Retrovirology

Research

The sequence of the CA-SP1 junction accounts for the differential sensitivity of HIV-1 and SIV to the small molecule maturation inhibitor 3-*O*-{3',3'-dimethylsuccinyl}-betulinic acid

Jing Zhou[1], Chin Ho Chen[2] and Christopher Aiken*[1]

Address: [1]Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA and [2]Department of Pathology, Duke University Medical Center, Durham, NC, USA

Email: Jing Zhou - jing.zhou@vanderbilt.edu; Chin Ho Chen - chc@duke.edu; Christopher Aiken* - chris.aiken@vanderbilt.edu

* Corresponding author

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Abstract

Background: Despite the effectiveness of currently available antiretroviral therapies in the treatment of HIV-1 infection, a continuing need exists for novel compounds that can be used in combination with existing drugs to slow the emergence of drug-resistant viruses. We previously reported that the small molecule 3-*O*-{3',3'-dimethylsuccinyl}-betulinic acid (DSB) specifically inhibits HIV-1 replication by delaying the processing of the CA-SP1 junction in $Pr55^{Gag}$. By contrast, SIVmac239 replicates efficiently in the presence of high concentrations of DSB. To determine whether sequence differences in the CA-SP1 junction can fully account for the differential sensitivity of HIV-1 and SIV to DSB, we engineered mutations in this region of two viruses and tested their sensitivity to DSB in replication assays using activated human primary $CD4^+$ T cells.

Results: Substitution of the P2 and P1 residues of HIV-1 by the corresponding amino acids of SIV resulted in strong resistance to DSB, but the mutant virus replicated with reduced efficiency. Conversely, replication of an SIV mutant containing three amino acid substitutions in the CA-SP1 cleavage site was highly sensitive to DSB, and the mutations resulted in delayed cleavage of the CA-SP1 junction in the presence of the drug.

Conclusions: These results demonstrate that the CA-SP1 junction in $Pr55^{Gag}$ represents the primary viral target of DSB. They further suggest that the therapeutic application of DSB will be accompanied by emergence of mutant viruses that are highly resistant to the drug but which exhibit reduced fitness relative to wild type HIV-1.

Background

The advent of highly active antiretroviral therapy has had a tremendous impact on the treatment of HIV infection. Combinations of drugs targeting the viral reverse transcriptase and protease enzymes allow for potent inhibition of viral replication to undetectable levels in many infected individuals. Despite these successes, the continuous administration of these drugs over many years leads to eventual treatment failure, and the drugs are often poorly tolerated. Novel inhibitors targeting additional steps in the viral life cycle could prove to be useful additions to the current arsenal of HIV therapies.

Retroviruses must undergo proteolytic maturation at a late step of replication (for a review, see [1]. For HIV-1, the viral protease (PR) cleaves the Gag precursor $Pr55^{Gag}$ into

the mature processed proteins MA, CA, SP1, NC, SP2, and p6. Cleavage occurs in a temporally regulated fashion, with processing between CA and SP1 representing the final step. Release of SP1 is essential for proper capsid condensation and function: mutations that prevent release of SP1 result in noninfectious virions containing unstable cores [2].

We and others have recently described the mechanism of action of 3-*O*-{3',3'-dimethylsuccinyl}-betulinic acid [DSB; referred to as YK-FH312 and PA-457 in other studies [3,4]], a small molecule inhibitor of HIV-1 replication [4,5]. The compound acts at a late stage of the HIV-1 replication cycle and results in the accumulation of an intermediate in the processing of $Pr55^{Gag}$ due to delayed cleavage at the CA-SP1 junction. Although DSB potently inhibits HIV-1 replication, SIV is fully resistant to the drug, and a chimeric SIV virus encoding the CA and SP1 regions of HIV-1 was sensitive to the compound [5]. Point mutations in the CA-SP1 junction resulted in limited resistance to DSB, further underscoring its novel mechanism of action [4,5]. Here we report an analysis of the effects of additional substitutions in the CA-SP1 cleavage site on DSB sensitivity. As few as two mutations in the HIV-1 cleavage site were found to confer strong resistance to the drug, while three substitutions in this region of SIV rendered the virus highly sensitive to DSB.

