Zinc Ejection as a New Rationale for the Use of Cystamine and Related Disulfide-Containing Antiviral Agents in the Treatment of AIDS

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The highly conserved and mutationally intolerant retroviral zinc finger motif of the HIV-1 nucleocapsid protein (NC) is an attractive target for drug therapy due to its participation in multiple stages of the viral replication cycle. A literature search identified cystamine, thiamine disulfide, and disulfiram as compounds that have been shown to inhibit HIV-1 replication by poorly defined mechanisms and that have electrophilic functional groups that might react with the metal-coordinating sulfur atoms of the retroviral zinc fingers and cause zinc ejection. ^{1}H NMR studies reveal that these compounds readily eject zinc from synthetic peptides with sequences corresponding to the HIV-1 NC zinc fingers, as well as from the intact HIV-1 NC protein. In contrast, the reduced forms of disulfiram and cystamine, diethyl dithiocarbamate and cysteamine, respectively, were found to be ineffective at zinc ejection, although cysteamine formed a transient complex with the zinc fingers. Studies with HIV-1-infected human T-cells and monocyte/macrophage cultures revealed that cystamine and cysteamine possess significant antiviral properties at nontoxic concentrations, which warrant their consideration as therapeutically useful anti-HIV agents.

Introduction

All retroviruses encode a Gag polyprotein that is produced in the host cell during the late stages of the infectious cycle and is critical for viral assembly.[1] Approximately 2000 copies of Gag assemble at the cell membrane and bud to form an immature viral particle. Concomitant with budding, the Gag polyproteins are cleaved by the viral protease into several smaller proteins, including the matrix (MA, p17), capsid (CA, p24), and nucleocapsid (NC, p7) proteins.[2,3] During this maturation process, the matrix proteins form an icosahedral-like shell that remains associated with the viral membrane and helps anchor the extraviral glycoproteins, the capsid proteins condense to form the cone-shaped particle in the center of the virus that contains the viral RNA and essential enzymes, and the nucleocapsid proteins form a ribonucleoprotein complex with the RNA.[4−6]

As part of the Gag precursor protein, the NC domain functions in the recognition and packaging of the viral genome[7−11] and may be important for viral particle formation[12] and packaging of reverse transcription primer $tRNA^{Lys_3}$.[13] In the early stages of the virus replication cycle, the NC protein appears to be important for stabilizing the RNA,[14] recognition and annealing of $tRNA^{Lys_3}$ primer during reverse transcription,[15] and stabilizing proviral DNA.[16] NC is also capable of facilitating dimerization of nonhomologous viral RNA genomes.[17,18]

Except for the spumaviruses, all retroviral NC proteins contain one or two copies of a conserved CCHC zinc finger motif ($C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$; C = cysteine, H = histidine, X = variable amino acid residue).[19,20] Mutagenesis studies have demonstrated that the arrays play essential roles during several stages of the viral replication cycle (see, for example, refs 21−24), and spectroscopic studies of synthetic CCHC peptides[25−33] and native NC proteins[34−43] have shown that the arrays bind zinc with high affinity, leading to the formation of a compact, stable miniglobular fold. Furthermore, zinc-edge-extended X-ray absorption fine structure (EXAFS) studies have provided clear evidence that the arrays are populated with zinc in mature virions.[34,44] The arrays are thus attractive targets for potential drug intervention. Although these arrays are highly resistant to the removal of zinc by chelating agents such as EDTA, due to low dissociation constants (K_d ca. 10^{-12}−10^{-14} M),[31,32,37] they are susceptible to modification by antiviral agents that function by chemically modifying the metal-coordinating cysteine residues. Thus, we first demonstrated that the two zinc finger domains of the HIV-1 nucleocapsid protein are susceptible to modification by nitroso-containing antiviral agents,[45,46] and more recently we identified several new classes of zinc-ejecting agents with potential chemotherapeutic utility.[47−49] One of these compounds, a derivative of disulfide benzamide (DIBA),[48] is currently in clinical trials in the United States,[50] and another is an azoic-based compound[51] that is in clinical trials in Europe for advanced AIDS.[52]

In an attempt to expand the arsenal of retroviral zinc finger inhibitors, we searched for compounds that have been shown to inhibit HIV-1 replication by unknown mechanisms and that have functional groups that might electrophilically attack the metal-chelating sulfur atoms of the CCHC motif. This led to the identification of three disulfide-based compounds that fit these criteria, including disulfiram (also known as Antabuse or tetraethylthiuram disulfide, an FDA-approved drug that is widely used for the treatment of alcoholism), thiamine disulfide, and cystamine (see Figure 1). The present studies suggest that these antiviral agents function

