Multifaceted HIV integrase functionalities and therapeutic strategies for their inhibition

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Antiretroviral inhibitors that are used to manage HIV infection/AIDS predominantly target three enzymes required for virus replication: reverse transcriptase, protease, and integrase. Although integrase inhibitors were the last among this group to be approved for treating people living with HIV, they have since risen to the forefront of treatment options. Integrase strand transfer inhibitors (INSTIs) are now recommended components of frontline and drug-switch antiretroviral therapy formulations. Integrase catalyzes two successive magnesium-dependent polynucleotidyl transferase reactions, 3' processing and strand transfer, and INSTIs tightly bind the divalent metal ions and viral DNA end after 3' processing, displacing from the integrase active site the DNA 3'-hydroxyl group that is required for strand transfer activity. Although second-generation INSTIs present higher barriers to the development of viral drug resistance than first-generation compounds, the mechanisms underlying these superior barrier profiles are incompletely understood. A separate class of HIV-1 integrase inhibitors, the allosteric integrase inhibitors (ALLINIs), engage integrase distal from the enzyme active site, namely at the binding site for the cellular cofactor lens epithelium-derived growth factor (LEDGF)/p75 that helps to guide integration into host genes. ALLINIs inhibit HIV-1 replication by inducing integrase hypermultimerization, which precludes integrase binding to genomic RNA and perturbs the morphogenesis of new viral particles. Although not yet approved for human use, ALLINIs provide important probes that can be used to investigate the link between HIV-1 integrase and viral particle morphogenesis. Herein, I review the mechanisms of retroviral integration as well as the promises and challenges of using integrase inhibitors for HIV/AIDS management.

Combination antiretroviral therapy (cART) treats patients with a mixture of drugs to inhibit different steps of the HIV-1 replication cycle (1). Unique among animal viruses is the requirement for retroviruses to integrate their genetic information into the genome of the host cell that they infect. Integration is mediated by the viral protein integrase (IN), which is incorporated into fledgling viral particles alongside the other viral enzymes reverse transcriptase (RT) and protease (PR). PR initiates virus particle maturation by cleaving viral Gag and Gag-Pol polypeptide precursors into separate viral structural proteins and enzymes, which is required to form the viral core (reviewed in Ref. 2). The core consists of the viral ribonucleoprotein (RNP) complex, which contains two copies of the RNA genome bound by viral nucleocapsid, IN, and RT proteins, encased within a fullerenelike shell composed of the viral capsid protein (reviewed in Ref. 3). RT converts retroviral RNA into a single molecule of linear DNA containing a copy of the viral long terminal repeat (LTR) at each end (Figs. 1 and 2) (reviewed in Ref. 4). The linear DNA, comprised of U3 and U5 terminal sequences in respective upstream and downstream LTRs, is the substrate for IN-mediated viral DNA insertion into chromosomal DNA (5–7).

Four classes of antiretroviral drugs, nucleoside RT inhibitors (NRTIs), nonnucleoside RT inhibitors (NNRTIs), PR inhibitors (PIs), and IN strand transfer inhibitors (INSTIs) have in recent years comprised frontline cART formulations (8). Highlighting the success of the INSTI drug class, current guidelines recommend the use of a second-generation INSTI (dolutegravir (DTG) or bictegravir (BIC)) co-formulated with two NRTIs to treat most people living with HIV (PLHIV) who have not previously failed an INSTI-containing regimen (9, 10). INSTIs inhibit IN strand transfer activity and thus specifically block the integration step within the HIV-1 life cycle (11) (Fig. 1). A separate class of inhibitors, the allosteric IN inhibitors (ALLINIs),...
Mechanism of retroviral integration