Results

We previously reported that DSB specifically inhibits HIV-1 replication by delaying the processing of the CA-SP1 junction by the viral PR [5]. By contrast, SIVmac239 was completely resistant to the drug, while the chimeric virus SIV(HIV CA-p2) was sensitive. Additional studies demonstrated that DSB inhibited HIV-1 and SIV(HIV CA-p2) with equal potency (our unpublished results). HIV-1 and SIVmac239 exhibit a limited number of differences in the CA-SP1 junction (Fig. 1), suggesting that differences between these sequences may underlie the differential sensitivity of the two viruses to DSB. To test this hypothesis, we designed and constructed HIV-1 and SIV mutants containing the corresponding residues of the opposite virus. Obvious differences were the identities of the residues at the P2 and P1 positions; we therefore created an HIV-1 mutant (HIVm2) containing a substitution of L and M for the V and L residues found at these positions. In addition, we made the reciprocal substitutions in SIV (SIVm2). Finally, to determine whether an additional substitution in SIV might be required for conferring sensitivity to DSB, we added the corresponding substitution at the P4' position (SIVm3). Full-length proviral clones containing these mutations were constructed to facilitate production of virus stocks for analysis of DSB sensitivity.

Figure 1
Comparison of HIV-1 and SIVmac239 CA-SP1 Gag cleavage sites, and substitution mutants analyzed in this study. The HIVm2 mutant contains the P2 and P1 residues of SIV, while the SIVm2 and SIVm3 mutants contain substitutions of the corresponding HIV-1 amino acids. HIV-1 and SIV mutants were created in the pNL4-3 and pBR239E full-length molecular clones, respectively.

An HIV-1 mutant containing two substitutions at the CA-SP1 junction is highly resistant to DSB

Mutations in the SP1 region have been reported to inhibit HIV-1 assembly [6]. To produce virus stocks and to determine whether the mutations affected virus assembly, the mutant and wild type proviral clones were transfected into 239T cells and the culture supernatants were quantified for virus content by p24 ELISA (HIV-1 stocks) or reverse transcriptase (RT) activity (SIV stocks). In all cases, the mutations were found to have only minimal effects on virus assembly and release, and in the case of the HIVm2 and SIVm3 mutants, moderate enhancements of particle production were observed (Fig. 2, panels A and C). Immunoblot analysis using CA-specific antisera revealed that, in the absence of DSB, the CA-SP1 junction was processed efficiently for wild type and mutant viruses (Fig. 3A). As previously reported [3-5], in wild type HIV-1 particles produced in the presence of DSB, cleavage of CA-SP1 was impaired, resulting in reduced infectivity (Fig. 3). By contrast, the infectivity of the HIVm2 virus was slightly enhanced by the drug, indicating that alterations of P2 and P1 residues to those of SIV rendered HIV-1 resistant to DSB (Fig. 3B). Accordingly, immunoblot analysis further revealed no accumulation of CA-SP1 in HIVm2 particles produced in the presence of DSB (Fig. 3A). As a control for the immunoblotting experiments, we analyzed HIV-1 containing the L363F previously shown to render

Figure 2
Effects of CA-SP1 cleavage site substitution mutations on virus particle production and sensitivity to DSB. A and C: wild type and mutant virions were produced by transfection of 293T cells, and particles were harvested and quantified by p24 ELISA (A) or reverse transcriptase assays (C). Mean values of five (panel A) or four (panel C) independent determinations are shown. B and D: assays of viral infectivity. HIV-1 and SIV were assayed for infectivity on P4-CCR5 indicator cells. Shown are the mean values of triplicate determinations after normalizing for p24 (HIV-1) or RT (SIV) in the virus stocks. Error bars represent one standard deviation.

HIV-1 partially resistant to DSB[5]. As expected, this mutant also exhibited efficient cleavage of CA-SP1 junction in the presence or absence of DSB.