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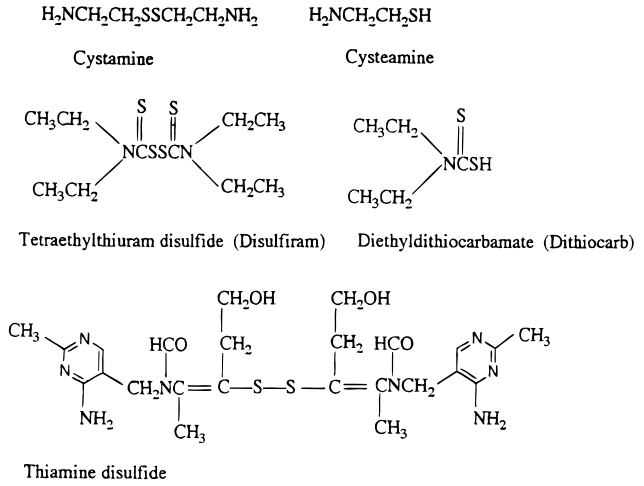

Figure 1. Structures of disulfide compounds and their thiol analogs used in the zinc-ejection studies.

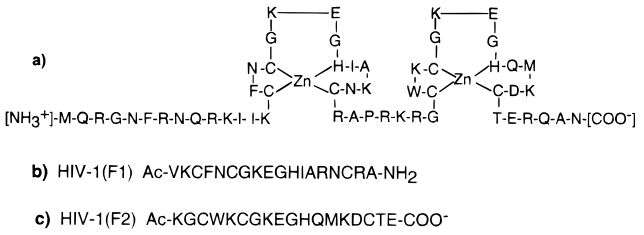

Figure 2. (a) Amino acid sequence and zinc coordination mode of HIV-1 nucleocapsid protein (MN strain). (b) Sequence of the synthetic peptide corresponding to the N-terminal zinc finger. (c) Sequence of the synthetic peptide corresponding to the C-terminal zinc finger.

by ejecting zinc from the HIV-1 NC protein. The reduced forms of disulfiram and cystamine, diethyldithiocarbamate (dithiocarb, dithiocarb sodium or Imuthiol) and cysteamine, respectively, were also evaluated since cysteamine was previously suggested to possess antiviral properties.[53]

Results

Interactions with Synthetic Zinc Finger Peptides. One-dimensional ^{1}H NMR spectroscopy was used to monitor potential interactions of cystamine, thiamine disulfide, and disulfiram with 18-residue synthetic peptides corresponding to the two CCHC zinc finger domains of the HIV-1 nucleocapsid protein (Figure 2). Both peptides (ZnF1 and ZnF2, which correspond to the amino- and carboxy-terminal zinc finger domains, respectively) were previously shown to bind zinc tightly and stoichiometrically, and structural studies by NMR demonstrated that the peptides adopt a fold identical to the corresponding regions of the native protein.[26,29,34,54,55] As illustrated in Figure 3, the addition of a 2-fold molar excess of cystamine, disulfiram or thiamine disulfide to ZnF1 resulted in the loss of signal due to zinc bound histidine, and the appearance of new peaks due to zinc-free histidine. In addition, the phenylalanine signal from the ring protons were broadened and shifted upfield (Figure 3), and the α proton signals downfield of water indicative of the folded structure disappeared (not shown) upon addition of the disulfide compounds. Similar results were obtained with ZnF2 (not shown). The spectral changes observed with the disulfide-reacted peptides are essentially identical to those observed upon treatment of the peptides with

