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Small molecule inactivation of HIV-1 NCp7 by repetitive intracellular acyl transfer

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Abstract

The zinc fingers of the HIV-1 nucleocapsid protein, NCp7, are prime targets for antiretroviral therapeutics. Here we show that S-acyl-2-mercaptobenzamide thioester (SAMT) chemotypes inhibit HIV by modifying the NCp7 region of Gag in infected cells, thereby blocking Gag processing and reducing infectivity. The thiol produced by SAMT reaction with NCp7 is acetylated by cellular enzymes to regenerate active SAMTs via a recycling mechanism unique among small molecule inhibitors of HIV.

Although antiretroviral therapy for HIV has been successful, newly acquired infections that harbor drug-resistant viruses are observed in treatment-intensive locations^{1–3}. Accordingly, there remains a need for new inhibitors of HIV. Among HIV-1 proteins, the nucleocapsid NCp7 is an attractive target for drug development^{4,5}. It is expressed in the Gag polyprotein, which is specifically cleaved by the viral protease in immature virions to form: matrix (MA), capsid (CA), NCp7, and p6. NCp7 contains two zinc fingers (ZFs) with the strictly conserved motif Cys-X₂-Cys-X₄-His-X₄-Cys that are flanked by residues highly conserved among viral clades^{6–8}. Intact ZFs are critical for NCp7 function, Gag processing, and infection^{8–11}.

Electrophilic molecules have been developed to eject coordinated zinc from NCp7 that show antiviral activity^{4,5}, yet their toxicity or lack of specificity has hampered their development into drugs¹². We have discovered that S-acyl-2-mercaptobenzamide thioesters (SAMTs) specifically eject zinc from the C-terminal ZF of NCp7 *in vitro*^{13,14}, resulting in irreversible unfolding of the NCp7 structure. SAMTs inhibit viral activity in cell-based assays, with EC₅₀ < 50 nM for inhibition of cell-to-cell HIV-1 transmission¹⁴. Although SAMTs react with selected cellular ZF motifs *in vitro*¹⁵, they show low cytotoxicity in cell culture and

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animal models^{16,17}. *In vitro* experiments using NCp7₃₂₋₅₅, containing the C-terminal ZF, revealed that reversible acyl transfer between the SAMT and sulfur side chain of Cys₃₆ is rapidly followed by irreversible intramolecular transfer of the acyl group to one of several proximal lysine residues⁷.

Since treatment of HIV-infected cells with NCp7 zinc ejectors results in accumulation of aggregated, unprocessed Gag polyprotein^{4,5}, we investigated whether a similar effect would occur upon SAMT treatment. For these experiments, we used SAMT-247 (**1**; Fig. 1a), a simple SAMT with good antiviral activity (EC_{50} =0.6–5.7 μ M) and low cellular toxicity (TC_{50} >100 μ M) in cultured and primary cells (Fig. 1a and Supplementary Table 1). Analysis of the virions from chronically HIV-infected H9 cells treated with SAMT-247, as described in the Supplementary Methods, demonstrated that treatment did result in the decrease of NCp7, the appearance of its precursor Gag (55kDa), and the accumulation of high molecular weight proteins containing NCp7 (Supplementary Fig. 1a,b). Immunoblotting with other Gag antisera demonstrated that the high molecular weight proteins were comprised of NCp7 and/or Gag (Supplementary Fig. 1c). HPLC purification of Gag from SAMT-treated virions indicated that ~20% of total Gag was modified by SAMT-247. The presence of unprocessed Gag polyprotein in the virions from SAMT-treated cells is consistent with other NCp7 inhibitors^{9,18} and demonstrates that proper zinc coordination is important for Gag processing. Interestingly, there was no evidence of partial Gag processing, suggesting that Gag was either processed fully or not at all. Exogenous HIV protease was unable to digest Gag in SAMT-treated virions (Supplementary Fig. 1b), suggesting that treated Gag exists in a non-functional complex, likely due to cysteine cross-linking, which renders the virus non-infectious.

We hypothesized that SAMT-247 would covalently modify the NCp7 region of Gag inside cells as observed for NCp7₃₂₋₅₅ *in vitro*⁷. Chronically-infected H9 cells were treated overnight with SAMT-247 that was ¹⁴C-labeled on the methyl carbon of the acetyl group (marked with an asterisk in Fig. 1a). Analysis of the virions by reducing gel electrophoresis showed specific radiolabeling of Gag (Fig. 1b), demonstrating that SAMT-247 promotes covalent acetylation of Gag intracellularly. Immunoblot analysis of a parallel experiment confirmed that the protein modified was Gag (Fig. 1c). Mass spectrometry analysis of Gag purified from chronically-infected H9 cells treated with unlabeled SAMT-247 showed modification of Gag residues 402–422 (NCp7 residues 22–42, Supplementary Fig. 2a). Following reduction and alkylation of the cysteine residues, the acetylation was still observed, indicating that modification occurred on a lysine residue. Tandem mass spectrometry identified the sites as Lys₄₁₃ and Lys₄₁₈ (Supplementary Fig. 2b), corresponding to Lys₃₃ and Lys₃₈ of NCp7, recapitulating our *in vitro* results⁷.

