Mechanism of action of allosteric HIV-1 integrase inhibitors

Dissertation

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Abstract

Human Immunodeficiency Virus (HIV)-1 is the etiological agent of the acquired immune deficiency syndrome (AIDS). HIV is a single stranded positive sense RNA virus which replicates through a DNA intermediate. An essential step in the replication cycle is the integration of the viral DNA into the host chromatin. The viral enzyme integrase (IN) catalyzes the integration of the viral DNA into the host genome. The cellular cofactor lens epithelial growth factor (LEDGF)/p75 plays a significant role in integration by acting as a bimodal tether between IN and chromatin. These interactions are crucial for HIV-1 replication and present attractive targets for antiviral therapy. There are currently 3 FDA approved active site integrase inhibitors. However, resistance remains a problem, emphasizing the need for inhibitors targeting novel sites or activities. Herein, I will present research aimed at dissecting the mechanism of action of novel allosteric HIV-1 IN inhibitors.

Chapter 1 introduces HIV-1 IN, the current inhibitors of IN and explores the molecular basis for allosteric inhibition of HIV IN. This chapter will first focus on the structural biology and function of HIV-1 integrase. Next, the role of LEDGF/p75 in replication and integration site selection will be discussed. Finally,
the chapter will discuss three mechanism of IN inhibition: (1) the mechanism of FDA approved active site inhibitors; (2) the basis for IN multimerization as a therapeutic target and (3) the interaction between LEDGF/p75 and IN as a drug target.

Chapter 2 presents the findings that novel allosteric HIV-1 IN inhibitors (ALLINIs) have a multimodal mechanism of action. We demonstrate that this class of compounds impairs both integrase-LEDGF/p75 binding and LEDGF/p75-independent integrase catalytic activities with similar IC$_{50}$ values, defining them as bona fide allosteric inhibitors of integrase function. 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 argue strongly that improved 2-(quinolin-3-yl) acetic acid derivatives could exhibit desirable clinical properties.

Chapter 3 elucidates the structural and mechanistic basis for this resistance to ALLINIs. Selection of viral strains under ALLINI pressure revealed an A128T substitution in HIV-1 IN as a primary mechanism of resistance. The A128T substitution did not affect inhibitor binding affinity but instead altered the positioning of the inhibitor at the IN dimer interface. Consequently, the A128T substitution had only a minor effect on the ALLINI IC$_{50}$ values for IN-LEDGF/p75 binding. Instead, ALLINIs markedly altered the multimerization of IN by promoting aberrant higher order WT (but not A128T) IN oligomers. We conclude that ALLINIs primarily target IN multimerization rather than IN-LEDGF/p75 binding.
Chapter 4 dissects the mechanism of resistance to the allosteric inhibitor BI-D. Selection of viral strains emerging under the inhibitor BI-D pressure has revealed H171T IN as a prominent resistant mutation that exhibits a ~68-fold resistance to BI-D treatment in infected cells. These results correlated with ~84-fold reduced affinity for BI-D binding to recombinant H171T IN CCD protein. The x-ray crystal structures of BI-D binding to WT and H171T IN CCD dimers coupled with binding free energy calculations revealed the importance of the $N_\delta$- protonated imidazole group of His171 for hydrogen bonding to the BI-D tert-butoxy ether oxygen and establishing electrostatic interactions with the inhibitor carboxylic acid, whereas these interactions were compromised upon substitution to Thr171. These findings reveal a distinct mechanism of resistance for the H171T IN mutation to ALLINI BI-D and indicate a previously undescribed role of the His171 side chain for binding the inhibitor.

Chapter 5 presents the design of small molecules that allowed us to probe the role of HIV-1 IN multimerization independently from IN-LEDGF/p75 interactions in infected cells. We designed pyridine-based molecules which allow these compounds to bridge between interacting IN subunits optimally and promote oligomerization. The most potent pyridine-based inhibitor, KF116, potently (EC$_{50}$ of 0.024 µM) blocked HIV-1 replication by inducing aberrant IN multimerization in virus particles, whereas it was not effective when added to target cells. These findings delineate the significance of correctly ordered IN structure for HIV-1 particle morphogenesis and demonstrate feasibility of exploiting IN
multimerization as a therapeutic target. Furthermore, pyridine-based compounds present a novel class of multimerization selective IN inhibitors as investigational probes for HIV-1 molecular biology.

Finally, chapter 6 summarizes the findings and significance of this dissertation research as well as provides future perspectives. In summary, this dissertation has: (I) summarized the structure, function and inhibition of HIV-1 integrase, (II) characterized the multimodal mechanism of ALLINIs, (III) studied the ALLINI resistant A128T IN substitution and (IV) H171T IN substitution and finally (V) characterized a multimerization selective IN inhibitor to understand the role of IN multimerization in HIV replication.
Dedication

This document is dedicated to my parents, Tim and Mary.
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Chapter 1: Introduction

1.1 HIV evolution and epidemiology

HIV-1 is divided into four groups, M, N, O and P, which each arose from independent zoonotic transmissions of the closely related simian immunodeficiency viruses (SIVs). Group M is the predominant group, the only one that has spread outside of West Africa and is responsible for the global pandemic. Groups N, O and P are less widespread and mainly limited to Cameroon with group O accounting for 1% of all HIV-1 infections, group N identified in less than 20 individuals and group P in only two individuals [1]. The zoonotic transmission of SIVcpz from chimpanzees gave rise to HIV-1 groups M and N, while group P arose from SIVgor zoonotic transmission from gorillas. Group O is genetically similar to SIVgor and thus likely to have been derived from transmission of SIVgor; however, because no strain has been identified to confirm this, it is also possible that group O is a result of SIVcpz transmission [2,3].

In 2011, an estimated 34.2 million people were HIV-positive with 2.5 million people newly infected [4]. Left untreated, HIV depletes CD4\(^+\) T cells leading to
acquired immunodeficiency syndrome (AIDS). As the number of CD4+ T cells declines, cell-mediated immunity is compromised rendering patients susceptible to opportunistic infections [5]. Some of the most common AIDS-associated opportunistic infections are Kaposi’s sarcoma, tuberculosis and recurrent pneumonia [6].

Advances in HIV antiretroviral therapy (ART) have changed HIV from a deadly prognosis to a manageable disease. The standard of care for HIV is highly active antiretroviral therapy (HAART), the combination of multiple mechanistically distinct antiretrovirals. There are six classes of FDA-approved ART that target four distinct steps in the replication cycle: entry, reverse transcription, integration and maturation (see below for detailed discussion). The FDA approved 3′-azido-3′-deoxythymidine (AZT), the first ART, in 1987, only four years after the identification of HIV [7]. Since then, 24 more ARTs have been approved [8]. In 1997, HAART started to become the standard of care as it reduces viral load and avoids the emergence of drug resistance mutations significantly better than monotherapy [8-10].

While great strides in ART have been made, the emergence of drug resistant strains remains a problem, particularly in under developed countries with reduced resources [11,12]. Resistance mutations to antiretrovirals continue to emerge due to the high rate of viral replication, low fidelity of HIV-1 reverse transcriptase and the high rate of recombination [13]. As such, there is a continued need for new ARTs that have novel mechanisms of action or novel targets.
1.2 Retroviral life cycle and mechanisms of inhibition

HIV is a positive sense single stranded RNA virus that infects CD4+ T cells. HIV replication begins when the viral envelope protein gp120 engages the CD4 receptor on the surface of a cell. This binding event results in a conformational change of gp120 to reveal a second binding site for the chemokine co-receptors CCR5 or CXCR4. Co-receptor binding allows the viral envelope protein gp41 to insert its N-terminal end into the host cell membrane and fusion of the virus and host cell membranes to occur (Figure 1.1 step 1, [14]). Currently, there is one FDA approved entry inhibitor, maraviroc, and one fusion inhibitor, enfuvirtide. Maraviroc is a CCR5 receptor antagonist and therefore inhibits gp120 binding to the CCR5 co-receptor. Enfuvirtide is a biomimetic peptide that binds gp41 preventing the fusion of the viral membrane with the cellular membrane [15].

After fusion occurs, the viral capsid core is released into the cytoplasm. The capsid core contains essential viral components for virus replication including the RNA genome, reverse transcriptase (RT) and integrase (IN). Once in the cytoplasm, the capsid core begins to disassemble. During or after capsid disassembly, the RNA genome is reverse transcribed into cDNA by RT (Figure 1.1 step 3). There are two classes of FDA approved RT inhibitors, nucleotide/nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). NRTIs mimic naturally occurring deoxynucleosides (dNTPs) and compete for incorporation during reverse transcription of the viral DNA genome by RT and terminate DNA chain
elongation [16]. Alternatively, NNRTIs bind allosterically and abrogate RT enzymatic activity by modulating the protein conformation [16].

The reverse-transcribed viral DNA genome is next imported into the nucleus and integrated into the host genome. The viral DNA associates with other viral and cellular proteins to form the pre-integration complex (PIC). The PIC is then imported into the nucleus where integrase (IN) catalyzes the integration of the viral DNA into the host chromatin through two separate reactions. In the first step, termed 3'-processing, IN cleaves a dinucleotide from both 3' ends of the viral DNA genome. In the second step, termed strand transfer, IN catalyzes a transesterification reaction to insert the viral DNA genome into chromatin to form the integrated provirus (Figure 1.1, step 4-5, [14]). Recently, the FDA approved integrase strand transfer inhibitors (INSTIs). INSTIs bind at the IN active site and selectively inhibit the second step of integration, strand-transfer [17].

The integrated provirus is transcribed by host RNA Pol II. HIV-1 mRNAs are transcribed as six different monocistronic or bicistronic splice variants: one that is unspliced, three that are singly spliced and 2 that are doubly spliced [18]. The doubly spliced mRNAs encode Tat, Rev and Nef and these three genes are the first to be translated [18]. Tat and Rev have functions relating to transcription and mRNA export respectively. Nef plays an important role in evasion of the immune system by down-regulating the cell surface expression of CD4 receptors and MHC-I [18].
Tat significantly enhances the RNA Pol II transcription of the provirus. Tat binds the HIV-1 transactivation response (TAR) element in the R region of the long terminal repeat (LTR). Tat recruits positive acting elongation factor (pTEFb). P-TEFb is comprised of cyclin T1 and CDK9. The CDK9 kinase hyperphosphorylates the C-terminal domain of RNA Pol II releasing it from the pause state, which significantly enhances transcription (Figure 1.1 step 6, [19]).

The ~9 kb unspliced and ~ 4kb single splice variants of the viral mRNA require Rev to export them from the nucleus. Each of the unspliced and singly spliced mRNAs variants contains the Rev response element (RRE). Rev oligomerizes on the RRE through an N-terminal arginine-rich motif. The C-terminal end of Rev engages the nuclear export factor Crm1 [14,18] to facilitate the export of unspliced and singly spliced mRNAs (Step 7). The singly spliced mRNAs encode for Env, Vif, Vpr, and Vpu while Gag-Pol is encoded by unspliced mRNA.

After being exported, the unspliced viral mRNAs are translated into the structural and enzymatic viral proteins Gag and Gag-Pol. Gag consists of four individual structural proteins including matrix (MA) or p17, capsid (CA) or p24, nucleocapsid (NC) or p7 and p6. Pol consists of three enzymatic proteins: protease (PR), RT and IN (Figure 1.1 Step 8). Gag and Gag-Pol remain as polyproteins throughout the assembly and budding stages. Nascent genomic RNA (gRNA) interacts with the NC protein of Gag and results in Gag multimerization. Matrix then interacts with phosphatidylinositol-4,5-bisphosphate
(PI(4,5)P2) which is located in the cellular plasma membrane as well as interacting with Env glycoproteins allowing for their incorporation into the plasma membrane (Figure 1.1 Step 9). Gag continues to accumulate at the plasma membrane and the C-terminal p6 domain of Gag recruits the cellular endosomai-sorting complex required for transport (ESCRT) proteins, which facilitate membrane budding and scission (Figure 1.1 Step 10, [20]).

After the virion buds from the progenitor cell, it generates an immature virion that must undergo PR catalyzed cleavage of the precursor polyproteins to yield a new infectious virus. PR cleaves Gag and Gag-Pol polyproteins into the structural MA, CA and NC proteins and enzymatic PR, IN and RT proteins. Following proteolytic cleavage, a structural change occurs inside of the virion. CA forms a conical core that encapsulates the gRNA as well as IN & RT enzymes. Following proteolytic cleavage, it is now a mature virion that is able to infect a naïve cell (Figure 1.1 step 11, [14]). The FDA has approved 9 PR inhibitors (PIs) that abolish protease activity. PIs bind in the PR active site and compete for peptide binding thus inhibiting catalytic activity resulting in immature virions that are unable to infect naïve cells [21].

1.3 Integrase structural biology and function

HIV IN catalyzes the integration of the reverse-transcribed viral DNA into host chromatin through two distinct steps. The first step, 3’-processing, occurs when IN removes a GT dinucleotide from the 3’-terminus of the viral DNA ends. This
processing reaction results in a reactive 3'-hydroxyl group that is used as nucleophile for the IN catalyzed second reaction, strand transfer. IN then catalyzes strand transfer in a staggered fashion through a transesterification reaction. Cellular DNA repair mechanisms complete gap repair to produce the integrated provirus (Figure 1.2, reviewed in [22]).

HIV IN is a 32 kDa protein comprised of three domains: an N-terminal domain (NTD), a catalytic core domain (CCD) and a C-terminal domain (CTD) (Figure 1.3). While the full-length recombinant protein has not been amenable to atomic-resolution structures due to its low solubility, structures have been determined for the individual domains as well as two domain structures [23-30]. The NTD contains an HHCC motif that coordinates a Zn$^{2+}$ ion. The Zn$^{2+}$ binding is critical for the correct structure of the NTD as well as the functional multimerization of full-length IN [31-33]. While the NTD NMR structure showed the domain as a dimer [24], the two-domain NTD-CCD crystal structure did not maintain these interactions and was tetrameric instead [29]. The CCD contains a D-D-E motif which coordinates Mg$^{2+}$ ions and is the active site for both 3'-processing and strand transfer [23,31]. The CCD crystal structure is seen as a dimer [23,25,26] and the interactions between the CCD subunits are preserved in both the NTD-CCD crystal structure [29] and the CCD-CTD crystal structure [30]. The CTD contains an SH3-like fold and is important for DNA binding. The CTD NMR structure was observed as a dimer [27,28]; however, the subunit interactions were completely lost in the two-domain CCD-CTD structure [30].
Full length IN assembles as a tetramer with viral DNA ends to form a stable synaptic complex (SSC) to catalyze integration [34,35]. While the crystal structure of the HIV-1 IN intasome has not been solved, the crystal structure of a related retroviral integrase, prototype foamy virus (PFV), has given clues to the structural organization of functional tetrameric HIV-1 IN [36,37]. The PFV IN intasome consists of two inner subunits that are responsible for tetramerization and viral DNA binding and two outer subunits that play a role in maintaining protein conformation. The CCDs of the inner subunits interact with viral DNA. The D-D-E active site of each subunit catalyzes the 3’-processing and strand transfer of one end of the viral DNA (Figure 1.4). The NTD and CTD of the inner subunits make additional viral DNA contacts. The outer and inner subunits interact through the CCD-CCD interface that is similarly seen in the single and two domain HIV-1 IN structures (Figure 1.4). The PFV IN intasome crystal structure has allowed for the generation of homology models of the HIV-1 intasome [38-40]. The majority of the interfaces seen in the PFV IN intasome structure are retained in each of the homology models including the interactions between the CCDs of the inner and outer subunits and the interactions between NTD-CCD of the opposing inner subunits. However, the conformation of the linker between the CCD and CTD of inner subunits has been modeled as either an unstructured flexible linker [38,39] or as a loop-helix-loop motif as is seen with PFV IN [40]. Together, these studies have aided in understanding the INSTI mechanism of action and aided in the design of second generation INSTIs [36], as well as understanding HIV biology and mechanism of retroviral integration.
1.4 Integration site selection and LEDGF/p75

The crystal structure of the PFV intasome showed that there is a severe bending of the target DNA during strand transfer. Consequently, PFV has a slight preference for integrating into pyrimidine (Y) – purine (R) dinucleotides at the center of the integration site. YR dinucleotides are the most flexible of the possible combinations, thus allowing for bending of target DNA [37]. HIV similarly prefers nucleotide sequences that will allow for flexibility and bending. At the center of the integration site, HIV prefers the sequence RYXRY. This variability of the central nucleotide sequence allows for either YR/RR or YY/YR to tolerate target DNA bending [41]. Additionally, in vitro, HIV has a loose preference for integration into naked DNA as compared to chromatized templates. This could be accounted for by the fact that the target DNA is more flexible than wrapped DNA.

While HIV has only slight integration site preferences for nucleotide sequence, HIV does exhibit a strong bias towards integrating in active gene bodies in cells [42]. In infected cells, HIV IN associates with the cellular protein lens epithelial derived growth factor (LEDGF)/p75 which acts as a bimodal tether to link PICs to chromatin and directs integration towards active genes [43-45]. Antagonism of the IN-LEDGF/p75 interaction in target cells through knockdown (KD) or knockout (KO) results in the loss of HIV infectivity [46,47].

LEDGF is encoded from the PSIP1 (positive cofactor 4 and serine/arginine-rich splicing factor 1 or PC4- and SFRS-interacting protein 1) gene. LEDGF exists as
two isoforms through alternative splicing, called p75 and p52. LEDGF/p75 is a 530 amino acid protein and LEDGF/p52 is a 330 amino acid protein. LEDGF/p75 and LEDGF/p52 share the same 325 N-terminal amino acids and differ at the C-terminal end (Figure 1.5). HIV-1 integrase interacts with the C-terminal end of LEDGF/p75 and as a result, LEDGF/p52 plays no role in HIV replication [48].

The N-terminal portion of LEDGF/p75 is involved in chromatin binding and the C-terminal portion is involved in multiple protein-protein interactions [48]. The N-terminus of LEDGF/p75 contains a Pro-Trp-Trp-Pro (PWWP) domain that binds both histone mark H3K36me3 and chromosomal DNA to create a high affinity interaction with mononucleosomes [49,50]. Additionally, the N-terminal end contains a nuclear localization signal (NLS), three charged regions (CR) and two AT-hook DNA binding motifs, which contribute to non-specific DNA binding [48]. Rescue of HIV-1 infectivity in LEDGF/p75 KD SupT1 cells or KO MEF cells using ΔPWWP LEDGF/p75 was unsuccessful [45,46]. Additionally, chimeras of LEDGF/p75 in which the N-terminal end has been replaced with other chromatin binding domains are able to support HIV replication as well as redirect integration to regions in which the respective chromatin domains bind [51-54]. Together, these studies demonstrate the necessity of tethering the PIC to chromatin for productive HIV integration as well as the role of cellular proteins in integration site selection.

At the C-terminal of LEDGF/p75, an 83-amino acid domain interacts with lentiviral INs, including HIV-1 IN, and is thus named the integrase binding domain
The LEDGF/IBD is comprised of 5 alpha-helices, of which 4 (α1, α2, α4 and α5) form a right-handed helical bundle which are connected by α3. A cluster of hydrophobic residues at the top of the LEDGF/IBD structure formed by the loops between α1-α2 and α4-α5 have been shown to interact with IN. Mutagenesis these amino acids, Ile366, Asp366 and Phe406 abrogated LEDGF/IBD-IN interactions while Val408 severely reduced binding. This confirmed that the V-shaped loop is critical for protein-protein interaction [56]. Biochemical studies have also demonstrated that the IN CCD in necessary and sufficient to bind LEDGF/p75 [57]. Additionally, the crystal structure of the LEDGF/IBD in complex with the HIV-1 IN CCD dimer revealed that the LEDGF/IBD binds at the CCD-CCD dimer interface. In the crystal structure the Ile366 of LEDGF/IBD forms hydrophobic interactions with HIV-1 IN residues Leu102, Ala128, Ala129, Try132, Thr174 and Met178. Additionally, LEDGF/IBD residues Phe406 and Val408 have hydrophobic interactions with HIV-1 IN Try131 and LEDGF/IBD Asp366 forms hydrogen bonds to the backbone amides of HIV-1 IN Glu170 and His171 [58].

Although the IN CCD is necessary and sufficient to bind LEDGF/IBD, the IN NTD contributes to the high-affinity binding of IN to LEDGF/p75 [57]. The crystal structure of the LEDGF/IBD in complex with HIV-2 IN NTD-CTD two domain structure have confirmed the role of the IN NTD in binding to LEDGF/p75. The α1 of the HIV-2 IN NTD and α4 of LEDGF/IBD create a charge-charge interface that contributes to the high-affinity IN-LEDGF/IBD binding. For example, the HIV-2 IN
Glu10 forms a salt bridge with Arg405 of LEDGF/IBD. The remaining negatively charged HIV-2 IN residues Glu6 and Glu13 and the positively charged LEDGF/IBD residues Lys401, Lys402, Arg405 contribute to the overall charge-charge interaction. The acidic residues at positions 6, 10 and 13 in HIV-2 IN NTD α1 are highly conserved among other lentiviruses. Furthermore, mutagenesis of these residues in HIV-2 or their HIV-1 counterparts has been shown to abrogate IN-LEDGF/p75 interactions [59].

While the LEDGF/IBD is necessary and sufficient for IN binding, as discussed above full length LEDGF/p75 is necessary to support HIV replication. In vitro, LEDGF/IBD inhibited LEDGF/p75 dependent IN catalytic activity [55]. Additionally, overexpression of LEDGF/IBD in target cells significantly inhibited HIV-1 replication, reinforcing the findings that the LEDGF/IBD is sufficient to bind IN but that the chromatin binding activity of LEDGF/p75 is necessary to support HIV replication [46,60]. Selection of virus strains resistant to LEDGF/IBD overexpression yielded mutations in the IN CCD dimer interface, A128T and E170G [61], which were previously shown to be important in IN-LEDGF/IBD binding [58]. These mutations affect both virus replication capacity and IN catalytic activity 2 to 4 fold compared to their WT counterparts, confirming the dependence on LEDGF/p75 for effective replication [61]. Taken together, these studies demonstrate the function of LEDGF/p75 as a bimodal tether in which the N-terminal end engages chromatin at histone mark H3K36me3 and the C-
terminal IBD binds HIV IN to both enhance integration efficiency and to direct integration towards active gene bodies.

1.5 HIV-1 integrase as a drug target

1.5.1 Active site IN inhibitors

The first INSTI, raltegravir (RAL), was approved by the FDA in 2007, seven years after the first report of diketo acids (DKA) as strand transfer inhibitors [62]. The first DKAs were discovered through a screen of more than 250,000 compounds for inhibitors of recombinant integrase in strand transfer assays. The most potent of the compounds inhibited recombinant integrase with an IC$_{50}$ of 80-150 nM and inhibited HIV replication with an EC$_{50}$ of 1-2 µM [62]. Biochemical studies revealed that DKAs bind to IN at the active site and compete with viral DNA for binding [63]. However, DKA binding to the active site alone is not sufficient to inhibit strand transfer; inhibitor sequestration of Mg$^{2+}$ is also important for inhibitory activity [64]. Subsequently, a structurally similar inhibitor from the same class was shown to inhibit replication of simian-human immunodeficiency virus (SHIV) in rhesus macaques [65]. From these studies RAL moved to clinical trials. While structurally distinct from the previously reported small molecules, RAL retains the Mg$^{2+}$ sequestration activity reported for DKAs [66,67].

Details of the mechanism of action of RAL were elucidated when the PFV intasome crystal structure soaked with RAL was solved [36]. PFV is sensitive to RAL in vitro and is therefore a good model to understand RAL mechanism of
The crystal structure revealed that the oxygen atoms of RAL responsible for chelating Mg$^{2+}$ are oriented towards the D-D-E active site residues. Additionally, RAL creates hydrophobic interactions with Y212 and stacking interactions with P214, which results in the deactivation of the intasome. Finally, RAL makes Van der Walls interactions with the CA nucleotides of the processed viral DNA ends and displaces them from the active site [36]. Taken together, the structural basis for INSTI activity reinforces the necessity of both the chelation of the divalent metal as well as competition for viral DNA binding in the IN active site.

While RAL has overcome many of the multidrug resistant viral strains [66], sequencing of virus from patients in virologic failure in a phase IIb clinical trial revealed the emergence of RAL-resistant mutations N155/H, Q148K/R/H, G140S and E92Q [69]. In 2012 the FDA approved a second INSTI, elvitegravir (EVG), for use in combination with two NRTIs. EVG inhibits the replication of HIV in cell culture from 0.26 – 1.3 nM [70]. However; EVG has considerable cross-resistance to RAL resistance mutants. Phase II clinical trials of EVG observed E92Q, Q148H/K/R and N155H mutations in patients experiencing virologic failure, each of which were observed for patients receiving RAL [71]. A second-generation INSTI, dolutegravir (DTG), was approved by the FDA in 2013. In the clinic with patients failing first-generation INSTIs, DTG remained active against INSTI resistance mutations Tyr143, His155, Gln148. However, if a mutation at Gln148 was combined with two or more additional resistance mutations, the
antiviral activity of DTG was compromised [72,73]. Collectively, these studies underscore the need for novel HIV IN inhibitors with novel mechanisms of action.

1.5.2 IN multimerization as a drug target

HIV-1 integrase acts as a tetramer to catalyze integration (discussed in 1.3). Modulation of the oligomeric state of IN renders IN inactive [74]. For example, the IN mutation K186A renders IN primarily dimeric and abolishes IN catalytic activity [75]. While inhibiting the tetramerization of IN could theoretically inhibit integration, inhibiting these protein-protein interactions is a challenge because of the large interactions between IN proteins (contact surfaces ~1500-3000 Å²), compared to the potential interactions a small molecule would have with a protein (contact surfaces ~300-1000 Å²) [76]. In the case of HIV IN, homogeneous time resolve fluorescence studies have estimated the $K_d$ of the dimer to be 68 pM [77]. It is for these reasons that studies to stabilize catalytically inactive forms of the IN tetramer rather than inhibit the formation of IN tetramers have been conducted.

The IN subunits in the SSC display a highly dynamic interplay allowing IN to correctly assemble with viral DNA. However, when LEDGF/p75 was allowed to bind IN, LEDGF/p75 restricted the exchange of IN subunits. [75]. Further studies compared the conformation of IN when bound to viral DNA or LEDGF/p75. The tetramer formed in the presence of viral DNA is significantly different than the tetramer formed in the presence of LEDGF/p75 [39]. Importantly, the tetramer
formed in presence of LEDGF/p75 was subsequently unable to engage viral DNA [39,78] and to form the SSC. Conversely, the pre-formed IN – viral DNA complex was able to then bind LEDGF/p75 [39]. These studies highlight the importance of the temporal assembly of the SSC in which a tetramer of IN engages viral DNA before interacting with LEDGF/p75 to carry out concerted integration. Furthermore, these biochemical studies help to clarify data in infected cells in which overexpression of LEDGF/IBD or LEDGF/IBD derived peptides inhibited integration [46,60,77-80]. Specifically, expression of LEDGF/IBD in LEDGF/p75 depleted cells further reduces HIV titer 555-fold, a significant increase from the 30-fold defect seen in LEDGF/p75 depleted cells alone [46,79]. These studies have suggested that the LEDGF/IBD must inhibit IN through a secondary mechanism such as stabilization of an inactive tetramer other than simply competition with endogenous LEDGF/p75.

