/M represent a very heterogeneous population of cells *in vivo* at different stages of maturation subjected to various stimuli (i.e., cytokines and others) able to modulate their metabolism (37). For this reason, therapeutic approaches must be effective against HIV replication in M/M, independent of their stage of maturation and grade of activation. Results that we report suggest that PMEA can be active against HIV in M/M when administered at doses able to reach active concentrations in the blood.

The potent antiviral activity of PMEA in M/M is in part accounted for by the low levels of the dATP pools present in M/M (compared with replicating cells). In the case of ddN such as didanosine and 2′,3′-dideoxyadenosine (but also zidovudine, zalcitabine, stavudine, and so on), the relatively

[2] P. Bagnarelli, A. Valenza, S. Menzo, R. Sampaolesi, P. E. Varaldo, L. Butini, M. Montroni, C. F. Perno, S. Aquaro, D. Mathez, J. Leibowitch, C. Balotta, and M. Clementi. Manuscript in preparation.

low levels of the dNTP pools in M/M compared with lymphocytes are sufficient to explain the increased antiviral efficacy of 2',3'-dideoxynucleosides in M/M, even in the presence of their reduced phosphorylation to the 5'-triphosphate moieties (38). Indeed, the ratio of ddN-triphosphates to dNTP (one of the most important factors affecting the inhibitory activity of these drugs) is markedly higher in M/M than in lymphocytes. In the case of PMEA, despite the limited phosphorylating ability of M/M, we found intracellular levels of PMEApp to be surprisingly higher than those found in T cells and fibroblasts. As a result of these two combined determinants, the ratio of PMEApp to dATP is far greater (≥2 orders of magnitude) in M/M than in the other cells tested. Consequently, PMEA is the most selective nucleoside analogue evaluated in our M/M system.

There are several hypotheses that might account for the increased phosphorylation of PMEA in M/M. The first is based on the phosphonate group linked to the acyclic alkyl side chain of the molecule, which makes PMEA able to bypass the first phosphorylating step required for nucleoside analogues (often a limiting factor in resting cells such as M/M). Another possibility is that the enzymes phosphorylating PMEA are more active in M/M than in other cells. Indeed, despite the similar mechanism of antiviral action, the metabolic pathway of activation of PMEA may be at least in part different from that of ddNs. Although recent investigations showed that both mitochondrial AMP kinase and 5-phosphoribosyl-1-pyrophosphate synthetase recognize PMEA as a substrate to be phosphorylated (39–41), the relatively low capacity of phosphorylation of PMEA by these enzymes, as reflected by their low $V_{max}$-to-$K_m$ ratios, suggests that other enzymatic pathways, unidentified but perhaps more represented in M/M than in other (replicating) cells, may play a major role. Finally, we cannot exclude that the transport of PMEA across the cellular membrane is more effective in M/M than in some other cells. Indeed, preliminary data suggest that the amount of authentic, unmetabolized PMEA is 6-fold lower in VERO cells than in M/M.[3] We may hypothesize that due to the markedly larger membrane surface of the M/M, more PMEA molecules may enter by endocytosis (42) (or by any other active transport across the cell membrane) than in the case of lymphocytes, fibroblasts, or other cell types (43). Further experiments are required to clarify this point.

It is worth noting that levels of both dATP and PMEApp are reported in this study as being ~2-fold higher in elutriated M/M than in 5-day adherent M/M. This is not surprising because elutriated M/M are less-differentiated cells; thus, DNA metabolism is somewhat more active. Nevertheless, the ratios of PMEApp to dATP in elutriated M/M are similar to those found in adherent M/M (0.94 and 0.75, respectively) (Tables 2 and 3); this strongly correlates to a similar $EC_{50}$ of PMEA (against both HIV and HSV-1) in these cell types.

Another point worth discussing is the potential antiviral efficacy of PMEA in M/M of the CNS. A real insight into the ability of PMEA to cross the blood-brain barrier in humans is not yet available. Data reported in this article show that PMEA is active in M/M (the major source of virus in the CNS) at concentrations far below (100–200-fold) those effective in lymphocytes (against HIV) and fibroblasts (against HSV-1). Thus, it is conceivable that PMEA may be active against HIV in M/M of the CNS even if the blood-brain barrier decreases the concentrations of PMEA in the cerebrospinal fluid to 10% (or even <10%) of those (active in lymphocytes) found in the blood. Only clinical investigation can provide a definitive answer to this issue. Nevertheless, it has recently been shown that PMEA administered systemically to lambs is able to interfere both with the replication of visna virus (an ovine retrovirus typically replicating in the M/M of the brain) and with the clinical outcome of its related disease (44). Furthermore, Naesens *et al.* demonstrated that systemic administration of [2,8-$^3$H]PMEA induces a limited but consistent presence of PMEA in the brain (45). Finally, PMEA administered systemically is able to inhibit the growth of an intracerebrally inoculated tumor induced by a murine retrovirus (26). Thus, these findings strongly suggest that PMEA may be effective against virus replication in the CNS, where HIV (as well as HSV-1) can induce lethal encephalitis.

In conclusion, overall the data suggest that PMEA has a potent effect in M/M against both HIV and HSV-1 and that its antiviral activity is related at least in part to its peculiar metabolism and relatively high ratios of PMEApp to dATP in these primary cells. The lack of detectable toxicity of PMEA at antivirally active concentrations, together with its pronounced antiviral effect, further supports the results of clinical studies directed toward assessing the potential role of PMEA (and its prodrugs) in the therapy of diseases induced not only by HIV but also by HSV-1.

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