leaf indicates that at least some of the xylem elements are capable of sustaining pressures below −1.5 MPa. This contradicts measurements made with the xylem pressure probe in which cavitation generally occurred at much higher pressures (>−0.4 MPa) (*9*, *11*, *12*). It is also at odds with predictions of the stability of water in the xylem based on measurements of cavitation thresholds in artificially constructed water columns subjected to centrifugal force (*14*). In the latter study, the mean cavitation threshold for distilled water in a glass tube was −0.26 MPa; stability below −1.0 MPa only occurred when stringent standards governing the purity of the water and cleanliness of all surfaces were observed. On the basis of these experimental results, Smith (*14*) concluded that xylem pressures more negative than −1 MPa are highly improbable. An alternative explanation consistent with the data presented here is that glass tubes are an inappropriate model system for assessing the stability of water under tension in the xylem.

The implications for water transport mechanisms proposed on the basis of xylem pressure probe measurements versus the balancing pressure method are profound (*15*). The balancing pressure technique indicates that hydrostatic gradients in the xylem are adequate to explain observed rates of water movement. The much smaller tensions measured by the xylem pressure probe require the existence of an additional, unknown mechanism for water transport in plants. Agreement between the balancing pressure and experimentally generated tension in the xylem provides empirical validation of the ability of the balancing pressure technique to measure negative xylem pressures, supporting the cohesion theory as the primary mechanism for water transport in higher plants.

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Inhibitors of HIV Nucleocapsid Protein Zinc Fingers as Candidates for the Treatment of AIDS

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Strategies for the treatment of human immunodeficiency virus–type 1 (HIV-1) infection must contend with the obstacle of drug resistance. HIV-1 nucleocapsid protein zinc fingers are prime antiviral targets because they are mutationally intolerant and are required both for acute infection and virion assembly. Nontoxic disulfide-substituted benzamides were identified that attack the zinc fingers, inactivate cell-free virions, inhibit acute and chronic infections, and exhibit broad antiretroviral activity. The compounds were highly synergistic with other antiviral agents, and resistant mutants have not been detected. Zinc finger–reactive compounds may offer an anti-HIV strategy that restricts drug-resistance development.

Successful therapeutic management of HIV-1 infection and the associated acquired immunodeficiency syndrome (AIDS) may be achieved by antiviral strategies targeted to retroviral features that are highly conserved and thus mutationally intolerant. Sequence analysis of retroviral components has revealed a highly conserved structural motif, termed the retroviral-type zinc finger, that is arranged in a peptide segment Cys-X_2-Cys-X_4-His-X_4-Cys (CCHC; X, any amino acid) and coordinated to zinc (*1*, *2*). The chelating residues (3 Cys, 1 His) and the spacing of the zinc finger array are absolutely conserved among all known lentiretroviruses and oncornoretroviruses, and mutations in the zinc-chelating residues result in noninfectious virus (*3*). Two such CCHC-type zinc fingers are contained within the HIV-1 p7 nucleocapsid (p7NC) protein, a maturational product of the $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ precursor polyproteins. Within the precursor polyproteins the fingers function in packaging of viral genomic RNA into progeny virions, whereas the same zinc fingers of the processed p7NC function in an early phase of retroviral infection (*3*, *4*).

The nucleophilic CCHC zinc finger donates electrons to the C-nitroso group of 3-nitrosobenzamide and certain other electrophilic groups (*5*), resulting in modification of the zinc-coordinating cysteine thiolates, ejection of zinc from the array, and inactivation of HIV-1 infectivity. Hence, electrophilic disulfide-substituted benzamides (DIBAs) discovered as active against HIV-1 by the National Cancer Institute's drug screening program were identified as potential zinc finger–reactive compounds. Molecular structures of five of the DIBA-type compounds are shown in Fig. 1. DIBA-1 and DIBA-2 are closely related congeners differing by only a single acetyl group, and DIBA-3 is a low molecular weight derivative of DIBA-1. DIBA-4 is a congener of DIBA-1 in which the *p*-aminophenyl sulfonamide moiety has been replaced with a DL-isoleucine residue, and DIBA-5 is a para-para positional isomer of DIBA-1 in which the spatial relation be-

tween the disulfide and the benzamide has been modified.

