A multimode, cooperative mechanism of action of allosteric HIV-1 integrase inhibitors.

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Background: 2-(quinolin-3-yl)-acetic-acid derivatives target HIV-1 integrase and inhibit viral replication.

Results: The compounds are allosteric integrase inhibitors (ALLINIs) that block integrase interactions with viral DNA and its cellular cofactor LEDGF, and cooperatively inhibit HIV-1 replication.

Conclusion: ALLINIs block multiple steps of HIV-1 integration.

Significance: These new properties of ALLINIs will facilitate their further development as potent antiretroviral compounds.

SUMMARY

The multifunctional HIV-1 enzyme integrase (IN) interacts with viral DNA and its key cellular cofactor LEDGF to effectively integrate the reverse transcript into a host cell chromosome. These interactions are crucial for HIV-1 replication and present attractive targets for antiviral therapy. Recently, 2-(quinolin-3-yl) acetic acid derivatives were reported to selectively inhibit the IN-LEDGF interaction in vitro and impair HIV-1 replication in infected cells. Here we show that this class of compounds impairs both IN-LEDGF binding and LEDGF-independent IN catalytic activities with similar IC50 values, defining them as bona fide allosteric inhibitors of IN function. Furthermore, we show that 2-(quinolin-3-yl) acetic acid derivatives block the formation of the stable synaptic complex (SSC) between IN and viral DNA by allosterically stabilizing an inactive multimeric form of IN. In addition, these compounds inhibit LEDGF binding to the SSC. This multimode mechanism of action concordantly results in cooperative inhibition of the concerted integration of viral DNA ends in vitro and HIV-1 replication in cell culture. Our findings, coupled with the fact that high cooperativity of antiviral inhibitors correlates with their increased instantaneous inhibitory potential, an important clinical parameter, argue strongly that improved 2-(quinolin-3-yl) acetic acid derivatives could exhibit desirable clinical properties.

INTRODUCTION

HIV-1 integrase (IN) is an important antiretroviral target due to its essential role in virus replication (1). Multimeric IN functions within the context of the preintegration complex (PIC) to catalyze pair-wise integration of the linear viral DNA ends synthesized by reverse transcription into a host chromosome in a two-step reaction (2). In the first step, termed 3'-processing, IN cleaves a GT dinucleotide from each 3'-terminus of viral DNA. Concerted transesterification reactions (DNA strand transfer) subsequently integrate both viral DNA ends into the host genome in a staggered fashion. Raltegravir (RAL), the clinically approved IN inhibitor, specifically impairs the second step of integration. While RAL results in significant reduction of viral loads in patients (3), HIV phenotypes resistant to this inhibitor evolve comparatively rapidly in the clinic (4). Therefore, there is a continued need for developing novel IN inhibitors with alternative mechanisms of action.

We previously proposed one such alternative mechanism with a small molecule inhibitor that
stabilizes interacting IN subunits into an inactive multimeric form (5). Our biochemical studies indicated that highly dynamic individual subunits of IN correctly assemble in the presence of viral DNA to form the functional nucleoprotein complex or intasome (6,7). Restricting the molecular movement of individual IN subunits within the multimer during its assembly with DNA compromised IN enzyme activity (5-7). These observations were further supported by detailed analysis of the available crystal structure of the prototype foamy virus (PFV) intasome (8) and corresponding molecular models of HIV-1 IN-viral DNA complexes (6,9). The organization of the individual IN subunits within the intasome indicates that cognate viral DNA plays a crucial role in their assembly into the functional complex. In contrast, the preformed IN tetramer in the absence of viral DNA would not allow binding of the two viral DNA ends as seen in both the crystal structures and molecular models (6,8,9). Therefore, premature multimerization of IN before it encounters cognate DNA presents an attractive avenue for antiviral drug development.

Recently, inhibitors targeting the interaction between HIV-1 IN and its key cellular cofactor LEDGF have been reported (10). LEDGF directly engages IN through its C-terminal IN binding domain (IBD) and tethers the viral protein to chromatin (11,12). Principal protein-protein contacts of the IN catalytic core domain (CCD) and N-terminal domain bound to the LEDGF IBD have been revealed in co-crystal structures (13,14), with the extended interacting interfaces between full length HIV-1 IN and LEDGF further defined by MS-based protein footprinting (7). Christ et al (10) exploited the co-crystal structure of the HIV-1 IN CCD bound to the LEDGF IBD (14) to rationally design inhibitors of this central protein-protein contact. That study revealed several 2-(quinolin-3-yl) acetic acid derivatives that potently inhibited the IN-LEDGF interaction in vitro as well as HIV-1 replication in infected cells (10). This class of compounds was termed LEDGINs, with one of the more potent inhibitors designated compound 6 (herein referred to as LEDGIN-6). Co-crystal structures of the LEDGIN-CCD complexes revealed that the compounds bind to the CCD dimer at the LEDGF binding pocket. Furthermore, selection of HIV-1 strains resistant to LEDGIN-6 identified an A128T resistance mutation that localized to the same pocket (10).

