Inactivation of HIV-1 Nucleocapsid Protein P7 by Pyridinioalkanoyl Thioesters

CHARACTERIZATION OF REACTION PRODUCTS AND PROPOSED MECHANISM OF ACTION*

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The synthesis and antiviral properties of pyridinioalkanoyl thioester (PATE) compounds that target nucleocapsid p7 protein (NCp7) of the human immunodeficiency virus type 1 (HIV-1) have been described previously (Turpin, J. A., Song, Y., Inman, J. K., Huang, M., Wallqvist, A., Maynard, A., Covell, D. G., Rice, W. G., and Appella, E. (1999) J. Med. Chem. 42, 67–86). In the present study, fluorescence and electrospray ionization-mass spectrometry were employed to determine the mechanism of modification of NCp7 by two lead compounds, N-[2-(5-pyridiniovaleroylthio)benzoyl]sulfacetamide bromide and N-[2-(5-pyridiniovaleroylthio)benzoyl]-4-(4-nitrophenylsulfonfonyl)aniline bromide (compounds 45 and 47, respectively). Although both compounds exhibit antiviral activity in cell-based assays, we failed to detect appreciable ejection of zinc from NCp7 under conditions in which previously described NCp7-active disulfides readily eject zinc. However, upon “activation” by Ag+, compound 45 reacted with NCp7 resulting in the zinc ejection from both zinc fingers. The reaction followed a two-step mechanism in which zinc was ejected from the carboxyl-terminal zinc finger faster than from the amino-terminal zinc finger. Both compounds covalently modified the protein with pyridinioalkanoyl groups. Compound 45 modified cysteines 36 and 49 of the carboxyl-terminal zinc finger. The results obtained herein demonstrate that PATE compounds can be constructed that selectively target only one of the two zinc fingers of NCp7, thus providing an impetus to pursue development of highly selective zinc finger inhibitors.

Development of drug-resistant HIV1 strains in response to therapy with inhibitors of the viral reverse transcriptase (1–3) and protease enzymes (4, 5) has necessitated the search for novel antiretroviral agents that are directed against new molecular targets. The involvement of HIV-1 NCp7 zinc fingers in multiple phases of the HIV-1 replication cycle and their mutationally non-permissive nature has provided incentives for choosing this protein as a target for antiretroviral therapy. Moreover, mutations or modifications of either the conserved zinc chelating or non-chelating residues have resulted in loss of NCp7-mediated activities, including rendering the HIV non-infectious (6–8). These observations gave impetus to explore several types of organic compounds that selectively target NCp7 protein. First among them being 3-nitrosobenzenamide (9) followed by a series of 2,2′-dithiobis(benzamide) disulfides (DIBA) (10) and azodicarbonamide (11) that inhibited a wide range of HIV-1 isolates. Even though 2,2′-dithiobis(benzamide) disulfides represent a new class of highly specific antiretroviral agents, the disulfide bond is susceptible to reduction in vivo, resulting in the loss of antiviral activity. To circumvent this problem, we synthesized novel pyridinioalkanoyl thioester (PATE) derivatives (12). Of various such compounds synthesized, two of them, compounds 45 and 47 (Fig. 1), showed superior antiviral activity. Both compounds were specific for NCp7, demonstrated antiviral activity in the presence of reduced glutathione, and showed minimal cytotoxicity. A close examination of their antiviral activities suggested that they act at different stages of viral replication. Compound 47 penetrated tumor necrosis factor-α induced U1 cells where it initiated Gag precursor cross-linking and inhibition of precursor processing. However, this compound was unable to initiate cross-linking of NCp7 protein within cell-free virions. In contrast, compound 45 did not inhibit the Gag precursor processing, and its anti-viral activity was ascribed to its ability to produce extensive cross-linking of NCp7 protein in cell-free virions (12).

The purpose of the current study was to determine the mechanism of action of PATEs and to determine the sites of modification on the NCp7 protein. For this purpose, a fluorescence-based assay using a zinc-specific fluorophore, Newport Green (NPG), and mass spectrometry were used to follow the kinetics of zinc ejection and to determine the site(s) of covalent modification on the target protein.