As shown in Table 1, DIBA-1 was active against HIV-1_{RF} in the initial CEM-SS cell-based screen with a median effective concentration (EC_{50}) of 2.3 μM; no cellular toxicity was observed at 200 μM. DIBA-2 (EC_{50} = 1.5 μM), DIBA-3 (EC_{50} = 0.4 μM), and DIBA-4 (EC_{50} = 1.9 μM) exhibited antiviral activity essentially equivalent to that of DIBA-1, and DIBA-5 was not active. DIBA-1, -2, -3, and -4 inhibited all other strains of HIV-1 tested (Table 1), including those selected for resistance to 3′-azido-2′,3′-dideoxythymidine [AZT, nucleoside inhibitor of reverse transcriptase (RT)], pyridinone, or nevirapine [non-nucleoside RT inhibitors (NNRTIs)]. The compounds also inhibited infection by a panel of clinical isolates of HIV-1 in human peripheral blood lymphocyte (PBL) cultures, monocytotropic strains of HIV-1 in monocyte-macrophage (Mono/Mϕ) cultures, and by HIV-2 and simian immunodeficiency virus (SIV). The combination of DIBA-1 and AZT resulted in an enhanced cytoprotective effect of ~70% (6), as compared with the synergistic actions of AZT with NNRTIs that typically demonstrate enhanced antiviral effects in the range of 10 to 50% (7). Synergy was observed with each of the active DIBA compounds in combination with AZT, 2′,3′-dideoxycytidine (DDC), various NNRTIs, or the KNI-272 protease inhibitor (8).

Addition of DIBA compounds to cultures of chronically infected H9/HIV-1_{SK1} cells resulted in concentration-dependent reductions in the production of virion-associated RT activity, p24, and infectious virus (Fig. 2A). Likewise, addition of DIBA-1 to cultures of latently infected U1 or ACH2 cells before stimulation with tumor necrosis factor–α (TNF-α), interleukin-6 (IL-6), or phorbol 12-myristate 13-acetate (PMA) also resulted in inhibition of late phase virion production (note the decreased amounts of supernatant RT in Fig. 2, B and C). Addition of various concentrations of DIBA-1 to cultures of 5 × 10^4 U1 cells that had been prestimulated with TNF-α (5 ng/ml) for 24 hours also resulted in a dose-

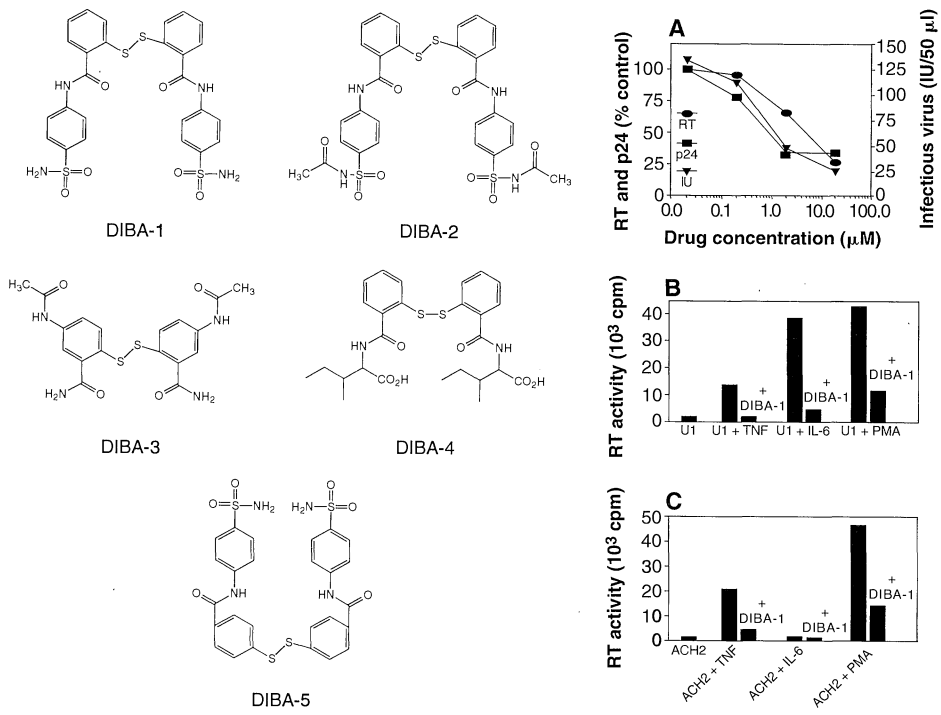

Fig. 1 (**left**). Molecular structures of the disulfide-substituted benzamide (DIBA) compounds. **Fig. 2** (**right**). Effects of DIBA-1 on the late phase of the HIV-1 infectious cycle. (**A**) Chronically infected H9/HIV-1_{SKI} cells (*17*) were cultured in the presence of DIBA-1, and cell-free supernatants were analyzed for virus content by RT assay and p24 levels. Infectious titers in the supernatant were quantitated by the HeLa/CD4/HIV-1 LTR/β-Gal system as described (*7*). Latently infected U1 cells (**B**) and ACH2 cells (**C**) (*22*) were pretreated with 20 μM DIBA-1 for 1 hour before the addition of TNF-α (100 U/ml), IL-6 (100 U/ml), or 10 nM PMA, and after 72 hours the culture supernatants were analyzed for virus content by RT assay.