Our interest in LEDGINs and hence the present studies were prompted by the observation that they bind at the IN dimer interface (10) adjacent to where we had previously mapped other small molecule inhibitors of IN multimerization (5). We accordingly sought to test the hypothesis that LEDGINs could allosterically modulate the dynamic interplay between IN subunits. In parallel experiments, we investigated the mechanism of action of another 2-(quinolin-3-yl) acetic acid derivative (Fig. 1A), which was patented by Boehringer Ingelheim as HIV replication inhibitor 1001 (15) (herein referred to as BI-1001). Remarkably, BI-1001 was derived from compounds identified via a fluorescence based high throughput screen (HTS) for IN 3’-processing activity, whereas, LEDGIN-6, which was reported to be highly selective for disrupting IN-LEDGF binding (IC50 = 1.37 µM), failed to inhibit 3’-processing activity (IC50 > 250 µM) (10). We have analyzed these two compounds in parallel experiments, and our data clarify that LEDGIN-6 and BI-1001 have identical antiviral mechanisms. These compounds potently inhibit not only IN-LEDGF binding but also LEDGF-independent IN catalytic function. Furthermore, we demonstrate that the key to inhibiting IN activities is through compound-mediated premature protein multimerization. Finally, we show that the inherent multimode mechanism of action of this class of inhibitors results in cooperative inhibition of concerted DNA integration in vitro and HIV-1 replication in infected cells.

EXPERIMENTAL PROCEDURES

Chemical Synthesis of IN Inhibitors: 2-(6-Chloro-2-methyl-4-phenylquinolin-3-yl)pentanoic acid (LEDGIN-6) was prepared in six steps from commercially available 2-amino-5-chlorobenzonitrile (Sigma-Aldrich) according to the scheme provided by Debyser and coworkers (10). 2-(6-bromo-4-(4-chlorophenyl)-2-methylquinolin-3-yl)-2-methoxyacetic acid (BI-1001) was synthesized in five steps from commercially available 2-amino-4’-chlorobenzophenone (TCI America) through slight modification of the procedures reported in the patent (15). The chemical structures of these compounds are shown in Figure 1A. Full experimental procedures and characterization data (1H and 13C NMR spectra) for the preparation of the compounds are provided in the Supplemental Data.

Construction of Flag-tagged Proteins: C-terminally FLAG-tagged LEDGF was constructed as described previously (16). N-terminally FLAG-tagged IN was constructed by PCR amplification of C-terminally His-tagged IN construct pKBIN6Hth (7) with T7T and InFlagN (5’-ggaatctcatgactaaagagcgatgacaaattttagatggaata gataaggecc-3’) primers. The C-terminal His-tag was then removed by insertion of a stop codon using site directed mutagenesis. Sequences of PCR-generated...
regions of plasmid DNA were verified by Sanger sequencing.

Preparation of Recombinant Proteins and DNA Substrates: Full-length proteins were expressed in Escherichia coli strain BL21 (DE3). Flag-tagged and tag-less INs were purified by loading the ammonium sulfate precipitate of cell lysate onto a phenyl sepharose column (GE Healthcare) and eluting bound IN with a decreasing ammonium sulfate gradient (800 mM to 0 mM) in a 50 mM HEPES, pH 7.5 buffer containing 200 mM NaCl, 7.5 mM CHAPS, 2 mM β-mercaptoethanol (BME). Peak fractions were pooled and loaded onto a heparin column (GE Healthcare), and IN was eluted with an increasing NaCl gradient (200 mM to 1 M) in a 50 mM HEPES, pH 7.5 buffer containing 7.5 mM CHAPS and 2 mM BME. Fractions containing IN were pooled and stored in 10% glycerol at -80 °C. His-tagged IN was purified as described previously (7, 17). Purified recombinant wild-type and Flag-tagged LEDGF/p75 were obtained as described previously (18). The blunt-end viral DNA substrate (~1 kb) for stable IN-viral DNA complex formation was obtained by PCR and purified by agarose gel electrophoresis as described previously (6).

In Vitro Integration Assays: IN 3′-processing and strand transfer activities were assayed using 32P-labeled blunt ended 21-mer or recessed end 19-mer synthetic double-stranded U5 DNA, respectively. 500 nM IN was preincubated with LEDGIN-6 or BI-1001 for 30 min on ice in 50 mM MOPS (pH 7.2) buffer containing 2 mM BME, 50 mM NaCl and 10 mM MgCl2. Then, 50 nM DNA substrate was added to the reaction and incubated at 37 °C for 1 h. The reactions were stopped with 50 mM EDTA. The reaction products were subjected to denaturing polyacrylamide gel electrophoresis and visualized using a Storm 860 Phosphorimager (Amersham Biosciences).