**EXPERIMENTAL PROCEDURES**

NCp7 Purification and Reconstitution—Recombinant NCp7 was purified using a pET3A-expressing NCp7 plasmid propagated in Escherichia coli strain BL21(DE3) pLYsE. Briefly, the cells were grown at 37 °C in the presence of 100 μM ampicillin and 34 μM chloramphenicol to an absorbance of 0.5 at 600 nm. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. After 3 h, the cells were harvested by centrifugation (13), lysed with buffer consisting of 50 mM Tris-HCl, pH 6.0, 10% (v/v) glycerol, 0.1 mM NaCl, 0.1 mM ZnCl2, 5 mM dithiothreitol, 2 mM EDTA, and a protease inhibitor mixture (Roche Molecular Biochemicals). After clearing the debris by centrifugation, the supernatant was acidified with acetic acid (to a final 10% v/v), centrifuged again, and loaded onto a reverse phase C8 HPLC column (Vydac, Hesperia, CA). The chromatogram was developed using a linear gradient of 0–55% acetonitrile, 0.04% trifluoroacetic acid.
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FIG. 1. Structures of the pyridinioalkanoyl thioesters used in this study and a schematic showing the sequence of NCp7. The apoprotein has a mass of 6369 ± 1 mass units.

NCp7 eluted at about 25% acetonitrile. The purity and integrity of the protein was confirmed by electrospray ionization mass spectrometry on a Finnigan MAT SSQ 7000 (San Jose, CA) mass analyzer. The apoprotein had a molecular mass of 6369 ± 1 Da (calculated mass is 6369.4 Da). Lyophilized NCp7 was reconstituted with 20 mM sodium phosphate buffer, pH 7.2, containing 10% (v/v) glycerol and 0.1 mM ZnCl₂. Excess zinc was removed using a Centricron-3 ultrafiltration unit (M₆ cut-off 3000 Da, Amicon), and the resulting reconstituted NCp7 was used in all the studies.

Peptide Synthesis—Peptides corresponding to amino-(12–20) and carboxyl-(22–52) terminal zinc fingers were synthesized by the solid phase method with Fmoc chemistry using an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, CA). Peptides were cleaved from the resin, and side chain protecting groups were removed by incubating in reagent K (trifluoroacetic acid/phenol/thiophenol/H₂O/EDT, 82.5:5:5:5:2.5) for 3 h at room temperature. The peptides were purified by HPLC on a pH-stable C-9 column (Vydac, Hesperia, CA). The masses of the peptides were confirmed using Finnigan MAT SSQ 7000 mass analyzer.

NCp7 Zinc Ejection Monitored by NPG Fluorescence—The zinc ejection assay buffer consisted of 10% (v/v) glycerol, 20 mM sodium phosphate buffer, pH 7.2. Zinc ejection was monitored by following the increase in the fluorescence of a zinc selective fluorophore, Newport Green (10 μM) (Molecular Probes, Eugene OR), in the assay buffer at room temperature. Under these conditions, NPG fluorescence increases linearly with increasing zinc concentration over the range 0.1 to 2 μM ZnCl₂. The effect of zinc concentration on NPG fluorescence was essentially unchanged upon increasing NPG to 20 μM, while it decreased 10% upon increasing the phosphate concentration to 30 mM. Lowering the phosphate concentration to 10 mM did not significantly affect NPG fluorescence. Therefore, 10 μM NPG and 20 mM phosphate were used as the standard condition. Zinc ejection was initiated by the addition of PATEs in dimethyl sulfoxide (5–80 μM) final concentration to NCp7 (1 μM) in assay buffer containing 10 μM NPG at room temperature (24 ± 1°C). The increase in fluorescence at 540 nm (λₑₓ = 490 nm) was monitored as a function of time using an Aminco Bowman Series 2 Luminescence Spectrometer. In experiments in which silver nitrate (20 mM) was added and incubated for 3 h at 37°C, mass spectrometric data for the clostripain peptides were obtained from collision-induced dissociation (CID) spectra using a Finnigan-MAT LCQ ion trap instrument equipped with electrospray interface (ESI) after introduction via a polymide-coated fused silica microcapillary reverse phase-HPLC system (14). The mass spectrometer was set for analyzing the positive ions and was operated on either double or triple play mode in which the instrument was set up to automatically acquire: 1) a full scan, 2) a ZoomScan of the (M+nH)⁺ ion above a preset threshold, and 3) a tandem MS/MS spectrum (relative collision energy = 50%) from that ion. ZoomScan was not monitored during the double play mode. The observed masses resulting from CID were compared with the predicted pattern of NCp7 fragmentation generated by the program Protein Prospector (University of California, San Francisco, CA).