The proof-of-concept studies that a small molecule can modulate integrase oligomerization came from the observation that tetra-acylated-chicoric acids bind at the CCD-CCD dimer interface and inhibit IN catalytic activity [81-83]. One of these compounds, N,O-bis(3,4,-diacetyloxycinnamoyl)-serinate (referred to as compound 1), selectively modifies Lys103 in the LEDGF/p75 binding pocket [83]. Interestingly, compound 1 inhibited the dynamic exchange of IN subunits [81] similarly to what was seen with LEDGF/p75 [75]. Additionally, compound 1 promoted protein-protein interactions for form inactive, tetrameric forms of IN [81]. Unfortunately, these compounds are cytotoxic and thus antiviral activity was
unable to be determined [82]. However, these studies provide an important proof of principle for small molecules modulating IN oligomerization as an effective allosteric IN inhibitor.

1.5.3 IN-LEDGF/p75 interaction as a drug target

The necessity of HIV IN to interact with LEDGF/p75 for successful replication makes the interaction an attractive therapeutic target. As mentioned earlier, RNAi knockdown of LEDGF/p75 significantly reduces HIV-1 replication in those cells [45-47,84,85]. Additionally, overexpression of the LEDGF/IBD can compete with LEDGF/p75 for binding and inhibit HIV replication [46,60].

While protein-protein interactions are difficult to target with small molecules, LEDGF/p75 and IN contains a small number of residues, or ‘hotspots’ which are necessary for binding [58]. Targeting these ‘hotspots’ with small molecules could feasibly inhibit IN-LEDGF/p75 binding [76]. As discussed in section 1.4, one such ‘hotspot’ occurs between a small v-shaped loop of LEDGF/p75 that projects into the IN CCD-CCD dimer interface [58]. Mutagenesis of LEDGF/p75 residues located in this loop, Ile365, Asp366, Phe406 or Val408, severely impairs or abolishes IN-LEDGF/p75 binding and virus replication [58]. Likewise, viruses containing IN mutations in the pocket formed by the CCD-CCD dimer interface at Ala128, Glu170, Gln168, Try131 and Try132, show a defect in LEDGF/p75 binding and have impaired infectivity [57,61,86,87]. Collectively, these studies highlight LEDGF/p75 as a valid target for antiretroviral therapy.
1.6 Summary of dissertation research

At the beginning of my dissertation research novel allosteric HIV-1 integrase inhibitors (ALLINIs) were discovered. Through a high-throughput screen for inhibitors of 3′-processing, Boehringer Ingelheim identified 2-(quinolin-3-yl) acetic acid derivatives [88-90]. Their lead compound, BI-1001 (herein referred to as ALLINI-1), inhibited IN 3′-processing activity with an IC$_{50}$ of 0.07 µM and inhibited virus replication in infected cells with an EC$_{50}$ of 0.45 µM [90]. Independently, Christ et al identified a similar compound, LEDGIN-6, through the rational design of inhibitors that bind in the LEDGF/p75 binding pocket and which block the IN-LEDGF/p75 interaction. LEDGIN-6 was reported to selectively inhibit the IN-LEDGF/p75 interaction with an IC$_{50}$ of 1.37 µM and to inhibit replication in infected cells with an EC$_{50}$ of 2.35 µM [91].

ALLINI-1 and LEDGIN-6 consist of the same quinoline scaffold with shared carboxylic acids at the 3 position and substituted benzene rings at the 4 position [90,91]. The crystal structure of the HIV IN CCD dimer soaked with LEDGIN-6 revealed that LEDGIN-6 binds at the CCD-CCD dimer interface [91]. This information, coupled with our previous findings with compound 1, show that small molecules that bind at the CCD-CCD dimer interface and thus modulate IN multimerization [81] led us to hypothesize that both ALLINI-1 and LEDGIN-6 may also stabilize IN subunits into an inactive conformation.
The aim of my dissertation research is to elucidate the mechanism of action of allosteric integrase inhibitors (ALLINIs) and determine the mechanism of resistance to these compounds by HIV. Chapter two describes my findings that ALLINI-1 and LEDGIN-6 have an identical mechanism of action in which they both inhibit IN-LEDGF/p75 binding and promote aberrant integrase multimerization with equal potency. Chapter three focuses on my work to dissect the mechanism of resistance conferred by the A128T IN substitution to ALLINI-1 treatment. In particular, we show that the A128T IN substitution confers resistance by altering the binding orientation of ALLINI-1 in the LEDGF/p75 binding pocket. This demonstrated that aberrant IN multimerization, and not inhibition of IN-LEDGF/p75 binding is the primary target of ALLINIs. Chapter four analyzes my studies into the mechanism of resistance of a more potent ALLINI, BI-D. This work revealed that the H171T IN substitution confers resistance to BI-D by reducing inhibitor binding and underscores the importance of the t-butyl ether of BI-D for potent inhibition of HIV replication. Chapter five describes my experiments with a novel multimerization selective IN inhibitor (MINI) and its mechanism of action. Finally, chapter six summarizes the significance of the presented research and discusses future perspectives.
Figure 1.1. The HIV-1 Replication cycle.
HIV enters a T-cell through binding a CD4 receptor and a co-receptor and fusion with the host cell membrane (Steps 1-2). The RNA genome is reverse transcribed by reverse transcriptase (step 3) and is then imported into the nucleus where HIV integrase (steps 4-5) inserts the reverse transcribed viral DNA into the host genome. Host RNA Pol II transcribes viral mRNA (step 6) and it is exported to the cytoplasm where it is translated into polyproteins that assemble at the cell plasma membrane (step 7-10). A new virion buds off and HIV protease cleaves the polyproteins to create a mature virion that can infect naïve CD4+ T-cells (step 11).
Figure 1.2 Catalytic activity of HIV-1 IN.
IN catalyzes the integration of through two steps. IN first catalyzes 3’-processing, the cleavage of a GT dinucleotide from the 3’ ends of the viral DNA. Next, IN catalyzes strand transfer, a transesterification reaction to insert the viral DNA into host nucleosomal DNA. Cellular enzymes complete gap repair to form a provirus.
Figure 1.3 IN structural organization.
IN is comprised of 3 domains, an N-terminal domain (NTD), a catalytic core domain C-terminal domain (CCD) and a C-terminal domain (CTD). The NTD contains an HHCC motif for the coordination of a Zn$^{2+}$ ion that is important for the multimerization of the full-length protein. The CCD contains the DDE active site and contributes to DNA binding. The CTD contributes to DNA binding as well as the multimerization of the full-length protein.
Figure 1.4 Crystal structure of PFV intasome.
The crystal structure of the PFV intasome (PDBID: 3L2Q,[36]). A tetramer of IN assembles on the viral DNA to form the intasome. The two inner subunits (purple and blue) form contacts with the viral DNA and account for the multimerization of IN. The outer subunits (teal and brown) provide
Figure 1.5 LEDGF/p75 structural organization. LEDGF/p75 acts as a bimodal tether to link PICs to active genes during integration. The N-terminal portion of LEDGF/p75 is responsible for binding chromatin. The PWPP domain binds H3K36me3 histones as well as DNA nonspecifically. The AT-hooks CR1, CR2 and CR3 each contribute to non-specific DNA binding. The C-terminal end harbors a integrase binding domain (IBD) which binds IN in a hydrophobic pocket at the CCD-CCD dimer interface.
Chapter 2: Multimode, cooperative mechanism of action of allosteric HIV-1 integrase inhibitors

2.1 Introduction

HIV-1 integrase (IN) is an important antiretroviral target due to its essential role in virus replication [92]. Multimeric integrase functions within the context of the preintegration complex to catalyze pair-wise integration of the linear viral DNA ends synthesized by reverse transcription into a host chromosome in a two-step reaction [93]. In the first step, termed 3′-processing, integrase 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, the clinically approved integrase inhibitor, specifically impairs the second step of integration. Although raltegravir results in significant reduction of viral loads in patients [94], HIV phenotypes resistant to this inhibitor evolve comparatively rapidly in the clinic [95]. Therefore, there is a continued need for developing novel integrase inhibitors with alternative mechanisms of action.

We previously proposed one such alternative mechanism with a small molecule inhibitor that stabilizes interacting integrase subunits into an inactive multimeric form [81]. Our biochemical studies indicated that highly dynamic individual
subunits of integrase correctly assemble in the presence of viral DNA to form the functional nucleoprotein complex or intasome [39,75]. Restricting the molecular movement of individual integrase subunits within the multimer during its assembly with DNA compromised integrase enzyme activity [39,75,81]. These observations were further supported by detailed analysis of the available crystal structure of the prototype foamy virus intasome [36] and corresponding molecular models of HIV-1 integrase-viral DNA complexes [38,39]. The organization of the individual integrase subunits within the intasome indicates that cognate viral DNA plays a crucial role in their assembly into the functional complex. In contrast, the preformed integrase 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 [36,38,39]. Therefore, premature multimerization of integrase before it encounters cognate DNA presents an attractive avenue for antiviral drug development.

Recently, inhibitors targeting the interaction between HIV-1 integrase and its key cellular cofactor lens epithelia derived growth factor (LEDGF)/p75 have been reported [91]. LEDGF/p75 directly engages integrase through its C-terminal integrase binding domain (IBD) and tethers the viral protein to chromatin [55,57]. Principal protein-protein contacts of the integrase catalytic core domain (CCD) and N-terminal domain bound to the LEDGF IBD have been revealed in co-crystal structures [58,59], with the extended interacting interfaces between full-length HIV-1 integrase and LEDGF/p75 further defined by MS-based protein
footprinting [75]. Christ et al. [91] exploited the co-crystal structure of the HIV-1 integrase CCD bound to the LEDGF IBD [58] 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 integrase-LEDGF/p75 interaction \textit{in vitro} as well as HIV-1 replication in infected cells [91]. This class of compounds was termed LEDGF-integrase inhibitors (LEDGINs), with one of the more potent inhibitors designated compound 6 (herein referred to as LEDGIN-6). Co-crystal structures of the LEDGIN-IN catalytic core domain (CCD) complexes revealed that the compounds bind to the CCD dimer at the LEDGF/p75 binding pocket. Furthermore, selection of HIV-1 strains resistant to LEDGIN-6 identified an A128T resistance mutation that localized to the same pocket [91].

Our interest in LEDGINs and hence the present studies were prompted by the observation that they bind at the integrase dimer interface [91] adjacent to where we had previously mapped other small molecule inhibitors of integrase multimerization [81]. We accordingly sought to test the hypothesis that LEDGINs could allostrically modulate the dynamic interplay between integrase subunits. In parallel experiments, we investigated the mechanism of action of another 2-(quinolin-3-yl) acetic acid derivative (Figure 2.1), which was patented by Boehringer Ingelheim as HIV replication inhibitor 1001 [89]. Remarkably, ALLINI-1 was derived from compounds identified via a fluorescence based high throughput screen for integrase 3′-processing activity, whereas LEDGIN-6, which was reported to be highly selective for disrupting integrase-LEDGF/p75 binding
(IC$_{50} = 1.37$ µM), exhibited IC$_{50}$ values of >250 µM and 19.5 µM for 3'-processing and strand transfer activities, respectively [91]. We have analyzed these two compounds in parallel experiments, and our data clarify that LEDGIN-6 and ALLINI-1 have identical antiviral mechanisms. These compounds potently inhibit not only integrase-LEDGF/p75 binding but also LEDGF/p75-independent integrase catalytic function. Furthermore, we demonstrate that the key to inhibiting integrase 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.

2.2 Methods

2.2.1 Chemical Synthesis of Integrase 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-chloro-benzonitrile (Sigma-Aldrich) according to the scheme provided by Debyser and co-workers [91]. 2-(6-Bromo-4-(4-chlorophenyl)-2-methylquinolin-3-yl)-2-methoxyacetic acid (ALLINI-1) was synthesized in five steps from commercially available 2-amino-4'-chlorobenzophenone (TCI America) through slight modification of the procedures reported in the patent [89]. Chemical structures of LEDGIN-6 and ALLINI-1 are shown in Figure 2.1.
2.2.2 Construction and purification of FLAG-tagged and tagless integrase

C-terminally FLAG-tagged LEDGF/p75 was constructed as described previously [96]. N-terminally FLAG-tagged integrase was constructed by PCR amplification of C-terminally His-tagged integrase construct pKBIN6Hthr [75] with T7T and In Flag N (5′-ggaattccatatggactacaagacgatgacaaatttttagatggaatagataaggccc-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.

2.2.3 Purification of His-tagged IN

Expression and purification of His$_6$-tagged IN was carried out as previously described ([97]). Briefly, His$_6$-tagged IN was expressed in *Escherichia coli* strain BL21 (DE3). His$_6$-IN was purified by loading cell lysate onto a Ni-NTA column (GE Healthcare) and eluted bound IN with increasing imidazole gradient (20 mM to 500 mM) in a buffer containing 50 mM HEPES, pH 7.4, 1 M NaCl, 7.5 mM CHAPS, 2 mM β-mercaptoethanol. 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 buffer containing 50 mM HEPES, pH 7.4, 7.5 mM CHAPS, 2 mM β-mercaptoethanol. Fractions containing integrase were pooled and stored in 10% glycerol at −80 °C.
2.2.4 Purification of FLAG-tagged LEDGF/p75

FLAG-tagged and tagless INs were purified by loading the ammonium sulfate precipitate of cell lysate onto a phenyl-Sepharose column (GE Healthcare) and eluting bound integrase 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. Peak fractions were pooled and loaded onto a heparin column (GE Healthcare), and integrase 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 β-mercaptoethanol. Fractions containing integrase were pooled and stored in 10% glycerol at −80 °C.

2.2.5 Preparation of DNA Substrates

The blunt-end viral DNA substrate (~1 kb) for stable integrase-viral DNA complex formation was obtained by PCR and purified by agarose gel electrophoresis as described previously [39].

2.2.6 In Vitro Integration Assays

Integrase 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 integrase was preincubated with LEDGIN-6 or ALLINI-1 for 30 min on ice in 50 mM MOPS (pH 7.2) buffer containing 2 mM β-mercaptoethanol, 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/p75-dependent concerted integration assays were carried out as described previously [59,98]. Briefly, 2 µM integrase was preincubated with increasing concentrations of LEDGIN-6 or ALLINI-1 at room temperature for 30 min in 22 mMHEPES (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 viral donor DNA (32-mer blunt-ended U5) and 600 ng of target (pBR322) DNAs were added. Samples were incubated at 25 °C for 5 min, and then LEDGF/p75 was added at a 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 of 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.

2.2.7 HTRF-based Integrase-LEDGF/p75 Interaction Assay

A previously described homogeneous time resolved fluorescence (HTRF) assay [96] was modified for the testing of inhibitors. Briefly, 10 nM N-terminally Histagged integrase was pre-incubated in a binding buffer (150 mM NaCl, 2 mM MgCl₂, 0.1% Nonidet P-40, 1 mg/ml BSA, 25 mM Tris (pH 7.4)) with the
tested compound for 30 min at room temperature, and then 10 nM C-terminally FLAG-tagged LEDGF/p75 was added to the reaction. 6.6 nM anti-His$_6$-XL665 and 0.45 nM anti-FLAG-EuCryptate antibodies (Cisbio, Inc., Bedford, MA) were then added to the reaction. After 4 h 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.

2.2.8 HTRF-based Integrase Multimerization Assay

Two separate preparations of His$_6$-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 MgCl$_2$, 0.1% Nonidet P-40, 1 mg/ml BSA. Test compounds were then added to the mixture and incubated for 2.5 h at room temperature. 6.6 nM anti-His$_6$-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 h. The HTRF signal was recorded as the emission ratio 655 nm / 620 nm multiplied by 10,000.

2.2.9 Crystallization and X-Ray Structure Determination

The HIV-1 integrase CCD (residues 50–212 containing the F185K mutation) was expressed and purified as described [25]. 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 sodium 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 ALLIN-1 was prepared by dissolving the compound in crystallization buffer supplemented with 10% dimethyl sulfoxide. 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 Raxis 4++ image plate detector at the Ohio State University Crystallography Facility. The intensity data integration and reduction were performed with HKL2000 program (Otwinowski Z., Minor W. 1997 in Methods in Immunology). Molecular replacement program Phaser [99] in the CCP4 package method was used to solve the structure. Coot [100] was used for the subsequent refinement and building of the structure. Refmac5 [101] of the CCP4 package was used for the restraint refinement. TLS [102] and restraint refinement was applied for the last step of the refinement. The crystal belonged to space group P3121 with cell dimensions a = b = 73.082 Å and 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

2.2.10 Stable Synapic Complex (SSC) Formation and SSC-LEDGF/p75 Binding Inhibition Assays

The previously reported methods [39] for assembly of the SSC and SSC-LEDGF/p75 binding were used for compound testing. Briefly, integrase was pre-incubated with the compound for 30 min at room temperature before adding viral
DNA to assemble the SSC [39]. In the SSC-LEDGF/p75 assay, the purified SSC was pre-incubated with inhibitor for 30 min at room temperature before addition of LEDGF/p75. SSC-associated integrase and LEDGF/p75 proteins were separated by spin-size exclusion chromatography and analyzed by SDS-PAGE. Proteins were visualized by Western blot using monoclonal antibodies against integrase (8G4, National Institutes of Health AIDS Research and Reference Reagent Program [103]) and against human LEDGF/p75 (BD Biosciences).

2.2.11 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, whereas HEK293T cells were maintained in Dulbecco's modified Eagle's medium modified to contain the same supplements. The concentration of HIV-1<sub>NL4-3</sub> in the supernatant of plasmid pNL4–3-transfected HEK293T cells was determined using a radionuclide-based exogenous assay for reverse transcriptase (RT) activity [104], and SupT1 cells (4 × 10⁴ per well of a 96-well plate) were infected with 5 × 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 raltegravir and saquinavir were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.
2.3 Results

2.3.1 LEDGIN-6 and ALLINI-1 inhibit IN-LEDGF/p75 binding and IN catalytic activity

Two 2-(quinolin-3-yl) acetic acid derivatives, LEDGIN-6 and ALLINI-1, 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 hot spot residues Ile-365 and Asp-366 in their contacts with the integrase-CCD dimer interface and has been reported to selectively inhibit the integrase-LEDGF/p75 interaction ($IC_{50} = 1.37 \mu M$) but not integrase 3'-processing activity ($IC_{50} > 250 \mu M$) [91]. Paradoxically (Figure 2.1), ALLINI-1 was identified via a high throughput screen for integrase 3'-processing activity [89]. Therefore, it was important to evaluate LEDGIN-6 and ALLINI-1 in parallel to dissect their mechanism of action.

We first compared the compounds for their ability to inhibit the integrase-LEDGF/p75 interaction using the HTRF-based assay (Figure 2.2). Both compounds effectively impaired integrase-LEDGF/p75 binding. In triplicate repeats of these experiments, ALLINI-1 consistently was several fold more potent than LEDGIN-6 (Figure 2.2). The compounds were next evaluated for their ability to inhibit integrase catalytic activities in the absence of LEDGF/p75. For these experiments, we chose to employ commonly used 3'-processing and strand transfer activity assays that enable reliable quantitation of $^{32}$P-labeled DNA substrates and reaction products. The 3'-processing assays, which were
conducted with a 21-mer blunt-ended DNA, revealed that LEDGIN-6 and ALLINI-1 inhibited integrase activity with 50% inhibitory concentration (IC\textsubscript{50}) values of 3.9 and 2.3 µM, respectively (Figure 2.3; results summarized in Table 2.1). The strand transfer reactions with pre-processed donor DNA substrates were also inhibited by LEDGIN-6 (IC\textsubscript{50} = 4.2 µM) and ALLINI-1 (IC\textsubscript{50} = 1.7 µM) (Figure 2.3, Table 2.1). Our results differ significantly from the previously reported LEDGIN-6 IC\textsubscript{50} values of >250 and 19.5 µM for inhibiting integrase 3'-processing and strand transfer activities, respectively [91].

2.3.2 ALLINI-1 and LEDGIN-6 have overlapping binding in the LEDGF/p75 binding pocket of HIV-1 IN

Our observations that the compounds inhibited integrase-LEDGF/p75 binding and inherent LEDGF/p75-independent integrase function equally well raised the question regarding the structural basis for their multimode mechanisms of action. We therefore solved the x-ray crystal structure of ALLINI-1 bound to the HIV-1 integrase CCD (Figure 2.4) and compared it with the previously reported co-crystal structure with LEDGIN-6 (Figure 2.4) [91]. Comparative analysis revealed nearly overlapping drug binding. However, we note one important difference: the ALLINI-1 methoxy group, which is absent in LEDGIN-6, forms an additional H-bond with integrase residue Thr-174. This interaction is likely to account for the superior potency of ALLINI-1 over LEDGIN-6 in both LEDGF/p75-dependent and -independent assays (Figure 2.2, 2.3; Table 2.1). Our discovery of relative potent inhibition of inherent integrase catalytic activities and confirmation that ALLINI-1
binds to the CCD dimer interface at the same position as LEDGIN-6, defines both compounds as bona fide allosteric inhibitors of the HIV-1 integrase enzyme. We next sought to determine the mechanistic basis for allosteric inhibition.

Co-crystal structures (Figure 2.4) [91] revealed that both compounds recapitulate the function of LEDGF/p75 hot spot residue Asp366, in that they engage main chain nitrogens of integrase residues Glu170 and His171, thus elucidating the mechanism for inhibition of integrase-LEDGF/p75 binding. However, this site is removed significantly from the presumed viral donor or host chromosomal target DNA binding sites on HIV-1 integrase [39,91]. Although the structures in Figure 2.4 lack the DNA substrates, comparing the inhibitor-CCD complexes with the apo-CCD dimer did not indicate any gross differences in the positions of the integrase active site residues. Instead, because both inhibitors establish extensive interactions with both CCD subunits of the dimer, we hypothesized that the compounds might deregulate integrase-integrase interactions critical for enzyme function.

2.3.3 ALLINI-1 and LEDGIN-6 promote IN multimerization

To test the hypothesis that ALLINI-1 and LEDGIN-6 deregulate integrase-integrase interactions, we designed an HTRF-based assay to monitor the integrase-integrase interaction. Anti-His\textsubscript{6}-XL665 and anti-FLAG-EuCryptate antibodies allow fluorescence energy transfer upon interaction of two full-length, wild type HIV-1 integrase proteins, one containing an N-terminal His\textsubscript{6} tag and the
other, an N-terminal FLAG tag (Figure 2.5). Compounds that inhibit integrase-integrase binding would accordingly decrease the HTRF signal, whereas those that promote multimerization by stabilizing the interacting integrase subunits would increase the signal. Representative data with LEDGIN-6 revealed a striking dose-dependent increase of the HTRF signal (Figure 2.5). As a control, we used raltegravir, which targets the integrase active site distal from the CCD dimer interface. The HTRF signal predictably remained at the background level with increasing raltegravir concentrations (Figure 2.5). The data in Figure 2.5 show that LEDGIN-6 and ALLINI-1 promoted integrase multimerization with IC$_{50}$ values of 11.3 and 4.9 µM, respectively (Table 2.1).

2.3.4 ALLINI-1 and LEDGIN-6 exhibit cooperative inhibition in vitro

LEDGF/p75 dependent integration assays and in virus replication in infected cells.

We next examined the compounds in an in vitro integration assay dependent on the LEDGF/p75-integrase interaction for effective stimulation of concerted integration [59,98]. Although 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 ALLINI-1 under these reaction conditions (Figure 2.7 and Table 2.1). 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/p75 binding. Conversely, in reactions where integrase-LEDGF/p75
binding (Figure 2.2) or integrase catalytic function (Figure 2.3) were monitored separately, Hill coefficients were ~1 (Table 2.1). To make sure that a cooperative mode of action in the concerted integration reaction was specific to LEDGIN-6 and ALLINI-1, we conducted control experiments with raltegravir. No cooperativity was observed under these conditions (data not shown), due presumably to the fact that raltegravir 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 ALLINI-1 EC_{50} and Hill coefficient values were determined and compared with those of control compounds raltegravir and saquinavir. As established previously [105], the protease inhibitor saquinavir displayed cooperative inhibition (m = 2.6) under conditions where raltegravir failed to reveal evidence of cooperativity (m = 1.1). Both LEDGIN-6 and ALLINI-1 displayed highly cooperative inhibition, yielding m-values of 3.9 and 3.7, respectively (Figure 2.8 and Table 2.1).

2.4 Discussion

Here, we investigated the mechanism of action of two prototypes of a growing number of small molecule compounds that bind HIV-1 integrase distal from the enzyme active site. In contrast to the previous report [91] indicating that LEDGIN-6 specifically inhibited the integrase-LEDGF/p75 interaction, we show conclusively that this inhibitor impairs both integrase-LEDGF/p75 binding and the inherent catalytic activities of integrase, which do not rely on LEDGF/p75, with
very similar IC<sub>50</sub> values. Although structurally similar, ALLINI-1 was more potent than LEDGIN-6, and the two compounds displayed an overlapping mechanism of action. We therefore conclude that LEDGIN-6 and ALLINI-1 belong to the same class of inhibitors. Because these 2-(quinolin-3-yl) acetic acid derivatives allosterically modulate integrase structure, we propose to name this class of compounds allosteric integrase inhibitors.

We show that the underlying basis of inhibition of LEDGF/p75-independent integrase catalytic function by allosteric integrase inhibitors is premature integrase multimerization (Figures 2.5 and 2.6). Although the functional intasome or SSC contains a tetramer of integrase stably bound to the two viral DNA ends [36,106], the highly dynamic interplay between free integrase subunits is critical for their productive assembly with viral DNA and hence functional SSC formation [75]. In the absence of cognate DNA, free integrase can also multimerize, but these preformed integrase multimers do not form the SSC and accordingly lack integrase catalytic function [39]. Previously, we showed that the LEDGF IBD promotes formation of integrase multimers [39,75]. Remarkably, the conformations of integrase multimers within integrase-LEDGF/p75 complexes formed in the absence of viral DNA and SSCs differ significantly, and the preassembled integrase-LEDGF IBD complex moreover lacks the ability to functionally integrate viral DNA ends [39,107]. Our findings that allosteric integrase inhibitors also modulate integrase multimerization and impair the formation of the SSC argue further for exploiting integrase multimerization as a
novel therapeutic target. Due to their molecular mimicry of LEDGF/p75 hot spot residues Ile-365 and Asp-366, we postulate that allosteric integrase inhibitors recapitulate the inhibitory activities of anti-integrase peptides derived from the tip of the corresponding helix-hairpin-helix IBD structure [78].

The allosteric integrase inhibitor mode of action of promoting, rather than inhibiting, integrase 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 integrase multimerization. The substituted benzene ring of the compounds primarily engages one integrase monomer through hydrophobic interactions, whereas the carboxylic acid group, and in the case of ALLINI-1, the nearby methoxy moiety, hydrogen bond with the second integrase molecule (Figure 2.4). Such compounds are likely to be effective during the early stages of HIV-1 replication when cognate DNA is unavailable for integrase until after viral DNA synthesis by reverse transcriptase (RT) is completed. LEDGIN-6 accordingly blocked the integration step of HIV-1 replication, and mutations in integrase conferred resistance to the compound [91].

In vitro concerted integration experiments and experiments in infected cells have revealed a cooperative mechanism of action of allosteric integrase inhibitors. High cooperativity of antiviral compounds is important because it strongly influences the instantaneous inhibitory potential, the key clinical parameter for a
retroviral drug that indicates the log reduction in a single round infectivity assay at clinical drug concentrations [105,108]. Inhibitors with high cooperativity or high instantaneous inhibitory potentials are particularly desirable for superior clinical outcomes. Comparative analysis [105,108] of current HIV therapies under clinically relevant conditions have revealed that protease inhibitors and non-nucleoside RT inhibitors, which 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 instantaneous inhibitory potential values, whereas inhibitors such as nucleoside RT inhibitors and raltegravir, that specifically target the active enzyme complexes, do not display cooperativity and thus have low instantaneous inhibitory potential values (~1).