LEDGF-dependent concerted integration assays were carried out as described previously (13, 17). Briefly, 2 µM IN was preincubated with increasing concentrations of LEDGIN-6 or BI-1001 at room temperature for 30-min in 22 mM HEPES (pH 7.4) buffer containing 25.3 mM NaCl, 5.5 mM MgSO4, 11 mM DTT, 4.4 µM ZnCl2. To this mixture 1 µM of the viral donor DNA (32-mer blunt ended U5) and 600 ng target (pBR322) DNAs were added. Samples were incubated at 25 °C for 5 min and then LEDGF was added at final concentration of 2 µM, after which reactions proceeded for 90 min at 37 °C. Integration reactions stopped by addition of 0.5% SDS and 25 mM EDTA were deproteinized by digestion with 40 µg proteinase K (Roche Applied Science) for 60 min at 37 °C. DNA products were separated in 1.5% agarose gels in Tris–acetate–EDTA buffer and visualized by staining with ethidium bromide.

HTRF-based IN-LEDGF Interaction Assay: A previously described homogeneous time resolved fluorescence (HTRF) assay (16) was modified for the testing of inhibitors. Briefly, 10 nM N-terminally His-tagged IN was pre-incubated in a binding buffer (150 mM NaCl, 2 mM MgCl2, 0.1% NP40, 1 mg/ml BSA, 25 mM Tris, pH 7.4) with the tested compound for 30 minutes at room temperature, and then 10 nM C-terminally Flag-tagged LEDGF was added to the reaction. 6.6 nM anti-6His-XL665 and 0.45 nM anti-FLAG-EuCryptate antibodies (Cisbio, Inc., Bedford, MA) were then added to the reaction. After 4 hours at 4 °C, the HTRF signal was recorded using a Molecular Devices M5 plate reader using 314 nm for excitation wavelength and 668 and 620 nm for the wavelength of the acceptor and donor emission respectively. The HTRF signal is defined as the emission ratio 665 nm/620 nm multiplied by 10,000.

HTRF-based IN Multimerization Assay: Two separate preparations of His-tagged and Flag-tagged INs (each at 10 nM final concentration) were mixed in 25 mM Tris, pH 7.4 buffer containing 150 mM NaCl, 2 mM MgCl2, 0.1% NP40, 1 mg/ml BSA. Test compounds were then added to the mixture and incubated for 2.5 hours at room temperature. 6.6 nM anti-6His-XL665 and 0.45 nM anti-FLAG-EuCryptate antibodies (Cisbio, Inc., Bedford, MA) were then added to the reaction and incubated at room temperature for 3 hours. The HTRF signal was recorded as above.

Stable Synaptic Complex (SSC) Formation and SSC-LEDGF Binding Inhibition Assays: The previously reported methods (6) for assembly of the SSC and SSC-LEDGF binding were used for compound testing. Briefly, IN was pre-incubated with the compound for 30 minutes at room temperature before adding viral DNA to assemble the SSC (6). In the SSC-LEDGF assay, the purified SSC was pre-incubated with inhibitor for 30 minutes at room temperature before addition of LEDGF. SSC-associated IN and LEDGF proteins were separated by spin-size exclusion chromatography and analyzed by SDS–PAGE. Proteins were visualized by western blot using monoclonal antibodies against IN (8G4, NIH AIDS Research and Reference Reagent Program (19)) and against human LEDGF (BD Biosciences).

Differential Scanning Fluorimetry: Differential scanning fluorimetry was performed on a LightCycler 480 96-well plate, Real-Time PCR instrument (Roche Applied Science, Indianapolis, IN) according to Nettleship et al. (20). Sypro orange was purchased from Invitrogen (Life Technologies).
Differential scanning fluorimetry is based on denaturation of the protein in the absence or presence of a ligand, exposing hydrophobic residues that can be detected with high sensitivity with a fluorescent dye. The melting temperature (Tm) of the protein is calculated from this data. After incubation of HIV-1 IN and inhibitor at room temperature for 1 hr in 50 mM MOPS, pH 7.2, 50 mM NaCl, 10 mM MgCl2, 2 mM BME and 1% DMSO, sypro orange was added to the final concentration of 0.1% (v/v). The mixture was subsequently heated in a LightCycler 480 from 30 to 90 °C in increments of 0.11°C/sec. Fluorescence intensity was measured using excitation/emission wavelengths of 483 and 610 nm, respectively. Changes in protein thermal stability (∆Tm) upon inhibitor binding were analyzed by using LightCycler 480 Software provided by the manufacturer. All assays were performed in duplicate.

