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11. HIV-1_{MN} (11.8 μg total protein) was treated for 1 hour at 37°C with 25 μM test compound. Samples were centrifuged to pellet the virus from the drug. Virus pellets were resolved by SDS-PAGE and analyzed by immunoblot with monospecific rabbit antisera to the purified NC proteins. The extent of p7NC cross-linkage was dependent on the length of time that virus was incubated with the compound and on the concentration of compound used. Cross-linkage did not occur when the incubation was done at 0° to 4°C, but occurred at 20°C and more rapidly at 37°C. In addition, cross-linkage of the NC protein was observed with purified equine infectious anemia virus and Moloney murine leukemia virus, retroviruses distantly related to HIV-1. AZT was unable to induce cross-linkage of p7NC molecules in HIV-1 virions.
12. For quantitative viral inactivation studies, 100 μl of stock virus [HIV-1_{RF}, median tissue culture infectious dose per milliliter ($TCID_{50}$/ml) = 1.25×10^5] were incubated with each compound for 1 hour at 37°C. Samples were then serially diluted and 10 replicates of each dilution placed in culture with 5×10^3 CEM-SS cells. After 1 week the cultures were assayed for p24 content and scored positive (absorbance >2 SDs above background levels) or negative for infection, and a $TCID_{50}$ viral infectious titer was calculated for each concentration of each compound. The infectious titer of the HIV-1_{RF} stock ($TCID_{50}$/0.1 ml = 1.25×10^4) was reduced to 1370, 1111, 1923, 5556, and 1.25×10^4 $TCID_{50}$/0.1 ml after treatment for 1 hour with 25 μM DIBA-1, -2, -3, -4, and -5, respectively, and reduced to 540, 139, 233, 3225, and 1.25×10^4 $TCID_{50}$/0.1 ml after treatment for 1 hour with 100 μM DIBA-1, -2, -3, -4, and -5, respectively. The inactivation of HIV-1 by DIBA compounds was also time-dependent, and 100 μM DIBA-1 reduced viral titers from 1.25×10^4 $TCID_{50}$/0.1 ml to 3226, 1333, and 139 $TCID_{50}$/0.1 ml after 15, 30, and 60 min of treatment, respectively. As an internal control, quantitative measurements of viral inactivation determined that AZT did not directly inactivate HIV-1 infectivity (infectious titer remained at 1.25×10^4 $TCID_{50}$/0.1 ml after treatment with 10 μM AZT for 60 min at 37°C).
13. Attachment of HIV-1 to fresh human PBLs, binding of gp120 to CD4, and the effects of compounds on HIV-1 p66/p51 RT [with the poly(rA).oligo(dT), rAdT, and the poly(rC).oligo(dG), rCdG, template-primer systems] and protease (with HPLC-based detection of cleavage of the Ala-Ser-Glu-Asn-Tyr-Pro-Ile-Val-Glu-amide substrate) were quantitated as described [W. G. Rice *et al., Proc. Natl. Acad. Sci. U.S.A.* **90**, 9721 (1993)]. None of the DIBA compounds inhibited virus binding, the interaction of gp120-CD4, or RT. DIBA-1 and DIBA-5 inhibited protease activity by 50% at 8.4 and 13.0 μM, respectively. As controls, AZT triphosphate inhibited RT activity [median inhibitory dose (ID_{50}) = 27 nM] against the rAdT template-primer system and nevirapine inhibited RT activity (ID_{50} = 39 nM) against the rCdG template-primer system, the KNI-272 protease inhibitor reduced protease activity (ID_{50} = 3 nM), dextran sulfate inhibited virion binding (ID_{50} = 1.8 μg/ml), and cosalane inhibited gp120-CD4 interactions (ID_{50} = 7.8 μM).
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18. Plasma concentrations of DIBA-4 were measured as a function of time after oral administration (250 mg/kg) in male $CD2F_1$ mice. DIBA-4 was formulated as a solution in dimethyl sulfoxide and delivered in a volume of 1.0 μl per gram of body weight, and groups of three to five mice were bled under mild anesthesia by retro-orbital puncture at the indicated times. Total drug concentration in plasma was determined by quantitative conversion of DIBA-4 and its mixed disulfides to the thiophenol monomer, which was then assayed by reversed-phase HPLC. Plasma samples (50 μl) were mixed with dithiothreitol (10 M, 0.5 μl), deproteinized, and resolved on a Nova-Pak C_8 column. The pharmacokinetic studies in mice were done in accordance with current NIH guidelines on the humane care and use of laboratory animals in research.
19. The bioavailable fraction of drug (f) was calculated as $f = CL \times AUC_{p.o.}/DOSE_{p.o.}$, where CL is the total plasma clearance determined after intravenous administration, $DOSE_{p.o.}$ is the oral dose, and $AUC_{p.o.}$ is the area under the plasma concentration–time profile from time zero to infinity after oral dosing. The value of $AUC_{p.o.}$ was estimated by the linear trapezoidal method to the last data point, with extrapolation to infinity by using the slope of the terminal log-linear phase [M. Gibaldi and D. Perrier, *Pharmacokinetics* (Dekker, New York, ed. 2, 1982), pp. 410–411].
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Prevention of SIV Infection in Macaques by (*R*)-9-(2-Phosphonylmethoxypropyl)adenine