Based on our observations, it is logical to propose that allosteric integrase inhibitors could affect the entire population of integrase 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 allosteric integrase inhibitors, which likely accounts for the Hill coefficient of ~2 observed in the concerted integration assay. Inhibition of integrase multimerization was also cooperative (Hill coefficient ~ 2, Figure 2.5, Table 2.1), due presumably to allosteric integrase inhibitors stabilizing integrase dimers and thus shifting the
equilibrium toward their further assembly into tetramers. Premature integrase multimerization could be a key contributor of the greater Hill coefficient (∼4) observed in replication in infected cells, although additional work with viral replication intermediates will be needed to reveal the details. It is nevertheless noteworthy that mutations in integrase 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, preintegration complex nuclear import, and integration in challenged target cells (reviewed in Refs. [109] and [110]).

Our findings, together with published results [91], argue strongly for further development of allosteric integrase inhibitors as well as studies to discover new inhibitors targeting integrase multimerization. Although the original goal of the rational design of small molecule inhibitors was to effectively compete with integrase-LEDGF/p75 binding [91], future efforts can consider enhancing allosteric integrase inhibitor properties to more tightly bridge the two integrase subunits that meet at the LEDGF/p75 binding cleft [58]. As an example, our co-crystal structure (Figure 2.4) indicates the significance of the ALLINI-1 methoxy group for establishing a unique H-bond with one of the integrase subunits. In general, structural analysis of the pocket at the integrase dimer interface reveals ample opportunities for further enhancing the ability of allosteric integrase inhibitors to more effectively engage both integrase subunits.
Discovery of new integrase multimerization inhibitors could proceed through a high throughput screen. Our method of identifying compounds that stabilize interacting integrase subunits (Figure 2.5) 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 integrase CCD dimer interface [81,111,112]. Significantly, two additional integrase domains (N-terminal and C-terminal domains) also are essential for functional protein multimerization [24,27], and new inhibitors targeting these unexploited protein-protein interfaces are likely to emerge from high throughput screen. As established here, integrase multimerization inhibitors can be expected to behave cooperatively to disarm integrase molecules in excess of the four that compose the heart of the DNA recombination machine. They moreover can be expected to be active against raltegravir-resistant virus and hence complementary to current antiretroviral therapies [91]. Our clarification of a cooperative mode of ALLINI action argues strongly that improved integrase multimerization inhibitors could exhibit desirable clinical properties.

2.5 Acknowledgements

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<th>LEDGIIN-6</th>
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<tr>
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<tr>
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<td>1.9 ± 0.2</td>
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<tr>
<td>Antiviral Activity</td>
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<td>3.9 ± 0.6</td>
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Table 2.1 Activities of LEDGIIN-6 and ALLINI-1. 
Average values with S.E. from the mean are shown for two or three independent experiments.
Figure 2.1. Chemical Structures of LEDGIN-6 and ALLINI-1.
LEDGIN-6 and ALLINI-1 share a common quinoline scaffold with substituted carboxylic groups at the 3 position, aromatic rings at the 4 position and halogens at the 7 position.
Figure 2.2. Effects of LEDGIN-6 and ALLINI-1 on the integrase-LEDGF/p75 binding.
Representative raw data of the inhibition dose response of LEDGIN-6 on the integrase-LEDGF/p75 interaction using the integrase-LEDGF/p75 HTRF assay. Each data point represents the mean of three independent experiments. Below, the curve fitting of dose-dependent inhibition of integrase-LEDGF/p75 binding by LEDGIN-6 (squares) and ALLINI-1 (circles). The average values from three independent experiments are shown.
Figure 2.3. Effects of LEDGIN-6 and ALLINI-1 on integrase 3′-processing and strand transfer activities.

(A) a representative gel image for 3′-processing inhibition by LEDGIN-6. The 21-mer DNA substrate (sub.) and 19-mer reaction product (prod.) are indicated. Lane 1, DNA load; lane 2, DNA+integrase 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.

(B) Curve fitting of the dose-dependent inhibition of integrase 3′-processing activity by LEDGIN-6 (squares) and ALLINI-1 (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+integrase 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 integrase strand transfer activity by LEDGIN-6 (squares) and ALLINI-1 (circles). The average values from three independent experiments are shown.
Figure 2.3. Effects of LEDGIN-6 and ALLINI-1 on integrase 3′-processing and strand transfer activities.
Figure 2.4. Structural analysis of the inhibitor-CCD complexes.

(A) The crystal structure of ALLINI-1 bound to the integrase CCD dimer. Surface views of individual integrase subunits are depicted in magenta and cyan. (B) Overlay of CCD-LEDGIN-6 (Protein Data Bank code 3LPU) and CCD-ALLINI-1 co-crystal structures. Schematic views of integrase subunits are colored as described in A; LEDGIN-6 and ALLINI-1 backbones are green and yellow, respectively. Compound oxygen and nitrogen atoms, as well as those of integrase residues Thr-174, Glu-170, and His-171, are colored red and blue, respectively (for simplicity, only main chain Glu-170 and His-171 atoms are shown). The inhibitor carboxyl groups H-bond (green and orange dashed lines for LEDGIN-6 and ALLINI-1, respectively) with the main chain nitrogens of Glu-170 and His-171, and to the Thr-174 side chain. The ALLINI-1 methoxy group forms an additional H-bond (black dashed line) with Thr-174.
Figure 2.5. Effects of LEDGIN-6 and ALLINI-1 on integrase multimerization.

(A) HTRF assay design. The assay monitors the interaction between two integrase molecules: one containing His$_6$ and the other containing the FLAG tag. The antibodies conjugated with Europium cryptate (Eu) 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 665:620 nm ratio. (B) Representative raw data for affects of LEDGIN-6 (black bars) and raltegravir (gray bars) on integrase (IN) multimerization. Each data point represents the mean of three independent reactions. (C) Curve fittings of dose-response affects of LEDGIN-6 (squares) and ALLINI-1 (circles) on integrase multimerization. The maximal HTRF signal, obtained at high compound concentrations, was set to 100%. The average values from three independent experiments are shown.
Figure 2.6. Effects of LEDGIN-6 and ALLINI-1 on SSC formation and the SSC-LEDGF/p75 interaction
(A) SDS-PAGE analysis of SSCs. (B) SDS-PAGE analysis of LEDGF/P75 interactions with the SSC.
Figure 2.7. Effects of LEDGIN-6 and ALLINI-1 on integrase concerted integration activity.
(A) Representative raw data of the inhibition dose response of LEDGIN-6 on LEDGF/p75-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 contains DNA markers (BIOLINE Quanti-Marker, 1kB); Lane 2 contains target DNA load; lane 3 contains IN activities in presence of target DNA and donor DNA substrates. Lanes 4-16 contain concerted integration in the presence of 1 mM to 240 nM LEDGIN-6. (B). Curve fitting of the inhibition dose responses of LEDGIN-6 (squares) and ALLINI-1 (circles) on LEDGF/p75-dependent concerted integration activity. The average values from two independent experiments are shown.
Figure 2.8. Dose-response curves of antiviral activities of saquinavir, raltegravir, LEDGIN-6, and ALLINI-1.
The average values from two to three independent experiments are indicated. 
RAL, raltegravir; SQV, saquinavir
Chapter 3: The A128T resistance mutation reveals aberrant protein multimerization as the primary mechanism of action of allosteric HIV-1 integrase inhibitors.

3.1 Introduction

HIV-1 integrase (IN) is an important therapeutic target, as its function is essential for viral replication [92]. IN catalyzes the insertion of the reverse-transcribed RNA genome into human chromatin in a two-step reaction [93]. During the initial step (termed 3'-processing), IN removes a GT dinucleotide from each 3' terminus of the viral DNA. The subsequent transesterification reactions (termed DNA strand transfer) covalently join the recessed viral DNA ends into the host genome. To carry out these reactions, highly dynamic IN subunits assemble in the presence of cognate DNA to form the stable synaptic complex or intasome [34,39,75]. Premature multimerization of HIV-1 IN by various ligands in the absence of cognate DNA restricts the functionally essential dynamic interplay between individual subunits and DNA and thus impairs IN catalytic activities [39,75,81,113].
A cellular protein, lens epithelium-derived growth factor (LEDGF)/p75, markedly enhances the integration process in vitro and in infected cells. LEDGF/p75 tethers stable synaptic complexes to chromatin via direct interactions of its N-terminal chromatin-binding domain with nucleosomes, whereas its C-terminal IN-binding domain links to HIV-1 IN [55,57]. Structural studies [58,59] have elucidated the principal interacting interfaces between the LEDGF/p75 IN-binding domain and the HIV-1 IN catalytic core domain (CCD). The cornerstone of this protein-protein interaction is a hydrogen bonding network between the side chain oxygen atoms of LEDGF/p75 Asp-366 and the IN backbone amides of Glu-170 and His-171 [58,59]. These findings have opened up new venues for antiviral drug discovery.

Using a structure-based rational drug design approach, Christ et al. [91] developed 2-(quinolin-3-yl)acetic acid derivatives, which inhibit the IN-LEDGF/p75 interaction in vitro and HIV-1 replication in cell culture. Paradoxically, the identical class of compounds has emerged from a high-throughput screen for IN 3’-processing activity [89]. Subsequent studies described in chapter 2 from our group and others demonstrated that 2-(quinolin-3-yl)acetic acid derivatives exhibit a multimodal mechanism of action by allosterically modulating the IN structure, which affects both IN-LEDGF/p75 binding and catalytic activity [96,114,115]. Accordingly, we have proposed to name this class of inhibitors allosteric IN inhibitors (ALLINIs). Structural studies have shown that the carboxylic acid of ALLINIs hydrogen bonds with the backbone amides of Glu-170 and His-171 and
thus occupy the principal LEDGF/p75-binding interface [91,96,115]. At the same time, the quinoline core and the substituted phenyl group of the inhibitor bridge the two IN subunits and promote allosteric multimerization of the protein. As a result, ALLINIs potently inhibit both IN-LEDGF/p75 binding and LEDGF/p75-independent IN catalytic activities.

The ability of ALLINIs to impair steps in the viral replication cycle that extend beyond IN catalytic function results in a highly cooperative inhibition of HIV-1 replication [115]. Along these lines, it was recently reported that HIV-1 particles made in the presence of ALLINIs are noninfectious [114]. Whereas the Food and Drug Administration-approved HIV-1 IN strand transfer inhibitor raltegravir (RAL) exhibits a Hill coefficient of 1, which is consistent with only a single or non-cooperative mode of action, ALLINIs impair viral replication with a Hill coefficient of $\sim4$, indicating a highly cooperative mode of action [115]. Compounds with a high cooperativity are particularly desirable for superior clinical outcomes because they enable stronger viral suppression at clinical drug concentrations. The cooperativity of antiviral drugs is indicative of their instantaneous inhibitory potential (IIP), the fold decrease in single round infectivity at clinically relevant concentrations which are 10 to 100-fold above the EC$_{50}$. Compounds with a higher degree of cooperativity and thus a higher IIP can achieve a higher percentage of viral suppression at lower concentrations even than compounds with the same EC$_{50}$ but reduced cooperativity. [105,108].
The A128T substitution in HIV-1 IN has been identified from cell culture assays as a primary mechanism for resistance to ALLINI compounds [89,91,96]. Ala-128 is located at the IN dimer interface in the pocket occupied by ALLINIs or LEDGF/p75. Here, we have investigated the structural and mechanistic properties for the resistance of A128T IN to ALLINIs. Strikingly, the A128T substitution only modestly affected ALLINI IC\textsubscript{50} values for IN-LEDGF/p75 binding but markedly altered the multimerization of IN in the presence of the inhibitors. As a result, the catalytic activities of the WT protein were potently inhibited by ALLINIs, whereas A128T IN exhibited significant resistance to the inhibitor. Furthermore, considerably higher concentrations of ALLINIs were required to inhibit the infectivity of the A128T mutant virus compared with the WT counterpart. Taken together, our studies highlight that aberrant IN multimerization is the primary target of this class of inhibitors and thus provide the structural foundations for the development of second-generation ALLINIs with increased potency and decreased potential to select for drug resistance.

3.2 Methods

3.2.1 Antiviral Compounds

ALLINI-1 was synthesized as described in 2.2.1. ALLINI-2 was synthesized as previously described [89]. Chemical structures of ALLINI-1 and ALLINI-2 are shown in Figure 3.1. Raltegravir and saquinavir were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.
3.2.2 Expression & Purification of Recombinant Proteins

LEDGF/p75 and WT and A128T IN recombinant proteins with His<sub>6</sub> or flag tags were expressed in Escherichia coli and purified as described in 2.2.2 - 2.2.4.

3.2.3 Protein-Protein Interaction Assays

Homogeneous time resolved fluorescence (HTRF)-based IN-LEDGF/p75 binding assays were carried out as described in 2.2.7. HTRF IN multimerization assays were performed as described in 2.2.8. The HTRF signal was recorded using a Perkin Elmer EnSpire multimode plate reader.

3.2.4 Solubility Assays

WT IN was diluted to a final concentration of 100 nM in buffer containing 25 mMTris (pH 7.4), 2 mM MgCl<sub>2</sub>, 0.1% Nonidet P-40, 1 mg/ml BSA, and either 150 or 750 mM NaCl. Increasing concentrations of ALLINI-1 or ALLINI-2 were then added to the mixture and incubated for 1 h at room temperature. The mixture was subjected to centrifugation for 2 min at 2000 × g. The supernatant was collected, and the pellet was washed three times with the same buffer. The supernatant and pellet fractions were analyzed by SDS-PAGE and IN was detected with anti-His antibody (Abcam).
3.2.5 3’-Processing, Strand Transfer and LEDGF/p75-dependent Integration assays

Gel-based LEDGF/p75-dependent and LEDGF/p75-independent integration assays were performed as described in 2.2.6. A recently reported time resolve fluorescence assay [96] was used to quantify 3’-processing and strand transfer activities. The HTRF-based LEDGF/p75 dependent integration assays were executed using 100 nM WT or A128T IN was incubated with 50 nM Cy-5 labeled donor DNA, 10 nM biotinylated target DNA and 100 nM LEDGF/p75. After the addition of europium-streptavidin, the HTRF signal was recorded using a Perkin Elmer EnSpire multimode plate reader.

3.2.6 Size Exclusion Chromatography

Experiments were performed with a Superdex 200 10/300 GL column (GE Healthcare) at 0.5 ml/min in elution buffer containing 20 mM HEPES (pH 6.8), 750 mM NaCl, 10 mM MgSO₄, 0.2 mM EDTA, 5 mM β-mercaptoethanol, 5% glycerol, and 200 µM ZnCl₂. IN (20 µM) was incubated with ALLINIs or Me₂SO (control) for 30 min and then subjected to size exclusion chromatography. The column was calibrated with the following proteins: bovine thyroglobulin (670,000 Da), bovine γ-globulin (158,000 Da), chicken ovalbumin (44,000 Da), horse myoglobin (17,000 Da), and vitamin B₁₂ (1,350 Da). Proteins were detected by absorbance at 280 nm. All the procedures were performed at 4 °C.
3.2.7 Crystallization and X-ray structure determination

The HIV-1 IN CCD was purification, crystallization and refinement was carried out as described in 2.2.9. Coordinates have been deposited in the Protein Data Bank with accession numbers 4JLH, 4GW6, and 4GVM.

3.2.8 Surface Plasmon Resonance

SPR experiments were performed using a Biacore T100 (GE Healthcare). A series S sensor chip NTA (GE Healthcare) was conditioned with 0.5 mM NiCl$_2$ at a flow rate of 10 µl/min for 1 min followed by a 1 min wash with 3 mM EDTA at a flow rate of 10 µl/min. 4 µg/mL WT His$_6$-IN CCD or A128T His$_6$-IN CCD in HBS-P, (GE Healthcare) was immobilized on the chip to 4400 response units (RU). Indicated concentrations of ALLINI-2 in HBS-P with 10% DMSO were flowed over the cell for 180s at a flow rate of 40 µl/min followed by a 5 minute dissociation. The chip was regenerated with 500 mM imidazole.

3.2.9 HIV-1 Virion Production and Infectivity Assay

HEK293T and HeLa TZM-bl cells were cultured in Dulbecco's modified Eagles medium (Invitrogen) 10% FBS (Invitrogen), and 1% antibiotic/antimycotic (Invitrogen) at 37 °C and 5% CO$_2$. Cultures of HEK293T cells (2 × 10$^5$ cells/well of a 6-well plate in 2 ml of complete medium) were transfected with 2 µg of pNL4–3 (WT or A128T mutant) at a 1:3 ratio of DNA to X-tremeGENE HP (Roche Applied Science) following the manufacturer's protocol. Twenty-four
hours post-transfection, cells were washed once with complete medium, and the culture supernatant was replaced with complete medium containing DMSO, RAL (250 nM), ALLINI-1, or ALLINI-2 (at the indicated concentrations). After 1 h, the culture supernatant was again replaced with fresh complete medium containing either DMSO or the indicated inhibitors. The virus-containing cell-free supernatant was collected after 24 h, and HIV-1 Gag p24 ELISA (ZeptoMetrix) was performed following the manufacturer's protocol. Virions equivalent to 2–4 ng of HIV-1 p24 was used to infect $2 \times 10^5$ HeLa TZM-bl cells in the presence of 8 µg/ml Polybrene (Sigma). HeLa TZM-bl cultures were extracted in 1× reporter lysis buffer (Promega), and virion infectivity was measured using the luciferase assay (Promega).

3.2.10 Antiviral Activity Assays

ALLINI EC$_{50}$ values were determined as described in 2.2.11

3.3 Results

3.3.1 A128T confers resistance to ALLINI-1 and ALLINI-2 treatment

To examine the effects of the A128T substitution on HIV-1 IN function, we compared the catalytic activities of purified recombinant WT and mutant proteins. The two proteins exhibited comparable levels of LEDGF/p75-independent and LEDGF/p75-dependent integration activities (Figure 3.2). Thus, the A128T substitution does not significantly alter the function of IN.
The A128T mutation has been shown to confer resistance to four different ALLINI compounds in cell culture assays [90,91,96,114]. To delineate the mechanism of drug resistance, the ALLINI-1 and ALLINI-2 compounds were synthesized (Figure 3.1). ALLINI-1 was identified by Boehringer Ingelheim through a high-throughput screen for IN 3'-processing activity [89], and its multimodal mechanism of action has been elucidated by our group [115]. In resistance studies under selective pressure of ALLINI-1 [90], the A128T substitution in IN was identified in both early and late stage viral passages. This single amino acid change resulted in 32-fold higher ALLINI-1 IC₅₀ values compared with the WT virus. In this study, we also examined ALLINI-2, a tert-butyl derivative of ALLINI-1. Similar to a published report [96] showing that the tert-butyl group increases the potency of this class of compounds, ALLINI-2 was ~10-fold more potent (IC₅₀ = 0.63 ± 0.3 µM) (Table 3.1) than ALLIN-1 (IC₅₀ = 5.8 ± 0.1 µM). Although we have not selected for the A128T mutation by serially passaging HIV-1 in the presence of ALLINI-2, the ability of this mutation to confer relative pan-tropic resistance to a number of different compounds, including those that harbor the tert-butyl moiety [96,114], gave us confidence that A128T would likely confer resistance to ALLINI-2. Supplemental Table 2 indeed shows that A128T conferred 19-fold resistance to ALLINI-2 in a spreading HIV-1 replication assay.
3.3.2 The A128T substitution has differences in levels of resistance between the activities of ALLINIs

We and others have shown that ALLINIs inhibit multiple functions of WT IN, including LEDGF/p75-independent catalysis and IN-IN multimerization, with similar IC$_{50}$ values [96,114,115]. Such a mechanism has been attributed to the fact that these compounds occupy the LEDGF/p75-binding pocket at the IN dimer interface. As a result, ALLINIs inhibit IN-LEDGF/p75 binding and also bridge two IN subunits and allosterically modulate their multimerization. In turn, the latter impairs the catalytic functions of WT IN.

Strikingly, we observed that the A128T substitution had markedly different effects on the IC$_{50}$ values for different assays in comparison with WT IN (Table 3.2). For example, the mutation conferred only an ~2-fold increase in IC$_{50}$ values for inhibiting the IN-LEDGF/p75 interaction, whereas the IC$_{50}$ values for the 3’-processing reaction increased by 287-fold and 1112-fold for ALLINI-1 and ALLINI-2, respectively. Thus, A128T IN was markedly resistant to ALLINIs in 3’-processing reactions, whereas these compounds remained potent inhibitors of A128T IN binding to LEDGF/p75 (Figure 3.3). The A128T substitution resulted in ~11.5- and 5-fold resistance to ALLINI-1 and ALLINI-2, respectively, in the strand transfer reactions (Table 3.2). In LEDGF/p75-dependent integration assays, the mutant protein exhibited ~12- and 25-fold resistance to ALLINI-1 and ALLINI-2, respectively (Table 3.2).
3.3.3 A128T IN is resistant to ALLINI induced IN multimerization

Interestingly, IN multimerization assays have revealed further striking differences between WT and mutant INs (Figure 3.4). Both WT and A128T INs exhibited a characteristic biphasic dose-response curve upon the addition of ALLINIs. The HTRF signal increase with increasing concentrations of inhibitor was due to the inhibitor-induced protein multimerization, yielding higher Förster resonance energy transfer (FRET). Although the exact nature of the descending curve was not clear, it could be explained by reduced accessibility of the fluorescent antibodies to their respective tags in the context of higher order IN oligomers. The assay uses anti-His$_6$-XL665 and anti-FLAG-europium-cryptate antibodies to monitor fluorescence energy transfer (HTRF signal) between His-IN and IN-FLAG proteins. During the initial multimerization of IN, when dimers and tetramers form, the affinity tags are sufficiently exposed to readily engage the antibodies. However, these interactions may be limited in higher order IN oligomers (see Figure 3.5) due to structural hindrance of the affinity tags and could thus account for the drop in HTRF signal. Figure 3.4 shows that the dose-dependent addition of ALLINIs to WT and A128T INs yielded different peak heights. These differences could be explained by WT and mutant INs adopting different oligomeric states (see below) or alternative conformations in the presence of the inhibitor.

To delineate between these possibilities, we performed size exclusion chromatography experiments (Figure 3.5). Due to the reduced sensitivity of
detection of SEC compared with the HTRF-based assays, elevated concentrations of IN was needed and therefore higher concentrations of ALLINIs were necessary. Tetramer and monomer peaks were detected with both WT and A128T INs in the absence of inhibitor, demonstrating that the substitution does not affect IN multimerization (Figure 3.5) or catalytic activities (Figure 3.2). Upon the addition of ALLINIs, the tetramer peak of WT IN was markedly reduced, and instead, new peaks corresponding to higher order oligomers were detected. In sharp contrast, the tetramer peak persisted upon the addition of ALLINI-1 or ALLINI-2 to A128T IN (Figure 3.5 and Tables 3.2–3.5). These findings are consistent with the results of the HTRF-based multimerization assays: the formation of higher order structures upon ALLINI addition resulted in greater HTRF signal strength compared with mutant IN and could also account for the downward slope of the WT IN curves at high compound concentrations (Figure 3.4).

3.3.4 ALLINI-IN complexes are soluble

We next examined whether the addition of ALLINIs to IN might promote the formation of insoluble aggregates. WT IN was incubated with increasing concentrations of ALLINI-1 or ALLINI-2 and then subjected to centrifugation. The results in Figure 3.6 show that, under our reaction conditions, the IN-ALLINI complexes remained soluble. The solubility (Figure 3.6) and HTRF-based IN multimerization (Figure 3.4) assays were carried out at two different NaCl concentrations (150 and 750 mM) and yielded very similar results, indicating that
the changes in the ionic strength of the buffer did not significantly affect the solubility of ALLINI-induced higher order IN oligomers. This supports the notion that higher order IN multimerization and not precipitation led to the decrease in HTRF signal seen in Figure 3.4.

### 3.3.5 Structural basis for A128T resistance

To elucidate the structural basis for how the resistance mutation affects ALLINI binding, we solved the crystal structures of the inhibitors bound to the WT and A128T IN CCDs (Figure 3.7). The overlay of these two structures shows that the hydrogen bonding network between ALLINIs and subunit 1 is fully preserved in both the WT and mutant proteins (Figure 3.7). These include the interactions of the carboxylic acid with the backbone amides of Glu-170 and His-171 and the methoxy group of ALLINI-2 with the side chain of Thr-174. Thus, ALLINIs effectively shield the access of the key LEDGF/p75 Asp-366 contact to its cognate hydrogen bonding partners on both WT and A128T INs. Accordingly, these compounds inhibited the binding of the cellular cofactor to WT and A128T INs with comparable IC₅₀ values (Figure 3.3, Table 3.2).

Interestingly, the A128T substitution affected the positioning of the quinoline group (Figure 3.7). ALLINIs were shifted down and inward (toward the protein) by ~2 Å as measured at the common bromine atom. Because the positioning of the carboxylic group remained intact, this resulted in the rotation of the rigid molecule by ~18° (as measured by the shift of the bromine atom with respect to the C3
atom (numbering according to Figure 3.1) in the quinoline ring). This change also caused the substituted phenyl group to shift downward by 0.8 Å at the chlorine atom. These structural changes could be explained by the substitution of Ala-128 with the bulkier and polar threonine, which could exert a steric effect and electronic repulsion of the compounds. The shifts of the quinoline and substituted phenyl groups that bridge the two monomers of the CCD could be the reason for the differential multimerization of WT and mutant INs.

We next examined direct ALLINI binding to the isolated CCD using surface plasmon resonance (SPR). ALLINI-2 bound to both WT and A128T CCDs albeit with slightly reduced (2.3-fold) affinity with the mutant protein (Figure 3.8). These findings together with available structural data (Figure 3.7) help to interpret the mechanism of resistance. It is logical to conclude that the A128T substitution does not affect inhibitor binding (Figure 3.8), thus ALLINIs are still able to inhibit IN-LEDGF/p75 binding. Conversely, the shift in inhibitor binding (Figure 3.7) adversely affects the ability of ALLINIs to promote aberrant IN multimerization.

3.3.6 A128T IN substitution causes resistance to ALLINI potency during particle production

A previous study [114] demonstrated that ALLINIs do not affect bulk viral particle production but nevertheless impair the infectivity of HIV-1 progeny virions. Here, we examined how the A128T substitution affects this aspect of ALLINI inhibition (Figure 3.9). For this, HEK293T cells were transfected with pNL4–3 (WT or
A128T mutant) and cultured for 24 h to ensure expression of the provirus. Next, we monitored the production of HIV-1 particles in the presence of increasing concentrations of ALLINIs. Twenty-four hours post-addition of the compounds, the virus-containing cell-free supernatant was harvested, and the amounts of viral particles produced were measured by p24 ELISA. The production of both WT and A128T IN viral particles was not affected by ALLINIs (Figure 3.9). Subsequently, we examined the infectivity of these progeny virions in a HeLa-based reporter cell line (TZM-bl) containing the HIV-1 LTR-luciferase reporter gene. For this, TZM-bl cells were infected with equivalent cell-free virions without any additional inhibitor being added to the target cells. Under these conditions, only 0.1% of the input ALLINIs was carried over from the producer cells to the target cells based on the supernatant volumes used for the infections. At the highest concentration of ALLINIs tested (100 µM ALLINI-1 treatment of the producer cells), only 100 nM inhibitor would carry over, which is well below the IC₅₀ value for the WT virus and thus will have negligible effects on the TZM-bl cell infections. The results in Figure 3.9 demonstrate that WT virions produced in the presence of the inhibitors lost their infectivity, with estimated IC₅₀ values of ~6 µM for ALLINI-1 and ~0.5 µM for ALLINI-2. In contrast, the A128T virions exhibited a marked resistance to ALLINIs (Figure 3.9). Control experiments with RAL showed no effects of this inhibitor on viral production or infectivity.
3.4 Discussion

ALLINIs are a growing class of new anti-HIV-1 compounds. Importantly, these inhibitors are complementary to all Food and Drug Administration-approved antiretroviral agents, including the IN strand transfer inhibitors RAL and elvitegravir. Whereas the IN strand transfer inhibitors specifically interact with the IN-viral DNA complex [36], ALLINIs target a clinically unexploited IN dimer interface at the LEDGF/p75-binding site. Consequently, ALLINIs allosterically modulate IN multimerization and impair IN-LEDGF/p75 binding [96,114,115].