**Crystallization and X-ray Structure Determination:** The HIV-1 IN CCD (residues 50-212 containing the F185K mutation) was expressed and purified as described (21). The protein was concentrated to ~ 8 mg/ml and crystallized at 4 °C using the hanging drop (2 µl) vapor diffusion method. The crystallization buffer contained 10% PEG 8K, 0.1 M Na cacodylate, pH 6.5, 0.1 M ammonium sulfate, and 5 mM DTT, and cubic shaped crystals reached 0.1- 0.2 mm within 4 weeks. A soaking buffer containing 5 mM BI-1001 was prepared by dissolving the compound in crystallization buffer supplemented with 10% DMSO. The protein crystal was soaked in the buffer for 12 h at 4 °C before flash-freezing it in liquid N2. Diffraction data were collected at 100 F on a Rigaku Rcryst / P3121 with cell dimensions a = b = 73.082 Å, c = 64.808 Å, with one 18 kDa monomer in the asymmetric unit. The structure was refined to 2.45 Å with Rcryst / Rfree = 0.2308/0.2763.

**Antiviral Activity Assays:** CD4-positive SupT1 T cells were grown in RPMI 1640 medium supplemented to contain 10 % fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin while HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium modified to contain the same supplements. The concentration of HIV-1NL4-3 in the supernatant of plasmid pNL4-3-transfected HEK293T cells was determined using a radionuclide-based exogenous assay for reverse transcriptase (RT) activity (27), and SupT1 cells (4 x 10⁵ per well of a 96-well plate) were infected with 5 x 10⁵ ³²P counts per minute in 200 µl. The effective concentration of compound required to inhibit 50% (EC₅₀) of HIV-1 replication was determined after 5 days using the WST-1 assay (Roche Applied Science) to quantify cell viability. Control compounds RAL and saquinavir (SQV) were obtained from the NIH AIDS Research and Reference Reagent Program.

**Curve Fittings:** The fitted dose-response curves were obtained as follows. Reaction yields in the absence of the inhibitors were considered 100%. The IC₅₀ and the Hill slope parameter m (28), which is analogous to the Hill coefficient n (29), for each reaction were determined from a respective dose-response curve using a modified Hill equation (Equation 1) and the Origin software (OriginLab, Inc., MA). All fitted curves displayed a R² of 0.97 or greater.

Equation 1:

\[ y = \frac{x^n}{k^n + x^n} \]

where x is the inhibitor concentration, y is the percentage of inhibition, k is IC₅₀ (or EC₅₀ for antiviral measures) and n (or m for the virus data) is the Hill slope (28).

**RESULTS**

Two 2-(quinolin-3-yl) acetic acid derivatives, LEDGIN-6 and BI-1001, which potently inhibit HIV-1 replication, were discovered using two different approaches. LEDGIN-6 emerged through rational structure-based design to spatially mimic the interactions of LEDGF IBD hotspot residues Ile365 and Asp366 in their contacts with the IN CCD dimer interface, and has been reported to selectively inhibit the IN-LEDGF interaction (IC₅₀ = 1.37 µM) but not IN 3’-processing activity (IC₅₀ > 250 µM) (10). Paradoxically (Fig. 1A), BI-1001 was identified via a HTS for IN 3’-processing activity (15). Therefore, it was important to evaluate LEDGIN-6 and BI-1001 in parallel to dissect their mechanism of action.

We first compared the compounds for their ability to inhibit the IN-LEDGF interaction using the HTRF-based assay (Fig. 1B). Both compounds effectively impaired IN-LEDGF binding. In triplicate
To test this hypothesis we designed an HTRF-based assay to monitor the IN-IN interaction. Anti-6His-XL665 and anti-FLAG-EuCryptate antibodies allow fluorescence energy transfer upon interaction of two full-length, wild type HIV-1 IN proteins, one containing an N-terminal 6His tag and the other, an N-terminal FLAG tag (Fig. 4A). Compounds that inhibit IN-IN binding would accordingly decrease the HTRF signal whereas those that promote multimerization by stabilizing the interacting IN subunits would increase the signal. Representative data with LEDGIN-6 revealed a striking dose dependent increase of the HTRF signal (Fig. 4B). As a control we used RAL, which targets the IN active site distal from the CCD dimer interface. The HTRF signal predictably remained at the background level with increasing RAL concentrations (Fig. 4B). The data in Figure 4C show that LEDGIN-6 and BI-1001 promoted IN multimerization with IC50 values of 11.3 µM and 4.9 µM, respectively (Table 1).

To further test the notion that LEDGIN-6 and BI-1001 stabilize interacting IN subunits, we monitored the melting temperatures of free IN protein and protein-inhibitor complexes. Figure 5 shows that IN complexes with LEDGIN-6 and BI-1001 were significantly more stable to thermal denaturation than free IN. The k_d values obtained from these experiments for LEDGIN-6 and BI-1001 were 8.0 µM and 5.3 µM, respectively. In control experiments, increasing concentrations of RAL did not affect IN stability (Fig. 5A). A logical interpretation of data of Figure 5 is that LEDGIN-6 and BI-1001 stabilize interacting IN subunits (Fig. 4) and thus increase thermostability of the multimer.