Table 1. Range of antiviral action of DIBAs. Anti-HIV studies with lymphocyte-derived cell lines were done with the XTT cytopathicity assay (*21*). Data are presented as the percent control of XTT values for the uninfected, drug-free control. EC_{50} values reflect the drug concentration that provides 50% protection from the cytopathic effect of the virus. Antiviral assays with fresh human PBLs and Mono/Mϕ cultures were done as described (*5*, *17*); EC_{50} values for these cultures indicate the drug concentration that provided a 50% reduction in viral p24 production. SI and NSI, syncytia-inducing and nonsyncytia-inducing strains of HIV-1, respectively. AZT^R, Pyr^R, and Nev^R indicate strains of HIV-1 that are resistant to AZT, pyridinone, or nevirapine, respectively.

Cell type	Virus strain	EC_{50} (μM)			
		DIBA-1	DIBA-2	DIBA-3	DIBA-4
CEM-SS	HIV-1_{RF}	2.3	1.5	0.4	1.9
CEM-SS	HIV-1_{IIIb}	2.8	5.2	0.4	1.6
	Drug-resistant				
MT-4	HIV-1_{6R}(AZT^R)	1.9	1.9	1.6	0.3
MT-4	HIV-1_{A17}(Pyr^R)	0.6	8.9	2.4	5.1
CEM-SS	HIV-1_{N119}(Nev^R)	2.2	4.6	2.3	3.5
	Clinical isolates				
PBL	HIV-1_{WEJO} (SI)	3.5	5.2	7.5	0.4
	HIV-1_{BAKI} (SI)	0.3	7.5	1.8	7.1
	HIV-1_{VIHU} (NSI)	3.6	9.9	5.2	9.3
	HIV-1_{WOME} (SI)	4.0	8.2	5.7	4.3
	Other				
Mono/Mϕ	HIV-1_{ADA}	5.8	9.4	17.5	35.0
CEM-SS	HIV-2_{ROD}	1.6	3.0	1.0	2.6
CEM-SS	SIV	14.6	3.4	2.1	2.7

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dependent decrease in virion-associated p24 production, and a 50% decrease was observed with 5.7 μM DIBA-1. DIBA-2, -3, and -4 were similarly active, whereas DIBA-5 was inactive, and evaluation of AZT and dextran sulfate revealed no inhibitory effects on the U1 and ACH-2 cellular systems (8). Thus, the DIBA-type compounds effectively blocked production of virus from previously infected cells by inhibiting an event during the late phase of infection.

Coordination of the p7NC CCHC motifs with zinc results in intrinsic fluorescence because of exposure of a tryptophan residue in the second finger to the aqueous environment (2). Consequently, ejection of zinc from the fingers can be detected by measuring the quenching of this fluorescence. The fluorescence intensity of purified p7NC (363.8 ± 7.9, mean ± SD; n =3) was readily quenched after 30 min by DIBA-1 (81.0 ± 1.4) and DIBA-2 (38.3 ± 0.5), and less so by DIBA-3 (233.3 ± 3.7) and DIBA-4 (216 ± 5.4), but DIBA-5 (357.3 ± 5.2) was unreactive, as was AZT (9). These data indicated that only those DIBA compounds with antiviral activities evoked the release of zinc from the fingers.

The chemical mechanism of action of the compounds on the zinc finger was further investigated by analysis of the reaction products by high-performance liquid chromatography (HPLC) separation (Fig. 3). Only the DIBA compounds that inhibited HIV-1 replication (but not the inactive DIBA-5 isomeric congener) elicited a decrease in the amounts of native p7NC protein (peak A) and a concomitant production of a new protein (peak B). Protein that eluted in peak B was reduced with 2-mercaptoethanol and reanalyzed by HPLC; it eluted as p7NC, indicating that the zinc finger cysteine thiolates of the p7NC in this peak had become cross-linked by disulfide bonds. A preparation of fully reduced monomeric DIBA, formulated by reduction of dimeric DIBA-1 with 2-mercaptoethanol, was unreactive with the zinc finger. These findings differ fundamentally from the effects of antioxidants, which exert antiviral effects only when added to cultures in millimolar concentrations and in their reduced form (10).