Mutations at the CA-SP1 junction render SIV sensitive to DSB

To determine whether sequences at the CA-SP1 junction can result in SIV sensitivity to DSB, we produced the

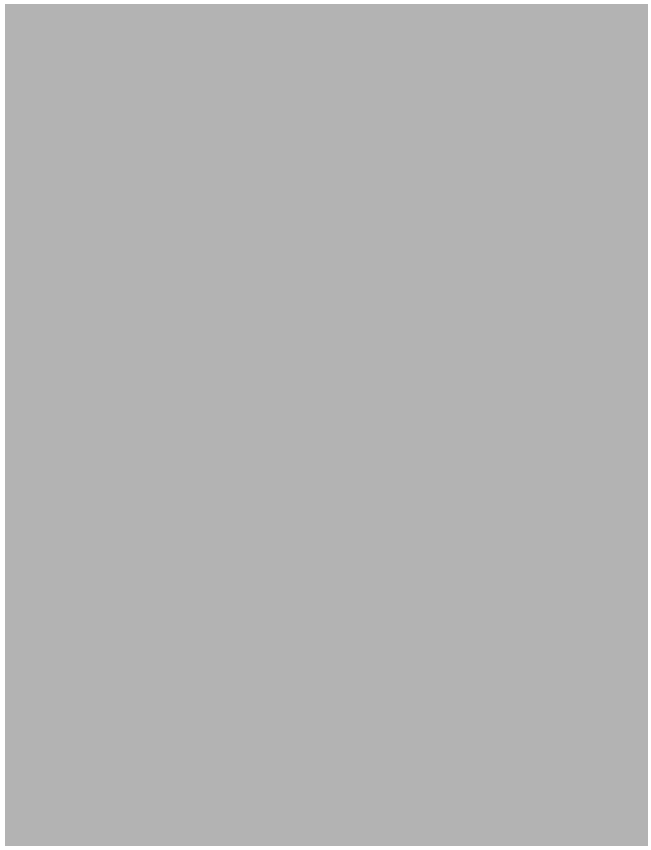

Figure 3
Mutations at the CA-SP1 junction render HIV-1 resistant to DSB, as revealed by single-cycle infection assays. Viruses were harvested from transfected 293T cells cultured in the presence of the indicated concentrations of DSB. Panel A: immunoblot analysis of viral lysates. Viral supernatants (1 ml) were pelleted, lysed, and subjected to SDS-PAGE and immunoblotting using polyclonal antisera to HIV-1 CA. Panel B: effects of DSB on HIV-1 infectivity. Viral supernatants were assayed for infection of P4-CCR5 indicator cells. Infectivity was calculated after normalizing for the p24 content of the inocula. Values shown are normalized against the infectivity of the respective vehicle-treated virus. The absolute infectivity values of the control viruses are shown in Fig. 2B. Results are representative of two independent experiments.