The IN A128T substitution has been identified from cell culture assays as a primary mechanism of HIV-1 resistance to numerous ALLINIs [90,91,96,114]. Here, we elucidated the structural and mechanistic basis for this resistance. The alanine-to-threonine substitution affects positioning of the core quinoline and substituted phenyl ring of ALLINIs that bridge the two IN subunits, whereas the hydrogen bonding network between the inhibitor and the protein that closely mimics the IN-LEDGF/p75 interaction remains intact. As a result, the A128T substitution shows marked resistance to ALLINI-induced aberrant multimerization of IN compared with its WT counterpart, whereas the compound remains a potent inhibitor of the A128T IN binding to LEDGF/p75.

We have shown that ALLINIs promote aberrant higher order multimerization of WT IN, but not A128T IN. Although previous studies have attributed the HTRF signal increase to IN dimerization [96], the size exclusion chromatography data in
Figure 3.5 clarify that the addition of ALLINIs to the WT protein promotes the formation of higher order oligomers. As a result, the catalytic activities of the WT protein are fully compromised. In sharp contrast, A128T IN is remarkably resistant to ALLINIs in the 3’-processing assays (287- and 1112-fold for ALLINI-1 and ALLINI-2, respectively) and exhibits 5–11-fold resistance in strand transfer assays. How does one explain the differential levels of resistance of A128T IN for 3’-processing and strand transfer activities? The HTRF assays coupled with size exclusion chromatography indicate that ALLINIs stabilize a tetrameric form of A128T IN. Of note, IN tetramers formed in the absence and presence of viral DNA adopt distinct conformations [39]. Although preformed tetramers are known to be active in 3’-processing, the strand transfer reactions require individual IN monomers to assemble in the presence of viral DNA to correctly engage target DNA [39,75]. Parallels can be drawn with our earlier results demonstrating the importance of highly dynamic interplay of individual IN subunits for productive integration [39,75]. The IN tetramers stabilized by the LEDGF/p75 IN-binding domain are active in 3’-processing reactions but fail to catalyze concerted HIV-1 integration. Similarly, IN tetramers prematurely stabilized by ALLINIs are likely to be different from the fully functional tetramers in the intasome formed in the presence of DNA substrate.

To understand the 5–11-fold resistance of A128T IN in the strand transfer assays, we analyzed the HTRF data in Figure 3.4. Accurate measurements of ALLINI IC₅₀ values from these assays were complicated due to biphasic curves
and differing peak heights for WT and A128T INs. Still, the analysis of the initial ascending curves enabled us to estimate the IC\textsubscript{50} values for WT versus A128T IN: 2.27 ± 0.13 µM versus 17.81 ± 1.46 µM for ALLINI-1 and 0.070 ± 0.008 µM versus 0.68 ± 0.06 µM for ALLINI-2. The ∼10-fold increase in estimated IC\textsubscript{50} values for the initial multimerization phase for A128T IN compared with its WT counterpart could explain the observed resistance of mutant IN in the strand transfer assays (Table 3.2).

The differential multimerization of WT and A128T INs induced by ALLINIs correlates with the differences in infectivity of HIV-1 progeny virions. The treatment of producer cells with the inhibitors impairs WT HIV-1 infectivity, with estimated IC\textsubscript{50} values of ∼6 µM for ALLINI-1 and ∼0.6 µM for ALLINI-2, whereas A128T HIV-1 exhibits marked resistance to these compounds (Figure 3.9). In turn, these results correlate well with the inhibitory activities of these compounds with respect to WT and A128T HIV-1 replication in spreading assays (Table 3.1) [115].

In conclusion, our findings that the A128T substitution did not significantly alter ALLINI IC\textsubscript{50} values for IN-LEDGF/p75 binding but substantially affected IN multimerization in the presence of the inhibitors indicate that allosteric IN oligomerization is the primary target of these inhibitors in infected cells. Our structural data showing that the A128T substitution repositions the quinoline ring of ALLINIs at the IN dimer interface provide a path for rationale development of
second-generation ALLINI compounds with decreased potential to select for drug resistance.

3.5 Acknowledgements

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<table>
<thead>
<tr>
<th>Virus</th>
<th>RAL</th>
<th>SQV</th>
<th>ALLINI-1</th>
<th>ALLINI-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.0040 ± 0.0035</td>
<td>0.014 ± 0.010</td>
<td>6.85 ± 1.9</td>
<td>0.63 ± 0.30</td>
</tr>
<tr>
<td>A128T_IN</td>
<td>0.0053 ± 0.0038</td>
<td>0.0096 ± 0.0087</td>
<td>&gt;100</td>
<td>12.2 ± 6.4</td>
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<td>Fold Change</td>
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<td>0.7x</td>
<td>&gt;15x</td>
<td>19x</td>
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Table 3.1. Antiviral activity of RAL, SQV, ALLINI-1 and ALLINI-2 against NL4-3 and NL4-3\textsubscript{A18T_IN}.
Average values with S.E. from the mean are shown for three independent experiments.

<table>
<thead>
<tr>
<th>IN-LEDGF/p75 binding</th>
<th>ALLINI-1</th>
<th>ALLINI-2</th>
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<tr>
<td>WT</td>
<td>1.9 ± 0.5</td>
<td>0.3 ± 0.02</td>
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<tr>
<td>A128T</td>
<td>4.0 ± 0.6</td>
<td>0.78 ± 0.06</td>
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<td>Fold Change</td>
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<th>Strand Transfer</th>
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<th>ALLINI-2</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.4 ± 0.09</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>A128T</td>
<td>4.61 ± 0.45</td>
<td>0.89 ± 0.19</td>
</tr>
<tr>
<td>Fold Change</td>
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<table>
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<th>LEDGF/p75 – dependent integration</th>
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<tr>
<td>WT</td>
<td>1.29 ± 0.34</td>
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<td>A128T</td>
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<tr>
<td>Fold Change</td>
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</table>

Table 3.2: Effects of the A128T Substitution on the IC\textsubscript{50} values of ALLINIs.
Means ± S.E. are shown for at least three independent experiments.
### Table 3.3: Summary of size exclusion chromatography result for ALLINI-1 interactions with WT IN

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Elution Volume (mL)</th>
<th>Estimated MW (kDa)</th>
<th>Estimated oligomeric state</th>
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<tr>
<td>1</td>
<td>16.5</td>
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<td>2</td>
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<td>3</td>
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<td>17.7</td>
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<tr>
<td>4</td>
<td>13.8</td>
<td>79.8</td>
<td>tetramer</td>
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<tr>
<td>5</td>
<td>7.85</td>
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<td>higher order oligomer</td>
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### Table 3.4: Summary of size exclusion chromatography result for ALLINI-1 interactions with A128T IN

<table>
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<th>Peaks</th>
<th>Elution Volume (mL)</th>
<th>Estimated MW (kDa)</th>
<th>Estimated oligomeric state</th>
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<td>3</td>
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<td>4</td>
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### Table 3.5: Summary of size exclusion chromatography result for ALLINI-2 interactions with WT IN

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<th>Estimated MW (kDa)</th>
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<td>6</td>
<td>10.0</td>
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<td>higher order oligomer</td>
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### Table 3.6: Summary of size exclusion chromatography result for ALLINI-2 interactions with A128T IN

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<tr>
<td>4</td>
<td>13.3</td>
<td>102.2</td>
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Figure 3.1. Chemical Structures of ALLINI-1 and ALLINI-2
Figure 3.2. Catalytic activities of WT and A128T INs.

A. Strand transfer reaction products (STP; upper panels) and 3′-processing products (19-P; lower panels). Lane 1, 21-mer DNA substrate (21-S) without IN; lanes 2–4, increasing concentrations (0.5, 1, and 2 µM) of WT IN added to the reactions; lanes 5–7, increasing concentrations (0.5, 1, and 2 µM) of A128T IN added to the reactions.

B. Concerted integration results. The positions of 32-mer donor and supercoiled (SC) target DNA substrates, as well as full-site (FS) and half-site (HS) integration products, are indicated. Lane 1, DNA markers (Bioline Quanti-Marker, 1 kb); lanes 2 and 3, target DNA; lane 4, WT IN (2.4 µM) added to donor and target DNA substrates without LEDGF/p75; lanes 5–7, LEDGF/p75 added with decreasing concentrations (2.4, 1.2, and 0.6 µM) of WT IN; lane 8, A128T IN (2.4 µM) added to donor and target DNA substrates without LEDGF/p75; lanes 9–11, LEDGF/p75 with decreasing concentrations (2.4, 1.2, and 0.6 µM) of A128T IN.
Figure 3.3. Effects of ALLINI-1 and ALLINI-2 on 3’-processing activities and IN-LEDGF/p75 binding of WT and A128T INs.

(A) Dose-response effects of ALLINI-1 on 3’-processing activities of WT (black) and A128T (red) INs. (B) Dose-response effects of ALLINI-1 on IN-LEDGF/p75 binding for WT (black) and A128T (red) INs. (C) Dose-response effects of ALLINI-2 on 3’-processing activities of WT (black) and A128T (red) INs. (D) Dose-response effects of ALLINI-2 on IN-LEDGF/p75 binding for WT (black) and A128T (red) INs.
Figure 3.4 Effects of ALLINI-1 and ALLINI-2 on multimerization of WT and A128T INs.
Shown are the dose-response effects of ALLINI-1 and ALLINI-2-induced multimerization of WT (black) and A128T (red) INs. The HTRF signal observed due to the dynamic exchange of IN subunits in the absence of the inhibitor is considered 100% base line. The mean values of three independent experiments are shown.
Figure 3.5 Size exclusion chromatography demonstrating differential multimerization of WT and A128T INs in the presence of ALLINI-1 and ALLINI-2.

Shown are the elution profiles of 20 µM WT IN in the absence and presence of (A) 80 µM ALLINI-1 or (B) 40 µM ALLINI-2. The elution times of estimated oligomeric states for indicated peaks of WT IN +/- ALLINI-1 in table 3.3, A128T IN +/- ALLINI-1 in table 3.4, WT IN +/- ALLINI-2 in table 3.5 and A128T IN +/- ALLINI-2 in table 3.6.
Figure 3.5. Size Exclusion Chromatography demonstrating differential multimerization of WT and A128T INs in the presence of ALLINI-1 or ALLINI-2
Figure 3.6. Neither ALLINI-1 nor ALLINI-2 affect solubility of WT IN.

WT IN was incubated with the indicated concentrations of (A) ALLINI-1 or (B) ALLINI-2 and then subjected to centrifugation. The supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE, and IN was detected by anti-His antibody.
Figure 3.7. Overlay of crystal structures of (A) ALLINI-1 or (B) ALLINI-2 bound to A128T and WT IN CCDs.
Ala-128 and its corresponding ligand ALLINI-1 are colored yellow, whereas Thr-128 and the respective (A) ALLINI-1 or (B) ALLINI-2 molecule are colored magenta. Hydrogen bonds between ALLINI-1 molecules and the backbones of Glu-170 and His-171 are shown by yellow (for WT IN) and magenta (for A128T IN) dashed lines. Subunits 1 and 2 are colored cyan and gray, respectively.
The binding kinetics of ALLINI-2 interactions with WT IN CCD (A), A128T IN CCD (B), WT IN. These results yielded the following $K_d$ values: $0.722 \pm 0.2 \, \mu\text{M}$ for WT IN CCD; $1.66 \pm 0.2 \, \mu\text{M}$ for A128T IN CCD. $RU$, response units.
Figure 3.9. Effects of ALLINI-1 and ALLINI-2 on WT and A128T HIV-1 p24 production and infectivity.

(A-B) HEK293T cells with WT or A128T IN provirus. HIV-1 particles were produced in the presence of inhibitor at indicated concentrations for 24h and cell free Gag was measured by HIV-1 p24 ELISA. Neither (A) ALLINI-1 or (B) ALLINI-2 effected p24 production. (C-D) WT or A128T IN cell-free virus equivalent to 4ng HIV-1 p24 was used to infect TZM-bl cells and luciferase expression measured 48 h post-infection. The luciferase signal obtained in absence of the inhibitor (DMSO alone) for WT and 128T IN was set to 100%. The average values from at least triplicate infections are shown and error bars represent S.D. Both ALLINI-1 (C) and ALLINI-2 (D) inhibited infection of WT NL4-3HIV-1 but were markedly resistant to A128T IN NL4-3HIV-1.
Chapter 4: The mechanism of H171T resistance reveals the importance of $N_\delta$-protonated His171 for the binding of allostERIC inhibitor to HIV-1 integrase.

4.1 Introduction

Rapid evolution of HIV-1 phenotypes conferring resistance to current antiretroviral therapies is a major clinical problem. The multifunctional nature of HIV-1 integrase (IN) provides attractive and unexploited targets for developing complementary antiretroviral compounds to enhance the treatment options for HIV-1 infected patients. During the early stage of HIV-1 replication, IN mediates integration of the reverse transcribed viral genome into human chromatin. This activity proceeds in two steps with the first step, termed 3’ processing, occurring when IN cleaves a GT dinucleotide from the 3’ ends of the viral DNA. The second step, a transesterification reaction termed strand transfer, inserts the processed viral DNA ends into host chromosomal DNA [93]. Three clinically approved antiretroviral drugs raltegravir (RAL), elvitegravir (EVG) and dolutegravir (DTG) inhibit IN strand transfer activity and are collectively referred to as IN strand transfer inhibitors or INSTIs [17]. Importantly, HIV-1 mutations that confer cross-
resistance to both RAL and EVG have been identified in patients [116-118]. While the second generation INSTI, DTG, appears to exhibit a higher genetic barrier to resistance, substitutions in IN that confer low-level resistance to DTG have been identified [119].

IN catalytic activities depend on the correct assembly of the stable synaptic complex (SSC) or intasome, where individual IN subunits engage the viral DNA ends to form the fully functional IN tetramer [34]. Each of the three IN domains, the N-terminal domain (NTD), the catalytic core domain (CCD) and the C-terminal domain (CTD), contribute to the assembly of the SSC through protein-protein and protein-DNA interactions [36-40]. Unliganded IN subunits exhibit highly dynamic interplay with the inhibition of this exchange through the stabilization of subunit-subunit interactions prior to their binding to viral DNA results in the loss of enzymatic function [39,75]. Initial studies with the small molecule inhibitor tetra-acetylated-chicoric acid have shown that the inhibitor binds at the IN dimer interface and promotes the incorrect multimerization of IN, which in turn compromises IN catalytic activity in vitro [81]. These findings have provided important proof-of-concept for a new mechanism for inhibition of IN activity through the modulation of its multimeric state.

Integration in infected cells is significantly enhanced by the cellular chromatin associated protein LEDGF/p75 which acts as a bimodal tether to link the lentiviral preintegration complex to active genes [43-46,58,120]. LEDGF/p75 association with chromatin is mediated through its N-terminal segment containing the PWWP
domain, which selectively recognizes the H3K36me3 histone mark as well as non-specifically engages nucleosomal DNA [49,50,121]. LEDGF/p75 also binds the IN tetramer through its C-terminal integrase binding domain (IBD) by inserting a small loop into a v-shaped cavity located at the HIV-1 IN CCD dimer interface [33,56,58,59]. LEDGF/p75 Asp366 establishes a pair of hydrogen bonds with IN Glu170 and His171 backbone amides, whereas LEDGF/p75 Ile365 and Leu368 engage in hydrophobic interactions with both IN subunits [56,58]. In addition, the LEDGF/IBD α-helix 4 forms electrostatic interactions with α-helix 1 of the IN NTD [59]. Antagonism of HIV-1 IN interaction with LEDGF/p75 through knockout (KO) of the cellular Psip1 gene, which encodes for LEDGF/p75 protein, resulted in marked decrease of HIV-1 infectivity [45,47,85]. Additionally, overexpression of the LEDGF/IBD, which is capable of both competing with endogenous LEDGF/p75 as well as inhibiting the formation of the SSC by stabilizing incorrect IN multimers [75], was able to potently inhibit HIV-1 replication [46,60]. These studies have established the importance and molecular basis of the interaction of HIV-1 IN with LEDGF/p75 and have highlighted the primary LEDGF/p75 binding pocket at the IN CCD-CCD dimer interface for anti-HIV-1 drug development.

Multifunctional allosteric IN inhibitors (ALLINIs) have been discovered that potently inhibit HIV-1 replication (reviewed in [122-124]). These compounds were identified through two separate methods, including a high throughput screen for 3'-processing inhibitors or through the rational design of inhibitors that block the IN-LEDGF/p75 interaction [88-91,125]. ALLINIs bind to HIV-1 IN in the principal
LEDGF/p75 binding pocket and bridge between two IN subunits [91,96,115,126-129]. ALLINIs share several common structural features including a central quinoline ring and the carboxylic acid moiety on a modifiable one-carbon linker attached to position 3 of the central ring. Similar to LEDGF/p75 residue Asp366, the ALLINI carboxylic acid forms hydrogen bonds with Glu170 and His171 backbone amides of one IN subunit. Additionally, the quinoline ring extends to form hydrophobic contacts with the second IN subunit akin to LEDGF/p75 residue Leu368. However, unlike LEDGF/p75, potent ALLINIs also contain a tert-butoxy ether oxygen at the modifiable carbon, which forms an additional hydrogen bond with the side chain of IN residue Thr174 [91,96,115,128].

ALLINIs inhibited both IN-LEDGF/p75 binding and LEDGF/p75 independent assembly of functional SSCs in vitro [91,96,114,115]. The latter inhibitory activity has been attributed to the ability of ALLINIs to prematurely stabilize interacting IN subunits and promote aberrant higher order protein multimerization [96,114,115]. Consistent with these observations, in infected cells ALLINIs impaired a step at or prior to 3'-processing and could reduce LEDGF/p75 mediated integration into active transcription units [96,130]. Unexpectedly though, the primary activity of ALLINIs occurs during the late stage of HIV-1 replication [127-129,131,132]. Virions produced in the presence of ALLINIs exhibited an eccentric morphology characterized by the electron dense material being mislocalized outside of the capsid core and were furthermore defective for reverse transcription during the subsequent round of infection [128,131-133]. This phenotype is similar to the one
caused by certain HIV-1 IN mutations, which are typed as class II, suggesting that IN structure may play a yet unidentified role during HIV-1 maturation [40,128,132-136]. Potential contributions of LEDGF/p75 and its interactions with IN during the late stage of HIV-1 replication are unlikely due to the observations that fully infectious virus particles were formed in LEDGF/p75 KO or knockdown cells [47,128,131]. Consistent with this view, LEDGF/p75 over expression did not affect ALLINI potencies in virus producer cells [131]. Instead, ALLINI induced aberrant IN multimerization has been shown to correlate with the inhibition of correct particle assembly. Further support for this notion has been provided by the recent development of multimerization selective IN inhibitors or MINIs. These compounds are not effective inhibitors of IN-LEDGF/p75 binding and instead potently induce aberrant IN multimerization during virus particle production and result in eccentric, non-infectious particles (discussed in chapter 5, [130]).

Genotyping HIV-1 in cell culture under the selective pressure of the archetypal ALLINI, ALLINI-1, and its several analogs have identified substitutions in the IN coding sequence near the inhibitor binding sites [90,91,96]. Of these, the A128T IN substitution was the most prevalent mutation with HIV-1NL4-3(A128T \text{ IN}) displaying marked resistance to respective ALLINI compounds [90,91,96,126]. Interestingly, crystallographic studies have revealed that ALLINI-1 is still able to bind A128T IN CCD by maintaining all hydrogen bonding interactions, but that the quinoline ring bridging the two IN subunits was slightly shifted compared with the wild type (WT) protein [126]. Consequently, ALLINI-1 was unable to promote
aberrant multimerization of recombinant A128T IN, whereas it maintained its ability to inhibit IN-LEDGF/p75 binding \textit{in vitro} \cite{126}. The results of these studies are consistent with the interpretation that aberrant IN multimerization rather than IN-LEDGF/p75 binding is the primary target of this inhibitor in infected cells.

Selection of HIV-1 variants in the presence of ALLINI BI-D (Figure 4.1), a more potent analog of ALLINI-1, did not result in the A128T mutation but instead revealed several amino acid changes near the inhibitor binding sites including Y99H, L102F, A/T124D, and H171T \cite{90}. Of these, HIV-1 bearing the single amino acid H171T substitution was found to be one of the most prominent mutations persisting at the highest concentration of BI-D tested \cite{90}. Here, we have investigated the mechanism of resistance for the H171T IN mutation. Our findings show that unlike A128T, the H171T IN substitution causes resistance to BI-D by reducing the binding affinity of the inhibitor to IN. Our structural studies have elucidated a previously undescribed role of the His171 side chain for hydrogen bonding with BI-D \textit{tert}-butoxy ether oxygen, which is compromised upon the H171T substitution. Since LEDGF/p75 lacks a \textit{tert}-butoxy moiety, the H171T substitution has minimal effects on IN-LEDGF/p75 binding and accordingly, HIV-1NL4-3(H171T IN), replicated in cells at WT levels. These findings have uncovered the structural and mechanistic basis for H171T IN resistance to BI-D and are expected to facilitate in the development of second generation ALLINIs with increased potency and decreased potential to evolve drug resistance.
4.2 Methods

4.2.1 Antiviral compounds, plasmids, and DNA constructs.

BI-D and LEDGIN-6 were synthesized as previously described \[115,137\]. Plasmid pNL4.3/Xmal \[138\] encodes for replication competent HIV-1\textsubscript{NL4-3}. Plasmids pNLX.Luc.R\textsuperscript{−} \[139\] or pNL4-3.Luc.Env\textsuperscript{−} \[140\] encodes for single-round HIV-1\textsubscript{NL4-3} carrying the luciferase reporter gene. Vesicular stomatitis virus G glycoprotein was encoded by pCG-VSV-G \[138\]. The H171T substitution was introduced into the IN coding region of pNLX.Luc.R\textsuperscript{−}, pNL4-3.Luc.Env\textsuperscript{−} and pNL4-3/Xmal using PCR-site directed mutagenesis (Agilent) and verified by dideoxy sequencing.

4.2.2 Cells, viruses and antiviral assays

Parental HEK293T and HEK293T LEDGF/p75 KO cells \[47\] were grown in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Invitrogen), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Gibco). SupT1 cells were maintained in RPMI medium 1640 containing 10% FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin. HIV-Luc was pseudotyped by cotransfecting HEK293T cells with either pNLX.Luc.R\textsuperscript{−} or pNL4-3.Luc.Env\textsuperscript{−} and with pCG-VSV-G using PolyJet DNA transfection reagent (SignaGen Laboratories).

To determine antiviral activity during virus production, DMSO or the inhibitor was added at indicated concentrations during media exchange at 18-20 hrs post-
transfection. Cell-free supernatants were measured for p24 content utilizing a commercial p24 ELISA kit (Advanced Biosciences Laboratories). SupT1 cells were infected in triplicate with HIV-Luc normalized for p24 content (5 ng/mL p24). Luciferase values were determined 48 hrs post-infection.

To assess antiviral activity during the early stage of HIV-1 replication, DMSO or the inhibitor was added at the indicated concentrations to the target cells 30 min before or at the time of infection. Luciferase values, expressed as relative light units were determined 48 hrs post-infection.

4.2.3 Electron microscopy

Cell-free HIV-1\textsubscript{NL4-3} from transfected HEK293T cell supernatants was concentrated via ultracentrifugation at 4°C for 2 hrs in a Beckman SW41 rotor at 32,000 rpm prior to fixation in 2% paraformaldehyde and submission to the Harvard Medical School Electron Microscopy core facility. Images were taken with a JEOL 1200EX microscope equipped with an AMT 2 k charge-coupled device camera. Virus particles (100 per experimental sample) were counted by eye.

4.2.4 Expression and purification of recombinant proteins

WT HIV-1 IN, H171T HIV-1 IN and LEDGF/p75 recombinant proteins with His\textsubscript{6} or FLAG tags were expressed in \textit{E. Coli} and purified as described in 2.2.2 - 2.2.4.
4.2.5 Crystallization and X-ray crystal structure determination

The HIV-1 IN CCD was purification, crystallization and refinement was carried out as described in 2.2.9. Coordinates have been deposited in the Protein Data Bank with accession number 4TSX.

4.2.6 Binding free energy calculations

The absolute binding free energies between the various forms of IN CCD dimer and BI-D were calculated using the double decoupling method (DDM) [115,138-141] in explicit solvent (TIP3P water model [142] plus counterions) at 300 K. The protein molecules are modeled by the Amber ff99sb-ILDN force field [143], and the ligand BI-D is described by the Amber GAFF [144] parameters set. The partial charges of the ligands are obtained using the AM1-BCC method [145]. A DDM calculation involves two legs of simulation, in which a restrained ligand is gradually decoupled from the receptor binding pocket or from the aqueous solution. In each leg of the decoupling simulations, the Coulomb interaction is turned off first using 11 lambda windows, and the Lennard-Jones interactions are then turned off in 17 lambda windows. The two decoupling free energies $\Delta G_{\text{gas}}^{*}\rightarrow \text{complex}$ and $\Delta G_{\text{gas}}\rightarrow \text{water}$ associated with the two legs of the DDM cycle were determined using thermodynamic integration (TI). The Hamiltonian derivative $\langle \partial U/\partial \lambda \rangle \lambda$ at a series of $\lambda$ from 0 to 1 were collected and integrated to obtain the free energy difference. For absolute binding free energy calculations, the MD simulation at each $\lambda$ was performed using the GROMACS [146,147]
version 4.6.4 for 15 ns; the last 10 ns was used for the calculation of binding free energy.

4.2.7 Size exclusion chromatography

Size exclusion chromatography experiments were carried out as described in 3.2.6.

4.2.8 Dynamic light scattering

DMSO, 0.12 µM BI-D, or 10 µM BI-D was incubated with 200 nM WT or H171T IN in 50 mM HEPES, pH 7.4 buffer containing 2 mM DTT, 2 mM MgCl2 and 1 M NaCl. Dynamic light scattering (DLS) signals were recorded at room temperature after 15, 20 and 30 minutes using a Malvern Nano series Zetasizer instrument.

4.2.9 Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were carried out with WT His<sub>6</sub>-IN CCD or H171T His<sub>6</sub>-IN CCD at indicated BI-D concentrations as described in 3.2.8.

4.2.10 Catalytic activities of recombinant INs

The homogeneous time resolved fluorescence (HTRF)-based LEDGF/p75 dependent integration and LEDGF/p75 independent integration assays were carried out as described in 3.2.5 with minor modifications. LEDGF/p75 independent assays were executed by incubating 400 nM WT or H171T IN with
50 nM Cy-5 labeled donor DNA and 10 nM biotinylated target DNA. For LEDGF/p75 dependent integration assays, 100 nM WT or H171T IN was incubated with 50 nM Cy-5 labeled donor, 10 nM biotinylated target DNA and 50 nM or 100 nM LEDGF/p75. After the addition of europium-streptavidin, the HTRF signal was recorded using a Perkin Elmer EnSpire multimode plate reader.

### 4.2.11 HTRF-based assay for IN-LEDGF/p75 interactions

The IN-LEDGF/p75 binding assay described in 2.2.7 was modified to monitor LEDGF/p75 binding to WT and H171T INs. Briefly, C-terminally FLAG-tagged LEDGF/p75 (0.01 – 100 nM) was titrated in the binding buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 0.1% Nonidet P-40, 1 mg/ml BSA) containing 10 nM N-terminally His6- WT or H171T HIV-1 IN. Anti-His6-XL665 and anti-FLAG-EuCryptate antibodies (Cisbio, Inc., Bedford, MA) were added to the reaction and the HTRF signal vs LEDGF/p75 concentration curves were fitted to Hill Equation to identify the Kd values for IN-LEDGF/p75 binding.

