Inhibition of HIV-1 Maturation via Drug Association with the Viral Gag Protein in Immature HIV-1 Particles

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The small molecule 3-O-(3',3'-dimethylsuccinyl)-betulinic acid (DSB) potently inhibits human immunodeficiency virus, type 1 (HIV-1) replication by interfering with proteolytic cleavage of the viral Gag protein at a specific site. Here we have demonstrated that the antiviral mechanism involves the association of DSB with Gag at a 1:1 stoichiometry within immature HIV-1 particles. The binding was specific, as mutations in Gag that confer resistance to DSB inhibited the association, which could be competed by DSB but not by the inactive compound betulinic acid. The addition of DSB to purified immature viral cores inhibited the cleavage of Gag at the CA-SP1 junction in vitro, thus reproducing the effect of the drug when present during maturation of HIV-1 particles. Based on these findings, we propose a model in which a trimer of DSB associates with the CA-SP1 junction of adjacent subunits within the Gag polymer. The model may explain the ability of highly similar compounds to specifically target the seemingly unrelated steps of HIV-1 maturation and virus entry.

We and others have recently described the novel antiviral mechanism of 3-O-(3',3'-dimethylsuccinyl)-betulinic acid (DSB), referred to alternatively as YK-FH312 or PA-457 (5–7). This compound was originally identified as a chemical derivative of betulinic acid, a plant-derived natural product, and was shown to potently inhibit HIV-1 replication (8, 9). DSB is active against a wide variety of HIV-1 isolates, including viruses that are resistant to drugs that target PR. Similar to PR inhibitors, DSB acts late in the virus life cycle, resulting in incompletely matured HIV-1 particles that are poorly infectious. However, the mechanism of DSB is distinct from that of drugs that inhibit the protease directly and thus prevent cleavage of all of its substrates. By contrast, DSB inhibits processing of a single cleavage site in the Gag polyprotein, the CA-SP1 junction. The specificity of DSB is determined by the sequence of the CA-SP1 junction, suggesting that the target of the drug is the viral Gag protein itself (10). However, DSB is inactive toward HIV-1 PR in vitro, even when recombinant HIV-1 Gag is the substrate. As a result, the biochemical target of DSB has not been defined.

Here we report that DSB was specifically incorporated into immature HIV-1 particles at a 1:1 stoichiometry with Gag. Mutations in the CA-SP1 junction of Gag that conferred viral resistance to DSB also prevented the association of the drug with particles. The addition of DSB to purified immature HIV-1 cores inhibited cleavage of Gag at the CA-SP1 junction by PR in vitro, recapitulating the effects of the drug on HIV-1 maturation. These data suggest that DSB inhibits maturation by associating with a binding site formed by oligomerization of Gag during the assembly of HIV-1 particles. Interference with virion maturation by specific association with Gag during virus assembly represents a novel mechanism for pharmacologic inhibition of HIV-1 replication.

**MATERIALS AND METHODS**

**Cells and Viruses**—293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. The viruses used in this study were based on the wild-type HIV-1 molecular clone pNL4-3 (11). Immature HIV-1 particles were derived from the protease-defective clone pNL-PR, which encodes substitutions of alanines for the three essential PR active site residues DTG. The DSB-resistant HIV-1 mutant HIVm2, containing two amino acid substitutions at the CA-SP1 junction, was previously described (10). HIVm2 PR was generated by PCR overlap splicing, resulting in transfer of the PR active site mutations from pNL-PR. Virus stocks were produced by calcium phosphate transfection of 293T cells (12), followed by culturing in the presence or absence of DSB. R9, a wild-type HIV-1 molecular clone, which mostly comprises viral sequences from pNL4-3, and its PR-inactivated mutant R9.PR have been described previously (13, 14). The CMV-Gag-opt vector was obtained from Dr. Paul Spearman and has been previously described (15). Mutations were introduced at the P2 (V to L) and P1 (L to M) positions of the CA-SP1 cleavage site by PCR-overlap mutagenesis to generate CMV-Gag-opt...
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M2. The wild-type SIVmac239 and DSB-sensitive mutant SIVm3 proviral constructs have been described previously (10). PR-defective versions of the SIV constructs were generated by engineering mutations in the PR coding region, resulting in substitutions of Asp→Ala and Thr→Ala at positions 134 and 135 of the Pol protein. These substitutions resulted in the release of immature SIV particles containing the unprocessed Gag protein. All PCR-amplified regions were sequenced to verify the correct substitutions and the absence of any additional mutations.