RESULTS

Zinc Ejection from NCp7 by the Pyridinioalkanoyl Thioesters—Since the PATEs analyzed in this study had a very high absorbance at 280 nm, the commonly used method of monitoring the rate of zinc ejection by following the decrease in tryptophan fluorescence (excitation at 280 nm) was not feasible (12, 15). Instead, a method of monitoring zinc ejection directly using NPG was adopted. NPG exhibits an increase in fluorescence upon binding to Zn²⁺. Upon the addition of NCp7, there was a small but definite increase in the NPG fluorescence, which remained constant over the monitored time course. Addition of either compound 45 or 47 (5–80 μM) (N-[2-(5-pyridiniovaleroyl)]benzoyl)sulfacetamide bromide and N-[2-(5-pyridiniovaleroyl)]benzoyl-4-(4-nitrophenylsulfonyl)aniline bromide, compounds 45 and 47, respectively) did not result in any apparent release of zinc from NCp7. Even after prolonged incubation (18 h) in the presence of these compounds, zinc remained bound to NCp7 as demonstrated by the subsequent rapid ejection of zinc upon addition of dithiane 1,1-dioxide (16), a previously established NCp7 inhibitor (Fig. 2). This result suggested that the PATEs alone may be considerably less reactive than the diisulfide and may require “activation” in vitro. Thioesters are selectively activated by silver ions, a method routinely employed in large segment condensation in peptide synthesis (17, 18). Therefore, we evaluated the effect of silver and other metal ions to “activate” the PATEs. Zinc ejection was initiated by the addition of compound 45 (5–80 μM) to NCp7 (1 μM) in zinc ejection assay buffer containing 10 μM NPG and 20 μM AgNO₃. The zinc ejection was rapid and appeared to be complete by 5 min. Silver ions alone were unable to eject zinc...
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Kinetics of Zinc Ejection by Compound 45—

Compound 45, in final concentrations of 5–80 \( \mu M \), was added to 1.0 \( \mu M \) NCp7, 20 \( \mu M \) Ag\(^{1+} \), and 10 \( \mu M \) NPG in zinc ejection assay buffer. The increase in NPG fluorescence was measured immediately. Panel A shows data obtained at three concentrations of compound 45 with symbols ■, ▼, and ● representing 5, 30, and 60 \( \mu M \) compound 45, respectively. The data were fit to the double-exponential equation: 

\[
y(t) = A + B[1 - \exp(-k_{1}\text{obs}t)] + C[1 - \exp(-k_{2}\text{obs}t)]
\]

and gives a good fit to the equation. Panel B shows a plot of the apparent rate constants \( k_{\text{obs}} \) versus the concentration of compound 45. Closed and open circles represent \( k_{1\text{obs}} \) and \( k_{2\text{obs}} \), for the fast and slow phases, respectively. The solid line represents the least-squares fit of the fast time constant to a steady state mechanism describing reversible formation of an intermediate followed by release of 1 eq of zinc. The dashed line represents the mean value of the slow time constant.