### 4.3 Results

#### 4.3.1 The H171T IN substitution does not affect HIV-1 replication or recombinant IN activities but causes resistance to BI-D in infected cells.

To assess the functional significance of the H171T IN substitution we introduced the mutation into both HIV-1NL4.3 and recombinant IN. As indicated in Figure 4.2, the H171T IN substitution did not significantly alter virus release from transfected cells as measured by p24 production or affect the infectivity of the mutant virus.
We next examined the biochemical properties of purified recombinant H171T IN. Size exclusion chromatography (SEC) experiments revealed that WT and H171T INs similarly formed tetramers and monomers (Figure 4.3). In an HTRF IN activity assay [126], the mutant IN protein exhibited near WT levels of catalytic function in the absence of LEDGF/p75 (Figure 4.3). Furthermore, LEDGF/p75 was able to stimulate the strand transfer activity of mutant IN similarly to WT IN (Figure 4.3). This suggested that even though the H171T IN substitution is within the IN-LEDGF/p75 binding pocket, IN retains the ability to effectively bind LEDGF/p75 in vitro and during virus infection. To test this directly, we compared LEDGF/p75 binding to WT and H171T INs utilizing a HTRF-based binding assay [126]. WT IN bound LEDGF/p75 with a $K_d$ of $3.3 \pm 0.3$ nM, whereas H171T IN bound LEDGF/p75 with a $K_d$ of $\sim 10.5 \pm 0.3$ nM, a 3.2-fold decrease in affinity (Figure 4.3).

Although the inhibition of particle maturation determines BI-D potency, the inhibitor displays a second, substantially weaker activity during the early phase of HIV-1 replication ([128], Table 4.1). To gain insight into the mechanism of BI-D action and the mode of H171T resistance, HIV-1$_{NL4-3(H171T\ IN)}$ was evaluated during both the early and late stages of the replication lifecycle. When drug exposure was limited to the acute phase of infection, BI-D $EC_{50}$ values increased from 1.17 µM for VSV-G pseudotyped HIV-1NL4-3 to 12.4 µM for the VSV-G pseudotyped HIV-1$_{NL4-3(H171T\ IN)}$, ~11-fold resistance (Table 4.1). However, the
H171T IN substitution resulted in significantly higher levels of resistance (~68-fold) when drug exposure was limited to the late stage of replication.

To examine the apparent differences in resistance levels between the early and late stages of replication, anti-viral potencies of BI-D were determined in LEDGF/p75 knock out (KO) cells. The complete removal of endogenous LEDGF/p75 increased BI-D potency during the acute phase of HIV-1\textsubscript{NL4-3} infection, but had minimal effect on the inhibitor activity when exposure was limited to the late stage. These results are consistent with other studies and indicate that during the early stage of replication, LEDGF/p75 is able to compete with ALLINIs for binding to IN and hence reduce inhibitor potency [128,137]. However, LEDGF/p75 expression levels did not detectably alter ALLINI potencies during the late stage of viral replication ([47,127-129,131,132], also see Table 4.1). The virus containing the H171T IN substitution still conferred resistance to BI-D in the absence of endogenous LEDGF/p75, with 28- and 45-fold resistance observed in the KO cells during the early and late stages, respectively.

The vast majority of virions produced in the presence of BI-D display eccentric core morphology, where the electron dense material normally situated within the conical core is mislocalized adjacent to a translucent capsid core and the viral membrane [128]. Consistent with these findings, when WT HIV-1\textsubscript{NL4-3} virions were produced in the presence of 0.18 µM BI-D (a dose equivalent to 2×EC\textsubscript{50}; Table 4.1), 77% of the virions displayed an eccentric morphology (Figure 4.4). However, at the same concentration of BI-D the H171T IN mutant virus resulted
in only 27% of the virions with eccentric core morphologies (Figure 4.4). When BI-D concentrations were increased to 12 µM, which corresponds to $2\times\text{EC}_{50}$ for HIV-1_{NL4-3} bearing the H171T IN substitution, the eccentric virion morphology for the mutant virus increased to 82% (Figure 4.4).

### 4.3.2 H171T IN substitution compromises BI-D binding to IN

We next wished to dissect the mechanism of the HIV-1_{NL4-3(H171T IN)} resistance to BI-D. SPR was used to compare BI-D binding to recombinant WT and H171T IN CCD proteins. Figure 4.5 shows that BI-D bound WT IN CCD with a $K_d$ of 0.123 µM, which is in good agreement with the antiviral activities of BI-D measured in cell culture (Table 4.1). BI-D binding to H171T IN CCD resulted in a significantly higher $K_d$ of 10.3 µM. Significantly, the observed ~84-fold decrease in the binding affinity of BI-D to the mutant IN CCD (Figure 4.5) roughly correlated with the ~68-fold decrease in the EC$_{50}$ value seen for antiviral activity against HIV-1_{NL4-3} bearing the H171T IN substitution (Table 4.1).

### 4.3.3 BI-D is unable to promote H171T IN multimerization

Previous studies have indicated that the primary mechanism of action of ALLINIs is through the promotion of aberrant IN multimerization [96,114,126-128]. Therefore, we compared the effects of BI-D on aberrant multimerization of WT and H171T INs using DLS. In the absence of inhibitor (DMSO control), peaks for soluble WT or mutant IN were not observed by this technique (Figure 4.6). Instead, a background signal corresponding to <1 nm diameter was detected.
both in the presence of IN and in the buffer alone sample, indicating that the reaction buffer contained small size particles. For inhibitor experiments, we examined two concentrations of BI-D: one (~0.120 µM) that correlated to the $K_d$ value of the inhibitor binding to WT IN CCD (~0.123 µM) and the other (10 µM) that correlated with the $K_d$ value for inhibitor binding to H171T IN CCD (~10.3 µM). Incubation of 0.12 µM BI-D with WT IN for 15 min resulted in a peak corresponding to particles with a diameter of 51 nm, which significantly exceeds an estimated diameter of 7.5 nm for the IN tetramer in the SSC [35]. The size of the oligomer continued to increase further to 106 nm and 142 nm diameter at 20 and 30 minutes, respectively (Figure 4.6A). In contrast, the same BI-D concentration (0.12 µM) failed to elicit higher order H171T IN oligomers even after 30 min incubation (Figure 4.6B). However, when the concentration of BI-D was increased to 10 µM, higher order oligomerization of H171T IN was detected in a time dependent manner (Figure 4.6D). As expected, 10 µM BI-D also induced higher order oligomers of WT IN (Figure 4.6C). This suggests that at lower concentrations, BI-D is unable to promote higher order IN oligomerization of H171T IN likely due to the decreased affinity of the inhibitor binding to the mutant protein (Figure 4.5). However, under conditions of increased inhibitor, BI-D is able to bind H171T IN (Figure 4.5) and promote aberrant IN multimerization (Figure 4.6D). This indicates that the H171T IN substitution confers resistance to BI-D by decreasing inhibitor binding affinity and hence correspondingly decreasing aberrant IN multimerization.
4.3.4 Structural basis for H171T IN resistance to BI-D

To understand the structural basis for the reduced binding affinity of BI-D to H171T IN, we solved the crystal structure of BI-D in complex with H171T CCD dimer (Figure 4.7A) and compared it to the complex of inhibitor bound to WT CCD dimer ([128], also see Figure 4.7B). As expected (Figure 4.5) with the high concentration of BI-D (~5 mM) used in the crystallographic experiments, the inhibitor bound to H171T CCD dimers. Furthermore, the H171T substitution did not detectably affect the inhibitor position within the binding pocket (compare Figure 4.7 A and B). BI-D hydrophobic interactions with IN CCD subunit 2 as well as hydrogen bonding between the inhibitor carboxylic acid, and backbone amides of subunit 1 were fully preserved in both crystal structures. Furthermore, the Thr174 side chain similarly hydrogen bonded to the tert-butoxy ester oxygen in both the WT and mutant IN structures.

Importantly, though, we observed differential interactions of His171 and Thr171 side chains with the inhibitor. The imidazole group of His171 formed both an electrostatic interaction with the carboxylic acid and a hydrogen bond with the tert-butoxy oxygen of BI-D (Figure 4.7B). However, the side chain of Thr171 which establishes a novel hydrogen bond with the carboxylic acid was unable to form a hydrogen bond with the tert-butoxy moiety of BI-D (compare Figure 4.7A and B).
4.3.5 Importance of Protonated His171 N_δ for BI-D Binding

To understand how these structural differences contributed to the markedly reduced ability of the inhibitor to bind H171T IN, we performed absolute binding free energy calculations for the interactions between WT and H171T IN CCD dimers with BI-D (Table 2 and Additional file 1: Figure S2). N_δ-, N_ε- and doubly (N_δ- and N_ε-) protonated forms of His171 were considered in our calculations. Table 4.2 shows that the doubly-protonated form of His171 and the N_δ-protonated form of His171 have similar calculated ΔG_{bind} (cal) of -11.2 kcal/mol and -10.1 kcal/mol respectively; whereas a significantly weaker ΔG_{bind} (cal) value of -5.9 kcal/mol was obtained for the N_ε-protonated form of His171. The calculated ΔG_{bind} (cal) for the IN containing Thr171 was -6.7 kcal/mol (Table 4.2). For comparison, Table 4.2 also shows the ΔG_{bind} (exp) values determined using experimental K_d values for BI-D binding to WT and H171T IN CCDs (Figure 4.5). Comparison of ΔG_{bind} (cal) and ΔG_{bind} (exp) suggests that BI-D binding to WT IN preferentially stabilizes the doubly protonated form and/or the N_δ-protonated tautomer states of His171, while the N_ε-protonated tautomer is a relatively minor species in the inhibitor-bound complex. We also decomposed the computed binding free energy ΔG_{bind} (cal) into contributions from electrostatic and non-polar interactions. We compared the results for both BI-D binding to the H171T IN CCD and its WT counterpart (data not shown), which suggests that the less favorable electrostatic interactions between BI-D and Thr171 primarily contribute to the lower K_d value for the inhibitor binding to the mutant protein. Significantly,
comparisons between the MD simulated structures obtained with different protonation states of His171 and the crystal structure of BI-D in complex with the WT IN CCD dimer are consistent with the trend in the computed binding free energies (Table 4.3 and Figure 4.8).

The simulations also revealed the importance of hydrogen bonding interactions between the ether oxygen on the tert-butoxy moiety on BI-D and the protonated Nδ- on His171. In contrast, to the mutant IN CCD, the hydroxyl group of the Thr171 side chain does not form such interactions with the ether oxygen on the inhibitor (Figure 4.7). This result is supported by the published observations that ALLINIs that contain an oxygen ether linkage are considerably more potent inhibitors of HIV-1\textsubscript{NL4.3} with WT IN than those lacking such an oxygen atom (reviewed in [123]).

To further test this notion we have examined the effects of the H171T IN substitution on the antiviral activities of LEDGIN-6, which lacks the tert-butoxy group ([91,115], also see Figure 4.1). LEDGIN-6 was only ~3.4-fold less potent with respect to HIV-1\textsubscript{NL4.3(H171T IN)} (EC\textsubscript{50} of 41.4 ± 6.4 µM) (data not shown) versus WT HIV-1\textsubscript{NL4.3} (EC\textsubscript{50} of 12.2 ± 2.9 µM) [115]. For comparison, BI-D, which unlike LEDGIN-6 contains the tert-butoxy group, was significantly more sensitive (~68-fold, Table 4.1) to the H171T IN substitution in HIV-1\textsubscript{NL4.3} likely due to the disruption of the hydrogen bonding between the tert-butoxy ether oxygen and Nδ-protonated His171.
4.3.6 Effect of H171T IN substitution on IN-LEDGF/p75 binding

Next, we wanted to understand the structural basis as to why the H171T substitution had significantly less effect on LEDGF/p75 binding (~3.2-fold) compared with BI-D binding (~84-fold) to recombinant HIV-1 IN. Comparison of available crystal structures of BI-D or LEDGF/IBD bound to WT HIV-1 IN CCDs [58,128] revealed that the hydrogen bonding between the protonated Nδ on His171 and the tert-butoxy ether oxygen of BI-D is unique to the inhibitor because LEDGF/IBD does not similarly contact IN.

To examine LEDGF/IBD interactions with the mutant IN we simulated the H171T change in the available crystal structure [58], which revealed that unlike BI-D, LEDGF/IBD made the same number of hydrogen bonds with WT and H171T mutant INs (Figure 4.9). In particular, LEDGF/p75 D366 can hydrogen bond with the side chain of Thr171 replacing the lost electrostatic interaction that occurred with His171. To further test these observations, we performed relative binding free energy calculations for LEDGF/IBD binding to WT and H171T IN CCDs. Free energy perturbation analysis allowed us to calculate that

\[ \Delta \Delta G_b = \Delta G_b(H171T) - \Delta G_b(WT) = 1.08 \text{ kcal/mol}. \]

Since \( K_d = e^{\Delta G_b/RT} \), \( \frac{K_d(H171T)}{K_d(WT)} = e^{[\Delta G_b(H171T)-\Delta G_b(WT)]/RT} = e^{\Delta \Delta G_b/RT} \), the calculated \( \Delta \Delta G_b \) of 1.08 kcal/mol translates into a relative binding affinity ratio \( \frac{K_d(H171T)}{K_d(WT)} = 6.2 \). Experimentally, the \( K_d \) of LEDGF/p75 binding to wild type and H171T mutant INs are ~3.3 nM and
10.5 nM respectively, i.e. \( \frac{K_d(H171T)}{K_d(WT)} = 3.2 \), which is in good agreement with the calculated relative affinity ratio of 6.2.

### 4.4 Discussion

Published studies have shown that ALLINIs are anchored to the IN dimer interface through their key pharmacophore, the carboxylic acid, hydrogen bonding with the backbone amides of IN residues Glu170 and His171. Furthermore, the Thr174 side chain has been implicated in hydrogen bonding with the ALLINI tert-butoxy ether oxygen [96,115,126]. However, the contributions of the His171 side chain for interacting with this class of inhibitors have not been previously elucidated. Yet, genotyping of HIV-1\textsubscript{NL4-3} variants under increasing selective pressure of ALLINI BI-D has revealed the H171T IN substitution as a key amino acid substitution. In addition, this variant persists at the highest inhibitor concentration tested suggesting that the amino acid side chain change at position 171 contributes to the resistance to BI-D [90]. The present studies provide mechanistic and structural clues for these observations. We show that HIV-1\textsubscript{NL4-3} containing the H171T IN substitution confers ~68-fold resistance to BI-D. Significantly, this level of resistance in infected cells correlates closely with ~84-fold reduced binding affinity of the inhibitor to recombinant H171T IN CCD as compared with its WT counterpart. Crystallographic experiments and binding free energy calculations have indicated that N\textsubscript{δ}-protonated or doubly protonated forms of the imidazole ring of His171 can engage in both electrostatic interactions with BI-D carboxylic acid as well as
hydrogen bonding with the tert-butoxy ether oxygen of the inhibitor. These interactions are compromised by the H171T substitution, with the Thr171 side chain forming a less electrostatically favorable hydrogen bond with the BI-D carboxylic acid and lacking any additional interactions with the tert-butoxy ether oxygen (Figure 4.7).

In contrast with the significant reduction in BI-D binding affinity, the H171T IN substitution only minimally reduced LEDGF/p75 binding affinity to recombinant H171T IN. In infected cells, where endogenous LEDGF/p75 levels significantly exceed what is needed for HIV-1 integration, HIV-1_{NL4-3} with the H171T substitution was not compromised for HIV-1 replication. The MD simulations and binding free energy calculation have revealed important differences between BI-D and LEDGF/IBD for their binding to HIV-1 IN. The N_{δ} protonated His171 hydrogen bonds the tert-butoxy ether oxygen of BI-D, which is compromised upon the H171T IN substitution. In contrast, such interactions are not formed between WT IN and LEDGF/IBD. Accordingly, the H171T IN change minimally affects the IN-LEDGF/p75 binding. Furthermore, unlike BI-D which engages only a small pocket at the CCD-CCD dimer interface, LEDGF/p75 establishes additional extensive interactions with HIV-1 IN, which extend beyond the CCD-CCD dimer interface and include strong electrostatic interactions between positively charged residues along LEDGF/IBD α-helix 4 and a number of acidic residues of α-helix 1 of IN [59]. HIV-1 seems to exploit these structural
differences between BI-D and LEDGF/p75 interactions with IN during the process of evolution of the H171T IN escape mutation.

The mechanism for H171T IN resistance is distinct from the previously described mechanism of resistance for the A128T IN escape mutation under the selective pressure of related, archetypal inhibitor ALLINI-1 [126]. The A128T IN substitution does not significantly reduce ALLINI-1 binding to HIV-1 IN CCD with all hydrogen bonding and electrostatic interactions of ALLINI-1 with HIV-1 IN being fully preserved in WT and A128T INs [148]. Instead, the substitution of Ala with bulkier and polar Thr repositioned ALLINI-1 at the IN CCD dimer interface and reduced its ability to effectively bridge between two IN subunits. Consequently, bound ALLINI-1 failed to induce aberrant multimerization of recombinant A128T IN and accordingly HIV-1 NL4-3 containing the A128T IN substitution exhibited marked resistance to ALLINI-1 [126]. In contrast, the H171T IN substitution was able to resist BI-D through decreasing the ability of the inhibitor to bind IN. However, at high BI-D concentrations, BI-D is able to bind, effectively bridging two IN subunits, and inducing aberrant IN multimerization. Collectively these findings provide important structural and mechanistic details for a novel mechanism of resistance.

Analysis of our crystal structure of BI-D bound to HIV-1 IN CCDs has revealed 13 residues (Gln95, Tyr99, Leu102, Thr124, Thr125, Trp132, Ala128, Ala129, Ala169, Glu170, His171, Lys173 and Met178) that are within 5 Å of the inhibitor. Of these only IN amino acids 124 and 125 are polymorphic with Thr
predominating at both positions in clade B, whereas the majority of clade C strains contain Ala124 and Ala125 [149]. Recent studies [125] have shown that these polymorphic substitutions only modestly affected the antiviral potencies of various ALLINIs. We also note close structural similarity between BI-D and BI-224436, the first ALLINI to advance into phase 1a clinical trials ([125], also see Figure 1.1). These two compounds exhibit similar antiviral activities in cell culture with an EC$_{50}$ range of 51-90 nM for BI-D and 11-27 nM for BI-224436 with respect to different viral strains [125,128,150]. However, the latter compound has been chosen for clinical trials due to its excellent pharmacokinetic profile in rats [125]. These two compounds differ only in the substituted ring system, with BI-D and BI-224436 containing bicyclic and tricyclic arenes, respectively ([125], also see Figure 1.1). While the crystal structure for BI-224436 bound to HIV-1 IN CCDs has not been published, based on its close structural similarity with BI-D we predict that both bi- and tricyclic arenes would similarly dock in the hydrophobic pocket that encompasses Leu102, Ala128, Ala129, Trp132 and Met178. At the same time the key interactions between the N$_\delta$-protonated imidazole group of His171 with the tert-butoxy ether oxygen and electrostatic interactions with the inhibitor carboxylic acid are likely to be equally important for both BI-D and BI-224436 binding to HIV-1 IN. Therefore, our findings are expected to facilitate in the development of improved IN inhibitors for potential clinical use.
4.5 Acknowledgements

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<table>
<thead>
<tr>
<th>Receptor</th>
<th>ΔG_{bind} (cal)</th>
<th>ΔG_{bind} (exp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT IN (His171-doubly-protonated)</td>
<td>-11.2</td>
<td>-9.5</td>
</tr>
<tr>
<td>WT IN (His171- Nδ-protonated)</td>
<td>-10.1</td>
<td></td>
</tr>
<tr>
<td>WT IN (His171- Nε-protonated)</td>
<td>-5.9</td>
<td></td>
</tr>
<tr>
<td>H171T IN</td>
<td>-6.7</td>
<td>-6.8</td>
</tr>
</tbody>
</table>

Table 4.2 Binding free energy calculations for BI-D interactions with WT and H171T mutant IN CCDs. Unit: kcal/mol. ΔG_{bind} (cal) represent calculated values for BI-D binding to His171 containing either Nδ-, Nε- or doubly protonated tautomer states and H171T IN CCDs. ΔG_{bind} (exp) values have been calculated based on the experimental data.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>RMSD (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>His171-doubly-protonated</td>
<td>0.53</td>
</tr>
<tr>
<td>His171-Nδ-protonated</td>
<td>0.57</td>
</tr>
<tr>
<td>His171-Nε-protonated</td>
<td>0.50</td>
</tr>
<tr>
<td>The H171T mutant</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Table 4.3 Heavy RMS deviation between the simulated bound BI-D and the IN CCD crystal structures.
Figure 4.1 Chemical Structures of BI-D, LEDGIN-6 and BI-224436
Figure 4.2. Effects of the H171T IN substitution on HIV-1 replication.

(A) p24 production of HIV-1NL4-3 and HIV-1\textsubscript{NL4-3(H171T IN)} plotted as percent WT with standard deviations shown for n = 3 independent experiments. (B) Single round infection of HIV-1NL4-3 and HIV-1\textsubscript{NL4-3(H171T IN)} determined by luciferase expression and plotted as percent WT infectivity with standard deviations shown for three independent experiments.
Figure 4.3. Effects of the H171T IN substitution on recombinant IN activities.

(A) SEC of WT and H171T INs. The peaks corresponding to tetrameric (T) and monomeric (M) forms of IN are indicated. (B) HTRF-based LEDGF/p75 independent integration assay and (C) HTRF-based LEDGF/p75 dependent integration assays showing stimulation of WT and H171T IN activities at indicated LEDGF/p75 concentrations. Total HTRF signal is plotted with standard deviation for three independent experiments shown. (D) HTRF-based assays to determine LEDGF/p75 binding affinities for WT (opened boxes) or H171T (closed circles) INs. Error bars indicate standard deviation for three independent experiments.
Figure 4.4. Concentration dependent effects of BI-D on viral core morphology for HIV-1\textsubscript{NL4-3} and HIV-1\textsubscript{NL4-3(H171T IN)}.
(A) Representative images of mature, eccentric and immature virion morphologies as visualized by electron microscopy. (B) Quantitation of counted virions (100 for WT or H171T per experiment). Virions were produced in the presence of DMSO, 0.18 µM BI-D or 12.2 µM BI-D as indicated. Graphed are averages and standard deviation for n = 2 independent experiments.
Figure 4.5. SPR analysis of BI-D interactions with WT and H171T mutant IN CCDs.

SPR binding kinetics for BI-D interactions with (A) WT IN CCD and (B) H171T IN CCD at indicated inhibitor concentrations. Binding affinities are summarized in C.

<table>
<thead>
<tr>
<th></th>
<th>$k_d$ (µM)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT IN</td>
<td>0.123±0.02</td>
<td>--</td>
</tr>
<tr>
<td>H171T IN</td>
<td>10.3±2.3</td>
<td>83.7x</td>
</tr>
</tbody>
</table>
Figure 4.6. DLS analysis of BI-D induced oligomerization of recombinant WT and the H171T INs.

Shown are the size distributions (%) of IN after DMSO treatment (blue) or BI-D treatment after 15 minutes (red), 20 minutes (green) and 30 minutes (yellow) incubation. BI-D treatments include (A) WT IN +0.120 µM BI-D, (B) H171T IN +0.120 µM BI-D, (C) WT IN +10 µM BI-D and (D) H171T IN +10 µM BI-D. The peak with the diameter size of <1 nm detected in these samples has also been observed for the buffer alone sample indicating that small size particles unrelated to IN or BI-D were present in our preparations.
Figure 4.7. Crystal structures of BI-D bound to WT and H171T CCD dimers. Panel A is the H171T CCD dimer and panel B is the WT CCD dimer. BI-D is colored green and individual IN subunits are colored yellow and cyan. Oxygen atoms are shown in red and nitrogen atoms are in blue. Black dash-lines indicate hydrogen bonding interactions, whereas the magenta dash-line shows the electrostatic interaction between the protonated Nδ- on His171 and the carboxylic acid of BI-D. The arrow indicates the hydrogen bond between the protonated Nδ- on His171 and the ether oxygen on the tert-butoxy (B), which is absent in the H171T IN CCD structure (A).
Figure 4.8. MD simulated structures obtained using different His171 protonation states.
The simulated structure at 15 ns is superimposed onto the X-ray crystal structure. The His171 side chain is indicated by the red arrows for the MD simulation (MD) and the crystal structure (X-ray).
Figure 4.9. MD simulations structures of IN – LEDGF/IBD binding. The MD Simulations reveal similar hydrogen bonding between LEDGF/p75 Asp366 with the WT (left) and the H171T mutant (right) IN CCDs.
Chapter 5: A new class of multimerization selective inhibitors of HIV-1 integrase.

5.1 Introduction

HIV-1 integrase (IN) is an important therapeutic target as its function is essential for viral replication. A tetramer of IN assembles on the viral DNA ends to form the stable synaptic complex (SSC) and catalyzes two reactions necessary for the integration of linear viral DNA into the host chromatin [93]. IN initially removes a GT dinucleotide from the 3′-terminus of each viral DNA end (3′-processing), and then integrates recessed viral DNA ends into the target DNA in a staggered fashion through concerted transesterification reactions (DNA strand transfer). IN is comprised of three domains: the N-terminal domain (NTD) which contains the Zn-binding motif (HH-CC type), the catalytic core domain (CCD) which contains the DDE catalytic triad that coordinates essential Mg2+ ions, and the C-terminal domain (CTD) which contains an SH3-like fold (reviewed in [151]). Each of these domains contributes to protein multimerization [29,30,152,153].

In infected cells, IN assembles with a number of viral and cellular proteins to form a larger nucleoprotein complex termed the preintegration complex (PIC). Cellular chromatin associated protein LEDGF/p75 is a key binding partner of HIV-1 IN.
and promotes effective tethering of PICs to active genes during integration [43-46,52,58,120]. The C-terminal domain of LEDGF/p75, termed the Integrase Binding Domain (IBD), stabilizes the IN tetramer by engaging the IN CCD dimer interface and the NTD of another dimer [57-59,75]. The N-terminal portion of LEDGF/p75, which contains a PWWP domain, nuclear localization signal, AT hooks and highly charged regions, associates with chromatin through engaging both nucleosomal DNA and the trimethylated H3 tail (H3K36me3) [49,50,121], which is an epigenetic marker for active genes and positively correlates with HIV-1 integration sites [154].

The catalytic function of HIV-1 IN has been exploited as a therapeutic target. Three HIV-1 IN inhibitors, raltegravir (RAL), elvitegravir (EVG) and dolutegravir (DTG), are currently in clinical use [17,92]. These inhibitors bind at the enzyme active site and inhibit DNA strand transfer (termed IN strand transfer inhibitors or INSTIs). HIV-1 IN mutations that alter amino-acids near the INSTI binding site can confer resistance to first generation INSTIs RAL and EVG, and have emerged in patients receiving treatment [116-118]. Second generation INSTI DTG retains significant activity against many RAL and EVG resistance mutations and appears to have a higher genetic barrier to resistance. However, IN mutations that confer low-level resistance to DTG have been recently reported [119]. Thus the development of small molecules that impair IN function with different mechanisms of action while retaining potency against INSTI resistant viruses is an important goal.
One such mechanism is to target IN multimerization. We have reported studies demonstrating a highly dynamic nature of HIV-1 IN subunit-subunit interactions and that such flexibility is important for the assembly of the SSC [75]. Various ligands that bind at the IN CCD dimer interface can bridge interacting IN subunits and promote aberrant IN multimerization [78,81,83,112]. The term “aberrant IN multimerization” is used here to refer to the formation of catalytically inactive IN oligomers that differ from the correctly assembled catalytically active tetramer found in the SSC. A significant advantage of stabilizing rather than destabilizing interacting subunits is that small molecule inhibitors do not have to overcome the energy barriers associated with the formation of large protein-protein interfaces. In proof-of-concept studies [81] a small molecule inhibited IN activity in vitro by binding at the IN CCD dimer interface, stabilizing the interacting subunits and promoting aberrant IN multimerization.