Virus-containing supernatants were harvested 36–48 h after transfection of 293T cells and were clarified by passing through 0.45-μm cellulose acetate syringe filters. For production of viruses in the presence of DSB, 24–36 h prior to harvesting, the culture medium was replaced with fresh medium to which the drug was added from a 1 mg/ml stock solution in Me2SO to the desired final concentrations. Virus stocks were quantified by p24 antigen capture ELISA (16) after heating in 1% SDS to disrupt immature virions.

Sucrose Density Gradient Purification of HIV-1 Particles—Supernatants of transfected 293T cells were concentrated by ultracentrifugation through a layer of 20% sucrose at 100,000 rpm in a Beckman SW32Ti rotor) on linear density gradients of 30–70% sucrose in STE. Fractions (1 ml volume) were harvested, and viruses were pelleted after the addition of 0.5 ml of STE to reduce the solution density. Viral pellets were resuspended in phosphate-buffered saline and assayed for the presence of p24 and DSB.

LC/MS-based Assay of DSB—To quantify the levels of DSB present in HIV-1 particles, suspensions of virions were extracted with 2 ml of a 3:1 (v/v) solution of chloroform and methanol. Prior to extraction, a fixed quantity (50 pmol) of a betulinic acid derivative (LH91; compound 4 in Ref 9) was added as an internal control for extraction efficiency. LH91 has an additional CH2 in the side chain of DSB, thus allowing for simultaneous detection of both molecules by mass spectrometry. The organic layer was collected and dried by evaporation under a gentle stream of nitrogen. The dried samples were dissolved in methanol (100 μl), and samples were analyzed by tandem LC/MS with electrospray ionization using a ThermoElectron Quantum Discovery triple quadrupole mass spectrometer operating in the selected reaction monitoring mode. The instrument was operated in negative ion mode using nitrogen for both the sheath nebulizing gas and auxiliary drying gas. The ion transfer capillary was maintained at 325 °C, and 15 electron volts of ion source collision-induced dissociation energy was used to facilitate solvent declustering. The primary ionization gave simple molecular anions [M − H]− for both DSB and the internal standard LH91. Collision-induced dissociation was conducted using argon as the collision gas with 42 electron volts of collision energy. Under these tandem MS conditions, the primary fragmentation process was the loss of the acyl group as either a neutral species or as a low mass anion (Fig. 1A). Chromatographic separations were done on a XDB C8 reversed phase column (2.1 × 150 mm) from Agilent Technologies using an acetonitrile/isopropanol/water gradient with 20 μM ammonium formate at pH 6.3. Quantitative studies were conducted using selected reaction monitoring; m/z 583→437 at 42 electron volts (DSB) and m/z 597→437 at 42 electron volts (LH91). Calibration curves were linear over the concentration range 4–500 nM DSB (correlation r = 0.98–0.99). The signals were quantified by integrating the area under the DSB and LH91 peaks from the chromatogram, and DSB signals were normalized against the area obtained for the LH91 internal standard. The ratio of DSB to LH91 was converted to absolute DSB levels based on the volume of sample analyzed by this technique.

Radiolabeling of DSB—DSB was labeled with tritium by reduction of the C-20 methylene group using a previously described procedure (18). Briefly, a mixture of DSB and 5% Pd-C with tritium gas in methanol was stirred overnight at room temperature (performed by Moravek Biochemicals, La Brea, CA). The reaction mixture was filtered, and the filtrate was concentrated and purified to 99% purity by reversed phase high pressure liquid chromatography (HPLC). DSB was dried, weighed, and dissolved at a concentration of 50 μg/ml in ethanol, and the concentration was verified by HPLC with known quantities of dihydro-DSB. The specific radioactivity (~20 Ci/mmol) was determined by scintillation counting. For procedures involving quantitation of [3H]dihydro-DSB, the quantity of DSB was converted directly from counts/min 3H by counting a known quantity of the stock [3H]dihydro-DSB, thereby eliminating the need to correct for counting efficiency.

In Vitro Digestion of Purified Immature HIV-1 Cores—Immature HIV-1 cores were produced by detergent treatment of PR-defective virions and purified by equilibrium sedimentation, as previously described (14). Cores (800 ng of Gag protein in 40 μl) were incubated in the presence and absence of DSB (2.5 μg/ml) for 2 h at 37 °C. Recombinant HIV-1 PR (a generous gift of Dr. David A. Davis, National Cancer Institute, NIH) was added to a final concentration of 1.12 μM in 40 μl of protease digestion buffer (50 mM MES, 100 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA, pH 6.0) and incubated at 37 °C. Samples were removed at various times and analyzed by SDS-PAGE and immunoblotting. The blots were probed with CA- and MA-specific antibodies, and signals were quantified using the LiCor Odyssey system following probing with an IR dye-conjugated secondary antibody.