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The efficacy of pre- and postexposure treatment with the antiviral compound (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) was tested against simian immunodeficiency virus (SIV) in macaques as a model for human immunodeficiency virus (HIV). PMPA was administered subcutaneously once daily beginning either 48 hours before, 4 hours after, or 24 hours after virus inoculation. Treatment continued for 4 weeks and the virologic, immunologic, and clinical status of the macaques was monitored for up to 56 weeks. PMPA prevented SIV infection in all macaques without toxicity, whereas all control macaques became infected. These results suggest a potential role for PMPA prophylaxis against early HIV infection in cases of known exposure.

An urgent need for new antiretroviral drugs has become evident as more people worldwide are exposed to and become infected with HIV. Currently, 3′-azido-3′-deoxythymidine (AZT; also called zidovudine) is the most widely used antiviral agent in both single and combination strategies for the treatment of acquired immunodeficiency syndrome (AIDS). Unfortunately, AZT has limited efficacy against HIV infection, and treatment can lead to drug toxicity and the emergence of drug-resistant strains of the virus (*1*). Drugs that are more efficacious and less toxic than AZT are clearly needed.

Several acyclic nucleoside phosphonate analogs have been developed that exhibit activity against retroviruses in vitro (*2*). Initial phosphorylation is not required for activation of these reverse transcriptase inhibitors, and therefore they may have activity in a wider range of cell types as compared with AZT (*2*, *3*). One of these compounds, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), has shown efficacy against SIV in macaques but with mild-to-moderate toxic side effects in the form of skin lesions (*3*–*5*). A related acyclic nucleoside phosphonate, (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA), has shown potent in vitro efficacy against HIV-1 and Moloney murine sarcoma virus (*6*), as well as SIV (Table 1). These in vitro results prompted us to design an efficacy study of PMPA against SIV in macaques as a model for evaluating HIV therapies (*7*).

Thirty-five age-matched, naïve, juvenile, long-tailed macaques (*Macaca fascicularis*) were housed individually in a biological safety level 3 animal facility (*8*). Each macaque was inoculated intravenously with 1 ml of a 10^3 cell culture infectious dose (equivalent to 10 times the 50% monkey

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infectious dose) of an uncloned SIV_{mne} virus stock (*9*, *10*). PMPA was administered subcutaneously at a dose of 20 or 30 mg per kilogram of body weight once daily for 4 weeks (*11*). Doses were adjusted weekly according to body weight. To determine the pre- and postexposure efficacy of PMPA, we divided the macaques into five treatment groups (Table 2). Group 1 was treated with PMPA at 20 mg/kg starting 48 hours before SIV inoculation ($n = 5$); groups 2 to 4 were treated with PMPA at 30 mg/kg starting either 48 hours before inoculation ($n = 10$), 4 hours after inoculation ($n = 5$), or 24 hours after inoculation ($n = 5$). Group 5 was treated with physiological saline starting 48 hours before SIV inoculation (control, $n = 10$).