Recently, quinoline-based allosteric IN inhibitors (referred to here as ALLINIs) have been reported that target the clinically unexploited LEDGF/p75 binding site at the IN CCD dimer interface and potently inhibit HIV-1 replication in cell culture [91,96,114,115,123,124,155]. An ALLINI carboxylic acid hydrogen bonds with one IN subunit, potentially mimicking an interaction with LEDGF/p75. The quinoline-ring, another key structural feature of ALLINIs, engages another subunit of IN through hydrophobic interactions [91,96,115]. The initial report [91] suggested that these compounds selectively impair the IN-LEDGF/p75 interaction. However, follow up studies [96,114,115,128] have demonstrated that
the quinoline-based ALLINIs inhibit both IN-LEDGF/p75 binding and LEDGF/p75-independent activities with similar IC₅₀ values in vitro. The underlying mechanism for inhibiting the catalytic function of IN has been shown to be promoting aberrant IN multimerization [96,114,115]. This multimodal mechanism of action of ALLINIs concordantly resulted in cooperative inhibition of HIV-1 replication in cell culture [115,155]. Selection of viral strains emerging under ALLINI pressure revealed changes near the inhibitor binding site on IN. In particular, A128T substitution is most common and has been shown to confer marked resistance to the majority of ALLINIs [90,91,96,126].

Studies on the antiviral mechanism of action of ALLINIs have unexpectedly revealed that these compounds inhibit both early and late stages of HIV-1 replication [91,96,114,115,123,124,128,131,132]. In fact, different ALLINIs were 3- to 40-fold more potent when added to producer cells versus target cells [128,131,132]. In target cells ALLINIs did not affect reverse transcription but did impair HIV-1 integration. The treatment of producer cells with ALLINIs resulted in virions with conical cores that were devoid of electron dense material, likely corresponding to ribonucleic acid-protein complexes (RNPs). Strikingly, RNPs were mislocalized between the core and viral membrane [128,131,132]. The viral particles produced in the presence of ALLINIs entered target cells normally but were defective for subsequent reverse transcription in target cells. A single substitution in HIV-1 IN at the inhibitor binding site which confers resistance to quinoline-based ALLINIs was able to overcome these late stage affects.
Furthermore, ΔIN viruses transcomplemented with wild type Vpr-IN were fully susceptible to ALLINI inhibition, whereas Vpr-IN containing a substitution in IN at the inhibitor binding pocket exhibited marked resistance [155]. Taken together, these studies demonstrate that ALLINIs target IN during the late stages of HIV-1 replication and compromise the assembly of electron dense cores. However, dissecting the exact mechanism of action of the quinoline-based ALLINIs has been complicated because they both inhibit IN-LEDGF/p75 binding and promote aberrant IN multimerization with similar potency in vitro [96,114,115].

Here we report design of small molecules that allowed us to probe the role of HIV-1 IN multimerization independently from IN-LEDGF/p75 interactions. We named these selective multimeric IN inhibitors or MINIs. For this, we have exploited the available X-ray crystal structures of quinoline-based ALLINIs bound to the HIV-1 IN CCD dimer to modify the existing scaffold. In particular, we have altered the quinoline moiety and made a series of modifications to enhance potency specifically for modulating IN multimerization without significantly affecting IN-LEDGF/p75 binding. These studies have resulted in a new class of pyridine-based MINIs, which potently modulate IN multimerization but are not effective inhibitors of IN-LEDGF/p75 binding. Using these new compounds as investigational probes we have delineated the significance of ordered IN structure for the formation of correctly assembled virus particles. Furthermore, MINIs inhibited the HIV-1 variant with the A128T IN substitution, which confers
resistance to the majority of ALLINIs. These findings indicate the feasibility of our rational modification approaches to improving IN inhibitors and present a novel class of multimerization selective IN inhibitors as investigational probes.

5.2 Methods

5.2.1 Antiviral compounds

Syntheses of KF115 and KF116 are described elsewhere [130]. ALLIN-1 was synthesized as described previously [115]. GS-B was a kind gift from Gilead Sciences Inc. (California, USA). Raltegravir (RAL, Cat. No. 11680), Nevirapine (NVP, Cat. No. 4666) and Saquinavir (SQV, Cat. No. 4658) were obtained from National Institutes of Health AIDS Research and Reference Reagent Program.

5.2.2 Crystallization and X-ray structure determination

The HIV-1 IN CCD was purification, crystallization and refinement was carried out as described in 2.2.9. Coordinates have been deposited in the Protein Data Bank with accession number 4O0J, 4O55 and 4O5B.

5.2.3 In vitro protein-protein interaction assays

HTRF-based IN multimerization and IN-LEDGF/p75 binding assays were carried out as described in 2.2.7. The HTRF signal was recorded using a Perkin Elmer Multimode EnSpire plate reader.
5.2.4 DLS

Dynamic Light Scattering (DLS) was carried out as described in 4.2.8 using 1 µM KF116 or DMSO. DLS signals were recorded at room temperature using a Malvern Nano series zetasizer instrument.

5.2.5 Cells, viruses, transfections and infections

HEK293T and HeLa TZM-bl cells were cultured in Dulbecco’s modified eagle medium (Invitrogen), 10% FBS (Invitrogen) and 1% antibiotic (Gibco) at 37°C and 5% CO2. MT-4 cells were cultured in RPMI 1640 (Invitrogen), 10% FBS (Invitrogen) and 1% antibiotic (Gibco) at 37°C and 5% CO2. The design and construction of HEK293T cell line, in which all alleles of the PSIP1 (LEDGF) gene were deleted using site-specific gene targeting with transcription activator-like effector nucleases (TALENs), has been reported elsewhere [47]. The TALEN-derived PSIP1 knockout (KO) cells lack 42 kb of PSIP1, with the deletion extending from exon 2 to exon 14, and thus lack all exons that encode the IBD and any protein domains N-terminal to it. They were cultured in Dulbecco's modified eagle medium (Invitrogen), 10% FBS (Invitrogen) and 1% antibiotic (Gibco) at 37°C and 5% CO2.

All viral stocks were generated by transfecting HEK293T cells with plasmid DNA and X-tremeGENE HP (Roche) transfection reagent at 1:3 ratio following manufacturer's protocol. Twenty-four hour post-transfection, the culture supernatant was replaced with fresh complete medium after washing once with
complete medium. Forty-eight hour post-transfection, the virus containing supernatant was collected, filtered through 0.45 µm filter and, if needed, concentrated by ultracentrifugation. Replication competent HIV-1 was generated using pNL4-3 [156]. Luciferase reporter HIV-1 (HIV-1-Luc) pseudotyped with VSV-G, was generated using pNL4-3.Luc.Env− [140] and pMD.G (VSV-G envelope plasmid) [157]. HIV-1 subtype C was generated using the infectious molecular clone pMJ4 [158].

5.2.6 Antiviral and cytotoxicity assays

For early stage experiments, the indicated concentrations of the test inhibitor or diluent control (DMSO) were added directly to the target cells and the cells were infected with untreated virions. Briefly, HeLa TZM-bl cells (2×10^5 cells/well of a 6-well plate in 2 ml of complete medium) were pre-incubated with the indicated concentrations of the test inhibitor or diluent control (DMSO) for 2 h. The cells were then infected with HIV-1 virions equivalent to 4 ng of HIV-1 p24 as determined by HIV-1 Gag p24 enzyme-linked immunosorbent assay (ELISA, ZeptoMetrix) following manufacturer's protocol. Two hours post-infection the culture supernatant was removed, washed once with complete medium, and then fresh complete medium was added with the inhibitor concentration maintained. The cells were cultured for 48 h and the cell extracts were prepared using 1× reporter lysis buffer (Promega). Luciferase activity was determined using a commercially available kit (Promega).
For late stage experiments, the progeny virions were prepared in the presence of the indicated concentrations of the test inhibitor or diluent control (DMSO) and were then used to infect untreated target cells. The antiviral assays for late stage experiments were performed using the procedure previously described [131].

For full replication cycle experiments, progeny virions were prepared in the presence of the test inhibitor or diluent control (DMSO) at given concentrations. Target cells were pre-incubated for 2 h with matching inhibitor concentrations. Target cells were infected and luciferase activity was determined 48 h post infection as described above.

The cytotoxicity assays were performed as described previously [159]. The fitted dose-response curves were generated to calculate EC$_{50}$ or CC$_{50}$ using Origin software (OriginLab, Inc.)

5.2.7 qPCR analysis

Levels of HIV-1 early reverse transcription products, late reverse transcription products (LRT), 2-LTR circles and integrated proviruses (Alu-PCR reactions) were quantified using primers, probes, and quantitative PCR (qPCR) conditions described previously [160,161]. Briefly, HEK293T cells (2×10$^5$ cells/well of a 6-well plate in 2 ml of complete medium) were infected with VSV-G pseudotyped HIV-1 virions equivalent to 2 µg of HIV-1 p24 as determined by HIV-1 Gag p24 ELISA (ZeptoMetrix) following manufacturer's protocol. All viral stocks were treated with 60 U/ml DNaseI (Ambion) prior to infections to avoid plasmid DNA
contamination. Infected cells were harvested at the indicated times post-infection and genomic DNA was isolated using DNeasy Blood & Tissue kit (Qiagen). For Alu-PCR reactions in HEK293T cells to quantify integrated provirus, genomic DNA was harvested from cells 7 days post-infection to eliminate unintegrated viral DNA and was used as the template for initial amplification. TaqMan-based qPCR reactions were prepared using iQ Supermix (Biorad) using primer-probe sets as described previously [160,161]. All reactions were normalized to GAPDH using SYBR-green (iQ SYBR Green Supermix; Biorad) based qPCR and primers as described previously [160]. Relative quantification analysis was performed using the 2^{ΔΔCT} method [162]. qPCR was performed using the CFX96 real-time system.

5.2.8 Immunoblotting

Standard Western blotting procedures were used with the following antibodies: HIV-1 Gag (a gift from Kathleen Boris-Lawrie), HIV-1 RT (NIH Cat. No. 7373), HIV-1 IN (a gift from Paul Bieniasz & Michael Malim), GAPDH (Serotech AHP 1628T), and LEDGF/p75 (BD Biosciences 611714).

5.2.9 Transmission Electron Microscopy

HIV-1 virion morphology was analyzed using thin-section transmission electron microscopy (TEM). HEK293T cells (2x10^6 cells/100 mm culture dish in 10 ml of complete medium) were transfected with HIV-1 provirus (pNL4-3) and cultured for 24 h to ensure expression of the provirus as described above. Next, the cells
were treated with the indicated inhibitor or diluent control (DMSO) for 1 h. The culture supernatant was replaced with fresh complete medium containing either the inhibitor or DMSO, and cells were then cultured for another 24 h to allow for the production of HIV-1 particles in the presence of the inhibitor or diluent control. Cells were harvested, washed twice with 1× PBS, and the cell pellets were fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer pH 7.4 containing 0.1 M sucrose), post-fixed in 1% osmium tetroxide in the same buffer, en bloc stained in 2% uranyl acetate in 10% ethanol, dehydrated in graded ethanols, infiltrated, and embedded in Epon resin. TEM sample preparation, negative staining and imaging were performed at Campus Microscopy & Imaging Facility (Ohio State University). Images were taken with FEI Tecnai G2 Spirit transmission electron microscope.

5.2.10 HIV-1 viral core analyses using sucrose gradient fractionation

Sucrose density gradient fractionation was carried out as described previously [163] with some modifications. Briefly, HIV-1 virions were produced in the presence of the inhibitor or diluent control (DMSO) and cell-free virions were concentrated over a 25% sucrose cushion by ultra-centrifugation at 28,000 rpm for 2 h at 4°C in SW41 rotor (Beckman). Pelleted virions were resuspended in 300 µl STE buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA), lysed with 0.5% Triton X100 for 2 minutes at room temperature. Lysed virions were loaded on 30–70% linear sucrose density gradients in STE buffer, and ultra-centrifuged at 28,500 rpm for 16 h at 4°C in SW41 rotor (Beckman). Twenty-one 0.5-ml
fractions were collected from the top of the gradient and subjected to SDS-PAGE and immunoblotted with HIV-1 Gag antisera to monitor the distribution of HIV-1 capsid.

5.2.11 Resistance Selection

Resistance selection was carried out as previously described [159]. Briefly, selection of resistance to HIV-1 was carried out in MT-4 cells with the inhibitor added at a final concentration corresponding to its antiviral EC$_{50}$. Infections were initiated with HIV-1$_{NL4-3}$ at a MOI $\sim$0.01. Cultures were split one-third every 3 to 4 days depending on the proliferation status of the cells. The progression of infection was monitored by the appearance of HIV-1-induced cytopathic effect (CPE, syncytia formation). When peak CPE was reached, the cell-free culture supernatant was used to infect fresh MT-4 cells in the presence of an equal or 2-fold higher inhibitor concentration. Successive viral passages were generated by repeating this procedure.

5.2.12 PCR Amplification, cloning and sequencing of viral DNA

Clonal sequencing of viral passage was carried out at passage 5 and 10, respectively, as described previously [159]. Total DNA isolated was from MT-4 cells infected with virus from each passage using DNeasy Blood & Tissue kit (Qiagen) and was used for the PCR amplification of a 2765 base pair viral DNA fragment spanning nucleotide 372 of the RT gene to nucleotide 127 of the Vpr gene. The PCR reaction was performed using the Platinum High Fidelity PCR
SuperMix (Invitrogen) following manufacturer protocol. The PCR product was gel-purified and cloned into the pCR-XLTopo (Invitrogen). Next, 96 transformed bacterial colonies (12 from DMSO control and 84 from KF116) were cultured in a SeqPrep HP 96 Plate (EdgeBio). Plasmid DNA isolation and sequencing were carried out at Ohio State University's Plant-Microbe Genomics Facility. The entire IN gene was sequenced using the following three primers: INseq15′-GTCTACCTGGCATGGGTACC-3′, INseq2 5′-GTATCTTGATTAGCAGCTCATG-3′, and INseq3 5′-CTAGTGAGATGTGTACTTCTG-3′. Sequencing reads were analyzed using BioEdit Sequence Analyzer Editor. Resistance selection under diluent control (DMSO) was used to identify naturally occurring polymorphisms. The inhibitor-specific mutations were documented based on their order of appearance and rate of emergence, as well as patterns/copurifying mutations.

5.2.13 Isolation of integration sites using 454-pyrosequencing

Isolation of HIV-1 integration sites was performed using ligation-mediated PCR as previously described [164,165] with some changes. Briefly, HEK293T cells (2x10⁵ cells/well of a 6-well plate in 2 ml of complete medium) for the indicated treatment were infected with VSV-G pseudotyped HIV-1 virions equivalent to 2 μg p24 based on HIV-1 p24 ELISA. Cells were harvested 10 days post-infection, and the genomic DNA was purified using DNeasy Blood & Tissue kit (Qiagen). Genomic DNA was fragmented with dsDNA Fragmentase (NEB) and then linkers were ligated. The provirus-host DNA junctions were amplified by nested PCR using bar-coded primers. The PCR products were gel-purified and sequenced on
the 454 GS-Junior (Roche). Six independent infections were performed for each sample and samples were separately bar coded with the second pair of PCR primers to enable pooling of the PCR products for sequencing.

5.2.14 Bioinformatics & statistical analyses of integration site distribution

The analysis and statistical methods for HIV-1 integration site distribution have been previously described [166]. Briefly, the reads from the 454 sequencing run were decoded by requiring a perfect match to the sample barcode and were subsequently trimmed. Next, the collection was trimmed and quality filtered by requiring a 95% match to the long terminal repeat (LTR) primer, and 100% match to the flanking LTR region. Finally, only those sequences were considered as authentic integration sites that began within 3 bp of the LTR end and showed best unique alignments to the human genome by BLAT (hg18, version 36.1, >98% match score). The HIV-1 integration sites were matched to computationally-generated random control sites. Fisher's exact test was used to compare the distribution of integration sites with respective matched random controls (MRCs). The frequency of integration of the indicated drug-treated sample with the diluent control (DMSO) sample was compared using Fisher's exact test. Statistical analysis was performed using R (http://www.r-project.org).

The association of HIV-1 integration sites to the genomic features was performed as described previously [154,166]. Briefly, the relationship between the integration site frequencies relative to the matched random controls for each of
the annotated genomic feature was quantified using receiver operator characteristic (ROC) curve area method. The ROC value of each comparison is represented as a tile in the heatmap. Statistical methods and tests to determine whether the ROC areas calculated were significantly different from one another or from 0.5 (matched random controls) have been described previously [167].

5.2.15 RNA isolation and analyses

Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen) and subjected to on-column DNase digestion following the manufacturer's protocol. Virion RNA was isolated from pelleted virions using TRIzol LS (Invitrogen), treated with TURBO DNase (Ambion), and subjected to acid phenol extraction (Life Technologies) followed by ethanol precipitation. Complementary DNA (cDNA) was synthesized using Omniscript reverse transcriptase (RT) kit (Qiagen), random hexamers and 100 ng of RNAs following the manufacturer's protocol. 10% of the RT reaction was used for SYBR-green (iQ SYBR Green Supermix; Biorad) based qPCR using previously described [168] HIV-1 gag and gapdh: gag forward 5′-GTAAGAAAAAGGCACAGCAAGCAGC, gag reverse 5′-CATTTCCTGGAGGTTTCTG, GAPDH forward 5′-CATCAATGACCCCTTCATTGAC, and GAPDH reverse 5′-CGCCCCACTTGATTGTTGGA. Standard curves to calculate absolute gag RNA copy numbers were generated using HIV-1 provirus plasmid (pNL4-3) in the range of $10^2$ to $10^8$ copies. qPCR reactions using cellular RNA were normalized
to GAPDH and relative quantification analysis was performed using the $2^{-\Delta\Delta CT}$ method [162].

**5.2.16 Virion protein processing**

HEK293T cells (2x10^6 cells/100 mm culture dish in 10 ml complete medium) were transfected with HIV-1 provirus (pNL4-3) and cultured for 24 h to ensure expression of the provirus as described above. Next, the cells were treated with the indicated inhibitor or diluent control (DMSO) for 1 h. The culture supernatant was replaced with fresh complete medium containing either the inhibitor or DMSO, and cells were then cultured for another 24 h to allow for the production of HIV-1 particles in the presence of the inhibitor or diluent control. The virus containing cell-free supernatant was collected, filtered through 0.45 µm filter, and the amounts of viral particles produced was measured by HIV-1 Gag p24 ELISA (ZeptoMetrix) following manufacturer's protocol. Equivalent amounts of virions (~5 µg p24 based on HIV-1 p24 ELISA) were concentrated over a 25% sucrose cushion by ultra-centrifugation at 28,000 rpm for 2 h at 4°C in SW41 rotor (Beckman). Pelleted virions were lysed in 100 µL RIPA buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Triton-X, 1% deoxycholic acid, and 2 mM PMSF. Indicated amounts of concentrated virions were resolved by SDS-PAGE (4–12%, Invitrogen) and immunoblotted.
5.2.17 Virion-associated IN cross-linking assay

The IN cross-linking assay was performed as described previously [131]. Briefly, concentrated virions equivalent to 50 ng of HIV-1 p24 were cross-linked with 50 µM bis(sulfosuccinimidyl)suberate (BS³) cross-linking reagent (Thermo Scientific). Subsequent cross-linked reaction products were quenched and resolved by SDS-PAGE (4–12%, Invitrogen) and immunoblotted.

5.2.18 Virion-associated reverse transcriptase (RT) assay

Virions generated in the presence of inhibitor or diluent control (DMSO) was concentrated over 25% sucrose cushion. The virion-associated RT activity assay was performed as described previously [169,170] with minor changes. Briefly, equivalent amounts of concentrated virions (based on HIV-1 p24 ELISA) were incubated with RT buffer, containing 50 mM Tris (pH 7.8), 75 mM KCl, 5 mM MgCl₂, 2 mM DTT, 0.05% Nonidet P-40 (v/v), 5 µg/ml Poly r(A). Poly d(T)₁₂₋₁₈, and 10 µCi of α⁻³²P dTTP, in 35 µl final volume. The reactions were incubated at room temperature for 3 h. Duplicate 10 µl of the final reactions were blotted onto a 96-well DEAE Filtermat paper (Wallac-Perkin Elmer), dried, washed and processed as described previously.

5.2.19 tRNA\textsuperscript{Lys3} primer extension assay

Total viral RNA, which serves as a source of tRNA\textsuperscript{Lys3} primer annealed to the viral RNA template, was extracted as described above. The relative amount of
viral RNA template from the virions produced in the presence of inhibitor or diluent control (DMSO) was determined using quantitative PCR as described above. The initiation of reverse transcription from total viral RNA was measured using an in vitro HIV-1 reverse transcription reaction, which extends tRNA\textsuperscript{Lys3} primer on viral RNA template by +6-nt, as described previously [171,172] with minor modifications. Briefly, 10\textsuperscript{7} copies of total viral RNA were incubated with 50 ng of purified HIV-1 RT (NIH Catalog # 3555) at 37°C for 15 min in 20 µl of RT buffer containing 50 mM Tris-HCl (pH 7.5), 60 mM KCl, 3 mM MgCl\textsubscript{2}, 5 mM DTT, 10 U of RNaseOut (Invitrogen), 200 µM dCTP, 200 µM dTTP, 50 µM ddATP and 5 µCi of [\textalpha\textsuperscript{32}P] dGTP. The reaction was stopped by adding 180 µl of termination buffer (100 µl isopropanol, 10 µl NaOAc, 70 µl ddH\textsubscript{2}O and 1 µg linear acrylamide) and incubating the samples at −80°C for 1 h to precipitate reverse transcription product. The final reaction products were resolved by PAGE (6% polyacrylamide, 7M urea) and detected by the phosphorimager analysis.

5.2.20 Virion RNA packaging

HEK293T cells (2x10\textsuperscript{6} cells/100 mm culture dish in 10 ml of complete medium) were transfected with HIV-1 provirus (pNL4-3) and cultured for 24 h to ensure expression of the provirus as described above. Next, the cells were treated with the indicated inhibitor or diluent control (DMSO) for 1 h. The culture supernatant was replaced with fresh complete medium containing either the inhibitor or DMSO, and cells were then cultured for another 24 h to allow for the production of HIV-1 particles in the presence of the inhibitor or diluent control. The virus
containing cell-free supernatant was collected, filtered through a 0.45 µm filter, and concentrated over a 25% sucrose cushion by ultra-centrifugation at 28,000 rpm for 2 h at 4°C in SW41 rotor (Beckman). In parallel, aliquots of cell-free supernatant were used to measure the amount of viral particles produced using HIV-1 Gag p24 ELISA (ZeptoMetrix) following manufacturer’s protocol. RNA was isolated from the pelleted virions and subsequent cDNA preparations were used for gag quantitative PCR as described above. Relative virion RNA packaging was calculated by normalizing virion Gag RNA copy numbers to Gag p24.

5.2.21 Viral entry assay

The viral entry assay [173] with some modifications was used to measure the incoming viral genomic RNA upon viral fusion and entry. Briefly, HEK293T cells (2×10^5 cells/well of a 6-well plate in 2 ml of complete medium) were infected with DNase (60 U/ml, Ambion)-treated HIV-1 virions equivalent to 0.5×10^7 viral RNA copies for 30 min at 4°C. The infected cultures were then incubated at 37°C and 5% CO₂ for 1 h. Next, infected cells were harvested and total cellular RNA was isolated using RNeasy mini kit (Qiagen) and subjected to on-column DNase digestion following the manufacturer’s protocol. cDNA synthesis and qPCR using HIV-1 gag and GAPDH primers were performed as described above. The Gag qPCRs were normalized to GAPDH as described above.
5.2.22 Viral genomic RNA stability assay

The stability of incoming viral genomic RNA was measured as described previously [173] with minor modifications. Briefly, HEK293T cells (2×10^5 cells/well of a 6-well plate in 2 ml of complete medium) were infected with DNase (60 U/ml, Ambion)-treated HIV-1 virions equivalent to 2 µg p24 based on HIV-1 p24 ELISA for 30 min at 4°C. The infected cultures were then incubated at 37°C and 5% CO₂. Infected cells were harvested at the indicated times post-infection and total cellular RNA was isolated using RNeasy mini kit (Qiagen) and subjected to on-column DNase digestion following the manufacturer’s protocol. cDNA synthesis and qPCR using HIV-1 gag and gapdh primers were performed as described above. The Gag qPCR were normalized to GAPDH as described above. In order to avoid any contamination of leftover HIV-1 proviral plasmid DNA from transfections to produce cell-free virions, which might interfere with gag qPCRs, 1 µM NVP was added to the cell culture. Briefly, cells were pre-incubated with 1 µM NVP for 2 h and subsequent viral infections were performed in the presence of NVP. The cells were cultured in the presence of NVP for indicated times before analysis.

5.3 Results

5.3.1 Rational Design and structure-activity relationship

The archetypal ALLINI, ALLINI-1 (Figure 5.1) is composed of four structural elements: quinoline ring, substituted benzene ring, carboxylic acid and a methoxy
group [89,115]. Optimization of the ALLINI-1 scaffold has led to the development of GS-B, one of the most potent ALLINIs reported to date [96]. As shown in Figure 5.1, GS-B possesses an optimized benzene ring and a sterically bulky tert-butoxy ether moiety, which together are responsible for the enhanced antiviral activity (IC$_{50}$ of ~5.8 µM for ALLINI-1 vs IC$_{50}$ of ~0.026 µM for GS-B) [115,131]. During the course of optimization [96], however, two of the four key structural elements, the quinoline ring and carboxylic acid were not modified. In the present study, we have modified the quinoline ring as explained below.

A comparison of the available crystal structures of ALLINI-1 and LEDGF/IBD bound to HIV-1 IN CCD dimers [58,115] shows that ALLINI-1 binds at the LEDGF/p75 binding site and bridges between two IN subunits (compare Figure 5.2A and 5.2B), providing a candidate structural explanation for the multimodal mechanism of action of ALLINIs. The compounds both promote aberrant IN multimerization and inhibit IN-LEDGF/p75 binding with very similar IC$_{50}$ values in vitro [96,114,115]. Both LEDGF/p75 and ALLINI-1 interactions with the IN CCD dimer are anchored by hydrogen bonding interactions with subunit 2 through the main chain nitrogens of IN residues Glu-170 and His-171. An additional key hydrogen bond is also formed, however, between the ether oxygen of ALLINI-1 and Thr-174 of subunit 2 ([115,126], also see Figure 5.2B).

In contrast with subunit 2, interactions with subunit 1 are primarily hydrophobic in nature as observed with LEDGF/p75 Ile-365 and Leu-368 (Figure 5.2A) or the substituted benzene ring of ALLINI-1 (Figure 5.2B). The rigid and planar
quinoline scaffold of ALLINIs significantly limits its interactions with subunit 1, although its projection towards Ala-128 of subunit 1 ultimately results in marked resistance to the majority of ALLINIs through emergence of A128T substitution [90].