The overall yield of the reaction, as indicated by the plateau value of the relative fluorescence shown in Fig. 3A, is distinctly lower at the lowest concentration of compound 45 and increases with increasing concentration to a limiting value. The kinetic model described by Equations 1 and 2 contains sequential irreversible reactions preceded by a reversible binding step. For reactions containing limiting amounts of NCp7, this model predicts that the eventual extent of the reaction should be the same, regardless of the concentration of compound 45, although the rate of the reaction is dependent on compound 45 concentration. The low amount of relative fluorescence initially established in a reaction containing 5 \( \mu M \) compound 45 increases rapidly to the limiting plateau value upon subsequent addition of compound 45 to a final concentration of 60 \( \mu M \) (data not shown). This result indicates that the limiting plateau values obtained at low compound concentrations do not result from irreversible adsorption of the protein or precipitation of silver ion. The lower plateau values obtained at low compound concentration may be the consequence of the requirement for activation of the compound, which for unknown reasons is less efficient at low concentrations. We note, however, that similarly reduced plateau values have been reported for inactivation of NCp7 by lower concentrations of the unrelated disulfide benzamides, which do not require activation (15).

To identify which zinc finger of NCp7 reacted rapidly with compound 45, peptides corresponding to the amino- and carboxyl-terminal zinc fingers (residues 13–30 and 32–52, respec-

The large separation in the time constants of the initial fit to the zinc release data suggested that the two phases were largely distinct and could be considered separately. Applying a standard steady-state assumption for the intermediate I results in,

\[
k_{\text{obs}}^{-1} = k_{-1} + k_{2}\frac{1}{[D]} \frac{1}{k_{0}}
\]

in which \([D]\) is the concentration of compound 45. The inverse of the observed rate constant is indeed linear with the inverse of the concentration of compound 45 for the five highest concentrations and exhibits a distinctly non-zero intercept (not shown). From this analysis, \( k_{-2} = 0.125 \text{ s}^{-1} \) and \( k_{2}/(k_{-1} + k_{2}) = 2.2 \times 10^{4} \text{ M}^{-1} \) for the faster phase of zinc ejection. The expected dependence of the rate constant \( k_{\text{obs}}^{-1} \) on the concentration of compound 45 is shown in Fig. 3B as a solid line and gives a good fit to the observed rate constants for the faster phase. The observed rate constants are somewhat faster than expected for the two lowest concentrations of compound 45, the sort of deviation expected when pseudo-first order conditions are not strictly met. The value of \( k_{0}^{-1} \) is determined from the average value of \( k_{2\text{obs}}^{-1} \) and is shown in Fig. 3B as a dashed line, indicating the lack of dependence of the observed rate constant on the concentration of compound 45. Application of the kinetic model described by Equations 1 and 2 without assuming a steady state did not lead to a significantly improved fit to the data.
Compound 45, in final concentrations of 5–80 μM, was added to 2.0 μM amino- or 1.0 μM carboxyl-terminal zinc finger peptide, in zinc ejection assay buffer containing 20 μM Ag⁺ and 10 μM NPG. The increase in NPG fluorescence was monitored immediately. Panel A, zinc ejection from the NH₂-terminal zinc finger peptide by compound 45. Symbols, • and ▲ represent zinc ejection by dithiane 1,1-dioxide (40 μM) and compound 45 (40 μM), respectively. Panel B, zinc ejection profiles obtained with carboxyl-terminal zinc finger peptide. The symbols ▲, ■, and ● represent the zinc ejection in presence of 10, 20, and 40 μM compound 45. The solid lines represent the fit to a single exponential characterized by the rate constant k_{e,j}. Panel C, dependence of the observed rate constant on the concentration of compound 45. The observed rate constants determined are indicated as filled circles. The solid line represents the least-squares fit to a steady state mechanism.