The clinical and virologic parameters of the macaques were monitored for up to 56 weeks postinoculation (PI). Blood samples were collected on a weekly basis during treatment, biweekly for the next 2 months, and then every 4 to 8 weeks until the end of the study. Clinical evaluations included complete blood counts, blood chemistry profile (including liver and renal functions), and lymphocyte subset analysis (*12*).

Table 1. In vitro efficacy of PMPA, PMEA, and AZT against SIV_{mne}. Control or SIV-infected C-8166 target cells were incubated in the presence of PMPA, PMEA, or AZT at different concentrations (0.0001 to 500 μM) for 7 days. All experiments were conducted in triplicate. A dose-response curve was generated for each antiviral agent. The expression of SIV antigen in cells was measured by an immunofluorescence assay (*24*), from which the 50% inhibitory concentration (IC_{50}) of the antiviral agent against SIV was calculated. Cell viability was determined by the trypan blue exclusion method (*24*), from which the 50% cytotoxic concentration (CC_{50}) of the antiviral agent was calculated. The selectivity index is the ratio of CC_{50} to IC_{50}.

Antiviral agent	IC_{50} (μM)	CC_{50} (μM)	Selectivity index
PMPA	1.5	>500	>333
PMEA	1.7	65	38
AZT	0.005	5	1000

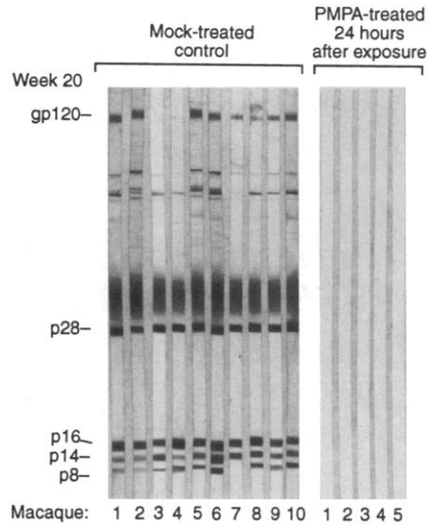

Fig. 1. Protein immunoblot analysis of SIV-specific antibody response in macaques 20 weeks postinoculation (PI) with SIV_{mne}. Mock-treated control macaques ($n = 10$) and macaques treated with PMPA starting 24 hours PI ($n = 5$) are represented. Antibodies to *env* protein gp120, *gag* proteins p28, p16, and p8 and *vpx* protein p14 were detected in the control macaques. None of the macaques treated with PMPA starting 24 hours PI showed SIV-specific antibodies. Identical analyses were performed at various time points on all of the PMPA-treated macaques, and SIV-specific antibodies were never detected.

EDTA-anticoagulated blood was separated into cellular and plasma fractions by centrifugation for virology and serology studies. Plasma p27 antigenemia was measured by a commercial SIV core antigen capture assay (*13*). Cell-free virus titers were determined by a limiting dilution assay of the plasma after treatment with polyethylene glycol (*14*). Peripheral blood mononuclear cells (PBMCs) were isolated from the cellular fraction of the blood by Ficoll-Hypaque gradient centrifugation (9). Cell-associated virus load in PBMCs was determined by a limiting dilution assay of 10^6 PBMCs. For macaques with an undetectable virus load in 10^6 PBMCs, cultures of 5×10^6 PBMCs were assayed (*3*). Plasma and PBMC cultures were maintained for 5 weeks, and supernatants were sampled weekly for SIV p27 by the SIV core antigen capture assay. Detection of proviral DNA in PBMCs was accomplished by nested polymerase chain reaction (PCR) techniques (*15*). SIV-specific antibodies in plasma were detected by immunoblotting (*10*) and SIV antibody titers were determined by an immunofluorescence antibody assay (9).

