Azodicarbonamide inhibits HIV-1 replication by targeting the nucleocapsid protein

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Nucleocapsid p7 (NCp7) proteins of human immunodeficiency virus type 1 (HIV-1) contain two zinc binding domains of the sequence Cys-(X)$_2$-Cys-(X)$_4$-His-(X)$_4$-Cys (CCHC)[1,2]. The spacing pattern and metal-chelating residues (3 Cys, 1 His) of these nucleocapsid CCHC zinc fingers are highly conserved among retroviruses[1,3,4]. These CCHC domains are required during both the early[5,6] and late[7,8] phases of retroviral replication, making them attractive targets for antiviral agents. Toward that end, we have identified a number of antiviral chemotypes that electrophilically attack the sulfur atoms of the zinc-coordinating cysteine residues of the domains[9-14]. Such nucleocapsid inhibitors were directly virucidal by preventing the initiation of reverse transcription[15] and blocked formation of infectious virus from cells through modification of CCHC domains within Gag precursors[16]. Herein we report that azodicarbonamide (ADA) represents a new compound that inhibits HIV-1 and a broad range of retroviruses by targeting the the nucleocapsid CCHC domains. Vandevelde et al.[17] also recently disclosed that ADA inhibits HIV-1 infection via an unidentified mechanism and that ADA was introduced into Phase I/II clinical trials in Europe for advanced AIDS. These studies distinguish ADA as the first known nucleocapsid inhibitor to progress to human trials and provide a lead compound for drug optimization.

The National Cancer Institute's AIDS drug discovery initiative recently found that ADA (Fig. 1a) inhibits the HIV-1$_{RF}$-induced cytopathicity of CEM-SS cells in a standard cell-based antiviral assay[12] with an $EC_{50} = 38$ µM (concentration providing 50% cytoprotection), while the concentration causing 50 % cell death (IC_{50}) was >200 µM. ADA inhibited infection by the HIV-1$_{WEJO}$ clinical isolate in fresh human peripheral blood lymphocyte cultures ($EC_{50} = 45$ µM) and the HIV-1$_{Ba-L}$ monocytotropic strain in fresh human monocyte/macrophage cultures ($EC_{50} = 110$ µM), as well as a host of HIV-1 isolates resistant to various reverse transcriptase inhibitors[13] (EC_{50} ranged from 15–65 µM). In

addition, ADA exerted antiviral activity against HIV-2$_{ROD}$ ($EC_{50} = 65$ µM) and SIV ($EC_{50} = 45$ µM). Interestingly, the reduced form of ADA, biurea (Fig.1a), showed no anti-HIV-1 activity. These data illustrate that ADA is broadly inhibitory to retroviruses, although the concentrations required to exert an antiviral effect are relatively high.

The potential interaction of ADA with the nucleocapsid (NCp7) CCHC zinc fingers was evaluated using a Trp37 fluorescence assay that measures the rate of zinc ejection from the C-terminal zinc finger of the HIV-1 NCp7 protein[14]. ADA promoted a time-dependent decrease in relative fluorescence as zinc was ejected from the purified NCp7 protein (Fig. 1b), whereas biurea and AZT did not. To investigate the relative reactivities of the compound with the N-terminal and C-terminal fingers of the NCp7 protein we performed ^{1}H NMR studies. These analyses demonstrated a time-dependent ejection of zinc from both zinc finger domains by ADA (Fig. 1c). Zinc was lost from each finger at equivalent rates, as indicated by a shift of the definitive zinc-bound histidine proton signals to the zinc-free histidine proton signal positions. The final spectrum is essentially identical to that of the apoprotein obtained upon incubation of the protein with the metal chelating agent EDTA[3,18]. Of note is that the zinc-free protein gives rise to multiple, broad signals due to heterogeneous disulfide bond formation among the Cys residues of the fingers. Thus, ADA interacts with and causes zinc ejection from both zinc finger domains of the HIV-1 NCp7 protein.