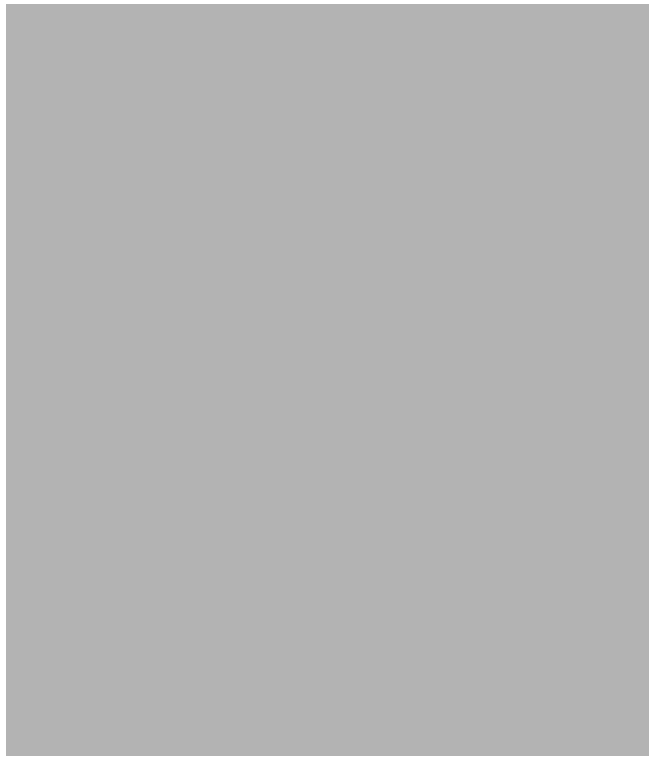

Figure 4
Mutations at the CA-SP1 junction confer DSB sensitivity to SIV, as revealed by single-cycle infection assays. Wild type and mutant SIV viruses were harvested from transfected 293T cells cultured in the presence of the indicated concentrations of DSB. Panel A: immunoblot analysis of viral lysates using a monoclonal antibody specific for SIV CA. Panel B: effects of DSB on SIV infectivity. Viral supernatants were assayed for infection of P4-CCR5 indicator cells. Infectivity was calculated after normalizing for the RT content of the SIV inocula. Values shown are normalized against the infectivity of the vehicle-treated virus stock. The absolute infectivity values of the control viruses are shown in Fig. 2D. The results are representative of two independent experiments.

SIVm2 and SIVm3 virions in 293T cells in the presence and absence of DSB. Production of the triply substituted mutant SIVm3 in the presence of DSB resulted in a small but significant accumulation of uncleaved CA-SP1, while this effect was not observed in wild type SIV (Fig. 4A). The infectivity of SIVm3 was less than wild type SIV (Fig. 2D), and was reduced by approximately 70% when the particles were produced in the presence of DSB (Fig. 4B). These results suggest that sequences in the CA-SP1 region of Gag determine the sensitivity of HIV-1 and SIV to DSB. In additional experiments, we tested whether the two substitutions at the P2 and P1 positions were sufficient to confer DSB sensitivity. The SIVm2 mutant virus was unaffected by DSB, both in single-cycle infectivity assays (Fig. 4B) and in immunoblot analyses (Fig. 4A). However, SIVm2 was found to be incapable of maintaining a spreading infection in CEMx174 and primary T cells (data not shown), possibly due to a reduced efficiency of particle production (Fig. 2C). Therefore this mutant was not analyzed in studies of virus replication.

Figure 5
The sequence of the CA-SP1 junction accounts for the differential sensitivity of HIV-1 and SIV to DSB. Viruses were harvested from transfected 293T cells, and equal quantities of p24 were used to inoculate cultures of activated primary $CD4^+$ T cells. Cultures were maintained in the indicated concentrations of DSB and supernatants were monitored periodically for p24 production (A and B) or RT activity (C and D). Panel A: wild type HIV-1; Panel B: HIVm2; Panel C: wild type SIV; Panel D: SIVm3. Data shown are representative of duplicate growth curves. Similar results were obtained in two independent experiments.

Replication of HIVm2 is highly resistant to DSB

The HIV-1 inhibitory effect of DSB is most pronounced in continuous replication assays, probably because nascent virions are highly infectious yet most sensitive to the delay in core maturation induced by the compound [5]. To further analyze the effects of the CA-SP1 cleavage site mutations on DSB sensitivity, we assayed the growth of wild type and mutant viruses in primary $CD4^+$ T cells purified

by positive selection from peripheral blood. T cells were activated using mitomycin C-killed allogeneic PBMCs and staphylococcal enterotoxin B, and cultured in IL-2-containing medium. As previously reported, HIV-1 replication in this system is highly efficient and reproducible, thus reducing the donor-to-donor and sample-to-sample variability often observed in cultures of PHA-activated PBMCs [7]. Titration of DSB in cultures inoculated with the HIVm2 mutant revealed that this virus was not inhibited by DSB at concentrations as high as 100 ng/ml (Fig. 5B). By contrast, DSB potently inhibited the replication of wild type HIV-1, with an IC_{50} of approximately 6 ng/ml (Fig. 5A). Interestingly, low concentrations of DSB actually resulted in a significant increase in the yields of HIVm2 virions at the peak of the growth curves, an effect we previously observed with wild type SIV [5]. Thus, when the P2 and P1 residues of the HIV-1 CA-SP1 cleavage site were replaced by the corresponding amino acids of SIV, the response of HIV-1 to DSB strongly mimicked that of SIVmac239. Comparison of the untreated control cultures further demonstrated that the two amino acid substitutions resulted in a significant replication delay relative to wild type HIV-1, possibly owing to the small but detectable reduction in infectivity observed for the mutant virus in single-round infection assays (Fig. 2B). In additional studies, we observed that HIVm2 replicated efficiently in the presence of DSB concentrations of up to 1.6 mg/ml, suggesting that the mutant virus is completely resistant to the compound, like wild type SIV.