Integration proceeds through the stable synaptic complex (SSC) or intasome comprising a tetramer of IN acting on the two ends of linear viral DNA substrate that we and others have shown is resistant in vitro to chaotropic agents, such as high concentrations of salt (6,8,30). We therefore next tested whether IN multimers assembled in the presence of LEDGIN-6 and BI-1001 retained the ability to form the SSC. In low ionic strength buffers, IN forms both stable and non-specific complexes with viral DNA, and subsequent treatment of the reaction mixture with high NaCl, followed by spin-column chromatography, yields the purified SSC (6). Since the limited amounts of the SSC recovered from these experiments did not permit us to carry out
extensive dose dependent analysis of LEDGIN-6 and BI-1001 activities, we tested two relatively high inhibitor concentrations (100 µM and 200 µM). We first pre-incubated LEDGIN-6 and BI-1001 with IN, and then supplied viral DNA to the reaction. Data of Figure 6A show that both compounds effectively inhibited SSC formation. Collectively, our findings argue that LEDGF-6 and BI-1001 stabilize IN multimers (Fig. 4 and 5), which are then incapable of forming the SSC (Fig. 6A) and accordingly lack catalytic function (Fig. 2).

Effective pair-wise integration of HIV-1 DNA ends during infection requires the interaction of the SSC with LEDGF. While data of Figure 1 shows that LEDGIN-6 and BI-1001 impaired IN-LEDGF binding, it was important to examine whether these compounds also inhibited the interaction between LEDGF and the pre-assembled IN-viral DNA complex. Both compounds effectively impaired this interaction (Fig. 6B and data not shown). Data of lanes 5 and 6 of Figure 6B moreover revealed that the treatment of the preassembled SSC with LEDGIN-6 did not dissociate IN from viral DNA. Comparative analysis of the data in Figures 6 A and B thus reveals the importance of order-of-addition. Addition of inhibitors to free IN impair its ability to assemble with viral DNA (Fig. 6A, lanes 4-7), whereas the preformed IN-viral DNA complex remains stable upon treatment with LEDGIN-6 or BI-1001 (Fig. 6B, lanes 4-6). The inhibitors, however, still effectively block SSC-LEDGF binding (Fig. 6B, lanes 4-6). Taken together, our results reveal that LEDGIN-6 and BI-1001 can disrupt at least two intermediate steps along the pathway of concerted HIV-1 DNA integration, namely: i) proper IN-IN multimerization and hence formation of the basic catalytic SSC and ii) the downstream interaction of the SSC with the LEDGF integration targeting host factor.

We next examined the compounds in an in vitro integration assay dependent on the LEDGF-IN interaction for effective stimulation of concerted integration (13,17). While inhibition of pair-wise integration products was expected, the dose-response curves yielded Hill coefficients of ~2, indicating cooperative modes of action for LEDGIN-6 and BI-1001 under these reaction conditions (Fig. 7 and Table 1). As alluded to above, we propose that such cooperativity is likely due to the ability of these compounds to impair two steps in the reaction pathway: inhibition of SSC formation, and subsequent SSC-LEDGF binding. Conversely, in reactions where IN-LEDGF binding (Fig. 1) or IN catalytic function (Fig. 2) were monitored separately, Hill coefficients were ~1 (Table 1). To make sure that a cooperative mode of action in the concerted integration reaction was specific to LEDGIN-6 and BI-1001, we conducted control experiments with RAL. No cooperativity was observed under these conditions (data not shown), due presumably to the fact that RAL impairs only the strand transfer step of HIV-1 integration.

To determine whether cooperative inhibitor action in vitro extended to the physiologically relevant condition of HIV-1 replication, LEDGIN-6 and BI-1001 EC₅₀ and Hill coefficient values were determined and compared to those of control compounds RAL and SQV. As established previously (28), the protease inhibitor SQV displayed cooperative inhibition (m = 2.6) under conditions where RAL failed to reveal evidence of cooperativity (m = 1.1). Both LEDGIN-6 and BI-1001 displayed highly cooperative inhibition, yielding m values of 3.9 and 3.7, respectively (Fig. 8 and Table 1).

DISCUSSION

Here, we investigated the mechanism of action of two prototypes of a growing number of small molecule compounds that bind HIV-1 IN distal from the enzyme active site. In contrast to the previous report (10) indicating that LEDGIN-6 specifically inhibited the IN-LEDGF interaction, we show conclusively that this inhibitor impairs both IN-LEDGF binding and the inherent catalytic activities of IN, which do not rely on LEDGF, with very similar IC₅₀ values. While structurally similar, BI-1001 was more potent than LEDGIN-6, and the two compounds displayed an overlapping mechanism of action. We therefore conclude that LEDGIN-6 and BI-1001 belong to the same class of inhibitors. Because these 2-(quinolin-3-yl) acetic acid derivatives allosterically modulate IN structure, we propose to name this class of compounds allosteric IN inhibitors, or ALLINIs.