Determination of the selectivity of ADA for the nucleocapsid CCHC zinc fingers required evaluation of the compound against an array of other molecular targets. As shown in Fig. 1d, ADA did not inhibit the attachment of HIV-1 to host cells or the enzymatic activities of HIV-1 p66/p51 reverse transcriptase (RT), protease or integrase. In contrast, antiviral agents used as controls for each of the in vitro molecular target assays effectively inhibited their respective targets. In addition, 100 µM ADA demon-
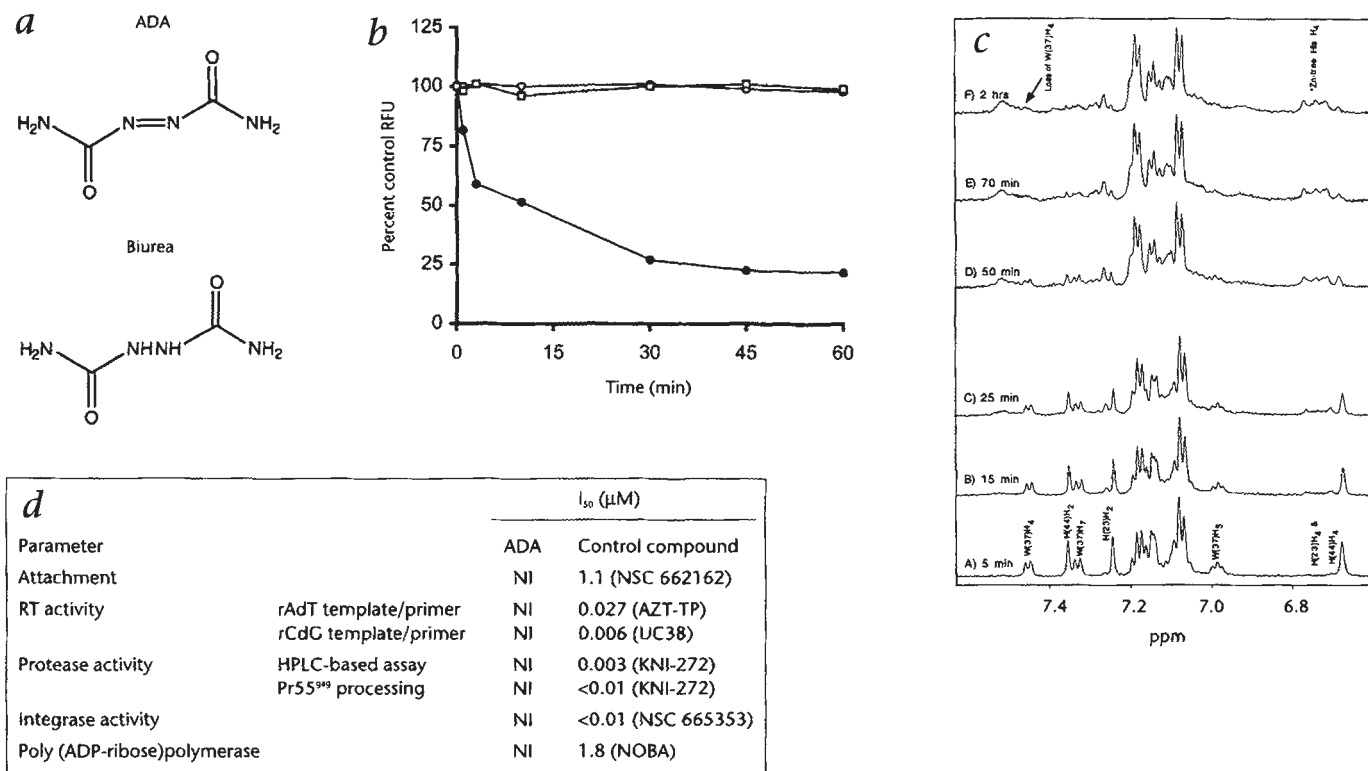
Fig. 1 ADA affects the NCp7 zinc finger domains but not other molecular targets. *a*, Structures of ADA and biurea are shown in the *trans* form. *b*, Relative fluorescence units (RFU) in the Trp37 fluorescence assay were measured over a 60 min period after exposure of NCp7 protein to 25 μM of ADA (●) or biurea (○), or 10 μM AZT (□). *c*, The ^{1}H NMR spectra depict the time-dependent alterations in the NCp7 zinc fingers after reaction of 1 mM NCp7 with 1 mM ADA at 20 °C, 30 mM sodium acetate, pH 7.0. D_2O was utilized as the solvent. NMR signals indicating the zinc-bound or zinc-free forms of the fingers are the delta proton (H_2) and epsilon proton (H_4) signals of the His residues in finger one [H(23)] and in finger two [H(44)]. As zinc is lost from the fingers the H(23) and H(44) proton signals are shifted from a diagnostic zinc-bound position to a diagnostic zinc-free position (see the *Zn-free His H_4). Five minutes after addition of ADA, well resolved peptide aromatic (W) and histidine proton signals are observed. Fifteen minutes after reaction, some broadening of the aromatics and loss of intensity of the His delta and epsilon signals from both zinc fingers indicate partial loss of zinc and folded structure. After 25 minutes approximately one third of the protein has lost the coordinated zinc, and NMR signals indicative of zinc finger unfolding begin to appear. Fifty percent unfolded protein is observed at the 50 minute time point, and very little zinc-coordinated protein remains by 70 minutes. Based on the complete loss of zinc-bound histidine delta resonance and broadening of the tryptophan peaks, all of the protein exists in the unfolded state after two hours. These data indicate that both zinc fingers react at approximately equivalent rates based on the loss of signal from the zinc-bound form of H(23)H_2 and H(44)H_2. *d*, ADA was tested for its ability to inhibit a variety of parameters involved with HIV-1 replication, as well as the enzymatic activity of poly (ADP-ribose)polymerase. I_{50} values (drug concentration providing 50 % inhibition of the indicated activity) were derived from graphs in which each point represented the mean of at least three replicates. NSC 662162 is an inhibitor of virus attachment, AZT-TP and UC38 inhibit HIV-1 RT, KNI-272 inhibits HIV-1 protease, NSC 665353 (ISIS 5320) inhibits HIV-1 integrase[13] and 3-nitrosobenzamide (NOBA) inhibits poly (ADP-ribose)polymerase[9]. NI indicates that no inhibition was observed at the high test concentration of 100 μM.