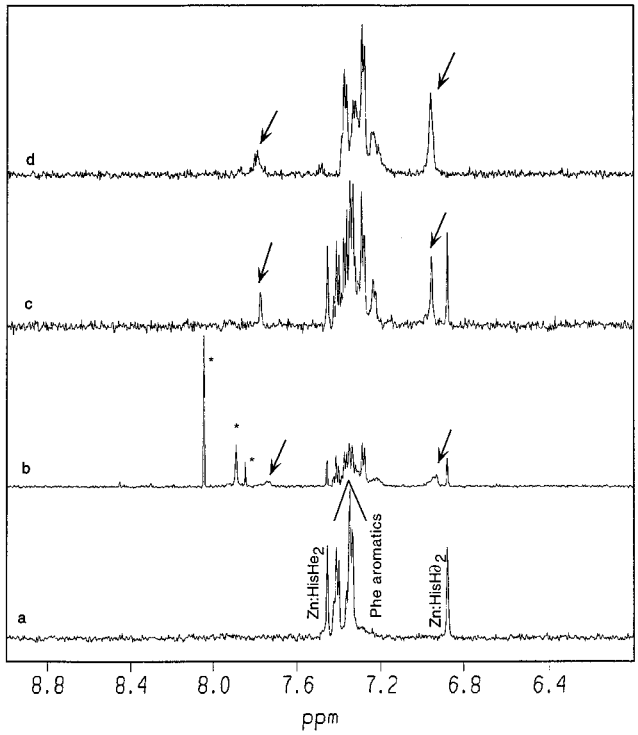

Figure 3. 600-MHz ^{1}H NMR studies of the addition of disulfide compounds to the synthetic peptide corresponding to N-terminal zinc finger from HIV-1 nucleocapsid protein. (T = 37 °C, pH = 7.0, 150 mM Na acetate). (a) NMR spectrum of the histidine and phenylalanine aromatic protons of ZnF1 in D_2O. (b) 48 h after the addition of a 2-fold excess of thiamine disulfide to ZnF1, a partial reaction is observed. Peaks corresponding to Zn-free histidine protons are marked with arrows. Broadening of the Phe aromatic signal is also observed. The peaks corresponding to thiamine disulfide aromatic protons are marked with an asterisk (*). (c) The addition of 2-fold cystamine at a time point of 21 h. More than 50% of the peptide is estimated to be zinc-free. (d) A complete dissociation of zinc from the peptide is observed after the addition of disulfiram. This spectrum was taken at 65 h after the initial reaction. Only Zn-free His and broad Phe aromatic protons can be observed.

large molar excesses of high-affinity Zn-chelating agents such as EDTA.

Similar ^{1}H NMR studies revealed that the reduced form of disulfiram, dithiocarb, was unable to remove Zn^{2+} from the peptide even after prolonged incubation. Cysteamine (the reduced form of cystamine) was also found to be ineffective at zinc ejection. However, the addition of cysteamine led to chemical shift changes for several peptide ^{1}H NMR signals, indicative of a weak cysteamine−zinc finger peptide interaction. Interestingly, cysteamine oxidizes to cystamine with half-life of only 1 day in the presence of ZnF1, compared to the measured half-life of 13 days in the absence of ZnF1. These findings suggest that the retroviral zinc finger is capable of catalyzing the oxidation of cysteamine to cystamine.

To further explore the nature of the cysteamine−ZnF1 interaction, ^{1}H NMR spectra were obtained for solutions containing ZnF1 with both cysteamine and cystamine. Zinc ejection from ZnF1, which proceeds rapidly upon addition of cystamine (see Figure 3c), was inhibited by the simultaneous addition of cysteamine. The combined findings indicate that cysteamine is capable of binding to ZnF1 but does not directly cause zinc ejection. They

further suggest that cysteamine and cystamine compete for the same "reactive site" on the peptide surface.

Interactions with Intact NC. Recent NMR and fluorescence studies have suggested that the CCHC zinc fingers interact with each other via hydrophobic packing, forming a single globular domain.[37,40,42,43] Although our laboratory has been unable to confirm this proposal, it remains possible that the reactivity of the CCHC zinc finger domains in the intact protein may differ relative to the reactivities of the peptides. Therefore, the reactivity of the full-length, recombinant HIV-1 NC protein was monitored by ^{1}H NMR using the same approach as was employed for the peptides. The δ and ϵ protons of His 23 and His 44 are resolved in the ^{1}H NMR spectra obtained at 37 °C, allowing direct measurement of the relative rates of zinc ejection from the two domains.

The results with the full NC protein were identical to those obtained with the truncated finger peptides. No appreciable difference in zinc-ejection rates between the two zinc fingers was observed with any of the disulfide compounds studied (Figures 4−6). As expected on the basis of the peptide results, cysteamine and dithiocarb were ineffective in terms of their ability to eject zinc from full-length NC. In addition, the intact protein was readily able to catalyze the oxidation of cysteamine to cystamine.

Prior to its oxidation, cysteamine was also able to inhibit the zinc-ejection reaction by cystamine with the complete protein as it did with the peptides. A 50-fold excess of cystamine and cysteamine was added to the NC protein and the reaction mixture monitored by NMR over a period of 1 month. More than 85% of the NC protein remained intact after 2 weeks of incubation. However, after a majority of the cysteamine in solution had been oxidized to cystamine (>3 weeks), zinc ejection occurred readily and the protein was converted entirely to the apo form.

***In Vitro* Activities.** NMR studies with cystamine, thiamine disulfide, and disulfiram indicated that each of these compounds promote metal ejection from the HIV-1 NC protein zinc finger domains. To evaluate the relative potential of these compounds as antiviral agents, each was tested for *in vitro* anti-HIV-1 activity against HIV-1_{RF} replication in CEM-SS cells by the XTT-based cytoprotection assay and against HIV-1_{ADA} replication in fresh human monocyte/macrophage cultures.

The data in Table 1 reveal that disulfiram does not exert *in vitro* anti-HIV activity. Thiamine disulfide exerted anti-HIV activity at concentrations close to 500 μM, consistent with earlier findings.[56] However, the compound was cytotoxic at concentrations above 500 μM, thus prompting our interpretation of poor efficacy as an antiviral agent. The most promising results were obtained for cystamine, which was found to inhibit HIV-1 replication for a lymphocytotropic strain (HIV-1_{RF}) in proliferating T-cell cultures and for a monocytotropic strain (HIV-1_{ADA}) in primary monocyte/macrophage cultures. In both cases, inhibition occurred at concentrations significantly lower than the cytotoxic concentrations (Table 1).