Following SAMT reaction with NCp7, a thiol (mercaptobenzamide thiol, MT-1, **2**) is generated. Surprisingly, we found that MT-1 has similar antiviral activity as SAMT-247 (Fig. 1a) even though it is inactive *in vitro*¹³. We hypothesized that MT-1 was activated upon entering cells to form a reactive compound, possibly through acetyl transfer from acetyl-CoA to form SAMT-247. Indeed, incubating MT-1 with acetyl-CoA *in vitro* resulted in time-dependent formation of SAMT-247 (Supplementary Fig. 3a) and metal ejection from NCp7¹³. To determine if this reaction occurred intracellularly, chronically-infected H9 cells were treated with ¹⁴C-acetate and SAMT-247 or MT-1. Analysis of the virions produced by reducing gel electrophoresis demonstrated that treatment with both MT-1 and SAMT-247 resulted in radiolabeling of Gag, just as observed for treatment with ¹⁴C-SAMT-247 (Fig. 1d). Likewise, treatment of HIV-infected H9 cells with SAMT-247 or MT-1 in the presence of 2-¹⁴C-pyruvate, which is converted to ¹⁴C-acetyl-CoA by pyruvate dehydrogenase, resulted in radiolabeling of Gag (Supplementary Fig. 3b). Virions from cells treated with MT-1 also showed similar accumulation of aggregated protein and inhibition of Gag

processing as cells treated with SAMT-247 (Fig. 1e,f and Supplementary Fig. 3c). The antiviral activity observed for MT-1 was specific for this molecule, as a slightly different thiol (MT-2, **3**) did not radiolabel Gag in the presence of ^{14}C -acetate or ^{14}C -pyruvate (Fig. 1d and Supplementary Fig. 3b). Furthermore, MT-2 showed no antiviral activity, nor did a third thiol, MT-3 (**4**), containing a methyl ester group in place of the terminal amide (Figure 1a). Virions from cells treated with MT-2 or MT-3 showed only background levels of protein aggregation and did not appreciably affect Gag processing (Fig. 3e,f and Supplementary Fig. 3c).

To further demonstrate the intracellular acetylation of MT-1, we designed a prodrug derivative in which a para-hydroxybenzyl ester was used to mask the sulfur atom (mercaptobenzamide thioether, MTE-1, **5**, Fig. 2a). Inside the cell, the oxygen ester of MTE-1 can be cleaved by esterases to yield an intermediate that breaks down via 1,4-elimination¹⁹, releasing MT-1 for acetylation to form SAMT-247. As expected, MTE-1 did not react with cell-free NCp7₃₂₋₅₅, but in cytoprotection assays using cultured and primary cells, it had antiviral activity similar to SAMT-247 (Fig. 2b). A non-cleavable control compound (MTE-2, **6**) showed significantly reduced activity in all cell types (Fig. 2b). As observed for MT-1 and SAMT-247, treatment of HIV-infected H9 cells with MTE-1 resulted in Gag aggregation and defects in its processing, whereas treatment with the non-cleavable MTE-2 had no effect (Fig. 2c and Supplementary Fig. 4a). Moreover, virions from cells treated with MTE-1 and ^{14}C -pyruvate showed radiolabeling of Gag similar to MT-1 and SAMT-247 (Supplementary Fig. 4b). Thus, the effects of MTE-1 treatment were similar to those of the SAMTs, confirming our hypothetical mechanism of action that the thiol produced by reaction with NCp7 can be recycled intracellularly to form an active thioester.

To investigate the importance of the recycling mechanism for SAMT activity, we next tested an activated oxygen ester compound that was not re-acetylated following reaction with NCp7 *in vitro* (Supplementary Fig. 5a, ester-7, **7**). *In vitro*, ester-7 ejected coordinated metal from NCp7₃₂₋₅₅ and covalently modified it like SAMT-247 (Supplementary Fig. 5b). However, it did not inhibit HIV replication in cell-based assays. Furthermore, treatment of HIV-infected H9 cells with ester-7 did not induce protein aggregation or affect Gag processing (Supplementary Fig. 5c,d). Thus, the lack of cellular activity of ester-7 shows that viral inhibition is lost when re-acetylation cannot occur, demonstrating the importance of the recycling mechanism for SAMT antiviral activity.