strated no inhibition of the enzymatic activity of poly (ADP-ribose)polymerase (PARP) that contains a Cys-(X)$_2$-Cys-(X)$_{28}$-His-(X)$_2$-Cys zinc binding domain, while a control compound (3-nitrosobenzamide, NOBA)[9] effectively inhibited PARP by modifying the zinc finger domain (Fig. 1*d*). These studies correlate the anti-HIV-1 activity of ADA with modification of the NCp7 zinc fingers, but not with any other molecular target. They also demonstrate the selectivity of ADA for the retroviral nucleocapsid zinc finger domain.

The virucidal activity of ADA was tested by treatment of an undiluted stock of HIV-1$_{RF}$ with various concentrations of compound. Concentrations of ADA above 10 μM caused complete inactivation of virus (Fig. 2*a*), and analysis of viral proteins by western blotting revealed a concentration-dependent decrease in the amounts of detectable virion NCp7 following treatment of HIV-1$_{MN}$ virions with ADA (Fig. 2*b*). The NCp7 protein resolved as high molecular weight aggregates due to the forma-

tion of disulfide bonds between the zinc fingers of closely associated NCp7 molecules within the virus. Viral inactivation was confirmed by PCR analysis, wherein HIV-1$_{IIIB}$ virions treated with >25 μM ADA failed to initiate intracellular synthesis of proviral DNA after incubation with CEM-SS cells (Fig. 2*c*). Thus, ADA inactivates cell-free HIV-1 virions, and this action correlates directly with modification of the NCp7 protein.