For further virologic studies, inguinal lymph node biopsies were performed on each macaque at 16 or 26 weeks PI. Two PMPA-treated macaques (groups 1 and 2) were euthanized 40 weeks PI for complete necropsy and tissue survey (*16*). The lymph node biopsies and tissues taken at necropsy (25 different tissues including lymphoid tissue and major organs) were evaluated for the presence of SIV by coculture, PCR, and immunohistochemistry (5, 9).

When PMPA treatment was administered starting 48 hours before, 4 hours after, or 24 hours after inoculation with SIV_{mne}, none of the macaques became

Table 2. Virus, antibody, and clinical status of macaques inoculated with SIV_{mne} and treated with PMPA or mock-treated.

Group	PMPA treatment*		Virus load		SIV DNA§ in PBMCs	SIV-specific antibody‖	Lymph node biopsy¶	Clinical status#
	Time started	Dose (mg/kg)	Plasma†	PBMCs‡				
1 ($n = 5$)	48 hours preinoculation	20	–	–	–	–	–	Four healthy, 56 weeks; one euthanized at 40 weeks, healthy
2 ($n = 10$)	48 hours preinoculation	30	–	–	–	–	–	Nine healthy, 36 to 56 weeks; one euthanized at 40 weeks, healthy
3 ($n = 5$)	4 hours postinoculation	30	–	–	–	–	–	Healthy, 36 weeks
4 ($n = 5$)	24 hours postinoculation	30	–	–	–	–	–	Healthy, 36 weeks
5 ($n = 10$)	Control, mock-treated	None	+	+	+	+	+	Transient rash, diarrhea, enlarged lymph nodes 20 to 36 weeks PI

*A single daily dose of PMPA was administered subcutaneously for 28 to 30 days. †Cell-free virus load was determined in polyethylene glycol–treated plasma samples (*14*); (–) not detected; (+) detected (for the control macaques, cell-free SIV was persistently detected at dilutions of 10^{-2} to 10^{-3}). ‡Cell-associated (PBMC) virus load was determined by a limiting dilution assay; for the PMPA-treated macaques, 5×10^6 PBMCs were cocultured; for the control macaques a minimum of 10 to 10^3 PBMCs were needed to detect SIV. §SIV DNA in PBMCs was detected by PCR (*3*, *15*). ‖SIV antibody titers and SIV-specific antibodies were detected by immunofluorescence antibody assay and by immunoblotting (*9*, *10*). ¶Inguinal lymph nodes were biopsied from each macaque at 16 or 26 weeks postinoculation (PI) and examined for the presence of SIV by coculture, PCR, and immunohistochemistry. #The clinical status of the treated macaques was monitored for 36 to 56 weeks PI; two healthy macaques were euthanized at 40 weeks PI for complete necropsy; mock-treated macaques were monitored for 20 to 36 weeks PI.

infected (Table 2). SIV was not detected in plasma or PBMCs, and SIV DNA was not detected in PBMCs throughout the entire study (Table 2). Similarly, lymph node biopsies taken from the PMPA-treated macaques were virus free. The lymphoid tissues and major organs of two healthy, PMPA-treated macaques euthanized 40 weeks PI were also free of SIV infection. SIV-specific antibodies were not detected in any of the PMPA-treated macaques (Fig. 1). Clinical signs of PMPA toxicity did not occur, nor were there any significant changes in complete blood counts or blood chemistries during or after the 4-week treatment regimen. Additionally, the absolute number of $CD4^+$ and $CD8^+$ cells did not differ significantly between the PMPA-treated macaques.

By comparison, 2 of 10 mock-treated control macaques had detectable virus 1 week PI, and all 10 macaques were persistently viremic 3 weeks PI. Cell-free and cell-associated virus load in these control animals were high. Plasma dilutions of 10^{-2} to 10^{-3} were persistently SIV positive, and as few as 10 to 10^3 PBMCs were sufficient to detect SIV (Table 2). Antibody titers to SIV in the control macaques were first detected 3 weeks PI, and SIV-specific antibodies persisted throughout the study (Fig. 1). Inguinal lymph node biopsies taken from the control macaques 16 weeks PI were also positive for SIV by isolation, PCR, and immunohistochemistry (Table 2).