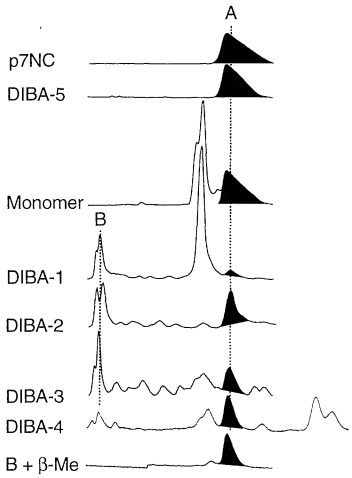

Fig. 3. Actions of the DIBAs on the HIV-1 p7NC protein zinc fingers. The p7NC protein was diluted to 40 μg/ml in 10 mM sodium phosphate buffer (pH 7.0), treated with 25 μM of each DIBA compound, and the products of the reaction analyzed by reversed-phase HPLC on a Waters μ-Bondapak C-18 column. Elution positions for the reactants and products are indicated: p7NC resolved in peak A and p7NC products from the reactions resolved in peak B. Shaded areas indicate the proteinaceous material. DIBA-3 and -4 yielded mixed disulfide covalent adducts that influenced the chromatographic character of the modified protein; depending on the reaction conditions, mixed disulfides could also be observed with DIBA-1 and -2.

To determine whether the DIBA compounds could also gain access to and interact with the p7NC protein sequestered within the enveloped virion, we treated sucrose gradient–purified HIV-1 with each compound, followed by SDS–polyacrylamide gel electrophoresis (PAGE) separation of the viral proteins under nonreducing conditions and immunoblot analysis for the p7NC protein. Compounds that ejected the zinc from the p7NC protein (DIBA-1, -2, -3, and -4) resulted in intermolecular disulfide bond formation among the cysteine thiolates of the viral p7NC proteins, causing the p7NC protein to resolve as an aggregate (11), and cross-linkage of the virion p7NC correlated with the ability of the compounds to inactivate HIV-1 infectivity in a concentration-dependent manner (12). Furthermore, mechanistic studies determined that the antiviral mode of action of DIBAs correlated with a specific attack on the zinc fingers but not with inhibition of virion attachment, RT, or protease (13).

We have previously isolated resistant mutants by coculture with nucleoside RT inhibitors (3TC) (14), various NNRTIs (such as nevirapine, thiazolobenzimidazole, and oxathiin carboxanilide) (15), protease inhibitors (KNI-272) (16), and inhibitors of viral attachment and fusion (cosalane) (17) within three to six passages (~2 months in culture). However, attempts to isolate mutants resistant to the DIBA compounds from HIV-1–infected cultures have been unsuccessful after more than a year in passage.

Pharmacokinetic studies in mice revealed that DIBA-1 and DIBA-2 were rapidly eliminated from plasma after intravenous injection, and systemic availability upon oral dosing proved to be negligible (<0.5%) for both compounds. DIBA-3 was not considered a viable candidate for development because of its poor chemical stability under physiologic conditions. However, DIBA-4 did exhibit appealing oral bioavailability properties. A single oral dose of 250 mg per kilogram of body weight, which appeared to be well tolerated by the mice, provided a peak total drug concentration (18) of 103 μM in plasma at 34 min after administration. Thereafter, plasma concentrations decreased slowly, remaining within the range effective against HIV-1 in vitro for at least 12 hours. Analysis of the plasma profile suggested that the absorption of DIBA-4 was complex, being neither a purely first-order nor zero-order process. The systemic availability of oral DIBA-4 was 39.8% (19).

The retroviral CCHC zinc finger motif stands as a rare conserved feature against a background of extreme variation among retroviral components (20). Albeit this fact suggests that mutational circumvention of reagents that selectively target the retroviral zinc finger may be difficult for the virus to achieve, only long-term studies of infected cultures and clinical experience with the DIBA-type compounds can fully address this issue. Nevertheless, our studies should provide a powerful impetus for utilization of the zinc finger as an antiretroviral target, and this concept should be included in strategies for the development of effective drugs for the treatment of HIV infection and other retrovirus-based diseases.

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6. Five concentrations of DIBA-1 were tested in all combinations with nine concentrations of AZT for their effects in the XTT cytoprotection assay, and data were analyzed according to the three-dimensional model of M. N. Prichard and C. Shipman Jr. [*Antiviral Res.* **14**, 181 (1990)]. Effects of the drug combinations were calculated on the basis of activities of the compounds tested individually. Within the resultant three-dimensional plot the flat plane (having a defined value of zero) identified the condition of additivity, while the peak extending above the plane of additivity to a level of ~70% revealed an enhanced antiviral effect (enhanced protection from HIV-1–induced cytopathicity); any value below the plane would represent antagonism between the two compounds, but that was not observed.
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367 ± 22 (mean ± SD, $n = 3$) at 30 min after addition, indicating no effect on the zinc coordination.
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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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