Replication of SIVm3 is potently inhibited by DSB

The modest reduction in infectivity of SIVm3 by DSB observed in single-round infection assays suggested that the replication of this virus in primary T cells might also be inhibited by DSB. To test this hypothesis, we assayed replication of wild type and mutant SIV in primary T cells cultured in the presence of a range of DSB concentrations (Fig. 5, panels C and D). Relative to wild type SIV, replication of the SIVm3 mutant was delayed by approximately 8 days, with a markedly reduced virus output at the peak of replication. However, by contrast to wild type virus, replication of SIVm3 was inhibited by DSB with an IC_{50} of approximately 12 ng/ml. This sensitivity was comparable to that of wild type HIV-1. We conclude that as few as three substitutions in the CA-SP1 cleavage site can render SIV highly sensitive to DSB.

Mutations at the CA-SP1 junction determine the differential sensitivity of HIV-1 and SIV to the delay in CA-SP1 cleavage induced by DSB

We previously demonstrated that DSB results in a significant delay in cleavage of the CA-SP1 site in HIV-1 Gag, and that a single mutation (L363F) at the P1 position prevented the processing impairment and resulted in significant level of resistance [5]. To further probe the molecular basis for the DSB sensitivity of SIVm3, we performed pulse-chase analysis of Gag processing in particles produced in the presence and absence of DSB. As shown in Fig. 6B, DSB had no detectable effect on processing of Gag in wild type SIV. By contrast, SIVm3 particles exhibited a modest delay in processing of the CA-SP1 junction when the drug was present during virion maturation. Phosphorimager quantitation of the radioactive proteins on the gels further confirmed that CA-SP1 cleavage in SIV particles further confirmed these observations (Fig. 7). These observations are consistent with the small but detectable accumulation of CA-SP1 observed in immunoblots of the SIVm3 virions, and support the conclusion that the DSB sensitivity of SIVm3 results from delayed processing of the CA-SP1 cleavage site in the mutant particles. In the absence of DSB, SIVm3 was markedly delayed in CA-SP1 processing relative to wild type SIV, suggesting that the reduced replicative capacity of this virus may result from a delay in virus maturation. In an analogous manner, the DSB-resistant HIV-1 mutant HIVm2 exhibited moderately delayed kinetics of processing of the CA-SP1 junction relative to wild type HIV-1 (Figs. 6A and 7) and reduced replicative efficiency. However, CA-SP1 processing was not affected by DSB in these particles, consistent with results of previous studies of HIV-1 mutants that exhibit partial resistance to DSB [4,5]. Collectively, these results demonstrate that sequences at the CA-SP1 junction control the sensitivity of this cleavage site to DSB-delayed cleavage by PR. They further suggest that the cleavage sites of HIV-1 and SIV are recognized optimally by the cognate viral proteases.

Discussion

In this report, we demonstrate that the differential sensitivity of HIV-1 and SIVmac239 to DSB is governed primarily by sequences at the CA-SP1 cleavage site of $Pr55^{Gag}$. DSB is a potent inhibitor of HIV-1 replication that acts by a unique mechanism. However, the mechanism remains incompletely understood, as a direct binding interaction between the compound and its putative target has not yet been detected. Several lines of evidence indicate that DSB inhibits HIV-1 replication by targeting the CA-SP1 junction. First, HIV-1 is highly sensitive to the compound, while SIVmac239 is completely resistant. A chimeric SIV containing the HIV-1 CA and SP1 coding sequences exhibited DSB sensitivity equivalent to that of wild type HIV-1 [5]; Zhou and Aiken, unpublished results), demonstrating that the determinants of sensitivity map to the CA and SP1 coding sequences of *gag*. Second, we show in the present study that DSB delays the processing of the CA-SP1 junction when present during HIV-1, but not SIV, maturation. Finally, selection of HIV-1 for replication in moderate concentrations of DSB resulted in mutations at the P1 and P1' positions of this cleavage site, either of which conferred modest resistance to the compound.