To enhance the interactions of the inhibitor with subunit 1, we have developed pyridine-based compounds, KF115 and KF116 (Figure 5.1), through dissociation of the fused quinoline moiety. We hypothesized that the rotatable bond connecting the pyridine ring with the benzene ring in KF115 or with the benzimidazole ring in KF116 would allow these structural motifs to adopt suitable orientations to optimally engage subunit 1.

To test our hypothesis we have solved the crystal structures of KF115 and KF116 bound to HIV-1 IN CCD dimer (Figure 5.2C and 5.2D). The results in Figure 2 show that KF115 and KF116 maintain hydrogen bonding with Glu-170 and His-171, and Thr-174 of subunit 2. However, interactions of KF115 and KF116 with subunit 1 significantly deviate from the ALLINIs and LEDGF/p75. In particular, the following two principal differences are noteworthy

i. Pyridine-based KF115 and KF116 establish more extensive interactions with subunit 1 compared with their quinoline-based ALLINI counterparts or LEDGF/p75. KF115 has significantly better overall hydrophobic interactions with subunit 1 compared with ALLINIs or LEDGF/p75. Unlike the rigid quinoline scaffold in ALLINIs, the dimethylated benzene ring of
KF115 takes advantage of the more flexible single bond and adopts a suitable conformation to obtain maximal hydrophobic interactions of the aryl unit with IN subunit 1. As anticipated, the observed perpendicular conformation of the biaryl system responsible for projecting the aromatic ring toward subunit 1 is enforced by the presence of the methyl substituents on the central pyridine ring. Moreover, the benzimidazole ring of KF116, which adopts a similar conformation, engages subunit 1 through an additional hydrogen bonding interaction with the side chain of Thr-125. The additional interactions of KF115 and KF116 with subunit 1 were expected to significantly enhance their ability to bridge between two IN subunits and accordingly enhance aberrant IN multimerization.

ii. Interactions of the ALLINI quinoline group with IN subunit 1 closely mimic LEDGF/p75 Leu-368, whereas KF115 and KF116 lack this quinoline moiety. Accordingly, we hypothesized that these changes would reduce the specificity of KF115 and KF116 for affecting IN-LEDGF/p75 binding compared with their quinoline-based ALLINI counterparts.

To test our hypotheses and dissect the mode of action of KF115 and KF116, we have evaluated their IC$_{50}$ values for promoting IN multimerization and inhibiting the IN-LEDGF/p75 interaction in vitro. The data in Table 5.1 and Figure 5.3 show that KF115 and KF116 were, ~50- and ~60-fold more selective for modulating IN multimerization compared with IN-LEDGF/p75 binding (selectivity ratio defined as IC$_{50}$ for inhibiting IN-LEDGF/p75 binding divided by EC$_{50}$ for aberrant IN multimerization).
multimerization, Table 5.1). In control experiments, the archetypal ALLINI, ALLINI-1, exhibited comparable potency for inhibiting IN-LEDGF/p75 binding compared with aberrant IN multimerization (0.2-fold selectivity ratio). Of note, all multifunctional ALLINIs reported to date have been shown to exhibit comparable potencies for inhibiting IN-LEDGF/p75 binding and promoting aberrant IN multimerization ([96,114,115,126,128], also see Table 5.1).

We next investigated whether the high degree of selectivity of KF115 and KF116 for modulating IN multimerization could affect the antiviral potency of these compounds. The results in Table 5.1 show that KF115 and KF116 inhibited HIV-1 replication in infected cells with an IC₅₀ value of ~0.121 µM and ~0.024 µM, respectively, whereas no cytotoxicity was detected with 100 µM (the highest concentration tested) of either of these compounds. Accordingly, the selectivity indexes (defined as CC₅₀ for cytotoxicity divided by EC₅₀ for antiviral activity) for KF115 and KF116 are >826 and >4,000, respectively (Table 5.1). Since KF115 and KF116 IC₅₀ values for antiviral activities in infected cells correlate closely with EC₅₀ for promoting aberrant IN multimerization but not with IC₅₀ for inhibiting the IN-LEDGF/p75 binding, we conclude that the antiviral potencies of KF115 and KF116 are determined primarily through promoting aberrant IN multimerization. Accordingly, we refer to KF115 and KF116 as multimeric IN inhibitors or MINIs. KF116 was chosen for further mechanistic studies due to its more potent antiviral activities.
IN residue Thr-125, which forms a hydrogen bond with KF116 (Figure 5.2D), predominates in clade B and is present in HIV-1\textsubscript{NL4-3}. However, the majority of clade C strains contain Ala at this position. Therefore, we examined the antiviral activities of KF116 with respect to HIV-1 clade C using the infectious molecular clone pMJ4, which contains IN residue Ala125 [158]. KF116 inhibited HIV-1\textsubscript{MJ4} replication with an IC\textsubscript{50} of 0.127 ± 0.01 µM. The observed differences in KF116 IC\textsubscript{50} values for HIV-1\textsubscript{NL4-3} (~0.024 µM) versus HIV-1\textsubscript{MJ4} (~0.127 µM) could be due to the presence of Thr or Ala respectively at IN residue 125. This would be consistent with the crystallographic results (Figure 5.2D) where Thr125 was found to hydrogen bond with KF116, whereas Ala125 would lack the ability to form a hydrogen bond with KF116, resulting in decreased potency. Thus the strategy of linking the pyridine and benzimidazole rings through a rotatable bond allows the inhibitor to engage both interacting subunits even with residue variations at position 125, thereby preserving activity against subtype C.

5.3.2 Evolution of HIV-1 variants in the presence of KF116

The A128T IN substitution is the most prevalent mutation conferring resistance to most quinoline-based ALLINIs, including ALLINI-1 [90,91,96,128,174]. Structural and biochemical analyses have elucidated the underlying mechanism for HIV-1 A128T IN resistance to ALLINIs [174]. The biochemical studies showed that the A128T substitution has evolved to overcome ALLINI-1 induced IN multimerization rather than the inhibition of IN-LEDGF/p75 binding. In particular, our published crystal structures of ALLINI-1 bound to WT and A128T IN CCDs revealed that
the A128T substitution affected the positioning of the quinoline group through the bulkier and polar threonine exerting a steric effect and electronic repulsion to ALLINI-1 ([174], also see Figure 5.4A). Since KF116 lacks the quinoline moiety, we hypothesized that this inhibitor could escape the effects of threonine at position 128. Indeed, our crystal structures have revealed very similar binding of KF116 to WT and A128T IN CCDs (Figure 5.4B). Moreover, KF116 potently promoted aberrant IN multimerization of A128T IN in vitro and effectively impaired A128T IN HIV-1 NL4-3 replication in infected cells, whereas in control experiments the A128T IN HIV-1 NL4-3 exhibited marked resistance to ALLINI-1 (Figure 5.4C).

To select HIV-1 strains resistant to KF116, HIV-1 NL4-3 was passaged serially in MT-4 cells under increasing concentrations of the inhibitor as described [159]. Clonal sequencing of KF116-selected viruses after 5 and 10 successive passages revealed substitutions in HIV-1 IN (Figure 5.5A). A single T124N substitution emerged after 5 passages, with KF116 concentration reaching 0.8 µM. With further increases in KF116 concentrations, which reached 25.6 µM at passage 10, the T124N substitution within the viral pool diminished to ~3.7% and instead the triple (T124N/V165I/T174I) substitution in HIV-1 IN emerged (Figure 5.5A). As expected (Figure 5.4) the A128T substitution, which is sufficient to confer resistance to ALLINI-1 [90,126], was not observed with KF116. Figure 5.5B shows that all of the substitutions selected under KF116 pressure were
located within or near the KF116 binding thus paralleling the structural results (Figure 5.2D) in the context of infected cells

**5.3.3 KF116 affects HIV-1 virion core morphology and inhibits subsequent reverse transcription in target cells.**

To dissect the primary mechanism of KF116 inhibition, we have examined its effects on early and late stages of HIV-1 replication by adding the inhibitor to target or producer cells. When added to the producer cells KF116 inhibited HIV-1 replication with an IC$\textsubscript{50}$ of $\sim 0.03 \, \mu$M, which closely correlated with the IC$\textsubscript{50}$ values obtained in full replication cycle ($\sim 0.024 \, \mu$M, Figure 5.6). In contrast, KF116 was $\sim 2,000$-fold less effective in target cells (Figure 5.6). Since the secondary mechanism of action of KF116 in target cells is observed at the inhibitor concentrations that significantly exceed a clinically relevant (sub-micromolar) range, our mechanistic studies have focused on the primary mechanism of action of KF116 seen in producer cells.

The data in Figures 5.7, 5.8 and 5.9 demonstrate that KF116 treatment did not affect virus particle production, HIV-1 Gag/ Gag-Pol protein processing, and viral genomic RNA packaging. Examination of virion morphology with thin-section transmission electron microscopy revealed that treatment of virus-producer cells with KF116 impaired the formation of electron-dense cores and resulted in virions with conical cores that were devoid of electron dense RNPs (referred here to as eccentric cores). Instead, the RNPs were mislocalized between the core and viral
membrane (Figure 5.10A), similar to eccentric HIV-1 viral particles produced upon ALLINI treatments [128,131,174] or with select IN class II mutants [40,131,135,175]. Quantitative analysis of mature virions have revealed a marked increase in eccentric cores (~95%) upon KF116 treatments compared with virions produced in the absence of the inhibitor (~6%) (Figure 5.10B). Analytical sucrose density gradient fractionation of detergent-lysed virions and immunoblot analyses with HIV-1 Gag antisera have similarly revealed that the KF116 treatment resulted in reduction (>95%) of HIV-1 capsid (p24) in higher density fractions (compare fractions 18–20 in the absence and presence of KF116 in Figures 5.10C and 5.10D). These results suggest that the density of the viral cores decreased upon inhibitor treatment and are consistent with formation of an empty core due to mislocalization of the RNPs (Figure 5A).

To examine whether mislocalization of RNPs could affect the initiation of reverse transcription, the extension of tRNA\textsuperscript{Lys3} primer was measured using total RNA isolated from KF116 or DMSO treated virions and recombinant reverse transcriptase (RT). Figure 5.11A shows similar levels of extension products in the inhibitor treated and untreated control samples suggesting that KF116 did not significantly affect annealing of tRNA\textsuperscript{Lys3} primer to the cognate viral RNA template. Furthermore, experiments in Figure 5.11B have shown that KF116 had no effects on virion associated RT activities. These findings are consistent with a previous report showing that ALLINI GS-B did not detectably affect endogenous RT activity [131].
We monitored how KF116 treatment of virus-producer cells affected subsequent early replication steps in target cells. For these experiments we used 1.0 µM inhibitor, which would allow us to distinguish the primary mechanism of action of KF116 (IC$_{50}$ of ~0.030 µM) seen in producer cells from the weaker activity (IC$_{50}$ of ~50 µM) detected in target cells. Figure 5.12 shows that comparable levels of genomic RNA were detected in target cells when infected by equivalent numbers of particles prepared in the presence or absence of KF116 suggesting that the mislocalization of RNPs upon KF116 treatment does not significantly compromise viral entry. Next, we have examined levels of early and late RT products as well as 2-LTR circles and integrated proviruses using qPCR. In control experiments, the selective IN strand transfer inhibitor RAL was tested. Producing virions in the presence of RAL yielded no inhibitory effects in the target cells (Figure 5.13), but virions produced in the presence of KF116 exhibited sharply reduced levels of early and late RT products (Figure 5.13A and 5.13B). The markedly defective viral DNA synthesis resulted in reduced levels of 2-LTRs, integrated proviruses and subsequent viral expression (Figure 5.13C–E).

For control experiments KF116 or RAL was added to the target cells and the viral replication intermediates were monitored after infection with untreated virions (Figure 5.14). As expected, RAL selectively inhibited the integration step and increased the levels of 2-LTRs (Figure 5.14C and 5.14D). In contrast, treatment with 1 µM KF116 had no effects on reverse transcription, integration, levels of 2-
LTRs, or viral expression (Figure 5.14). These findings are consistent with the results in Figure 5.6 indicating that KF116 is not an effective inhibitor of HIV-1 when added to target cells.

5.3.4 KF116 promotes aberrant IN multimerization in vitro and in virus particles

Our subsequent efforts have focused on delineating the contributions of aberrant IN multimerization versus the potential inhibition of IN-LEDGF/p75 binding on the antiviral activities of KF116. Homogeneous time-resolved fluorescence (HTRF)-based IN multimerization assays were used to determine EC$_{50}$ values of KF116 and ALLINIs for promoting IN multimerization in vitro. However, this technique cannot delineate multimeric forms of IN formed in the presence of inhibitors [96,115] and some groups have proposed that ALLINIs stabilize an inactive dimer of IN [96], while others have suggested the formation of higher order IN multimers [174]. To better understand KF116 induced IN multimerization, we employed DLS, an optical method used for determining the diffusion coefficients of particles in solution [176,177]. Since the diffusion coefficient depends on the particle size and shape, DLS can be used to study particle aggregation [177-179]. In the absence of added inhibitor, 0.2 µM IN did not yield any signal presumably due to the relatively small size of the fully soluble protein (Figure 5.15A). In control experiment with only KF116, no signal was recorded indicating that the inhibitor does not self-aggregate in the aqueous solution (Figure 5.15A). Immediately upon addition of KF116 to IN, we observed the appearance of a
peak corresponding to a particle size with 142 nm diameter (Figure 5.15A). Within 8 minutes two peaks corresponding to larger particle sizes with 190 nm and 955 nm diameters were detected. Particle sizes increased further after 30 min (1106 nm diameter) and 120 min (1281 nm diameter) incubation of IN with KF116. These results indicate the equilibrium shift toward higher order oligomers in a time-dependent manner. While we cannot not delineate the shapes of these complexes or determine the exact multimeric states of IN in the complex with KF116, our results can be compared with reported volumes of functional IN tetramers. For example, atomic force microscopy experiments have revealed that IN tetramers in the stable synaptic complex have volumes ~220 nm$^3$ [35], which correspond to a diameter of ~7.5 nm if a spherical shape is assumed. Thus, our DLS results reveal that the sizes of IN oligomers formed in the presence of KF116 significantly exceed that of tetrameric forms of the protein [35,180]. Figure 5.15B shows schematic interpretation of the DLS results. In the absence of the inhibitor, IN is in a dynamic equilibrium between monomers, dimers and tetramers (for clarity only monomers and dimers are shown in Figure 5.15B). KF116 binds at the IN CCD dimer interface and stabilizes interacting IN subunits, which in turn shifts the equilibrium toward aberrant, higher order oligomerization. Such a mechanism is also consistent with the marked increases in HTRF signal observed upon KF116 addition to IN (Figure 5.3).

To monitor IN multimerization in virions from KF116 treated cells we used the bifunctional BS$^3$ cross-linking reagent [131]. Under the mild cross-linking
conditions used in our experiments we observed monomers and dimers of IN when virions from non-treated cells were subjected to cross-linking reactions (Figure 5.15C). When virions from KF116 treated cells were subjected to similar cross-linking reactions, the levels of IN dimers increased and new slower migrating bands were observed which likely corresponded to higher oligomeric states of IN. Collectively, these findings (Figure 5.15) demonstrate that KF116 promotes aberrant IN multimerization both in vitro and in virus particles.

5.3.5 KF116 does not effectively inhibit IN-LEDGF/p75 interactions in infected cells

To assess the effects of KF116 on IN-LEDGF/p75 interactions we used i) a LEDGF/p75 KO HEK293T cell line to determine the KF116 IC\textsubscript{50} values for target and producer cells; and ii) ligation-mediated PCRs coupled with 454-pyrosequencing experiments to examine whether KF116 affects LEDGF/p75-dependent targeting of HIV-1 integration site distribution on chromatin.

Previous studies [128,131] have shown that siRNA- or shRNA-based LEDGF/p75 knockdown (KD) does not significantly affect IC\textsubscript{50} values of quinoline-based ALLINIs when the inhibitor is added to the virus-producing HEK293T cells. In the present study we used a PSIP1 (LEDGF/p75 gene) KO HEK293T cell line in which site-specific gene targeting with TALENs was used to engineer a gene deletion spanning exon 2 to 14 [47]. These cells thus lack a 42 kb chromosome 9 segment that contains all exons encoding the IBD and LEDGF
protein domains N-terminal to it. Immunoblot analysis verified absence of LEDGF/p75 protein in the PSIP1 KO cells (Figure 5.16A). We compared the IC\textsubscript{50} values of KF116 in HEK293T cells containing wild type (WT) levels of LEDGF/p75 versus LEDGF/p75 KO cells by adding the inhibitor to target or producer cells (Figure 5.16B and 5.16C). In target cells KF116 exhibited significantly higher potency in LEDGF/p75 KO cells (IC\textsubscript{50} of ~1.4 µM) compared with WT cells (IC\textsubscript{50} of >50 µM) suggesting that LEDGF/p75 effectively competes with KF116 for binding to IN during early steps of infection. However, the IC\textsubscript{50} values of KF116 in target and producer PSIP1 KO cells still differed significantly (Figure 5.16B and 5.16C, compare IC\textsubscript{50}s of ~1.4 µM versus ~0.075 µM for target versus producer LEDGF/ p75 KO cells). When KF116 IC\textsubscript{50} values were examined in producer cells, they remained unaffected by the PSIP1 KO (IC\textsubscript{50} of ~0.069 µM for WT vs. ~0.075 µM for KO cells) indicating that the presence of LEDGF/p75 during late stage of HIV-1 replication does not influence KF116 potency (Figure 5.16).

LEDGF/p75 is the primary cellular factor that directs HIV-1 integration into active genes [42,181]. Therefore, potential inhibitors of IN-LEDGF/p75 interactions would be expected to result in reduced HIV-1 integration frequencies into active genes. We monitored HIV-1 integration site distributions in cells treated with KF116 or GS-B (KF116 EC\textsubscript{50} of ~0.024 µM vs. GS-B EC\textsubscript{50} of ~0.026 µM for full replication cycle). The concentrations of KF116 and GS-B were optimized so that sufficient integration sites would be generated for statistical analyses. For the
virus-producer cell treatments, two concentrations of KF116 or GS-B were tested, 0.1 µM and 0.2 µM. The progeny virions produced in the presence of 0.2 µM inhibitor were not sufficiently infectious to enable subsequent 454-pyrosequencing experiments. In contrast, treatments with 0.1 µM of KF116 or GS-B yielded ~77% reduction in target cell HIV-1 expression when compared to the untreated virions (expression here serves as a measure of provirus formation) and allowed us to monitor genome wide integration site distribution (Figure 5.17). As expected [42,181] with DMSO control experiments HIV-1 integration was significantly favored into active genes (RefSeq and known genes, Figure 5.17). When compared to the DMSO control, no statistically significant differences were observed in HIV-1 integration into active genes when KF116 or GS-B was added to producer cells.

Because of the reduced potencies of these inhibitors in target cells, 5 µM and 10 µM GS-B were used, which resulted in ~71% and ~88% inhibition of HIV-1 expression, respectively. Under these conditions statistically significant reduction of HIV-1 integration frequencies into RefSeq and known genes were observed (Figure 5.17 and Figure 5.18, P < 0.001). Therefore, we have also examined matching concentrations (5 µM and 10 µM) of KF116 in target cells. At these concentrations, KF116 did not significantly inhibit HIV-1 expression (Figure 5.6) or result in any significant changes in HIV-1 integration frequencies into RefSeq and known genes (Figure 5.17 and Figure 5.18). Taken together, our results
show that KF116 primarily inhibited HIV-1 replication with low nanomolar IC$_{50}$ values by inducing aberrant IN multimerization.

5.4 Discussion

5.4.1 Rational Design of MINIs

The quinoline-based ALLINIs are promising leads for the development of clinically useful HIV-1 IN inhibitors. However, the multifunctional nature of quinoline-based ALLINIs, which inhibit IN-LEDGF/p75 binding and promote aberrant IN multimerization with similar potencies in vitro, has caused controversy about their antiviral mechanism of action. For example, it has been proposed that these compounds act through selectively inhibiting IN-LEDGF/p75 binding in infected cells [91], whereas others studies argued that ALLINIs impair HIV-1 replication primarily through inducing aberrant IN multimerization [96,114,115,128]. Furthermore, the A128T mutation in IN has been shown to confer marked resistance to the majority of ALLINIs. Here we report a new class of pyridine-based MINIs by rationally modifying quinoline-based ALLINIs. We also note that independently from our present studies, Boehringer Ingelheim reported structurally related compounds as inhibitors of HIV-1 replication in a patent application [182]. However, the patent did not describe the mechanisms of action or structural basis for their interactions with HIV-1 IN.

Our structural and mechanistic studies have revealed the following differences between MINIs and ALLINIs. In vitro assays have shown that unlike ALLINIs,
which exhibit similar IC\textsubscript{50} values for inhibiting IN-LEDGF/p75 binding and promoting aberrant IN multimerization, pyridine-based KF115 and KF116 modulate IN multimerization but are not effective inhibitors of IN-LEDGF/p75 binding (Table 5.1). Our structural studies have provided a plausible explanation for these observations. Unlike the rigid, planar quinoline ring in ALLINIs, the pyridine-based design contains a rotatable single bond which allows the benzene ring of KF115 or the benzimidazole ring of KF116 to engage subunit 1 through rotation out of the plane of the pyridine core. These additional interactions allow KF115 and KF116 to bridge the two IN subunits more effectively than their ALLINI counterparts. Consequently, the pyridine-based compounds exhibit significantly higher selectivity for promoting aberrant IN multimerization. In the absence of an added ligand, interactions between IN subunits are highly dynamic [75]. Viral DNA ends mediate ordered IN multimerization by allowing individual subunits to assemble into a functional tetramer in the context of the SSC [39]. LEDGF/p75 bridges between both individual subunits and two IN dimers to stabilize a tetrameric form of IN [75]. In contrast, small pyridine-based compounds promote subunit-subunit interactions and shift the equilibrium to higher order oligomers. Our results (Figure 5.15) show that upon addition of KF116 the oligomeric state of IN increased in a time-dependent manner leading to aberrant IN multimerization.

The planar quinoline core found in ALLINIs extends toward the Ala-128 residue and allows the evolution of HIV-1 IN A128T escape mutation. In contrast, the
benzimidazole ring in KF116 is oriented perpendicular to the core pyridine ring (Figure 5.2), which enables this compound to avoid the steric effects of the A128T substitution seen with archetypal ALLINI, ALLINI-1 [126]. While a single A128T mutation was sufficient to confer marked resistance to a number of ALLINIs, selection of HIV-1 variants under genetic pressure of KF116 revealed the triple T124N/V165I/T174I mutant as the predominant variant. Thr-124 and Thr-174 lie in the KF116 binding site, while Val-165 is distant from the inhibitor-binding site and is involved in inter-protein interactions. Therefore, it is possible that T124N and T174I substitutions directly affect KF116 binding, while the V165I substitution could be a compensatory mutation to restore IN function and/or stability that may be compromised by the T124N and T174I substitutions. This hypothesis would suggest an increased genetic barrier imposed by KF116 in comparison to ALLINIs.

5.4.2 KF116 is not effective during early stages of HIV-1 replication

Comparative analyses of antiviral mechanisms of action of MINIs and ALLINIs are of interest for delineating the effects of aberrant IN multimerization versus inhibiting IN-LEDGF/p75 interactions in infected cells. The main differences between KF116 and GS-B were seen in target cells, where the IC$_{50}$ values of GS-B (~0.7 µM) [131] versus KF116 (>50 µM) differed markedly. The reduced activities of ALLINIs in target cells compared with activity against the full replication cycle can be explained by endogenous LEDGF/p75 competing with these inhibitors during early infection steps. Indeed, ALLINI IC$_{50}$ values in
LEDGF/p75 KD target cells correlated closely to their EC$_{50}$ values in full replication assay [128]. Our results in Figure 5.16 demonstrate that LEDGF/p75 also competes with KF116, but even upon removal of LEDGF/p75, the KF116 EC$_{50}$ in LEDGF/p75 KO target cells was still ~20-fold higher than the IC$_{50}$ in the full replication assay, implicating LEDGF/p75-independent inhibition of late steps as the primary mechanism of inhibition.

Previous reports have shown that addition of GS-B to target cells does not affect reverse transcription but blocks HIV-1 integration [96,131]. By monitoring the frequencies of deletions and insertions at the 2-LTR junctions Tsiang et al. concluded that GS-B affected HIV-1 replication at a step preceding 3’-processing by inducing IN multimerization, which in turn compromised the formation of the functional IN-viral DNA complex [131]. In vitro experiments have demonstrated that ALLINIs can potently inhibit both the formation of the SSC and the LEDGF/p75 binding to the SSC [115]. Therefore, we wanted to explore whether GS-B could also affect IN-LEDGF/p75 binding in infected cells. The treatment of target cells with 5 µM or 10 µM GS-B resulted in statistically significant reduction of HIV-1 integration in active genes (Figures 5.17 and 5.18). Since LEDGF/p75 is the primary cellular factor responsible for targeting HIV-1 integration to actively transcribed units [42,181], our findings indicate that the treatment of target cells with GS-B likely inhibits IN-LEDGF/p75 binding in the infected cells. These results extend previous observations invoking allosteric modulation of the IN structure by GS-B [96]. Collectively, our findings together with the published
results [96,114,115] argue for a multimodal mechanism of action of ALLINIs in the infected cells and are consistent with in vitro results. In contrast to GS-B treatment, the treatment of target cells with 5 µM or 10 µM KF116 had no measureable effect on the distribution of integration sites in the host chromatin indicating that KF116 is not an effective inhibitor of IN-LEDGF/p75 binding in infected cells. Taken together, the differential activities of KF116 and GS-B in target cells can be explained by the relative potency of GS-B compared to KF116 in impairing the IN-LEDGF/p75 interaction.

A recent report [183] has shown that the treatment of infected cells with suboptimal concentrations of RAL leads to aberrant HIV-1 integration, which results in significant rearrangements in the host genome. These findings have raised the concern that patients exposed to suboptimal doses of RAL could encounter significant unintended consequences. Our findings that KF116 at therapeutically relevant (submicromolar) concentrations does not detectably affect HIV-1 integration while effectively inhibiting morphogenesis of infectious virions during late stages of HIV-1 replication provide further impetus for developing MINIs for potential clinical applications.

The genome-wide HIV-1 integration site analysis and the experiments with PSIP1 gene KO cells allowed us to explore the role of potential IN-LEDGF/p75 interactions as a target for KF116 and GS-B during the late stage of HIV-1 replication. The treatment of virus-producer cells with 0.1 µM GS-B or 0.1 µM KF116 similarly inhibited HIV-1 expression by ~77%. Yet, the distribution of
remaining integration sites did not show any statistically significant changes from untreated cells. Moreover, complete loss of LEDGF/p75 protein in the virus-producer cells did not alter the IC$_{50}$ values of either GS-B or KF116 during the late stage of HIV-1 replication. Taken together, we conclude that both KF116 and GS-B affect viral particle maturation through promoting aberrant IN multimerization rather than inhibiting IN-LEDGF/p75 binding.

In summary, we have developed a new class of pyridine-based MINIs which allowed us to examine the role of IN multimerization independently from IN-LEDGF/p75 binding in infected cells. Our results highlight small molecule induced aberrant IN multimerization as a plausible antiviral mechanism. Furthermore, the rational approach of designing KF116 provides a path for further development of improved MINIs for potential clinical use with increased potency and higher genetic barrier for HIV-1 resistance.