We show that the underlying basis of inhibition of LEDGF-independent IN catalytic function by ALLINIs is premature IN multimerization (Fig. 4 and 5). While the functional intasome or SSC contains a tetramer of IN stably bound to the two viral DNA ends (8,31), the highly dynamic interplay between free IN subunits is critical for their productive assembly with viral DNA and hence functional SSC formation (7). In the absence of cognate DNA, free IN can also multimerize, but these preformed IN multimers do not form the SSC and accordingly lack IN catalytic function (6). Previously we showed that the LEDGF IBD promotes formation of IN multimers (6,7). Remarkably, the conformations of IN multimers within IN-LEDGF complexes formed in the absence...
of viral DNA and SSCs differ significantly, and the preassembled IN-LEDGF IBD complex moreover lacks the ability to functionally integrate viral DNA ends (6,32). Our findings that ALLINIs also modulate IN multimerization and impair the formation of the SSC argue further for exploiting IN multimerization as a novel therapeutic target. Due to their molecular mimicry of LEDGF hotspot residues Ile365 and Asp366, we postulate that ALLINIs recapitulate the inhibitory activities of anti-IN peptides derived from the tip of the corresponding helix-hairpin-helix IBD structure (33).

The ALLINI mode of action of stabilizing rather than inhibiting IN subunit-subunit interactions has a major advantage in that these small molecules do not have to overcome the high energy barrier created by large interfaces between interacting protein subunits. Instead, they stabilize the interacting subunits to promote premature IN multimerization. The substituted benzene ring of the compounds primarily engages one IN monomer through hydrophobic interactions, whereas the carboxylic acid group, and in the case of BI-1001 the nearby methoxy moiety, hydrogen bond with the second IN molecule (Fig. 3). Such compounds are likely to be effective during the early stages of HIV-1 replication when cognate DNA is unavailable for IN until after viral DNA synthesis by RT is completed. LEDGIN-6 accordingly blocked the integration step of HIV-1 replication, and mutations in IN conferred resistance to the compound (10).

In vitro concerted integration and ex vivo experiments have revealed a cooperative mechanism of action of ALLINIs. High cooperativity of antiviral compounds is important because it strongly influences the instantaneous inhibitory potential (IIP), the key clinical parameter for a retroviral drug that indicates the log reduction in a single round infectivity assay at clinical drug concentrations (28,34). Inhibitors with high cooperativity or high IIPs are particularly desirable for superior clinical outcomes. Comparative analysis (28,34) of current HIV therapies under clinically relevant conditions have revealed that protease inhibitors and non-nucleoside RT inhibitors, that affect large pools of protease or RT, respectively, exhibit intermolecular cooperativity due to the importance of multiple copies of these proteins for virus maturation and reverse transcription. Consequently, these inhibitors exhibit high IIP values, whereas inhibitors such as nucleoside RT inhibitors and RAL, that specifically target the active enzyme complexes, do not display cooperativity and thus have low IIPs values (~ 1).

Based on our observations it is logical to propose that ALLINIs could affect the entire population of IN molecules (estimated to be ~ 40 to 100 copies) produced in a single infectious cycle by promoting premature protein multimerization and thus impair the multiple functions of this key retroviral protein. Under simplified in vitro conditions, we identified two concerted integration intermediates that are effectively inhibited by ALLINIs, which likely accounts for the Hill coefficient of ~2 observed in the concerted integration assay. Inhibition of IN multimerization was also cooperative (Hill coefficient ~ 2, Fig. 4, Table 1), due presumably to ALLINIs stabilizing IN dimers and thus shifting the equilibrium toward their further assembly into tetramers. Premature IN multimerization could be a key contributor of the greater Hill coefficient (~4) observed under the ex vivo replication conditions, although additional work with viral replication intermediates will be needed to reveal the details. It is nevertheless noteworthy that mutations in IN can affect a variety of steps along the HIV-1 life cycle, including virus assembly and release from virus producer cells, and subsequent viral core uncoating, reverse transcription, PIC nuclear import, and integration in challenged target cells (reviewed in (35,36)).

Our findings, together with published results (10), argue strongly for further development of ALLINIs as well as studies to discover new inhibitors targeting IN multimerization. While the original goal of the rational design of small molecule inhibitors was to effectively compete with IN-LEDGF binding (10), future efforts can consider enhancing ALLINIs properties to more tightly bridge the two IN subunits that meet at the LEDGF binding cleft (14). As an example, our co-crystal structure (Fig. 3) indicates the significance of the BI-1001 methoxy group for establishing a unique H-bond with one of the IN subunits. In general, structural analysis of the pocket at the IN dimer interface reveals ample opportunities for further enhancing the ability of ALLINIs to more effectively engage both IN subunits.