The thiols of cystamine and disulfiram were also tested for antiviral activity using the cellular assays described above. Dithiocarb was found to have no antiviral activity. Cysteamine, on the other hand, did exhibit significant antiviral activity. However, the therapeutic index of cysteamine was lower than that of its disulfide, cystamine. This result is consistent with recent findings by Bergamini et al.[53]

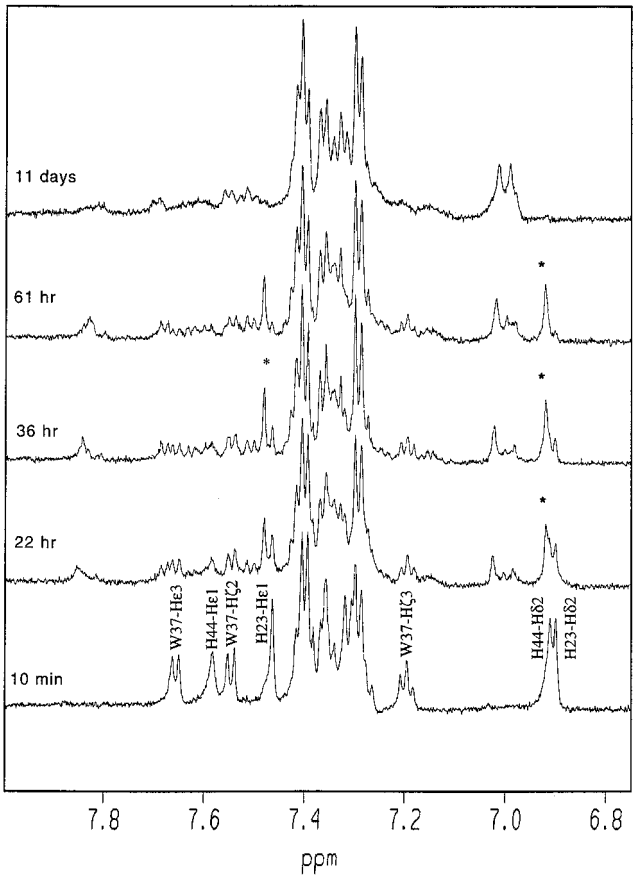

Figure 4. The time course with the addition of 2-fold cystamine to 1 mM NC at 37 °C, 150 mM sodium acetate, pH 7.0, in D_2O. ^{1}H NMR spectra shown are at time points of 10 min, 22 h, 61 h, and 11 days after the addition of cystamine. The gradual loss of zinc-bound histidine H_4 signal, to be replaced by a downshifted transient peak denoted by a star (*) and numerous peaks representing the zinc-free form. Zinc-bound H_2 signals from His 44 and His 23 are also lost over the time course, with the appearance of an transient signal (*) that is ultimately lost. These intermediate signals observed are thought to correspond to selective formation of internal disulfides due to different reaction rates by the zinc-coordinating cysteines. The final spectrum at 11 days with broadened line widths and complete disappearance of intermediates possibly represents cross dimerization or oligomerization via intermolecular disulfides by the apo-NC. The reaction rates for the N-terminal and C-terminal zinc fingers appear to be approximately equal based on the loss of intensity of the His-H^2 peaks for each finger.

To determine if any of the compounds evaluated in these studies might react with viral targets other than the NC zinc fingers, experiments were performed to determine their potential influence on the binding of HIV-1 virions to target cells and to test for inhibition of the HIV-1 reverse transcriptase and protease enzymes. Mechanistic studies revealed that none of the compounds inhibited attachment of HIV-1 to host target cells or the enzymatic activities of HIV-1 p66/p51 reverse transcriptase or protease (Table 1), in contrast to control compounds which demonstrated effective inhibition in the mechanistic assays.[49]