To determine if ADA could also affect the production of infectious virus from cells containing integrated proviral DNA, TNF-α stimulated U1 cells were treated with the compound and the production of viral proteins and infectious virus was monitored. No significant decrease in the levels of released virus-associated p24 and only very minor decreases in infectious units were observed in the absence of cellular toxicity (Fig. 3*a*). Inspection of viral proteins produced in U1 cells by western blot analysis (using NCp7 and p24 antisera) showed no detectable alterations after treatment with ADA (Fig. 3*b*). This indicated that ADA did

Fig. 2 ADA directly inactivates HIV-1. *a*, HIV-1$_{RF}$ was treated with the indicated concentrations of ADA and then titered in triplicate on HeLa-CD4-LTR-βgal cells. Values reflect the relative infectious units per ml (mean ± SD for triplicate wells) after treatment with each concentration of ADA. *b*, HIV-1$_{MN}$ was exposed to ADA or 10 μM AZT for 1 h at 37 °C and virus proteins were resolved by non-reducing 4–20 % SDS–PAGE, probed with NCp7 antisera, and visualized by chemiluminescence via HRP-conjugated goat anti-rabbit IgG. *c*, DNase treated HIV-1$_{IIIB}$ was incubated with ADA for 1 h at 37 °C. Treated virus was then adsorbed for 24 h onto CEM-SS cells. At 24 h the cells were lysed, proteinase K-treated and proviral DNA formation was determined by PCR amplification using primer pairs that identify late reverse transcription products (LTR/*gag*). Cellular β-globin expression was used as an internal amplification control. U1 cells containing two copies of integrated HIV-1 proviral DNA per cell were utilized as a positive control for infected cells, and the HIV-1 positive control plasmid was utilized as a positive control for HIV-1 DNA in the absence of cellular genes.
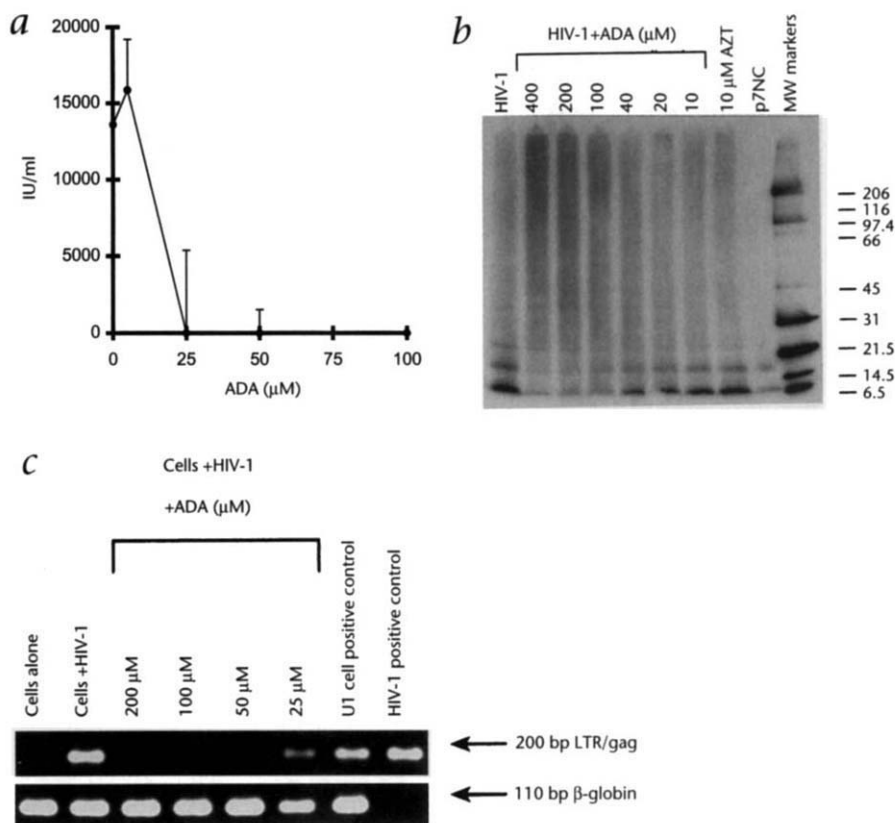

not significantly affect the Gag precursor polyproteins, as compared to the dramatic modification to intracellular Gag molecules previously observed with the 2,2'-dithiobisbenzamide (DIBA) compounds[16]. Thus, ADA does not effectively inhibit new virus production from U1 cells, and the slight decrease in the infectious titer of released virus particles could be due to the virucidal action of ADA after the particles are released from the cells.