Figure 6
Pulse-chase analysis of Gag processing in wild type and mutant HIV-1 and SIV particles. Virions were harvested at the indicated times from provirus-transfected 293T cells that were pulse-labeled with ^{35}S-labeled amino acids and cultured in the presence and absence of DSB (2.5 μg/ml). Particles were lysed and the Gag proteins immunoprecipitated using CA-specific monoclonal antibodies, and radioactive proteins in the immunoprecipitates analyzed by SDS-PAGE and autoradiography. Panel A: Analysis of HIV-1 and HIVm2; Panel B: Analysis of SIV and SIVm3. Similar results were observed in two independent experiments.

Previous studies reported that single substitutions at the P1 or P1' positions of the CA-SP1 junction render HIV-1 moderately resistant to DSB (approximately ten fold increase in the IC_{50}) [4,5]. The highly conserved nature of the CA-SP1 cleavage site in HIV-1 isolates, together with the specific differences in the corresponding SIV sequence, suggested that these sequences might fully account for the differential sensitivity of HIV-1 and SIV to DSB; this hypothesis proved correct. In the present study, we demonstrate that two mutations in the C-terminus of CA rendered HIV-1 fully resistant to DSB. Our data indicate that differences between the SIV and HIV-1 proteases do not contribute to the sensitivity to DSB. Our results thus invalidate other potential models for DSB action, such as alteration of protease substrate specificity by DSB binding to the viral protease. Our data further demonstrate that the differential sensitivity of HIV-1 and SIV to DSB is not due to intrinsic differences in the rates of processing of the CA-SP1 junction in these viruses (Fig. 7).