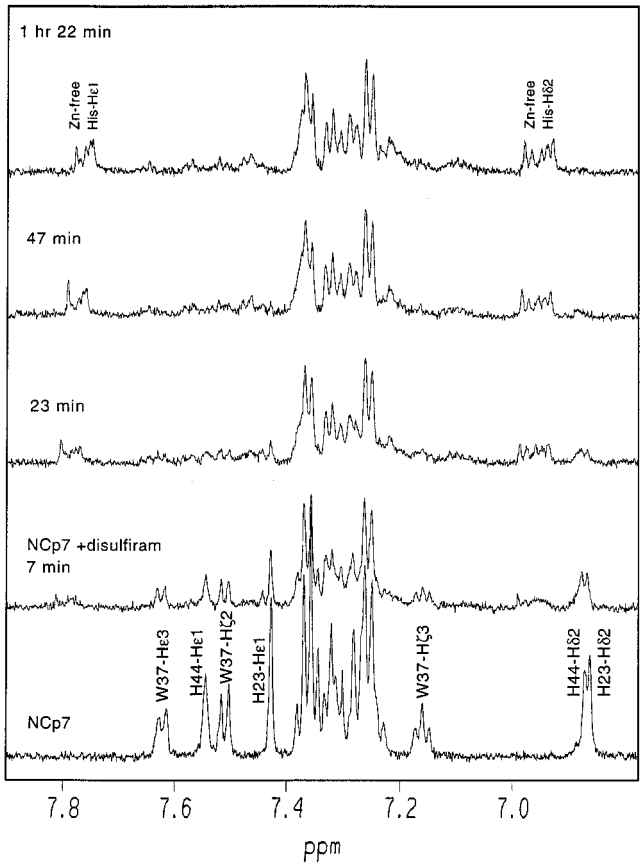

Figure 5. Rapid zinc-ejection reaction of disulfiram with NC. Reaction conditions are the same as in Figure 4. The first spectrum from the bottom is that of NC with no disulfide added. Time points are 7 min, 23 min, 47 min, and 1 h 22 min after the addition of disulfiram to purified NC protein.

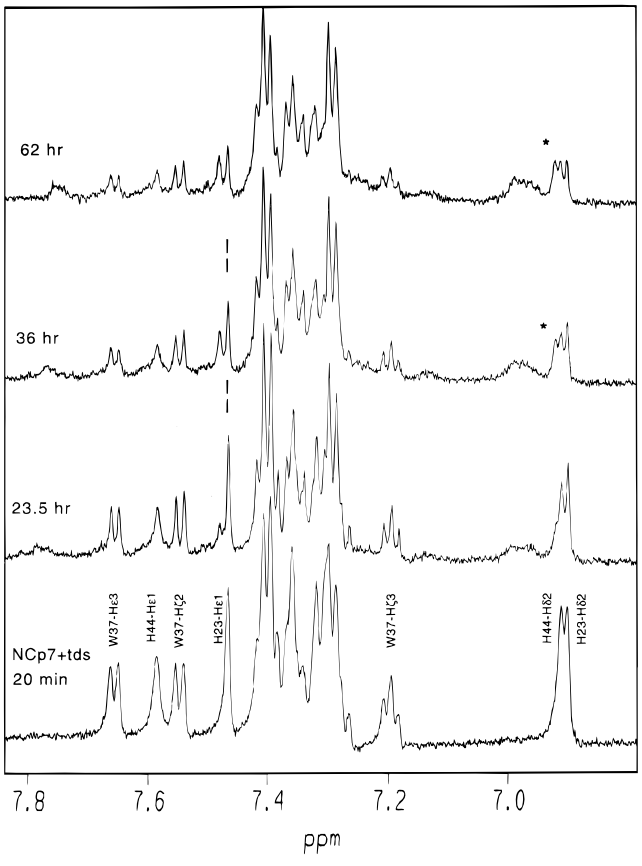

Figure 6. Zinc-ejection reaction of equimolar thiamine disulfide with NC, illustrating equal targeting of both N-terminal and C-terminal zinc fingers. Spectra include the time points at 20 min, 23 h 30 min, 36 h, and incomplete reaction at 62 h. This sample did not contain enough disulfide to react with the entire amount of protein. Thiamine disulfide converts to an inactive form as the zinc-ejection reaction takes place as determined by the shift of proton peaks not shown in this spectrum. Zinc-free histidine δ peaks are denoted by a star (*).

Discussion

The viability of the HIV-1 nucleocapsid protein as a target for chemotherapy was first demonstrated with the use of nitroso-containing antiviral agents.[45,46] We demonstrated that the nitroso agent, 3-nitrosobenzamide (NOBA), efficiently ejects zinc from the CCHC zinc finger domains of the NC protein. In addition, treatment of virions with nitroso compounds led to loss of viral zinc and infectivity, and treatment of HIV-1-infected peripheral blood lymphocytes led to loss of virus production at noncytotoxic doses.[45] It was further shown that NOBA functions by forming covalent adducts with the cysteine thiolates, leading to spontaneous oxidation of NC (in which the six cysteine residues form Cys−Cys disulfide bonds) and loss of zinc-binding ability.[57]

Later, we found that select electrophilic disulfide-based compounds could mediate a thiol−disulfide exchange with the nucleophilic cysteine sulfurs of the NC zinc finger domains and cause zinc ejection. One such class of compounds found in the National Cancer Institute's chemical repository was the disulfide benzamides (DIBAs, synthesized by Parke-Davis Pharmaceuticals), which were shown to exert anti-HIV activity through a direct action on the NC zinc finger domains. Specifically, the DIBAs were shown to eject zinc from the NC protein using a fluorescence-based assay, to inhibit both acute and chronic *in vitro* HIV-1 infections, to be highly synergistic in combination with other antiviral agents, and to be directly virucidal.[48] (Note that, although it was later suggested by Tummino et al.[58] that the fluorescence-based procedure for monitoring zinc ejection by the DIBA compounds was flawed