The exact site of the modification was determined by CID of the modified residues by tandem mass spectrometry. The NCp7 product produced by reaction with compound 45 was subjected to proteolytic digestion with clostripain, and the digest was analyzed by LC/MS/MS. Clostripain, which cleaves on the carboxyl side of arginine, is expected to cleave the protein into three large and three small peptides. Table II lists the ions and sequence positions obtained. The mass of the fragment corresponding to the carbonyl-terminal zinc finger, amino acids 32–52, indicated addition of two pyridinioalkanoyl groups (Δm = 162 ± 1 Da per group), respectively. Interestingly, although no appreciable zinc ejection was observed with compound 47 even after a 1-h incubation, a minor amount of NCp7 modified with one or two pyridinioalkanoyl groups was detected (Table I).

The results are tabulated in Table I. In the absence of any modification, NCp7 had an apparent molecular mass of 6396 ± 1 Da, corresponding to the apoprotein (zinc is lost from the protein under conditions used to separate species by HPLC). When silver ion was added to NCp7, the hydrophobicity of NCp7 decreased, as indicated by its earlier elution on a reverse phase C18 column, and the molecular mass increased to 6906 ± 1 Da, corresponding to apo-NCp7 with 5 bound silver ions. Since there are 4 dicarboxylic amino acids (three Glu and one Asp) plus the carboxyl terminus of the protein, it is expected that the silver ions form a salt with NCp7. Upon addition of a PATE, a new, slightly more hydrophobic peak was observed containing a mixture of modified peptides with molecular masses of 7066 ± 1 Da and 7026 ± 1 Da corresponding to apo-NCp7 with 5 silver atoms plus one or two pyridinioalkanoyl groups (Δm = 162 ± 1 Da per group), respectively. Interestingly, although no appreciable zinc ejection was observed with compound 47 even after a 1-h incubation, a minor amount of NCp7 modified with one or two pyridinioalkanoyl groups was detected (Table I).

Other metals were tested for their ability to promote zinc ejection and covalent modification of NCp7 by compound 45. Only Fe(II) (40 μM) was able to do so after 4 h of incubation, while Fe(III), Mn(II), and Ca(II) showed no effect. Ferrous ions, unlike silver, did not form a stable complex with NCp7 that was detected by MS and the resulting modified protein had a mass of 6531 ± 1 Da, corresponding to the addition of a single pyridinioalkanoyl group. In addition, the yield of the modified protein was much lower than that obtained when silver ions were used as the activator.

Identification of the Modified Residues by Tandem Mass Spectrometry—The NCp7 product produced by reaction with compound 45 was subjected to proteolytic digestion with clostripain, and the digest was analyzed by LC/MS/MS. Clostripain, which cleaves on the carboxyl side of arginine, is expected to cleave the protein into three large and three small peptides. Table II lists the ions and sequence positions obtained. The mass of the fragment corresponding to the carbonyl-terminal zinc finger, amino acids 32–52, indicated addition of two pyridinioalkanoyl groups per fragment (Δm = 162 ± 1 per group). Fragments corresponding to the amino-terminal zinc finger did not exhibit evidence of modification.

The exact site of the modification was determined by CID of the modified fragment. As shown in Fig. 5A, the CID spectrum of the +6 ion with a parent mass of 2875.82 Da contains a series of b ions that exhibit evidence of modification as of
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Modification of NCp7 by PATE compounds 45 and 47

NCp7 (1 μM) was incubated alone or with silver nitrate (10 μM) or iron (II) bromide (40 μM) and drug (40 μM) for various times in 20 mM sodium phosphate buffer, pH 7.2, and separated on a RP C-18 column. The fractions were lyophilized and the mass was determined using a Finnigan MAT SSQ-7000 Mass Analyzer.