Understanding how ligands interact with the NCp7 zinc fingers is of primary interest for the optimization of lead compounds. For this purpose, interatomic surface molecular modeling studies were initiated to explore how ADA associates with potential high affinity ligand attachment sites on the atomic surfaces of the two zinc fingers of the HIV-1 NCp7 protein[14,19]. Using this procedure, ADA docked with high apparent affinity to a single ligand attachment site (distal site) on the N-terminal zinc finger. Interestingly, ADA docked to two separate sites (proximal and lateral sites) on the C-terminal zinc finger. In addition, specific hydrogen bonding interactions between the terminal amine protons of ADA and the backbone residues of the

Fig. 3 Effects of ADA on virus production from TNF-α induced latently HIV-1 infected U1 cells. *a*, U1 cells were induced with 5 ng/ml TNF-α in the presence of indicated concentrations of ADA. Cell-free supernatants were analyzed for p24 content and infectious units and cell viability was determined by XTT dye reduction. Parameters are expressed as the percent of control values determined for cultures in the absence of ADA. U1 cell viability (▲); p24 in cell-free supernatants (■); HIV-1 infectious units per ml of cell-free supernatant (●). *b*, Whole cell lysates from TNF-α induced U1 cells (1 × 10^6) in the absence or presence of ADA

were prepared as described in the Methods. Total cell lysates were resolved on 4–20% SDS-PAGE and electroblotted onto PVDF membranes. Recombinant NCp7 and Pr55^{gag} were included as marker controls. Viral specific proteins were detected by chemiluminescence using NCp7 p24 antisera.
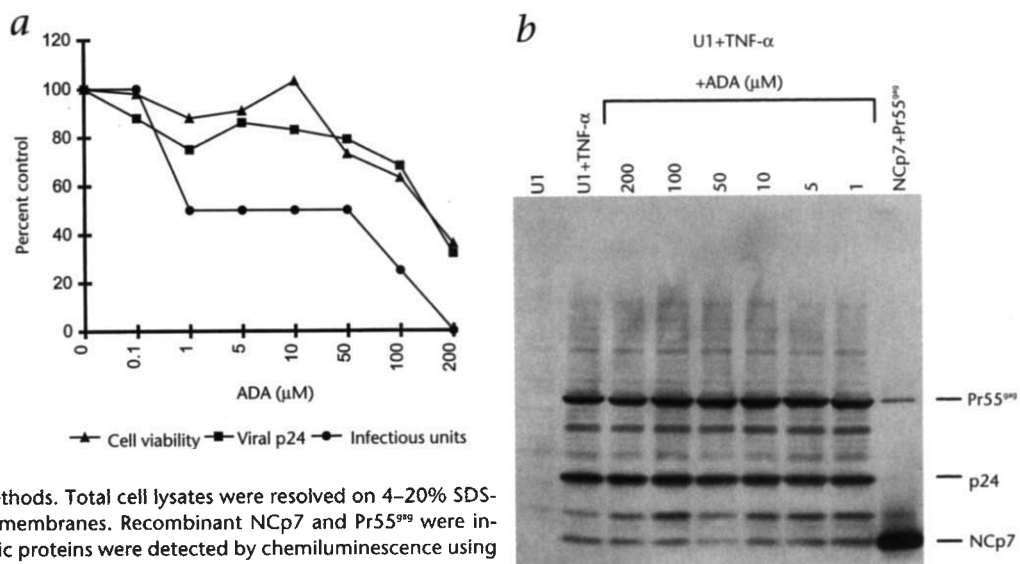
zinc finger domains were critical for productive ligand-protein associations. Thus, modeling studies may be useful in explaining the disparate zinc finger reactivities of closely related congeners and may assist in the design of analogs of ADA.