IN is a polynucleotidyl transferase composed of three conserved protein domains: the N-terminal domain (NTD) with conserved His and Cys residues (HHCC motif) that coordinate Zn$^{2+}$ binding for 3-helix bundle formation; the catalytic core domain (CCD), which adopts an RNase H fold and harbors the enzyme active site composed of invariant carboxylate residues (DDE motif); and the C-terminal domain (CTD), which adopts an SH3 fold (reviewed in Ref. 14). The role of the DDE residues in catalysis is to coordinate the positions of two divalent cations, which under physiological conditions are almost certainly magnesium, to deprotonate attacking oxygen nucleophiles and destabilize scissile phosphodiester bonds for one-step transesterification chemistry (15, 16). Similar functionalities exist across a large superfamily of polynucleotidyl transferases that includes related enzymes such as transposase proteins and RNase H (reviewed in Ref. 17).

Two different IN activities, 3’ processing and strand transfer, are required for integration (Fig. 2). During 3’ processing, IN prepares the linear reverse transcript for integration by hydrolyzing the DNA ends 3’ of conserved CA dinucleotides, which most often liberates a dinucleotide from each end (18–20). However, symmetrical DNA processing is not required for integration; the upstream terminus of spumaviral DNA is 5’-TG, obfuscating the need for U3 end processing by IN (21), whereas a trinucleotide is processed from the U5 end of some primate lentiviruses (22, 23), including HIV-2 (24). During strand transfer, IN uses the CA_{OH-3’} hydroxy groups to cut chromosomal DNA in a staggered fashion, which, due to the nature of SN2 chemistry, simultaneously joins the viral DNA ends to the 5’-phosphate groups of the dsDNA cut (6, 7, 15). The resulting gapped DNA intermediate with unjoined viral DNA 5’ ends is repaired by host cell machineries to yield the integrated provirus flanked by the sequence duplication of the host DNA cut, which for HIV-1 is most often 5 bp (25, 26) (Fig. 2).
Intasome structure and function

Integration in cells is mediated by the preintegration complex (PIC), which is a large nucleoprotein complex derived from the core of the infecting virion (27, 28). Within the confines of the PIC, IN functions as part of the intasome nucleoprotein complex, which is comprised of a multimer of IN and the viral DNA ends (29–35) (Figs. 1 and 3). A series of X-ray crystallographic and single-particle cryogenic electron microscopy (cryo-EM) structures determined over the past decade has clarified that the number of IN molecules required to build the intasome differs depending on the type of retrovirus (reviewed in Ref. 36). Seven retroviral genera are grouped into two sub-families of Retroviridae: Spumavirinae, solely harboring the spumaviruses, and Orthoretrovirinae, which encompass the lentiviruses, such as HIV-1, as well as α-, β-, δ-, ε-, and γ-retroviruses. X-ray crystal structures of the spumavirus prototype foamy virus (PFV) intasome provided initial high-resolution views of the functional IN-viral DNA architecture as well as critical insight into the mechanism of INSTI action (see below) (35, 37, 38).

The PFV intasome is composed of an IN tetramer with the following division of labor. Two extended, intertwined IN molecules (Fig. 3A, blue and green) harbor operational active sites and thus catalyze 3' processing and strand transfer activities, whereas the other two IN molecules (Fig. 3A, cyan) with non-operational active sites serve as bookends to truss the DNA-bound IN protomers together (35). The interwoven nature of the two catalytically active IN molecules, with their NTDs mutually swapped between CCDs, was observed previously in crystal structures of two-domain lentiviral IN NTD-CCD constructs in the absence of DNA (39, 40). Prior to these structures, the NTD from one IN protomer had been shown to function in trans with the active site of a separate IN molecule within the active HIV IN multimer (41, 42). The interwoven NTD-CCD arrangement at the heart of the machine leverages the participation of both viral DNA ends in intasome assembly and DNA recombination.