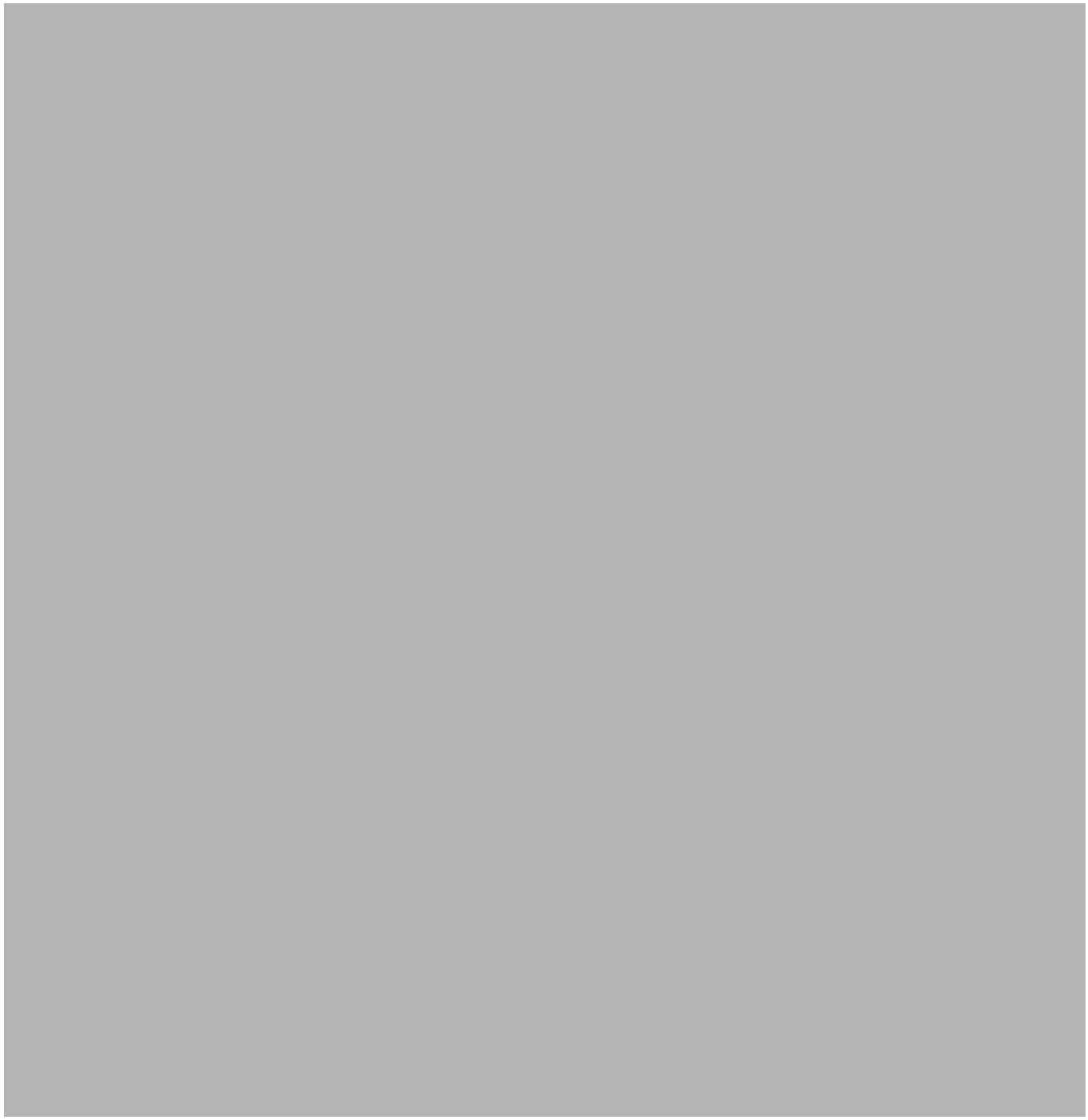

Figure 7
Quantitative analysis of the ^{35}S levels in the CA and CA-SP1 bands in the pulse-chase assays of Gag processing shown in Fig. 6. Dried gels were analyzed for radioactivity using a Fuji phosphorimager. Values shown represent the quantity of CA as a percentage of the sum of CA plus CA-SP1.

In this study, substitution of three amino acids in the CA-SP1 cleavage site were sufficient to render SIV replication highly sensitive to DSB in T cells. Interestingly, processing of the CA-SP1 junction in the SIVm3 virions was only moderately affected by DSB as compared to the more pronounced effects of the compound on cleavage of HIV-1

Gag. Relative to wild type SIV, the SIVm3 mutant also exhibited delayed processing in the absence of DSB, suggesting that the mutations have a deleterious effect on SIV infectivity and that even a modest delay in cleavage is sufficient to confer high sensitivity to the compound. It is also possible that additional residues in the HIV-1 CA-SP1 cleavage site, such as the serine residue at the P5' position, are necessary for optimal DSB binding to its target.

A plausible mechanism for DSB action involves binding of the compound to the CA-SP1 junction in $Pr55^{Gag}$ during HIV-1 assembly. However, we and others have failed to detect an effect of DSB cleavage on recombinant Gag or in HIV-1 virus-like particles *in vitro* [3-5]. These negative results suggest that DSB may be incorporated into a cavity formed by associated Gag molecules during HIV-1 particle assembly, where it subsequently interferes with binding of PR during maturation. Alternatively, the compound may be nonspecifically incorporated into virions, and may associate with a Gag processing intermediate transiently formed during maturation, such as CA-SP1 or MA-CA-SP1. Future studies will be aimed at testing these models.

DSB represents an especially promising candidate for antiviral therapy. The compound is highly potent against a variety of HIV-1 isolates, moderately soluble in aqueous solutions, and nontoxic at high concentrations. Although DSB-resistant mutant viruses are readily selected in culture, such mutants are significantly reduced in replication efficiency, indicating that mutant viruses are less fit than wild type. This was not unexpected, as the sequence of the CA-SP1 junction is highly conserved among HIV-1 isolates, and changes in the proximal half of the cleavage site could also affect the function of the CA protein. DSB acts through a mechanism that is distinct from currently approved antiretrovirals, suggesting that the compound is likely to be useful in combination with other classes of HIV-1 therapeutics.

Conclusions

Our results demonstrate that the differential sensitivity of HIV-1 and SIV to inhibition by DSB is determined by sequences at the CA-SP1 cleavage site in Gag. We conclude that the CA-SP1 junction represents the primary viral target of the inhibitor. Our results further demonstrate that strong resistance to DSB can result from as few as two amino acid changes in Gag, and that resistance is accompanied by a reduction in viral fitness.