In these studies we have evaluated the *in vitro* anti-HIV-1 activity of ADA and demonstrated that the molecular target of ADA is the retroviral CCHC zinc finger motif of the NCp7 protein. The Trp37 and ^{1}H NMR studies demonstrated that ADA interacts with both zinc finger motifs of the HIV-1 NCp7, promotes the ejection of zinc and results in the loss of native structure of the protein. Thus, ADA represents a novel category of azo-based NCp7 zinc finger inhibitors, distinct from the aromatic C-nitroso and disulfide-based compounds previously identified[9-14]. ADA inhibited a wide variety of retroviruses, including drug-resistant strains, clinical isolates and monocytotropic strains of HIV-1, as well as HIV-2 and SIV. The compound caused direct inactivation of cell-free HIV-1 virus particles through cross-linkage of viral nucleocapsid proteins. Yet, ADA had no inhibitory action on virus binding to target cells or the enzymatic activities of HIV-1 reverse transcriptase, protease or integrase, and there was no significant inhibition of HIV-1 replication in cytokine stimulated latently infected U1 cells. Moreover, ADA did not affect the zinc finger motif of the cellular poly (ADP-ribose)polymerase enzyme, demonstrating selectivity of ADA for the retroviral zinc finger domain.

Recently, Vandevelde *et al.*[17] reported that ADA inhibits the replication of HIV-1. We now correlate the antiviral mode of action of ADA with chemical modification of the NCp7 zinc finger motif. Furthermore, we show that the antiviral action of ADA is primarily due to the virucidal action of the compound via modification of NCp7 proteins within intact virions and subsequent blockage of proviral DNA synthesis. We were unable to detect any appreciable post-transcriptional antiviral effect of ADA with U1 cells that contain stably integrated proviral DNA. This lack of a post-transcriptional effect of ADA is in contrast with the marked post-transcriptional inhibition observed for other NCp7 inhibitors such as DIBAs[11,16]. Through modification of the CCHC zinc finger domains of Gag and Gag-Pol precursors, DIBAs promoted formation of disulfide linkages between these molecules, thereby preventing the HIV-1 protease from recognizing them as substrates and blocking the processing of precursors to mature viral proteins[12,16]. The inability of ADA to modify the zinc fingers of the viral precursor polyproteins effectively may be the result of rapid intracellular reduction of ADA to the inactive biurea compound. The minimal decline in the infectious titer of virus released from the ADA-treated U1 cells is likely due to inactivation of the virus by the residual compound in the media after the virus particles are released from the cell. Thus, we suggest that the antiviral activity of ADA is due to its virucidal activity rather than as an intracellular effector of Gag-associated zinc fingers.

In summary, we have provided experimental evidence that ADA exerts its anti-HIV-1 effects by targeting the zinc finger domains of the HIV-1 NCp7 protein and promoting zinc ejection. This information should facilitate the evaluation of the therapeutic potential of ADA, which is currently in Phase I/II clinical trials in Europe for advanced AIDS[17]. Moreover, ongoing molecular modeling and medicinal chemistry-directed analog synthesis and evaluation should hopefully provide for more efficacious and stable azoic-based nucleocapsid inhibitors.

Methods

Virus replication inhibition assays. Anti-HIV activity was determined with various cell types and HIV-1 isolates using the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) cytoprotection assay, as previously described[13]. Phytohemagglutinin-stimulated human peripheral blood lymphocytes and fresh monocyte-macrophage cultures were prepared and utilized in antiviral assays as previously described[13]. Experimental compounds were obtained from the NCI chemical repository. U1, HeLa-CD4-LTR-βgal and 174 × CEM cells were obtained from the AIDS Research and Reference Program (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD, USA).