RESULTS

DSB is a potent inhibitor of HIV-1 replication that acts during particle maturation. We have previously shown that sequencing of the CA-SP1 cleavage site in Gag determines the viral sensitivity to DSB (10). However, the compound did not inhibit the viral protease in vitro, even using recombinant HIV-1 Gag as the substrate. To reconcile these observations, we hypothesized that DSB specifically associates with the Gag protein during HIV-1 particle assembly. A prediction of this hypothesis is that DSB would be specifically incorporated into HIV-1 particles during their assembly, whereas mutations in Gag that confer resistance to the drug would prevent DSB incorporation. To assay HIV-1 incorporation of DSB, we developed a specific LC/MS-based method to detect the compound. The assay was found to be sensitive and quantitative (Fig. 1, A and B) and was therefore useful for detecting the association of DSB with HIV-1 particles.

Because DSB acts during virion maturation, the compound must be present at the time of particle production to be effective. To determine the levels of DSB incorporated into HIV-1 particles, cells were transfected with proviral DNA and cultured in the presence of the compound. Virus particles were harvested and concentrated by pelleting through a sucrose cushion and were further purified by equilibrium density-gradient sedimentation. Fractions containing virus particles were identified by an ELISA specific for the viral core antigen, and the DSB in the particles was extracted and analyzed by LC/MS. We first asked whether viruses containing unprocessed Gag protein would contain significant quantities of DSB. For this purpose, we analyzed immature HIV-1 particles produced from an HIV-1 proviral clone containing PR-inactivating mutations (PR−). Analysis of gradient fractions containing these particles revealed a strong DSB signal (Fig. 1C). As a con-

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DSB incorporation into immature HIV-1 particles. For this purpose, we produced \(^{3}H\)-labeled DSB by reductive tritiation of the methylene group at the 29 position. Previous studies have demonstrated that reduction of this site has minimal effects on DSB antiviral potency (9). To quantify incorporation of DSB into PR-defective HIV-1, we produced the virus in the presence of various concentrations of \(^{3}H\)-dihydro-DSB, purified the virions, and quantified the radioactivity (by liquid scintillation counting) and viral core antigen (by ELISA). Incorporation of the drug was nearly saturated at a concentration of 2 \(\mu\)g/ml (Fig. 2A). Quantitation of virus-associated DSB and Gag protein revealed that the levels corresponded to a stoichiometry of DSB:Gag of \(\sim 1\). We conclude that DSB is stoichiometrically associated with Gag in immature HIV-1 particles.

To further evaluate the specificity of DSB incorporation, we tested the ability of unlabeled DSB, or the poorly active parent compound betulinic acid (BA), to inhibit incorporation of \(^{3}H\)-dihydro-DSB during assembly of HIV-1 particles. For this purpose, we produced immature HIV-1 particles in the presence of a subsaturating concentration (0.5 \(\mu\)g/ml) of \(^{3}H\)-dihydro-DSB together with increasing doses of DSB or BA. Particles were then harvested, purified to remove unincorporated drug, and assayed for the levels of associated \(^{3}H\)-dihydro-DSB. Unlabeled DSB (but not unlabeled BA) inhibited incorporation of \(^{3}H\)-dihydro-DSB in a dose-dependent manner (Fig. 2B). These data provide additional evidence for the specific and saturable incorporation of DSB during the assembly of HIV-1 particles.

Previous studies have established that expression of Gag is sufficient for formation of immature virus-like particles (19). To further probe the requirements for DSB incorporation into HIV-1 particles, we quantified the incorporation of \(^{3}H\)-dihydro-DSB into particles generated by the expression of a codon-optimized Gag expression construct. This approach allows the production of HIV-1 particles without the requirement for expression of other viral proteins. DSB was associated with the
Gag virus-like particles, and mutations at the CA-SP1 junction that confer strong resistance to DSB also markedly inhibited association (Fig. 3). We conclude that Gag is sufficient for the specific association of DSB with immature HIV-1 particles.