| Reaction           | Time (min) | Zinc ejection | Mass       | Mass       | Mass       |
|--------------------|------------|---------------|------------|------------|------------|
| P7                 |            |               | 6369 ± 1   | 6368 ± 1   | 7226 ± 1   |
| P7 + compound 45   | 15         | –             | 6369 ± 1   | 6905 ± 1   | 7065 ± 1   |
| P7 + Ag            | 15         | +             | 6369 ± 1   | 6905 ± 1   | 7226 ± 1   |
| P7 + Ag + compound 45 | 15        |               | 6368 ± 1   | 6904 ± 1   | 7065 ± 1   |
| P7 + Ag + compound 47 | 15       |               | 6368 ± 1   | 6905 ± 1   | 7227 ± 1   |
| P7 + Ag + compound 47 | 60      | NO           | 6368 ± 1   | 6905 ± 1   | 7065 ± 1   |
| P7 + Ag + compound 47 | 75       | NO           | 6368 ± 1   | 6905 ± 1   | 7226 ± 1   |
| P7 + Fe²⁺ (40 μM)  | 240        | ND           | 6369 ± 1   | 6531 ± 1   | 6531 ± 1   |
| P7 + Fe²⁺ + compound 45 | 240      | ND           | 6369 ± 1   | 6531 ± 1   | 6531 ± 1   |
| P7 + Fe²⁺ + compound 47 | 240      | ND           | 6369 ± 1   | 6531 ± 1   | 6531 ± 1   |

* NO, not observed.

DISCUSSION

Understanding the detailed mechanism of action of an antiviral agent and identifying the possible site(s) of interaction on the target protein is important in developing highly optimized and specific drugs. In this study, the mechanism of NCp7 inhibition by two novel PATEs, compounds 45 and 47, was probed with fluorescence-based assays and mass spectrometric techniques. Although both compounds were very active in cell and target based assays in inhibiting HIV-1, and preliminary studies indicated that these compounds acted through modification of NCp7 zinc fingers (12), they failed to show any appreciable zinc ejection in vitro from purified, recombiant NCp7 or its synthetic peptides under the standard zinc ejection assay conditions. However, zinc ejection was evident upon “activation” with silver ions. Surprisingly, the zinc ejection was observed only with compound 45 and not with compound 47. A detailed analysis of the time course of zinc ejection produced by compound 45, in the presence of silver, suggested the formation of a loosely bound intermediate prior to zinc ejection. Zinc ejection followed a double exponential curve that can best be interpreted as the ejection of carboxyl-terminal zinc finger Zn²⁺ at a rate 15-fold greater than ejection of amino-terminal Zn²⁺ (Fig. 3). Further proof of the specificity of interaction and sequential release of Zn²⁺ from two fingers was obtained with synthetic peptides corresponding to amino- and carboxyl-terminal zinc fingers (Fig. 4). Interestingly, the complete lack of reactivity of the isolated amino-terminal peptide provides further support that the release of the second equivalent of zinc from intact NCp7 results from the collapse of the protein structure and not a direct attack of the PATE on the amino-terminal zinc finger. In accord with this observation, mass analysis of compound 45-treated NCp7 as well as the zinc finger peptides revealed that both modifications were present exclusively on the carboxyl-terminal zinc finger. Sequence data indicated that the peptide corresponding to carboxyl-terminal zinc finger (32–52) was modified at Cys⁴⁶ and Cys⁴⁹ as b and y ions with a mass shift of 162 ± 1 mass units were found. Various multiply charged internal fragments corresponding to these modified cysteines were also observed.

It has been previously demonstrated that 2,2′-dithiopyridine (20), disulfide benzamides (15), and 2,2′-dithiobi(st)benzamide (21), all disulfide-based inhibitors, follow a similar, sequential...
Fig. 5. Tandem mass spectrometry analysis of clostripain digest of NCp7 treated with compound 45. Panels A and B represent the full MSMS spectrum of the +6 (m/z 446.96) and +4 (m/z 696.73) charged ions, respectively. Observed b and y ions consistent with mass shift of +162 mass units are labeled with *. Ions formed as a result of dissociation of a thio-pyridinioalkanoyl group from cysteine are labeled as $x_m^*$, where $x$ represents b or y ions.
zinc ejection pathway. By comparing the rates of zinc ejection using Trp57 fluorescence (an indicator of carboxyl-terminal zinc ejection) and zinc-specific fluorophores (indicator of zinc ejection from the whole protein), it has been postulated that carboxyl-terminal zinc is released prior to amino-terminal zinc. Our results provide direct evidence of this differential zinc ejection. The greater reactivity of the carboxyl-terminal zinc finger toward attack by a variety of electrophiles has been attributed to a combination of steric factors and differences in nucleophilicity of the cysteines (22–24). Molecular modeling studies based on density-functional theory suggested that the thiolate of Cys49 is the most electron rich and the most reactive toward highly polarizable electrophiles (23). Molecular modeling studies show that the reactive sites of the carboxyl-terminal zinc finger lie in a more contiguous reactive surface compared with the amino-terminal finger (24). Docking of some antiviral agents, 2,2′-dithiobis(benzamide) disulfides-1 in particular, revealed that the structure of the carboxyl-terminal finger allows a significantly closer approach of the ligands to the cysteine thiolates, compared with the amino-terminal finger (24).