The results presented here support a model for SAMT inhibition of HIV in which SAMT reaction with NCp7 generates a thiol product that is acetylated by an intracellular mechanism that relies on acetyl-CoA (Fig. 2d). This reaction regenerates a thioester that can react with additional Gag proteins in a repeating cycle. Acyl transfer from the SAMT to the protein leads to loss of zinc coordination, protein aggregation and inhibition of Gag processing. Even small changes in Gag processing severely decrease HIV-1 infectivity^{20,21}. For example, a 30% block in processing results in a 3-log decrease in viral replication²⁰. Thus, rather than a simple 1:1 inhibitor:target inactivation model, a single molecule of SAMT could potentially inactivate numerous NCp7 ZFs in Gag or free NCp7, a unique mechanism for a small molecule inhibitor of HIV.

Despite extensive efforts, viral resistance to NCp7 inhibitors has not been observed in treated cells²², likely due to the absolute and unvarying requirement for cysteines and surrounding residues in the ZF motif. Because MT-1 relies on cellular pathways for activation, HIV-1 resistance via a host cellular mechanism could be difficult to generate as there is no selection pressure for the cell to mutate to accommodate the virus. Furthermore, mutation or down-regulation of proteins in the acetyl-CoA and pyruvate pathways is deleterious and associated with disease states^{23,24}. This SAMT-cell partnership mechanism

opens new avenues for development of inhibitors of HIV NCp7 and suggests ways to inhibit other disease-related proteins containing zinc fingers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SAMT-247 covalently modifies the Gag polyprotein and the thiol released by reaction is acetylated intracellularly to form an active thioester. (a) Chemical structure and antiviral activity of SAMT-247 and thiol compounds in μM . Cytoprotection was measured using the XTT assay in CEM-SS cells infected with HIV-1_{IIIIB} (SAMT-247) or HIV-1_{RF} (thiols). Toxicity was measured in uninfected CEM-SS cells. NA denotes not active. (b) Autoradiogram of virions from either uninfected (un.) or HIV-1 infected (HIV) H9 cells treated with 5 μCi ^{14}C -SAMT-247. The site of ^{14}C -labeling is denoted with an asterisk on the chemical structure in panel a. (c) Virions from infected H9 cells treated with 100 μM compound were separated and analyzed on a reducing gel. Proteins were immunoblotted with an antibody against NCp7. (d) Autoradiogram of virions from infected H9 cells treated with ^{14}C -acetate and either SAMT-247 or a thiol compound (100 μM). Un. denotes an uninfected control and – denotes infected cells not given a compound. (e) Virions from infected H9 cells treated with SAMT-247 or thiol compounds (10 or 100 μM) were separated and analyzed on a non-reducing gel. Proteins were immunoblotted with an antibody against NCp7. (f) Virions from infected H9 cells treated with SAMT-247 or thiol compounds (10 or 100 μM) were separated and analyzed on a reducing gel. Proteins were immunoblotted with an antibody against CA. **1:** SAMT-247; **2:** MT-1; **3:** MT-2; **4:** MT-3. See Supplemental Methods for experimental details.

Figure 2.

A thioether pro-drug confirms thiol acetylation and inhibits HIV. (a) Schematic of the enzymatic conversion of MTE-1 (leftmost panel) to MT-1 (rightmost panel). Shown in the inset is the chemical structure of MTE-2. (b) Antiviral activity of MTE-1 and MTE-2 in μM . Antiviral activity was measured using the XTT cytoprotection assay in CEM-SS cells infected with HIV-1_{IIIB}, monocyte-macrophages infected with HIV-1_{BaL}, and proliferating blood mononuclear cells (PBMCs) infected with HIV-1_{HT/92/599}. Toxicity was measured in the respective uninfected cells. (c) Virions from HIV-infected H9 cells treated with the thioether compounds (100 μM) were separated and analyzed on a reducing gel. Proteins were immunoblotted with an antibody against NCp7. (d) Model of SAMT intracellular recycling mechanism. Inside a cell, the SAMT-thioester reacts with the NCp7 domain of the Gag polypeptide, resulting in covalent modification of the protein and Gag aggregation, thereby inhibiting maturation of the virion. MT-1, released from reaction with Gag, can be re-acetylated intracellularly by acetyl-CoA to form a reactive thioester (SAMT-247) capable of reacting with the NCp7 domain of another molecule of Gag. In addition, introduction of the thioether prodrug (MTE-1) can enter this cycle through esterase-mediated release of the thiol. **1:** SAMT-247; **2:** MT-1; **5:** MTE-1; **6:** MTE-2. See Supplemental Methods for details.