The lack of activity of DSB with recombinant Gag or peptide substrates of HIV-1 PR suggests that DSB associates a binding site formed by the assembly of Gag into particles. This putative binding site might only be accessible during an intermediate stage of HIV-1 particle assembly or could also exist in the intact immature particle. We therefore asked whether DSB is capable of associating with preformed immature HIV-1 particles. Immature virions were produced by transfection of 293T cells with HIV-1 PR or HIVm2.PR proviral DNAs. DSB (0.5 \( \mu \)g/ml) was added to a sample of each virus and incubated for 2 h. The particles were concentrated by ultracentrifugation to remove free DSB, resuspended in a small volume of buffer, and purified by equilibrium density sedimentation (Fig. 4). DSB was present at high levels in immature HIV-1 particles but not when amino acids conferring resistance to the drug were present in Gag. We conclude that DSB is capable of specific association with immature HIV-1 particles following their release from cells.

A barrier to understanding the mechanism of DSB antiviral action has been the lack of detectable activity of the drug in vitro. The ability of DSB to specifically associate with preformed immature HIV-1 particles suggests an experimental approach to detect DSB activity in a cell-free system. Specifically, we hypothesized that the addition of DSB to assembled immature HIV-1 cores would inhibit cleavage of the CA-SP1 junction in Gag in vitro by exogenously added HIV-1 PR. To test this, viral cores were purified from immature HIV-1 particles by Triton X-100 treatment of virions followed by equilibrium sedimentation to remove the detergent. Our laboratory has previously used this approach to demonstrate the stable association of the HIV-1 envelope glycoproteins with immature HIV-1 cores (14). Following purification of the immature cores, DSB was added and incubated for 2 h to allow association with the cores, followed by the addition of purified recombinant HIV-1 PR and withdrawal of samples at various times for analysis. Cleavage of Gag was subsequently monitored by SDS-PAGE and immunoblotting using a CA-specific antibody. When added to the purified immature HIV-1 cores (HIV-1.PR-), DSB was observed to delay the processing of the CA-SP1 junction in Gag (Fig. 5A). By contrast, the drug had no detect-
able effect on the cleavage of Gag in the HIVm2.PR− cores (Fig. 5B). In previous studies, we showed that processing of the CA-SP1 junction occurred more slowly in HIVm2 virions than in wild-type HIV-1 particles (10), a difference that was also apparent in these in vitro assays (Fig. 5; compare PR− and HIVm2.PR− samples without DSB). However, we observed similar rates of processing of the MA-CA junction in the two core samples (data not shown), thereby excluding a global effect of the mutations on core structure or protease sensitivity. A slight accumulation of a Gag processing intermediate, likely corresponding to an MA-CA Gag processing intermediate, was observed for both viruses in the presence of DSB. This effect was not linked to the antiviral activity of the drug and is therefore an apparently nonspecific effect of the drug. Collectively, these results suggest that DSB associates with its functional target when added to immature HIV-1 cores in vitro, thus inhibiting PR-mediated cleavage of the CA-SP1 junction.

To determine whether the inhibition of Gag cleavage by DSB could be correlated with the incorporation of the compound into viral particles, we analyzed the cleavage of immature HIV-1 cores by recombinant PR in the presence of various concentrations of DSB. In these reactions, samples were removed at 1, 2, 4, and 8 h of incubation, and the cleavage of the CA-SP1 junction was then monitored by immunoblotting using a CA-specific antibody (Fig. 6A). The results revealed a dose-dependent inhibition of CA-SP1 cleavage by DSB. The magnitude of the effect of DSB was dependent on the incubation time, likely reflecting the observation that DSB delays (but does not fully inhibit) cleavage of CA-SP1 during HIV-1 maturation (5). Quantitative analysis of the immunoblot demonstrated that, in the 2-h incubation time, the drug had inhibited processing of CA-SP1 by ~50% at a concentration of 0.31 μg/ml (Fig. 6B). This is a concentration at which DSB was incorporated at a level that was approximately half-maximal (Fig. 2A) and is similar to the IC_{50} of the drug in transfected 293T cells (10). Thus, the potency of DSB inhibition of CA-SP1 cleavage in immature HIV-1 cores in vitro correlated well with the level of DSB incorporation into HIV-1 particles and its antiviral potency.