As mentioned earlier, zinc ejection was observed only with compound 45. However, mass spectrometry, which is more sensitive than the zinc ejection assay, revealed that compound 47 also modified NCp7 to a limited extent (Table I), resulting in addition of one or two pyridinoalkanoyl groups. The low reactivity of compound 47 toward NCp7 was suggested previously by its poor cross-linking of cell-free virions (12). However, compound 47 was active in initiating Gag precursor cross-linking (12). Since the structural context of the zinc fingers in NCp7 differs significantly from that of the zinc fingers in Pr55gag and Pr160 gag-pol, the observed differences in reactivity of the two compounds probably reflects the influence of local protein conformation.

Further experiments are needed to define the interaction of compound 47 with either NCp7 or the Pr55gag precursor.

Based on the results presented, we propose the following mechanism for the action of compound 45. As depicted schematically in Fig. 6, the reaction between compound 45 and NCp7 is initiated by the formation of a loosely bound complex followed by a relatively slow nucleophilic attack from the sulfur atom of the “reactive” Cys49 toward the carbonyl carbon of the activated PATE to form a new thioester bond in a transacylation reaction. Loss of cysteine coordination results in the release of zinc from the carboxyl-terminal finger facilitating a rapid attack by another molecule of compound 45 at Cys49. This process is followed by the slow structural collapse of NCp7, resulting in release of zinc from the amino-terminal finger, a process that is independent of concentration of compound 45.

This model includes several notable features. First, it is interesting to note that only two molecules of the antiviral compound are required to completely inactivate one molecule of NCp7 as opposed to three molecules of disulfide based inhibitors (15, 20, 21) due to the lack of reactivity toward the amino-terminal zinc finger. Second, both the need for activation and the initial formation of a noncovalent complex preceding transacylation are indicative of the lower reactivity of thioester compounds compared with disulfide agents. However, it should be noted that the NCp7 used in this study is a purified protein and is devoid of viral RNA normally associated with it. Under more native, in vivo conditions, the initial recognition step may be enhanced and additionally may result in activation of the PATE through well positioned amino acid side chains of NCp7 with consequent acceleration of the transacylation reaction without the requirement for an external activator.

Finally, a major concern with the utilization of NCp7 zinc fingers as anti-HIV targets has been the issue of selectivity of compounds for zinc fingers of target proteins. The PATEs were designed with this issue in mind, such that the compounds were selected for minimal chemical reactivity while maintaining anti-HIV activity (12). In this paper we have identified a PATE that reacts with only one of the two highly similar zinc finger domains of the NCp7 protein. These findings clearly illustrate that zinc fingers exhibit differential susceptibility to chemical entities. The exact susceptibility of various zinc fingers may be due to slight differences in chemical potentials of the sulfur atoms, their solvent accessibility, atomic surface geometric and hydrophobic/hydrophobic characteristics, and other factors (12, 23). Nevertheless, the proof now exists for selective targeting of zinc fingers, and efforts must be undertaken to further exploit those differences.

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![Fig. 6. Schematic showing proposed model for the mechanism of inactivation of NCp7 by compound 45.](image-url)
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