Four types of intasomes describe the ground states and product complexes associated with IN 3' processing and strand transfer activities. IN processes the viral DNA ends in the context of the initial stable synaptic complex (SSC), yielding the cleaved synaptic complex (CSC) after viral DNA hydrolysis. The target capture complex (TCC) describes the CSC bound to target or host DNA, whereas strand transfer yields the strand transfer complex (16, 35–37, 43). The overall conformation of the PFV intasome structure changes little as the complex morphs from the SSC to the strand transfer complex and promotes IN 3’ processing and strand transfer activities (16, 35, 37, 43, 44). Although integration occurs largely throughout animal cell genomes (45, 46), host DNA sequences that contort to fit the target DNA-binding interface within the CSC are preferred targets (37, 44, 47–49) (for a detailed review, see Ref. 50). Thus, from each end. After nuclear localization, the intasome interacts with host target DNA (gray lines with targeted green sequence) to promote DNA strand transfer. The DNA gaps that persist after strand transfer are repaired by host cell machinery to yield a target site duplication (thin green lines) flanking the integrated provirus.

Figure 2. DNA cutting and joining steps of retroviral integration. The linear viral reverse transcript (lavender lines; plus-strands shaded more darkly than same-colored minus-strands throughout the cartoon) contains a copy of the LTR at each end composed of cyan U3, yellow R repeat, and magenta U5 sequences. The upstream LTR is abutted by the primer-binding site (PBS; purple box), whereas the downstream element is abutted by the polypurine tract (PPT; lavender box). During 3’ processing, IN hydrolyzes the DNA adjacent to invariant CA dinucleotides, which for HIV-1 liberates the pGTOH dinucleotide.
strand transfer proceeds without gross rearrangements in intasome architecture.

Studies of additional retroviral intasomes unveiled a common structural feature at the hearts of the machines that was coined the conserved intasome core (CIC) (51) (Fig. 3, A–C). However, as mentioned, different viruses utilize different numbers of IN protomers to form the CIC. The tetrameric IN architecture of the PFV intasome defines the basic features of the CIC, including two active protomers with CCDs and NTDs swapped across a synaptic interface where two CTDs engage target DNA for integration (37) and two additional IN molecules that bookend the active subunits (35). Whereas four PFV IN molecules suffice to build the CIC, both MMTV and MVV require eight IN protomers. For MMTV, critical CTDs (magenta) are donated by flanking IN dimers, leading to an overall IN octamer. In MVV, flanking IN tetramers provide the critical CTDs, resulting in an overall IN hexadecamer. Gray coloring in B and C deemphasizes IN elements that do not compose the CIC. D–F, resected CCD and CTD domains from above green IN protomers, oriented to highlight the different CCD-CTD linker regions (dark gray). Associated magenta CTDs from separate IN protomers in E and F assume similar positions as the green CTD in D. Red sticks, DDE catalytic triad residues.

Figure 3. Retroviral intasome structures. A–C, representative intasomes from the spumavirus PFV (A; protein database (PDB) accession code 3OY9), β-retrovirus MMTV (B; PDB code 3JCA), and lentivirus MVV (C; PDB code 5M0Q) are color-coded to highlight the CIC. Green and blue, catalytically active IN protomers; cyan, supporting IN CCDs; black, DNA strands. Whereas four PFV IN molecules suffice to form the CIC, both MMTV and MVV require six IN protomers. For MMTV, critical CTDs (magenta) are donated by flanking IN dimers, leading to an overall IN octamer. In MVV, flanking IN tetramers provide the critical CTDs, resulting in an overall IN hexadecamer. Gray coloring in B and C deemphasizes IN elements that do not compose the CIC. D–F, resected CCD and CTD domains from above green IN protomers, oriented to highlight the different CCD-CTD linker regions (dark gray). Associated magenta CTDs from separate IN protomers in E and F assume similar positions as the green CTD in D. Red sticks, DDE catalytic triad residues.
DNA is monomeric (35), α- and β-retroviral INs are predominantly dimeric (52, 53), and lentiviral INs are predominantly tetrameric although with evidence for additional lower- and higher-order forms (40, 42, 51, 57–62). However, the relationship between protein behavior in solution and the multimeric state of IN in virions or during reverse transcription is largely unknown. Because HIV-1 IN binds genomic RNA in virions (63), it seems possible that RNA-bound IN may transfer to the DNA ends as they form during reverse transcription to initiate SSC formation. The IN tail region, which is composed of the amino acids C-terminal from the CTD SH3 fold, varies in length from about 5 residues in the lentivirus equine infectious anemia virus to 55 residues in MMTV. The tail region in α-retroviral IN, which is 19 residues, can regulate DNA-dependent IN octamer formation (64, 65). Although implicating a role for the region of IN in intasome assembly, tail regions are unresolved in all IN and intasome structures solved to date, limiting the interpretation of how the tail might regulate nucleoprotein complex formation.