HIV-1 expression from TNF-α-induced U1 cells. Fifty-thousand U1 cells per ml were induced with 5 ng/ml of recombinant TNF-α (Sigma Chemical Co., St. Louis, MO) in the presence of the indicated concentrations of ADA. Cell-free supernatants were collected 72 h later for p24 ELISA and infectious units measurements and cell viability determined by XTT dye reduction. Infectious units in supernatants derived from TNF-α-induced U1 cells were determined by serial dilution onto 174 × CEM cells (5 × 10³), as described[16]. For analysis of viral proteins by western blot, U1 cells were stimulated with 5 ng/ml TNF-α in the presence of various doses of ADA and incubated for 72 h total. Lysates from 1×10^6 cells were resolved by 4–20 % SDS-PAGE, blotted onto PVDF membranes (Millipore, Bedford, MA), reacted with NCp7 and p24 antisera, probed with HRP-conjugated goat anti-rabbit IgG and viewed with western blot Chemiluminescence Reagent (Dupont NEN, Wilmington, DE) as described[16].

Virus attachment and enzymatic assays. Binding of HIV-1$_{RF}$ to CEM-SS cells was measured by a p24-based assay[19]. The effects of ADA on the *in vitro* activity of purified HIV-1 p66/p51 RT was determined by measurement of incorporation of [³²P]TTP onto the poly(rA):oligo(dT) (rAdT) or [³²P]GTP onto the poly(rC):oligo(dG) (rCdG) homopolymer template/primer systems (Recombinant DNA Laboratory, NCI-FCRDC, Frederick, MD), as previously described[22]. HIV-1 protease activity was quantitated by a reversed phase HPLC assay utilizing the Ala-Ser-Glu-Asn-Tyr-Pro-Ile-Val-Glu-Amide substrate (Multiple Peptide Systems, San Diego, CA) as previously described[10,13]. Processing of purified recombinant HIV-1 Pr55^{gag} by purified recombinant HIV-1 protease enzyme was measured as described[16]. The *in vitro* effects of ADA on 3'-processing and strand transfer activities of purified HIV-1 integrase were performed according to Bushman and Craigie[20] with minor modifications. The ability of compounds to inhibit the generation of poly (ADP-ribose) polymer formation by poly (ADP-ribose)polymerase was performed with purified protein and activated DNA according to the manufacturer's instructions (Trevigen, Inc., Gaithersburg, MD) using an assay kit.

Biochemical and NMR zinc finger assays. Fluorescence measurements of the Trp37 residue in the C-terminal zinc finger of the recombinant HIV-1 NCp7 protein were performed as previously described[14]. ^{1}H NMR studies of the HIV-1 NCp7 protein[3,10] were performed with a 1 mM solution of each the NCp7 and ADA in 30 mM deuterated sodium acetate buffer, pH 7.0 in 99.5% D₂O. Data were collected with a Bruker DMX (600.14 MHZ, ^{1}H) spectrometer. The sample was maintained at 20 °C with low power presaturation during relaxation delay to suppress residual H₂O signal. Spectra were processed with Silicon Graphics computers using the FELIX software package (version 95.0; Biosym Technologies, Inc.).

Actions of ADA on intact virions. Viral inactivation was determined by treatment of HIV-1$_{RF}$ with various concentrations of ADA for 1 h at 37 °C, centrifuged (18,000g for 1 h at 4 °C) to remove compound from the virus, resuspended and diluted two-fold serially, and 200 µl of each dilution plated in triplicate onto HeLa-CD4-LTR-βgal cell monolayers. Following incubation, fixation and staining, blue-stained cells (representing a single infectious unit) in each well were counted and the infectious units per ml of sample (mean ± s.d.) calculated[13,15]. The ability of ADA-treated HIV-1 to mediate reverse transcription in host cells was evaluated by PCR-based detection of proviral DNA as described previously[29]. To determine the effect of ADA on the NCp7 protein in intact virions, purified HIV-1$_{MN}$ was exposed to ADA for 1 h at 37 °C and then centrifuged to pellet the virus from the drug. Virus pellets were solubilized and viral proteins were resolved by non-reducing 4–20 % SDS–PAGE, probed with rabbit antisera to NCp7 protein, and visualized by

chemiluminescence via HRP-conjugated goat anti-rabbit IgG[11-13,15].

Molecular modeling and docking studies. Methods for docking of ADA onto the HIV-1 NCp7 structure[4] were described previously[14,19].

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