5.4.3 KF116 primarily inhibits late stage of HIV-1 replication by promoting IN multimerization

The treatment of virus-producer cells with KF116 and GS-B resulted in comparable EC$_{50}$ values of 0.030 µM and 0.039 µM, respectively, which correlate closely with their respective EC$_{50}$ values in the full replication cycle. Previous studies have suggested that ALLINIs modulate IN multimerization during viral particle maturation [131,183]. However, IN-LEDGF/p75 binding in the late stage of HIV-1 replication could not be discounted.
KF116 promotes aberrant IN multimerization but is not an effective inhibitor of IN-LEDGF/p75 binding, allowing its use as a probe. The KF116 EC\textsubscript{50} value in the virus-producer cells closely correlated with the in vitro EC\textsubscript{50} values for aberrant IN multimerization but not for its IC\textsubscript{50} value for IN-LEDGF/p75 binding. Furthermore, viral particles prepared in the presence of KF116 displayed aberrant IN multimerization and mislocalization of viral conical cores lacking RNPs.

The genome-wide HIV-1 integration site analysis and the experiments with PSIP1 gene KO cells allowed us to explore the role of potential IN-LEDGF/p75 interactions as a target for KF116 and GS-B during the late stage of HIV-1 replication. The treatment of virus-producer cells with 0.1 µM GS-B or 0.1 µM KF116 similarly inhibited HIV-1 expression by ~77%. Yet, the distribution of remaining integration sites did not show any statistically significant changes from untreated cells. Moreover, complete loss of LEDGF/p75 protein in the virus-producer cells did not alter the IC\textsubscript{50} values of either GS-B or KF116 during the late stage of HIV-1 replication. Taken together, we conclude that both KF116 and GS-B affect viral particle maturation through promoting aberrant IN multimerization rather than inhibiting IN-LEDGF/p75 binding.

In summary, we have developed a new class of pyridine-based MINIs which allowed us to examine the role of IN multimerization independently from IN-LEDGF/p75 binding in infected cells. Our results highlight small molecule induced aberrant IN multimerization as a plausible antiviral mechanism. Furthermore, the rational approach of designing KF116 provides a path for
further development of improved MINIs for potential clinical use with increased potency and higher genetic barrier for HIV-1 resistance.

5.5 Acknowledgements

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Table 5.1. Inhibitory activities of IN inhibitors.
Data for IC$_{50}$ and EC$_{50}$ are given as the mean ± SD from at least three independent experiments.

CC$_{50}$ values of >100 µM indicates that the respective inhibitors were not cytotoxic at the tested concentrations up to 100 µM.

*a ALLINI-1 EC$_{50}$ value has been described elsewhere [115].
Figure 5.1 Chemical structures of ALLINIs and MINIs.
Figure 5.2. Crystal Structures of LEDGF/IBD (A), ALLINI-1 (B), KF115 (C) and KF116 (D) bound to HIV-1 IN CCD.
The IN subunit 1 and 2 are colored in cyan and green, respectively. LEDGF/IBD loop (amino acids 365–368) is shown in dark blue. Bi-1001 is shown in orange. KF115 is shown in red. KF116 is shown in magenta. The hydrogen bonds between the IN subunit and the LEDGF/IBD or the indicated inhibitors are shown by black dashed lines. Side chains of HIV-1 IN residues A128T and T125 in subunit 1, and E170, H171 and T174 in subunit 2 are shown.
Figure 5.3. *In vitro* activities of KF115 and KF116.
HTRF-based assays were performed to monitor KF115 and KF116 activities for
promoting aberrant IN multimerization and inhibiting IN-LEDGF/p75 binding. (A)
Dose dependent effects of KF115 on promoting aberrant IN multimerization. (B)
Dose dependent effects of KF115 on IN-LEDGF/p75 binding. (C) Dose
dependent effects of KF116 on promoting aberrant IN multimerization. (D) Dose
dependent effects of KF116 on IN-LEDGF/p75 binding. Bars represent mean ±
SD (n = 3). The results are summarized in Table 5.1
Figure 5.4. KF116 promotes allosteric IN multimerization of A128T IN in vitro and impairs A128T HIV-1NL4-3 replication in infected cells.

An overlay of crystal structures of WT and A128T IN CCDs bound to BI-1001 (A) or KF116 (B). (A): The sidechain of Ala-128 and its corresponding BI-1001 molecule are colored orange, whereas Thr-128 and its corresponding BI-1001 molecule are colored gray. (B): The sidechain of Ala-128 and its corresponding KF116 molecule are colored magenta, whereas Thr-128 and its corresponding KF116 molecule are colored gray. The hydrogen bonds between the inhibitors and IN subunits are shown by black dashed lines. Side chains of HIV-1 IN residues T125 in subunit 1, and E170, H171 and T174 in subunit 2 are shown. (C) Effects of BI-1001 or KF116 on A128T HIV-1 infectivity. HEK293T cells were transfected with HIV-1 provirus bearing a substitution in the IN gene (pNL4-3A128T). A128T HIV-1 particles were produced in the presence or absence of the indicated inhibitors. TZM-bl cells were infected with cell-free A128T virus equivalent to 4 ng of HIV-1 Gag p24 and luciferase assay was performed 48 hour post-infection. The luciferase signal obtained for the non-treated (DMSO) control was set to 100%. Bars represent mean ± SD (n = 3).
Figure 5.5. Genotype of HIV-1 variants selected in cell culture in the presence of KF116.

(A) Mutations in the HIV-1NL4-3 IN gene of resistant viruses selected with KF116. Clonal sequencing of viral passage was carried out at passages 5 and 10, respectively. Eighty-two clones from each viral passage were sequenced using three sequencing primers covering the entire IN gene. Percentage of IN mutations for a given passage are indicated. Passage 5 corresponds to 50 days of selection with the KF116 concentration reaching 0.8 µM. Passage 10 corresponds to 100 days of selection with the KF116 concentration reaching 25.6 µM. (B) Crystal structure of KF116 bound to HIV-1 IN CCD dimer indicating the Thr-124, Val-165 and Thr-174 residues. The IN subunit 1 and 2 are colored in cyan and green, respectively. KF116 is shown in magenta.
Figure 5.6. KF116 selectively impairs the late stage of HIV-1 replication. (A) Dose-response curves for KF116 antiviral activities during early stage, late stage or one full replication cycle. For early stage experiments, KF116 was added directly to the target cells and then these cells were infected with untreated virions. For late stage experiments, the progeny virions were prepared in the presence of KF116 and were then used to infect untreated target cells. For one full replication cycle experiments, KF116 was added to both producer and target cells. (B) EC50 values for the indicated antiviral assays. Results represent mean ± SD from three independent experiments.
Figure 5.7 KF116 does not affect virus production. HEK293T cells were transfected with HIV-1 provirus (pNL4-3). HIV-1 particles were produced in the presence of DMSO or 1 μM KF116. Virus-containing cell-free supernatant were harvested and cell-free Gag was measured by HIV-1 Gag p24 ELISA. Bar graph indicates HIV-1 Gag p24 production relative to non-treated (DMSO) sample. Bars represent mean ± SD (n = 3).
Figure 5.8 KF116 does not affect viral protein processing. HEK293T cells were transfected with HIV-1 provirus (pNL4-3). HIV-1 particles were produced in the presence of DMSO, 1 µM KF116, or 1 µM SQV. Virus-containing cell-free supernatant equivalent to 5 µg HIV-1 Gag p24 were subjected to ultra-centrifugation and pelleted virions were detergent-lysed. Indicated amounts of pelleted virions were subjected to SDS-PAGE and immunoblotted with HIV-1 Gag, RT and IN antibodies.
Figure 5.9 KF116 does not affect virion RNA packaging.
HIV-1 virions were produced in HEK293T cells in the presence of DMSO or 1 µM KF116, cell-free virions were harvested, concentrated by ultra-centrifugation, RNA was isolated from the pelleted virions, and subsequent cDNA preparations were used for Gag quantitative PCR. In parallel, aliquots of cell-free virus-containing supernatant were used to measure the amount of viral particles produced using HIV-1 Gag p24 ELISA. Virion RNA packaging was calculated by normalizing virion Gag RNA copy numbers to Gag p24. Bar graph indicates virion RNA packaging relative to non-treated (DMSO) sample. Bars represent mean ± SD (n = 3).
Figure 5.10. KF116 impairs formation of dense cores in HIV-1 virions.
(A) Representative thin-section electron micrographs of HIV-1 virions produced in the presence of DMSO or 1 µM KF116. (B) Quantitative analysis of mature virions prepared in the presence of DMSO or 1 µM KF116. Correctly matured electron dense cores are shown in black and eccentric virions lacking electron density are shown in gray. Standard errors determined from two independent experiments are shown. Images of at least 50 mature virions were examined from each experiment. (C) Sucrose density gradient fractionation of detergent-lysed HIV-1 virions produced in HEK293T cells in the presence of DMSO or 1 µM KF116. Cell-free virions were harvested, detergent-lysed, and separated on 30–70% linear sucrose density gradients. Twenty-one 0.5 ml fractions were collected from the top of the gradient and subjected to SDS-PAGE and immunoblotted with HIV-1 Gag antisera to monitor the distribution of HIV-1 capsid. Positions of Gag p24 (capsid) and Gag p17 (matrix) are indicated. (D) Quantitation of HIV-1 capsid (p24) signal intensity from (C) as measured by ImageJ software. Graph represents the relative distribution of HIV-1 capsid (p24) in each of the sucrose density gradient fractions.
Figure 5.10. KF116 impairs formation of dense cores in HIV-1 virions.
Figure 5.11. KF116 does not affect initiation of reverse transcription or virion-associated reverse transcriptase (RT) activity.

(A) HIV-1 virions were produced in HEK293T cells in the presence of DMSO or 1 µM KF116 and total viral RNA, which serves as a source of tRNALys3 primer annealed to the viral RNA template, was extracted. The initiation of reverse transcription from total viral RNA was measured using an in vitro HIV-1 reverse transcription reaction, which extends tRNALys3 primer on viral RNA template by +6-nt. The final reaction products were resolved by PAGE and detected by phosphorimager analysis. (Upper panel) A representative image of the 6-nt extension products is shown. (Lower panel) The bar graph represents quantification of bands from the upper panel using ImageJ software. The signal intensity obtained for the non-treated (DMSO) sample was set to 100%. Bars represent mean ± SD (n = 4).

(B) HIV-1 virions were produced in HEK293T cells in the presence of 1 µM KF116 or DMSO control. Cell-free virions were harvested and concentrated by ultra-centrifugation. Equivalent amounts of concentrated virions, based on HIV-1 p24 ELISA, were analyzed for virion-associated RT activity. Bar graphs indicate RT activity relative to non-treated (DMSO) control. Bars represent mean ± SD (n = 3).
Figure 5.12. KF116 does not affect viral entry in the target cells.
VSV-G pseudotyped HIV-1 virions were produced in the presence of 1 µM KF116 or DMSO control. HEK293T cells were infected with DNase-treated virions equivalent to 0.5×10⁷ viral RNA copies. Cells were harvested 1 h post-infection, total cellular RNA was harvested, and subsequent cDNA preparations were used for Gag or GAPDH quantitative PCR. The incoming viral genomic RNA in target cells was calculated by normalizing Gag RNA to GAPDH RNA. Bar graphs indicate incoming viral genomic RNA relative to non-treated (DMSO) control. Bars represent mean ± SD (n = 3).
Figure 5.13. Virions produced in the presence of KF116 are defective in reverse transcription.
VSV-G pseudotyped HIV-1-Luc produced in the presence of DMSO, 1 µM KF116, or 1 µM RAL were used to infect HEK293T cells. Infected cells were harvested at the indicated times and subjected to qPCR or luciferase assay. Graphs indicate the amount of PCR products relative to non-treated (DMSO) sample at 6 h post-infection for (A) early reverse transcription (Early RT), (B) late reverse transcription (Late RT) and (C) 2-LTR circles (2-LTRs) products. (D) Bar graphs indicate the integrated provirus relative to non-treated (DMSO) control at 7 days post-infection. (E) Aliquots of infected cells were harvested and luciferase assay was performed at 48 h post-infection. The luciferase signal obtained for the non-treated (DMSO) sample was set to 100%. All graphs represent mean ± SD (n = 3).
Figure 5.14. KF116 treatment in target cells has no effect early stages of replication.
HEK293T cells were treated with DMSO, 1 µM KF116, or 1 µM RAL and then infected with VSV-G pseudotyped HIV-1-Luc. Infected cells were harvested at the indicated times and subjected to qPCR or luciferase assay. Graphs indicate the amount of PCR products relative to non-treated (DMSO) sample at 6 h post-infection for (A) early reverse transcription (Early RT), (B) late reverse transcription (Late RT) and (C) 2-LTR circles (2-LTRs) products. (D) Bar graphs indicate the integrated provirus relative to non-treated (DMSO) sample at 7 days post-infection. (E) Aliquots of infected cells were harvested and luciferase assay was performed at 48 h post-infection. The luciferase signal obtained for the non-treated (DMSO) sample was set to 100%. All graphs represent mean ± SD (n = 3).
Figure 5.15. KF116 promotes IN multimerization *in vitro* and in HIV-1 particles.

(A) DLS analysis of KF116-induced multimerization of recombinant IN. Size distribution of IN at 2 minutes (blue), 8 minutes (red), 30 minutes (green) and 120 minutes (yellow) after addition of KF116. No detectable signal was recorded in control experiments with KF116 alone or IN+DMSO incubated for up to 120 minutes. (B) The schematic to show the inhibitor induced equilibrium shift toward aberrant IN oligomerization. (C) HIV-1 virions were produced in HEK293T cells in the presence of DMSO or 1 µM KF116, cell-free virions were harvested, detergent-lysed, and treated with BS3 cross-linking reagent. The indicated volumes of cross-linked reaction products were resolved by SDS-PAGE and immunoblotted with HIV-1 IN antibody. The bands corresponding to IN “monomer”, “dimer”, and “higher order oligomers” are indicated.
Figure 5.15. KF116 promotes IN multimerization \textit{in vitro} and in HIV-1 particles.
Figure 5.16. LEDGF/p75 expression does not affect KF116 potency during late stage of HIV-1 replication.

(A) Equivalent whole cell lysates from the clonal TALEN-derived PSIP1 KO cell line (indicated as “KO”) and parental wild type HEK293T cell line (indicated as “WT”) were subjected to SDS-PAGE and immunoblotted for LEDGF/p75 and a GAPDH control to verify knockdown of LEDGF/p75 protein. (B) Dose-response curves representing the antiviral assays performed in WT or KO cell lines under the indicated conditions of drug treatment. For producer cell treatment, the VSV-G pseudotyped HIV-1-Luc progeny virions were prepared in the indicated cell line in the presence of KF116 and were then used to infect untreated HEK293T cells. For target cell treatment, KF116 was added directly to the indicated cell line and the cells were infected with untreated VSV-G pseudotyped HIV-1-Luc virions. (C) EC50 values for the indicated antiviral assays. Results represent mean ± SD from three independent experiments.
Figure 5.16. LEDGF/p75 expression does not affect KF116 potency during late stage of HIV-1 replication.

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>EC$_{50}$ (µM)</th>
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<tbody>
<tr>
<td>WT producer cells</td>
<td>0.069 ± 0.008</td>
</tr>
<tr>
<td>KO producer cells</td>
<td>0.075 ± 0.002</td>
</tr>
<tr>
<td>WT target cells</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>KO target cells</td>
<td>1.4 ± 0.5</td>
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Figure 5.17. Effects of MINI KF116 and ALLINI GS-B on LEDGF/p75-dependent targeting of HIV-1 integration site distribution on chromatin.

(A) HIV-1 integration frequencies in RefSeq and known genes, comparing effects of KF116 and GS-B on LEDGF/p75-dependent targeting of HIV-1 integration. All the samples differed significantly from their respective matched random controls (using Fisher’s exact test, P<0.001). Significant deviation from the control non-treated (DMSO) sample was calculated using Fisher’s exact test and is denoted by asterisks (** P<0.001). (B) Heatmap summarizing HIV-1 integration frequencies relative to the genomic features. The columns depict HIV-1 integration site data sets for the indicated drug treatments. For experiments involving the drug treatment of virus-producer cells (indicated “Producer”), the progeny virions were prepared in the presence of 0.1 µM KF116 or 0.1 µM GS-B and were then used to infect untreated target cells. For experiments involving the drug treatment of target cells (indicated “Target”), 5 µM KF116 or 5 µM GS-B inhibitor was added directly to the target cells and the cells were infected with untreated virions. The rows depict the analyzed genomic features and the base pair values shown in the rows indicate the size of a given genomic interval used for the analysis. The relationship between HIV-1 integration site frequencies relative to matched random controls for each of the genomic feature was quantified using the receiver operator characteristic (ROC) curve area method. The color key shows enrichment (indicated in red) or depletion (indicated in blue) of a given genomic feature near integration sites. P values were calculated for the individual integration site data sets compared to the matched random controls, ***P<0.001; **P<0.01; *P<0.05.
Figure 5.17. Effects of MINI KF116 and ALLINI GS-B on LEDGF/p75-dependent targeting of HIV-1 integration site distribution on chromatin.
Figure 5.18. Effects of 10 μM GS-B or KF116 on LEDGF/p75-dependent targeting of HIV-1 integration site distribution on chromatin.

10 μM GS-B or 10 μM KF116 was added directly to the target cells and the cells were infected with untreated virions. (A) HIV-1 integration frequencies in RefSeq and known genes, comparing effects of KF116 and GS-B on LEDGF/p75-dependent targeting of HIV-1 integration. All the samples differed significantly from their respective matched random controls (using Fisher's exact test, P<0.001). Significant deviation from the control non-treated (DMSO) sample was calculated using Fisher's exact test and is denoted by asterisks (*** P<0.001). (B) Heatmap summarizing HIV-1 integration frequencies relative to the genomic features. The columns depict HIV-1 integration site data sets for the indicated drug treatments. The rows depict the analyzed genomic features and the base pair values shown in the rows indicate the size of a given genomic interval used for the analysis. The relationship between HIV-1 integration site frequencies relative to matched random controls for each of the genomic feature was quantified using the receiver operator characteristic (ROC) curve area method. The color key shows enrichment (indicated in red) or depletion (indicated in blue) of a given genomic feature near integration sites. P values were calculated for the individual integration site data sets compared to the matched random controls, ***P<0.001; **P<0.01; *P<0.05.
Figure 5.18. Effects of 10 μM GS-B or KF116 on LEDGF/p75-dependent targeting of HIV-1 integration site distribution on chromatin.
Chapter 6: Significance and future perspectives

6.1 Summary and significance of dissertation research

HIV-1 integrase (IN) is an essential enzyme in the virus life cycle and is therefore an ideal drug target. In 2007, the FDA approved the first integrase inhibitor, Raltegravir (RAL). RAL binds to the IN active site and inhibits the strand transfer step of integration through two mechanisms: (1) RAL chelates the Mg$^{2+}$ ion that is necessary for IN catalytic activity and (2) RAL displaces the reactive 3'-hydroxyl end of the viral DNA from the active site and thereby inhibiting integration [36]. While RAL has been effective in the clinic, patients receiving treatments have seen the development of resistance viral strains emerge, most prominently through IN substitutions G140S:Q148H and N155H and E92Q [69]. The FDA has since approved two more IN strand transfer inhibitors (INSTIs) since the approval of RAL: elvitegravir (EVG) in 2012 and a second-generation INSTI dolutegravir (DTG) in 2013. While EVG has considerable cross-resistance to RAL resistance variants, DTG displays an increased genetic barrier to resistance [184]. However, DTG does not overcome resistant strains that contain substitutions in IN at Gln148 in combination with 2 or more other resistance mutations [72,73]. To combat the continuously emerging resistance mutations, new inhibitors that target novel sites on IN are needed.
At the start of my dissertation research the discovery of allosteric integrase inhibitors (ALLINIs) had just been reported. Boehringer Ingelheim (BI) [89] and Christ *et al* [91] independently reported the discovery of 2-(quinolin-3-yl) acetic acid derivatives as inhibitors of integration. BI discovered their lead compound, ALLINI-1, through a high throughput screen for inhibitors of 3’-processing [89]. Conversely Christ *et al* discovered their lead compound, LEDGIN-6 through rational design of inhibitors of IN-LEDGF/p75 binding [91]. While these two compounds were built around the same 2-(quinolin-3-yl) acetic acid pharmacophore, they were reported to have two separate mechanisms of action; catalytic activity versus inhibition of IN-LEDGF/p75 respectively. Additionally, LEDGIN-6 had been shown to bind in the LEDGF/p75 binding pocket of the CCD-CCD dimer interface, where we had previously mapped inhibitors (such as compound 1) and showed that their mechanism of action is to alter the functional tetramerization of IN. For these reasons we set out to test the hypothesis that LEDGIN-6 and ALLINI-1 can modulate the dynamic exchange of IN subunits and thereby affect its catalytic activity.

The data presented in chapter 2 describes the mechanism of action of this class of allosteric integrase inhibitors (ALLINIs). ALLINI-1 and LEDGIN-6 both inhibit IN-LEDGF/p75 binding as well as LEDGF/p75-independent IN catalytic activity with equal potency. Homogeneous time resolve fluorescence (HTRF) assays revealed that both compounds promote aberrant IN multimerization. Importantly, these compounds were also active in antiviral activity assays. The high level of cooperativity seen in the antiviral and catalytic activity assays is reminiscent of
protease inhibitors. High levels of cooperativity often increase the instantaneous inhibitory potential (IIP), which indicates the log reduction in a single round infectivity assay at clinically relevant concentrations and is strongly correlated with clinical outcomes [105,108].

Chapters 3 and 4 show our work to elucidate the mechanisms of resistance to ALLINIs. The IN substitution A128T, which has emerged under selective pressure of ALLINI-1 while the H171T IN substitution has emerged under selective pressure of the significantly more potent ALLINI, BI-D. The A128T IN substitution confers >15-fold resistance to ALLINI-1 treatment. The single amino acid substitution was shown to only minimally reduce the inhibitor binding affinity but to significantly shift the inhibitor binding orientation in the binding pocket at the CCD-CCD dimer interface. This shift in inhibitor orientation significantly affects the ability of ALLINI-1 to promote aberrant IN multimerization. The BI-D resistant IN mutation, H171T, directly affects the binding affinity of BI-D to the CCD-CCD dimer interface. The H171T substitution compromises the hydrogen bond formed between the N$_\delta$-protonated imidazole group of H171 side chain and the tert-butoxy ether oxygen. Increased inhibitor concentrations overcome the reduced binding affinity, effectively bridging two IN subunits and are capable of inducing aberrant IN multimerization. Collectively these findings conclusively demonstrate that promotion of aberrant IN multimerization is the primary mechanism of action of ALLINI and have provided important structural and mechanistic details for separate mechanisms of resistance.
Surprisingly, studies on ALLINIs’ antiviral mechanism of action revealed that ALLINIs inhibit both the early and late stages of HIV-1 replication. The potency of ALLINIs is derived from inhibition of IN during virus maturation, rather than during the early stages where the catalytic function of IN is critical for replication [128,129,131,132]. Virions produced from cells that were treated with ALLINIs yielded predominantly eccentric morphologies characterized by the electron dense material corresponding to the ribonucleic acid-protein complexes (RNP), which is normally situated inside the capsid core, being mislocalized between an empty capsid core and the virus membrane [128,131,132]. Subsequent infection with these virions in untreated target cells showed a defect in production of early reverse transcription products [128,129,131,132]. While these studies convincingly demonstrate that ALLINIs inhibit the late stage of replication by inhibiting the correct formation of electron dense cores during virus maturation, the multimodal nature of quinoline-based compounds complicates dissecting the mechanism of inhibition during the late stage of replication. To address this question, we sought to develop mono-functional, multimerization selective IN inhibitors.

The data in chapter 5 describes the design of multimerization selective integrase inhibitors (MINIs) and their mechanism of action. The most potent MINI, KF116 inhibited HIV-1 replication with an EC$_{50}$ of 0.024 µM. Like ALLINIs, MINIs potently inhibited during the late stage of HIV-1 replication and caused the formation of virions with eccentric morphologies that were defective for reverse transcription after subsequent infection in untreated target cells. Unlike ALLINIs
which displayed some activity during the early stage of replication, KF116 was not potent in target cells (EC$_{50}$ ~ 50 µM). Because KF116 weakly inhibited IN-LEDGF/p75 binding \textit{in vitro}, LEDGF/p75 knockout cells were used to understand the role of any residual IN-LEDGF/p75 binding inhibition during virus production in cells. Knockout of LEDGF/p75 in infected cells during virus production had no effect on the EC$_{50}$ of KF116. This demonstrated that the antiviral activity seen during the late stage of replication by KF116 is due to the promotion of aberrant IN multimerization and not the inhibition of IN-LEDGF/p75 binding. The mechanisms of ALLINI and MINI inhibition are summarized in figure 6.1.

Recently, the first ALLINI to move to phase 1a clinical trials, BI-224436 was reported \cite{125,150,185}. Like the previously reported ALLINIs, BI-224436 has a quinoline scaffold. The antiviral activity of BI-224436 is from 7-15 nM \cite{125}. While the crystal structure of IN CCD-CCD dimers soaked with BI-224436 has not yet been solved, due to the structural similarity to other ALLINIs, it is also predicted to bind at the CCD-CCD dimer interface. Importantly, due to the vast similarities between ALLINI-1, BI-D and BI-224436, many of the same resistance mutations seen before may arise under BI-224436 selective pressure. Therefore, the detailed mechanism of action and mechanism of resistance to ALLINI-1 and BI-D may be exploited to generate second-generation compounds that are able to overcome this resistance.
6.2 Future Perspectives

6.2.1 Resistance to KF116

Resistance continues to be a problem in HIV-1 antiretroviral therapy; therefore there is a continued need for the design of inhibitors that overcome resistant strains. KF116 was shown to have an increased genetic barrier to resistance as a triple mutation in IN, T124N/V165I/T174I, arising after 10 passages under increasing KF116 selective pressure. These residues map to the CCD-CCD dimer interface. It is likely that T124N and T174I, both of which are at the inhibitor binding site contribute to increased resistance to KF116 as these substitutions are seen as single amino acid substitutions in earlier passages. However, the contribution of V165I, which is only observed in combination with T124N and T174I and which is adjacent to the inhibitor binding site, is unknown. It is possible that the V165I substitution increases the level of resistance to KF116 through currently unknown mechanisms but more likely V165I may be a compensatory mutation to increase the replicative fitness of T124N/T174I. Understanding the mechanism of resistance to KF116 is important in generating second-generation inhibitors that will be sensitive to viral strains containing these substitutions.

6.2.2 Role for integrase during virus maturation

IN mutants can be classified into two separate classes: class I mutants and class II mutants. Mutations that selectively inhibit only integration are defined as class I mutants. Class II mutants are those that display pleiotropic effects and have defects at steps other than or in addition to integration [135]. The eccentric
particle phenotype and subsequent reverse transcription defect observed with ALLINI or MINI treatment during the late stage of replication is reminiscent of many class II IN mutants [135,139,155,175,186-193]. This finding has generated renewed interest in the role of IN during the late stages of replication.

Viruses that harbor an IN deletion exhibit the class II phenotype with the eccentric virion morphology and a block at reverse[194,195], but can be partially rescued by the expression of VPR-IN or catalytically inactive VPR-IN_{D116A} [195]. This suggests that while IN catalytic activity is not required for proper core formation, IN plays an active role in assembling the mature virus particle. Understanding the function of integrase during the late stages of replication, the necessity of correctly ordered IN structure for correct particle maturation and how ALLINIs modulate this process are key questions moving forward in not only understanding HIV-1 biology, but in designing novel inhibitors to specifically target IN during particle maturation.
Figure 6.1. Overview of the mechanism of action of ALLINIs and MINIs.

(Top Panel) When introduced to a target cell, ALLINIs inhibit integration by promoting aberrant IN multimerization, which are then unable to form stable synaptic complexes as well as inhibiting IN-LEDGF/p75 binding.

(Bottom Panel) ALLINI or MINI treatment of producer cells promotes aberrant IN multimerization and results in eccentric virions characterized by mislocalized RNPs. These virions are defective for reverse transcription in the subsequent infection of naive target cells.
References