**INSTIs**

Research in the mid-1980s first established a role for the 3′ region of the pol gene, which encodes for IN, in retroviral replication (66–69), and the extension of this requirement to HIV-1 highlighted IN as a high-value antiviral target (70). However, a scant number of promising preclinical lead compounds were known by the time RT and PR inhibitors were administered to patients in cART formulations (71–73), calling into question whether IN inhibitors would ever make it to the clinic. Indeed, around this time, I can recall one of the more prominent researchers in our field espousing the view at a national meeting that clinical IN inhibitors were unattainable. The reasoning here was based on the observation that an equal number of IN, RT, and PR molecules are packaged into each virion particle, which one can estimate as 120 based on the 20:1 synthesis ratio of Gag to Gag-Pol (74) and circa 2,400 Gag molecules per virion (75). Per replication cycle, RT and PR catalyze roughly 19,400 and 12,900 chemical reactions, respectively. However, the same population of IN molecules performs only four chemical reactions. How then could one effectively inhibit IN in the face of this seemingly large excess of available enzyme? Fortunately, my colleague turned out to be incorrect. What was unknown at the time of our discussion was the utility of molecules designed to inhibit IN strand transfer activity. Whereas HIV-1 IN processes the viral DNA ends to yield the CSC concomitant with or soon after reverse transcription (30, 76) (Fig. 1), integration into chromosomal DNA does not occur until hours to days (76–79) or, in some extreme cases, weeks later (80). The comparatively long-lived CSC intasome replication intermediate is a pharmacological HIV-1 Achilles’ heel that is leveraged fully by the INSTI class of antiretroviral compounds.

Because HIV-1 IN purified from recombinant sources displayed 3′ processing and strand transfer activities in vitro (15, 20, 81, 82), systems to search for inhibitory molecules of HIV-1 IN activity were readily scalable (83–85). However, due to suboptimal assay designs, few early leads turned out to specifically inhibit HIV-1 integration under physiological conditions (86, 87). Consider the following example. A compound such as ethidium bromide that would likely score as a hit if test compounds were comixed together with IN and viral DNA is highly unlikely to specifically inhibit integration in infected cells. Numerous early compounds accordingly lacked specificity to inhibit integration during HIV-1 infection (reviewed in Ref. 88). A key turning point in IN inhibitor development came from reformulating the design of the in vitro assay to prebind IN to a synthetically preprocessed viral DNA end substrate (87) and screen for inhibitors of strand transfer activity, which led to the discovery of first-in-class INSTIs (11). Although these diketo acid compounds were never licensed to treat PLHIV, they nevertheless served as important molecules with which to probe INSTI mechanisms of action. INSTIs harbor two commonalities across otherwise diverse pharmacophores (Figs. 4A and 5). At the hearts of the compounds are three adjacent heteroatoms (most usually oxygen; red in Fig. 5, B and C), whereas a terminal halogenated benzene ring connects to the rest of the molecule via a flexible linker (Fig. 4A; blue in Fig. 5, B and C). The compounds avidly bind IN-viral DNA complexes yet failed to appreciably bind HIV-1 IN in the absence of viral DNA (89), and subsequent work revealed the importance of the terminal deoxyadenylate residue at the 3′ end of processed viral DNA in the regulation of INSTI binding and dissociation (90, 91). The conserved INSTI heteroatoms engage the divalent metal ions that are bound by the DDE active-site residues (35, 92).