Table 1. Antiviral and Mechanistic Activities of Disulfide Compounds

	antiviral assays[a] (μM)				mechanistic assays[b] I_{50} (μM)		
	XTT assay		Mo/Mϕ assay				
compound	EC_{50}	IC_{50}	EC_{50}	IC_{50}	binding	RT	protease
cystamine	56.2	172	4.3	>100	NI	NI	NI
cysteamine	41.6	71.0	53.7	>100	NI	NI	NI
thiamine disulfide	I	535	I	>100	NI	NI	NI
disulfiram	I	3.9	I	>100	NI	NI	NI
dithiocarb	I	>100	I	>100	NI	NI	NI

[a] Antiviral assays using the XTT cytoprotection procedure or using primary monocyte/macrophage (Mo/Mϕ) cultures were performed as previously described.[60,75,76] EC_{50} values indicate the drug concentration that protected 50% of CEM-SS cells from the cytopathic effects of HIV-1_{RF} for the XTT assays and provided a 50% reduction in viral p24 production by HIV-1_{ADA} in Mono/Mϕ cultures. "I" indicates that no antiviral activity was observed at or below the concentration of compound that caused cellular toxicity. IC_{50} values reflect the drug concentrations that elicit 50% cell death. [b] Binding of HIV-1 to CEM-SS cells, and the effects of compounds on HIV-1 reverse transcriptase (using both the rAdT and rCdG template/primer systems) and protease were quantitated as described in the Experimental Section. "NI" indicates that no inhibition was observed at the highest test concentration (100 μM).

due to potential fluorescence interference or light scattering by the DIBA compounds, that criticism is invalid because the samples were actually diluted prior to the fluorescence measurements in order to prevent artifactual fluorescence or light-scattering interference.) We subsequently demonstrated that modification of the NC protein by the DIBA compounds inhibits reverse transcription[59] and also prevents the formation of infectious virus from previously HIV-1-infected cells (chronic and latent infections) by modifying the zinc fingers of the NC domain within the Gag precursor polyprotein during the late phase of virus replication. Modification of the Gag polyprotein prevented the HIV-1 protease enzyme from recognizing it as a substrate, thereby preventing processing of the Gag to mature viral proteins.[60] Thus, we were the first to show that NC inhibitors, such as disulfide benzamides, cause zinc ejection from the NC zinc fingers, block the early phase of infection by preventing reverse transcription (without affecting the RT enzyme), and block the late phase of viral replication by preventing processing of the Gag molecules (without affecting the protease enzyme).

With a better understanding of the potential biological consequences of NC inhibitors, we began to search for other types of compounds that might inhibit HIV-1 replication by selective modification of the NC protein zinc fingers. One such azoic-based compound, azodicarbonamide (ADA), was found to inhibit *in vitro* HIV replication by targeting the NC protein and exerting a direct virucidal effect.[51] Interestingly, it had been previously reported by Vandevelde et al.[52] that ADA inhibits HIV-1 through an undetermined mechanism and that ADA had been taken to clinical evaluation in Europe for the treatment of advanced AIDS. Thus, ADA appears to be the first known NC inhibitor to enter clinical trials. These findings prompted us to determine if any other previously described anti-HIV agents might also mediate their mechanism of antiviral action by targeting the NC zinc fingers.

Treatment of HIV-1-infected lymphocytes and monocytes with cystamine has been shown to lead to the release of noninfectious virions that are defective with respect to their assembly and budding parameters,[61] and it was suggested that oxidation of free SH groups on cysteine residues of viral proteins might affect protein structure and function.[61] It was later proposed that the antiviral activity of cystamine may be due to an antioxidant effect, in which the tumor necrosis factor-α (TNF-α) mediated signal transduction pathway for virus synthesis is inhibited.[62] Neither of these hypotheses provide an adequate explanation for the cellular effects observed with the treatment of HIV-infected cells with cystamine, namely, the formation of noninfectious and RNA defective viral particles and the inhibition of proviral DNA formation in early infection.

The viral phenotype obtained upon treatment of infected cultures with cystamine is very similar to results obtained by Aldovini et al., wherein mutation of the cysteine residues of HIV-1 NC zinc fingers led to the production of noninfectious viral particles with normal protein content but which exhibited defective RNA packaging.[11] Cystamine was also found to inhibit proviral DNA formation in the early stages of viral infection. All of these findings are consistent with the interruption of NC zinc finger function during the early events in viral infection. We therefore hypothesized that the target of cystamine in HIV-infected cell lines may be the cysteine sulfur atoms of the CCHC zinc fingers.