Discovery of new IN multimerization inhibitors could proceed through HTS. Our method of identifying compounds that stabilize interacting IN subunits (Fig. 4) exhibits excellent statistical parameters (Z’=0.87) and could be exploited for screening large chemical libraries. The rationale for pursuing these studies is provided by the present and prior findings that a number of small molecule inhibitors interact with the IN CCD dimer interface (5,37,38). Significantly, two additional IN domains (N-terminal and C-terminal domains) are also essential for functional protein multimerization (39,40), and new inhibitors targeting these
unexploited protein-protein interfaces are likely to emerge from HTS. As established here, IN multimerization inhibitors can be expected to behave cooperatively to disarm IN molecules in excess of the four that compose the heart of the DNA recombination machine. They moreover can be expected to be active against RAL-resistant virus and hence complementary to current antiretroviral therapies (10). Our clarification of a cooperative mode of ALLINI action argues strongly that improved IN multimerization inhibitors could exhibit desirable clinical properties.

REFERENCES


FOOTNOTES
Abbreviations used are: IN, integrase; CCD, catalytic core domain; LEDGF, lens epithelium-derived growth factor; SSC, stable synaptic complex; RT, reverse transcriptase; ALLINI, Allosteric integrase inhibitors; IIP, instantaneous inhibitory potential; RAL, raltegravir; SQV, saquinavir; PFV, prototype foamy virus; IBD, integrase binding domain; HTS, high throughput screen; BME, β-mercaptoethanol ; HTRF, homogeneous time resolved fluorescence; 6His, hexahistidine.

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FIGURE LEGENDS
FIGURE 1. Effects of LEDGIN-6 and BI-1001 on the IN-LEDGF binding. A. Chemical structures of LEDGIN-6 and BI-1001. B. Representative raw data of the inhibition dose response of LEDGIN-6 on the IN-LEDGF interaction using the IN-LEDGF HTRF assay. Each data point represents the mean of three independent experiments. C. Curve fitting of dose-dependent inhibition of IN-LEDGF binding by LEDGIN-6 (black squares) and BI-1001 (gray circles). The average values from three independent experiments are shown.

FIGURE 2. Effects of LEDGIN-6 and BI-1001 on IN 3’-processing and strand transfer activities. A. A representative gel image for 3’-processing inhibition by LEDGIN-6. The 21-mer DNA substrate and 19-mer reaction product are indicated. Lane 1: DNA load; lane 2: DNA+IN without inhibitor; Lane 3: 25 mM EDTA was included in the reaction; the remaining lanes contained the following concentrations of LEDGIN-6: lane 4: 1 mM; lane 5: 500 μM; lane 6: 250 μM; lane 7: 125 μM; lane 8: 62.5 μM; lane 9: 31.25 μM; lane 10: 15.6 μM; lane 11: 7.8 μM; lane 12: 3.9 μM; lane 13: 1.95 μM; lane 14: 0.977 nM; lane 15: 488 nM; lane 16: 240 nM. B. Curve fitting of the dose-dependent inhibition of IN 3’-processing activity by LEDGIN-6 (black squares) and BI-1001 (gray circles). The average values from three independent experiments are shown. C. A representative gel image for strand transfer inhibition by LEDGIN-6. The 19-mer DNA substrate and strand transfer (ST) products are indicated. Lane 1: DNA load; lane 2: DNA+IN without inhibitor; Lane 3: 25 mM EDTA was included in the
reaction; the remaining lanes contained the following concentrations of LEDGIN-6: lane 4: 1 mM; lane 5: 500 µM; lane 6: 250 µM; lane 7: 125 µM; lane 8: 62.5 µM; lane 9: 31.25 µM; lane 10: 15.6 µM; lane 11: 7.8 µM; lane 12: 3.9 µM; lane 13: 1.95 µM; lane 14: 977 nM; lane 15: 488 nM; lane 16: 240 nM. D. Curve fitting of the dose-dependent inhibition of IN strand transfer activity by LEDGIN-6 (black squares) and BI-1001 (gray circles). The average values from three independent experiments are shown.

FIGURE 3. Structural analysis of the inhibitor-CCD complexes. A. The crystal structure of BI-1001 bound to the IN CCD dimer. Surface views of individual IN subunits are depicted in magenta and cyan. B. Overlay of CCD-LEDGIN-6 (PDB ID code 3LPU) and CCD-BI-1001 co-crystal structures. Cartoon views of IN subunits are colored as in A; LEDGIN-6 and BI-1001 backbones are green and yellow, respectively. Compound oxygen and nitrogen atoms, as well as those of IN residues Thr174, Glu170, and His171, are colored red and blue, respectively (for simplicity, only main chain Glu170 and His171 atoms are shown). The inhibitor carboxyl groups H-bond (green and orange dashed lines for LEDGIN-6 and BI-1001, respectively) with the main chain nitrogens of Glu170 and His171, and to the Thr174 side chain. The BI-1001 methoxy group forms an additional H-bond (black dashed line) with Thr174.