Methods

Cells and Viruses

Primary $CD4^+$ T cells were purified from human blood by positive selection, and were activated and cultured as previously described [7]. CEMx174 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. 293T cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and antibiotics. The wild type full-length HIV-1 and SIV molecular clones pNL4-3 [8] and pBR239E (an unpublished construct generously provided by Toshiaki Kodama), respectively, were used for these studies. Virions were produced by transfection of 293T cells using a calcium phosphate coprecipitation method, as previously described [9]. Hela-CD4/LTR-lacZ-CCR^+ (P4-R5) cells were used as target cells in single-cycle infection assays, as previously described [10]. HIV-1 p24 antigen was quantified by antigen-capture ELISA [11]. SIV stocks were quantified by reverse transcriptase assays of viral lysates, by a modification of a previously reported method [12]. Duplicate aliquots of virus supernatants (5 μl) were added to 20 μl of RT assay cocktail (50 mM Tris-HCl, pH 8.3, 60 mM KCl, 7 mM DTT, 10 μg/ml of poly rA, 5 μg/ml of oligo dT, 7 mM $MgCl_2$, .07% of Triton X-100, 40 μCi/ml of ^{3}H-TTP {60 Ci/mmol}). Reactions were incubated at 37°C for 2 h, and aliquots (5 μl) were spotted on DE-81 paper (Whatman) in a 96-well array. The filters were washed thrice in a solution containing 0.3 M NaCl and 30 mM sodium citrate (2X SSC) for 5 minutes, rinsed with ethanol, and dried. Filters were analyzed for radioactivity using a MATRIX Direct Beta Counter (Packard BioSciences).

Synthesis of DSB (3-O-{3',3'-dimethylsuccinyl} betulinic acid)

DSB was synthesized and purified as previously reported [13]. The identity of the product was confirmed by mass spectrometry and ^{1}H NMR spectroscopy.

Mutagenesis

Viral mutants were created by PCR segment overlap mutagenesis and cloned into pNL4-3 or pBR239E. Primers used to produce the HIVm2 mutant were: CATAAAGCGCGCCTTATGGCTGAAG (sense mutagenic primer) and TAAGGCGCGCTTTATGGCCGGG (antisense mutagenic primer). The PCR product was digested with SpeI and ApaI and used to replace the corresponding fragment in pNL4-3. Primers used to produce SIVm2 were: GAAGGCTCGAGTACTGGCAGAAGCCATGAAAGAG (sense) and GGCTTCTGCCAGTACTCGAGCCTTC (antisense). Primers used to produce SIVm3 were: GAAGGCTCGAGTACTGGCAGAAGCCATGAAAGAG (sense) and TGGCTTCTGCCAGTACTCGAGCCTTTC (antisense). The PCR products were cleaved with BamHI and SbfI and used to replace the corresponding fragment in pBR239E. The PCR-amplified regions of the resulting clones were sequenced to confirm the presence of the desired mutations and the absence of unwanted substitutions.

Pulse-chase assays of virus maturation

Provirus-transfected 293T cells were starved for 1 hour in cysteine- and methionine-free medium containing 10% dialyzed FBS, and pulse-labeled in the same medium containing ^{35}S-labeled cysteine and methionine (0.2 mCi/ml Pro-Mix, Amersham Biosciences, Inc.) for 20 min. The cells were then washed and cultured in nonradioactive complete medium, and culture supernatants were harvested at various times following the chase. Virus samples were lysed and Gag proteins immunoprecipitated in RIPA buffer using monoclonal antibodies specific for HIV-1 CA (183-H12-5C from Bruce Chesebro) and SIV CA (2F12 from Niels Pedersen). Immune complexes were collected using Protein A/G-conjugated agarose beads (Santa Cruz Biotechnology), and the labeled proteins separated by SDS-PAGE and visualized by autoradiography. Radioactivity in protein bands was quantified using a Fuji phosphorimager.

Competing Interests

None declared.

Authors' Contributions

JZ designed and performed the experiments, CHC provided DSB and helpful discussions, and CA conceived of the study and wrote the manuscript. All authors read and approved the final version of the paper.

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