We have confirmed the antiviral properties of cystamine and have provided definitive evidence that cystamine readily ejects zinc from the HIV-1 NC CCHC zinc fingers *in vitro*. Cystamine does not inhibit the binding of virions to target cells, nor does it inhibit the viral reverse transcriptase or protease enzymes. These data provide strong experimental evidence that the antiviral activity of cystamine is due to a zinc-ejecting mechanism. The results are highly reminiscent of those obtained previously for zinc-ejecting nitroso and aromatic disulfide-containing antiviral agents, which have been shown to eject zinc by undergoing electrophilic attack on the zinc-coordinated cysteine thiolates.[45−48]

Reports of the anti-HIV-1 activity of cysteamine indicate that it is ineffective in inhibiting *in vitro* replication of HIV-1 in chronically infected T-cell and macrophage cell lines, but that it possesses an antiviral activity that occurs prior to proviral DNA synthesis in acutely infected lymphocytes and macrophages and has an additive antiviral effect when used in combination with Zidovudine and didanosine. In contrast to cystamine, cysteamine was found to have an inhibitory effect only in the early phases of viral infection by suppressing proviral DNA formation. Clinical trials of cysteamine in combination with Zidovudine are reportedly in progress (National Institutes of Health AIDSTRIALS Database FDA/00583: A Phase II/III Study of Cysteamine and Zidovudine for the treatment of HIV disease). We have confirmed the anti-HIV activity of cysteamine, and demonstrate that its therapeutic index is below that of cystamine. In addition, our NMR studies reveal that cysteamine does not eject zinc either from the 18-residue zinc finger peptides or from the intact NC protein. However, chemical shift changes observed upon addition to cysteamine indicate that the compound binds to the zinc finger domains and, furthermore, that the zinc fingers catalyze the oxidation of cysteamine to cystamine. The observed antiviral activity of cysteamine may thus be due to the zinc-ejecting activity of the oxidation product, cystamine. Alternatively, since cysteamine is capable of interacting with the CCHC zinc fingers, it is also possible that this interaction disrupts an essential zinc finger function such as the recognition, packaging, or annealing of the viral genome.

Thiamine disulfide was previously shown to possess anti-HIV activity, and it was suggested that this agent inhibits HIV-1 replication by oxidation of cysteine residues of the HIV-1 Tat protein.[56] No clinical evaluations of the *in vivo* effects of thiamine disulfide on HIV-1 infection have been reported. Our NMR data demonstrate that thiamine disulfide is capable of ejecting zinc from the HIV-1 CCHC zinc fingers. However, the effective doses necessary for inhibitory activity in cell cultures is only slightly below the levels that produce toxicity, and the utility of thiamine disulfide as a new therapeutic protocol thus remains speculative.

Disulfiram and its primary *in vivo* metabolite, diethyl dithiocarbamate, have been clinically evaluated against HIV-1 infection because of their immunoregulatory properties.[63−70] These small-scale trials reported minor effects on disease progression in HIV-1 infected indi-

viduals, but no truly compelling data from larger scale trials of any conclusive benefit of disulfiram or dithiocarb have come forth. Disulfiram and several thiuram analogs scored positive as zinc ejectors in our fluorescence assay,[47] and disulfiram was effective at ejecting zinc from both of the NC zinc fingers in the NMR assay. Thus, it is possible that the benefits observed in the limited clinical trials were due to an antiviral rather than an immunoregulatory effect.

The combined results suggest possible therapeutic benefits of cysteamine, cystamine, thiamine disulfide, and disulfiram for the treatment of AIDS due to their ability to eject zinc from the HIV Gag and NC proteins. These compounds might be used as part of a multidrug regimen that includes nucleoside and non-nucleoside reverse transcriptase inhibitors and protease inhibitors, attacking the retrovirus from diverse directions, thereby reducing the possibility of emergence of resistant strains. Cystamine has not directly been used on humans, although it exists in the human body naturally as a metabolite in amino acid biosynthesis. Cystamine has been extensively studied in animals as a free radical scavenger and a radioprotectant.[71−73] Cysteamine has been used on humans, including in the long-term treatment of organic acidemias, and is known to be relatively nontoxic.[74] Its main side effects are gastrointestinal upset and its foul taste and odor associated with the extended use. Although thiamine disulfide and disulfiram did not demonstrate anti-HIV activity in our cellular system, they clearly have zinc-ejecting capability, and medicinal chemistry modification of these compounds may result in the discovery of other disulfides that are safe and effective.

Experimental Section

Protein Expression and Purification. The NC protein was overexpressed in *Escherichia coli* using the T7 promoter system and purified by ion exchange and gel filtration chromatography. Purity was assessed by SDS−PAGE. Details of the cloning and purification will be published elsewhere.