These findings show that the antiretroviral compound PMPA prevented the establishment of SIV infection in 25 of 25 macaques (100%) without toxicity when administered up to 24 hours after virus inoculation. These significant results in the macaque model are extremely encouraging compared with data obtained from efficacy studies of other nucleoside analogs such as PMEA and AZT. A previous study in four pretreated rhesus macaques showed that a daily dose of PMEA at 10 to 20 mg/kg for 4 weeks suppressed $SIV_{macBK28}$ replication without toxic side effects, although antibody to SIV was detected 1 to 2 weeks after treatment was stopped (*4*). More recently, it was shown that PMEA prevented SIV_{mne} infection in 10 of 12 pretreated, long-tailed macaques (83%) (*3*) and in 1 of 5 (20%) macaques treated 4 hours after inoculation (*17*). Toxic side effects were evident in the form of mild skin lesions (*3*). By contrast, a daily dose of AZT at 100 mg/kg for 4 weeks prevented SIV_{mne} infection in only 1 of 18 pretreated macaques (6%) (*18*) and in none of the macaques treated 4 hours after inoculation (*19*), and there were signs of hematologic toxicity as indicated by decreased erythrocyte counts and hemoglobin measures. Although other related studies confirm the limited efficacy of pre- and postexposure treatments with AZT against SIV in juvenile and adult macaques (*20*), a study in infant macaques showed that AZT prevented infection when treatment was initiated 2 hours before exposure to SIV but was ineffective after exposure (*21*). Hematologic toxicity in the form of thrombocytosis and anemia was also evident (*20*, *21*). A comparison of these data clearly shows that PMPA is more efficacious and less toxic than either PMEA or AZT. Additional studies are in progress to assess the upper limit for the time after virus inoculation that PMPA could protect macaques from SIV infection.

Currently, postexposure prophylaxis of AZT against HIV is widely accepted and is used in humans despite numerous reports of failure (*22*). In the PMPA study reported here, our pre- and postexposure findings in the SIV-macaque model indicate that PMPA is a promising candidate for anti-HIV treatment. Postexposure prophylaxis with PMPA could have a significant impact on preventing HIV infection in health care workers or others accidentally exposed to the virus. Furthermore, the results from several recent anti-HIV clinical trials indicate that combining nucleoside analogs, such as AZT, with lamivudine (3TC) or proteinase inhibitors can enhance antiviral efficacy (*23*). Therefore, PMPA may also have an important role in combination therapies or strategies against HIV.

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12. Specific lymphocyte subsets were determined by incubating EDTA-anticoagulated blood samples with a panel of mouse anti-human monoclonal antibodies. Cell percentages were calculated by flow-cytometric procedures with a FACScan (Becton Dickinson, Mountain View, CA). Cell percentage times the total leukocyte count gave the absolute number of each subset (*3*).
13. SIV Core Antigen Assay, Coulter Corp., Hialeah, FL. The lower detection limit of p27 was 50 pg/ml.
14. Plasma was thoroughly mixed with 12% polyethylene glycol (PEG)–8000 and refrigerated overnight at 4°C. The sample was centrifuged at 2000*g* at 4°C for 20 min. The pellet was washed twice and resuspended in culture medium. The suspension was serially diluted 10^{-1} to 10^{-4} and C-8166 cells were added for culture [K. E. Follis and C.-C. Tsai, *J. Med. Primatol.* **23**, 255 (1994)].
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16. Sodium pentobarbital overdose (90 mg/kg) was the method of euthanasia for all macaques.
17. PMEA (20 mg/kg) was administered once daily to five macaques starting 4 hours after inoculation with SIV_{mne}. Treatment continued for 28 days, and the clinical and virologic status of the macaques was monitored for 20 weeks (C.-C. Tsai, unpublished data).
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