Raltegravir (RAL) in 2007 was the first INSTI licensed by the United States Food and Drug Administration (FDA) (93) and elvitegravir (EVG) in 2012 became the second licensed INSTI (94). (Fig. 4A). Although prior work demonstrated the importance of divalent metal ion and viral DNA sequence for INSTI binding (90–92), the field lacked a detailed view of how INSTIs inhibited IN strand transfer activity. Fortuitously, both NRTIs and INSTIs, which target respective RT and IN active sites composed of invariant amino acid residues, inhibit a wide range of retroviruses (95–103) including spumaviruses (104, 105). Thus, the PFV intasome could serve as a model system to investigate INSTI mechanism of action. Co-crystal structures with RAL or EVG revealed that the halobenzyl groups assumed the position of the purine rings of the 3′-deoxyadenylate residue, supplanting the terminal nucleoside from the IN active site (Fig. 5, A and B). INSTI binding accordingly inactivates the intasome complex by displacing from the enzyme active site the DNA 3′-OH group that is required to cut chromosomal DNA for strand transfer activity (35).

Second-generation INSTI compounds physically expand upon first generation scaffolds while maintaining both metal-chelating and DNA-supplanting drug functions. Such modifications include increasing the length of the linker between the metal-chelating and halobenzyl moieties (106–108), increasing the number of central ring moieties to three (106, 107, 109), and, akin to EVG, derivatization of a second ring that lies distal from the halobenzyl group (110–113) (Fig. 4A). Second-generation INSTIs more fully occupy the IN active-site region that spans from the DNA-binding pocket on the one side to the connector sequence that links IN secondary structural elements β4 and α2 on the other (112–115) (Fig. 5C, β4-α2 connector).
Overlaying the structures of INSTI-bound PFV intasomes to those of the SSC and TCC yielded important insight into the mechanism of drug action (16, 112, 113). The RAL metal-chelating oxygen atom distal from the halobenzyl group coincided with the nucleophilic water molecule for IN 3' processing activity (red sphere in Fig. 5D), whereas the RAL-chelating oxygen proximal to the halobenzyl coincided with the scissile phosphodiester bond in viral DNA (Fig. 5D). For strand transfer, the halobenzyl-proximal oxygen coincided with the nucleophilic 3'-oxygen of processed viral DNA, whereas the distal RAL oxygen overlapped with the scissile phosphodiester bond in target DNA (16) (Fig. 5E). These observations first identified INSTIs as IN substrate mimics, which was subsequently expanded through the broader concept of substrate envelope. Previously espoused for HIV-1 PR and the mechanism of PI action, the substrate envelope is defined as the space occupied by the substrate (peptide in the case of PR; DNA for IN) in an enzyme active site. Because the enzyme must interact with the substrate for catalysis, drugs that interfere with enzyme–substrate interactions should be inhibitory and might impart relatively high resistance barriers (116–118). Indeed, second-generation INSTI elements distal from the halobenzyl groups coincide with the position of target DNA in PFV intasome structures (16, 112–114) (Fig. 5E), likely accounting for the competition between target DNA and INSTIs for binding to HIV-1 IN-viral DNA complexes (115). Compound modifications that further interfere with HIV-1 IN–substrate interactions could improve INSTI potency and increase the barrier to acquire drug-resistant mutations (119).