FIGURE 4. Effects of LEDGIN-6 and BI-1001 on IN multimerization. A. HTRF assay design. The assay monitors the interaction between two IN molecules: one containing 6xHis and the other the FLAG tag. The antibodies conjugated with Europium Cryptate and XL665 yield HTRF signal upon the protein-protein interaction. Europium Cryptate is excited at 320 nm, and emissions at 665 and 620 nm are measured. The HTRF signal is calculated from the 665 nm:620 nm ratio. B. Representative raw data for affects of LEDGIN-6 (black bars) and RAL (gray bars) on IN multimerization. Each data point represents the mean of three independent reactions. C. Curve fittings of dose-response affects of LEDGIN-6 (black squares) and BI-1001 (gray circles) on IN multimerization. Maximal HTRF signal, obtained at high compound concentrations, was set to 100%. The average values from three independent experiments are shown.

FIGURE 5. Effects of LEDGIN-6 and BI-1001 on IN thermostability. A. Representative raw data with LEDGIN-6 (black bars) and RAL (light bars). B. Curve fitting of the dose-response effects of LEDGIN-6 (black squares) and BI-1001 (gray circles) on IN thermostability. The average values from two independent experiments are shown.

FIGURE 6. Effects of LEDGIN-6 and BI-1001 on SSC formation (A) and the SSC-LEDGF interaction (B). A. SDS–PAGE analysis of SSCs. Lane 1: 1/10 of IN load; lane 2: protein markers: MagicMark XP Western Protein Standard (Invitrogen, Carlsbad, CA, USA); lane 3: SSC assembly without compound; lane 4: SSC assembly with 100 µM LEDGIN-6; lane 5: SSC assembly with 200 µM LEDGIN-6; lane 6: SSC assembly with 100 µM BI-1001; lane 7: SSC assembly with 200 µM BI-1001. IN was visualized by western blotting. B. SDS–PAGE analysis of LEDGF interactions with the SSC. Lane 1: 1/100 of LEDGF load; lane 2: SSC load; lane 3: protein markers: MagicMark XP Western Protein Standard (Invitrogen, Carlsbad, CA, USA); lane 4: SSC plus LEDGF; lane 5: SSC incubated with 100 µM LEDGIN-6 plus LEDGF; lane 6: SSC incubated with 200 µM LEDGIN-6 plus LEDGF. IN and LEDGF were visualized by western blot.

FIGURE 7. Effects of LEDGIN-6 and BI-1001 on IN concerted integration activity. A. Representative raw data of the inhibition dose response of LEDGIN-6 on LEDGF-dependent concerted integration activity. Positions of supercoiled (SC) target and 32-mer donor DNA substrates as well as half-site (HS) and full-site (FS) integration products are indicated. Lane 1: DNA markers (BIOLINE Quanti-Marker 1Kb); lane 2: target DNA load; lane 3: IN activities in the presence of LEDGF and target DNA and donor DNA substrates; the remaining lanes contained the following concentrations of LEDGIN-6: lane 4: 1 mM; lane 5: 500 µM; lane 6: 250 µM; lane 7: 125 µM; lane 8: 62.5 µM; lane 9: 31.25 µM; lane 10: 15.6 µM; lane 11: 7.8 µM; lane 12: 3.9 µM; lane 13: 1.95 µM; lane 14: 977 nM; lane 15: 488 nM; lane 16: 240 nM. Curve fitting of the inhibition dose responses of LEDGIN-6 (black squares) and BI-1001 (gray circles) on LEDGF-dependent concerted integration activity. The average values from two independent experiments are shown.

FIGURE 8. Dose-response curves of antiviral activities of SQV, RAL, LEDGIN-6, and BI-1001. The average values from two to three independent experiments are indicated.
Table 1. Activities of LEDGIN-6 and BI-1001

<table>
<thead>
<tr>
<th></th>
<th>IN-LEDGF Binding</th>
<th>3’-Processing</th>
<th>Strand Transfer</th>
<th>IN multimerization</th>
<th>Concerted Integration</th>
<th>Antiviral Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEDGIN-6 IC₅₀ (µM)</td>
<td>10.0 ± 0.4</td>
<td>3.9 ± 0.5</td>
<td>4.2 ± 0.6</td>
<td>11.3 ± 1.1</td>
<td>12.9 ± 0.8</td>
<td>12.2 ± 2.9</td>
</tr>
<tr>
<td>LEDGIN-6 Hill Coeff.</td>
<td>1.0 ± 0.03</td>
<td>0.9 ± 0.1</td>
<td>0.95 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>BI-1001 IC₅₀ (µM)</td>
<td>1.0 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>4.9 ± 0.3</td>
<td>5.4 ± 0.5</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>BI-1001 Hill Coeff.</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.3</td>
<td>3.7 ± 0.2</td>
</tr>
</tbody>
</table>

Average values with standard errors from the mean are shown for two or three independent experiments.
Fig. 5

A

IN Melting Temp., °C

50 54 58 62 66

0 0.25 0.5 1 2.5 5 10 25 50 100

Compound concentration, μM

B

Relative IN Thermostability (%) 0 50 100

0.1 1 10 100

Compound concentration, μM
A multimode, cooperative mechanism of action of allosteric HIV-1 integrase inhibitors
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