Peptide Synthesis. The 18-residue peptides HIV1-F1 and HIV1-F2 were synthesized using standard methods employing *t*-Boc amino acids and purified by HPLC. Results from amino acid analysis were consistent with the correct peptides. Zn-coordinated peptide was prepared as described previously.[26]

Materials. Experimental compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI). 3′-Azido-2′,3′-dideoxythymidine 5′-triphosphate (AZTTP) and the Uniroyal non nucleoside reverse transcriptase inhibitor, UC38, were obtained from the Developmental Therapeutics Program (DTP), Division of Cancer Treatment, Diagnosis and Centers (DCTDC), National Cancer Institute (NCI). CEM-SS cells were obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, MD) and were maintained in RPMI 1640, 10% FCS, 20 μg/mL gentamicin, 200 mM L-glutamate.

Anti-HIV-1 Assays. Determination of the anti-HIV activity of experimental compounds was performed with the CEM-SS human T-cell line and HIV-1_{RF} at an MOI = 0.01. The microtiter assay measures the concentration dependent capacity of compounds to protect the cells from cytopathic effects of the virus. Cytoprotection was quantitated by XTT dye reduction, as previously described.[75] Effective antiviral concentrations providing 50% cytoprotection (EC_{50}) and cellular growth inhibitory concentrations causing 50% cytotoxicity (IC_{50}) were calculated. 3′-Azido-2′,3′-dideoxythymidine (AZT) and dextran sulfate were utilized as positive control compounds for anti-HIV-1 activity.

Monocytes (>95% positive by nonspecific esterase staining) were cultured for 7 days in AIM V medium (GIBCO BRL, Rockville, MD) supplemented with 1% pooled AB + serum (Sigma Chemical Co., St. Louis, MO), after which the monocyte/macrophage (Mo/Mϕ) cultures were infected with the monocytotropic HIV-1_{ADA} viral strain (MOI = 0.1 to 0.01) as previously described.[60,76] After a 2 h virus adsorption, fresh medium containing the indicated concentration of reagent was added. Cultures were fed every 3 days by 1/2 volume replacement with replenishment of the test reagent. At 12 days of infection, the cells were analyzed for cell viability by XTT dye reduction, microscopic evaluation for multinucleated giant cell formation, and the level of virus-associated p24 in cell-free supernatants was quantitated. Effective antiviral concentrations providing 50% reduction in p24 production (EC_{50}) and causing 50% cytotoxicity (IC_{50}) were calculated. The p24 assays were performed using p24 ELISA kits obtained through the common services of the AIDS Vaccine Program (NCI-FCRDC, Frederick, MD).

Virus Attachment and Enzymatic Assays. Binding of HIV-1 to target cells was measured by a p24-based assay.[46] Various concentrations of experimental compounds were preincubated with a concentrated stock of virus (HIV-1_{RF}) for 2 h at 37 °C. The treated virus samples were then mixed with 2 $\times$ 10^5 CEM-SS cells and incubated for an additional 1 h at 37 °C, after which the unbound virus was washed away and the cell-associated virus was solubilized in 1% Triton X-100 and 1% BSA and analyzed by the p24 antigen capture assay.

The effects of compounds on the *in vitro* activity of purified recombinant HIV-1 p66/p51 reverse transcriptase (kindly provided by Steve Hughes, ABL-Basic Research Program, NCI-FCRDC, Frederick, MD) were determined by measurement of incorporation of [^{32}P]dTTP onto the artificial poly(rA):oligo(dT) homopolymer template/primer system or [^{32}P]dGTP onto the poly(rC):oligo(dG) system.[49] Samples were precipitated with 5% trichloroacetic acid (TCA), harvested onto glass fiber filters (Packard, Meridan, CT), washed with 10% TCA, and then quantitated on a Packard Matrix 9600 direct beta counter. 3′-Azido-2′,3′-dideoxythymidine 5′-triphosphate (AZTTP) and Uniroyal nonnucleoside RT inhibitor, UC38, served as positive controls. HIV-1 protease activity was quantitated by an HPLC assay utilizing the Ala-Ser-Glu-Asn-Tyr-Pro-Ile-Val-Glu-Amide substrate (Multiple Peptide Systems, San Diego, CA) as previously described.[46]

NMR Spectroscopy. ^{1}H NMR studies of the HIV-1 NC protein and analogous peptides were performed with approximately 1 mM protein in 150 mM deuterated sodium acetate buffer at pH 7.0 in 99.5% D_2O. The drug concentrations were typically 2-fold of the peptide and the protein, with the exception of samples containing both cysteamine and cystamine where higher drug concentrations were used. Data were collected with Bruker DMX and GE Omega PSG 600 MHz (^{1}H) utilizing Bruker triple resonance probes. Samples were maintained at 37 °C during and between the collection of spectra. The spectra contained 16K complex points and were processed using a squared-sinebell window function prior to Fourier transformation. Silicon Graphics computers with the Felix software package (version 95.0, Biosym Technologies, Inc., San Diego, CA) were utilized for data processing.

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