**DISCUSSION**

In this study, we provide compelling evidence that the mechanism of DSB antiviral activity involves association of the molecule with HIV-1 particles as they are formed. We showed that DSB specifically associates with immature HIV-1 virions when the drug is present during or following completion of particle formation. The maximum level of DSB in the particles corresponded to a stoichiometry of 1 molecule/molecule of Gag. The viral protease was not required for incorporation of DSB, as pseudovirions containing only the viral Gag proteins also incorporated DSB. Thus, DSB likely associates with Gag during and/or after particle assembly. Mutations in Gag that render HIV-1 resistant to DSB prevented association of the compound with the particles, thus linking the antiviral activity of DSB to HIV-1 particle association. Collectively, these results indicate that DSB associates with a specific binding site formed by the viral Gag protein within the virion. The putative binding pocket involves amino acids located at the CA-SP1 cleavage site, as substitutions in this region result in HIV-1 resistance to DSB resistance and reduced drug association with the mutant virions. Small molecule HIV-1 antivirals currently in clinical use target viral enzymes, including reverse transcriptase, integrase, and protease. The majority of these drugs act as competitive inhibitors of the viral enzyme, or in the case of nucleoside-based reverse transcriptase inhibitors, as substrates that interfere with chain elongation during reverse transcription. Our data indicate that DSB acts by binding to the viral Gag protein (a substrate of the viral protease) and interfering with its ability to serve as a substrate. It is also formally possible that DSB associates with Gag via a bridging event, although this seems less likely, as mutations in Gag confer strong resistance to the drug, yet do not affect HIV-1 particle formation (10). To our knowledge, the DSB antiviral mechanism is unprecedented; together with studies of other viruses (20, 21), this underscores the fact that small molecules need not target viral enzymes to be potent replication inhibitors.

Our results also demonstrated that DSB inhibits cleavage of the CA-
Recent structural studies indicate that the Gag protein forms a hexameric lattice within the immature HIV-1 particle (22). We imagine three possibilities for association of DSB with Gag in the particle. First, one molecule of DSB may bind each molecule of Gag when the protein is present in a specific conformation that is unique to the assembled particle. Second, DSB may associate with an interface between each pair of Gag molecules within the hexamer. Both of these hypotheses are consistent with the observed 1:1 stoichiometry of DSB association with Gag in immature HIV-1 particles. Nonetheless, we favor a third model in which DSB forms a trimer that is stabilized by intermolecular interactions of the hydrophobic betulinic acid core (Fig. 7). In this scenario, the CA-SP1 junction, representing the target of DSB, is also present as a trimer at each 3-fold axis of symmetry in the hexameric Gag lattice. This model is attractive in its ability to reconcile a variety of observations regarding DSB. First, DSB targets a specific site in Gag. Second, DSB does not inhibit cleavage of recombinant soluble Gag by PR in vitro, making it unlikely that DSB binds a monomer of the viral protein. The compound is therefore likely to recognize a Gag-Gag interface. Third, DSB is incorporated into immature particles at a 1:1 stoichiometry with Gag. Fourth, Gag forms a hexameric lattice in immature HIV-1 particles; nevertheless, emerging evidence indicates that a myristoylated MA-CA fragment trimerizes in solution (23) and that Gag can form trimers in solution upon the addition of specific phospholipids (2). The hexameric lattice of Gag also contains 3-fold axes of symmetry, further underscoring the possibility of trimeric regions of the polyprotein. Finally, the betulinic acid molecular scaffold on which DSB is built also serves as the core for other derivatives that specifically block HIV-1 entry (24). An enigma surrounding betulinic acid-based HIV-1 antivirals is how nearly identical molecules can specifically and potently block such seemingly unrelated steps in the virus life cycle as entry and maturation. Because the viral envelope protein that is the target of the betulinic acid-derived entry inhibitors is a homotrimeric protein, it is possible that binding of the compounds as trimers with their targets is a common feature of HIV-1 maturation inhibitors and BA-derived entry inhibitors. The model implies that DSB association with immature particles is cooperative; however, the hyperbolic binding of DSB is apparently inconsistent with this hypothesis. Nonetheless, it remains possible that DSB forms trimers in solution that associate non-cooperatively with the Gag polymer. The model may not prove to be accurate in all its aspects, but it makes specific predictions that can be tested in the future using structural and biophysical approaches.

DSB is the prototype of a new class of HIV-1 antivirals known as maturation inhibitors. The potency and selectivity of DSB, combined with its novel mechanism of action, make it attractive for therapeutic development, and the compound is currently in clinical trials as an HIV-1 therapy. Our results further clarify the target and mechanism of action of this interesting molecule and establish new assays that may be used in evaluating other compounds that may act by a related mechanism.

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