Second-generation INSTIs are currently undergoing extensive safety evaluation due to their planned global rollout for HIV/AIDS treatment. Although DTG was initially deemed safe for pregnant women (120), follow-up work highlighted a greater frequency of neural tube defect in infants born to Botswanan mothers who were taking DTG-containing cART since the time of conception (4 of 426; 0.94%) versus frequencies observed in the general population (86 of 87,755; 0.1%) or in infants from mothers taking other cART regimens (14 of 11,300; 0.12%) (121). Such observations prompted several regulatory agencies including the FDA and the World Health Organization in 2018 to issue alerts regarding possible increased risk of neural tube defect in infants born to mothers taking DTG-containing cART at the time of conception (122). Subsequent retrospective analyses have failed to detect a link between DTG usage and neural tube birth defect, although such studies were generally limited by sample size (123, 124). Retrospective analysis has also failed to detect an increase in the frequency of neural tube defect in infants born to mothers on RAL-based drug regimens (125). Recent follow-up work that increased the number of patients from 426 to 1,683 in the Botswanan cohort revised the neural tube defect frequency from 0.94% to 0.3%, which was still 0.2% greater than the frequencies observed in control populations (126). It will be informative to ascertain whether BIC, which is the other licensed second-generation INSTI and is structurally related to DTG (Fig. 4A), also influences neural tube defect frequency versus control populations.

**Mechanisms of HIV resistance to INSTIs**

Substitutions of HIV-1 IN residues Gln-148, Asn-155, or Tyr-143 were recognized early on as separate genetic pathways to clinical RAL resistance (127), and mutant viral strains har-
boring changes such as Q148H/G140S or N155H conveyed significant resistance to EVG as well (reviewed in Ref. 128).

Although structure-based studies with PFV intasomes informed the mechanisms of drug resistance (35, 38), partial amino acid identity between PFV and HIV-1 INs limits the extent of information that can be gleaned from the model system. PFV and HIV-1 IN are overall 18.4% identical, whereas their respective CCDs share 22.4% identity (35). Fortuitously, two of the three clinically relevant amino acids, Tyr-143 and Asn-155, are conserved as Tyr-212 and Asn-224 in PFV IN (Fig. 5A). The binding mode of RAL to the PFV intasome in particular informed the Tyr-143 resistance pathway, as the methyl-oxadiazole constituent of this drug stacked against the \( \text{p-} \)cresol side chain of IN residue Tyr-212 (Fig. 5B). Changes that would reduce the aromatic nature of HIV-1 IN residue Tyr-143 would accordingly result in loss of an important RAL binding contact. The structure accounted for the relative specificity of RAL resistance to Tyr-143 changes in IN (129, 130), as only RAL among the clinical INSTIs harbors the methyl-oxadiazole (Fig. 4A). Although Asn-224 similarly resides near the INSTI-binding pocket, it does not directly contact bound drugs. The mutant His side chain in the intasome structure derived from PFV IN N224H interacted with the 3′-deoxyadenylate-bridging phosphate, which was disrupted by second-generation......
IN INSTI MK-2048 binding (38). Although disruption of the His–DNA interaction could contribute to the mechanism of INSTI resistance to HIV-1 N155H (38), intasome structures with INS that share greater amino acid identity to HIV-1 are expected to more completely inform INSTI resistance mechanisms outside of the Tyr–143 pathway. Of note, although MVV is a lentivirus, its IN also shares limited amino acid sequence identity with HIV-1 IN (27.4% overall; 34.3% between CCDs). Given fast-paced advancements in single-particle cryo-EM (131, 132), one can optimistically expect comparatively high-resolution structures of INSTIs bound to the HIV-1 intasome in the not too distant future. Such structures should critically inform mechanisms of INSTI drug resistance as well as how to potentially improve INSTI potencies moving forward.

Clinical (133–135) as well as in vitro (136) studies have highlighted the superior resistance profiles of second-generation INSTIs such as DTG compared with predecessor first-generations compounds. Whereas selection of HIV-1 resistance in cell culture invariably leads to changes in IN that can confer >100-fold resistance to RAL and EVG, such resistance is much harder to come by for DTG, and the selected changes in IN, such as R263K, engender just a few-fold resistance to the compound (137). Whereas cART formulations typically comprise three distinct compounds, such observations inspired clinical evaluation of DTG as a monotherapy or as dual therapy in conjunction with an NRTI, NNRTI, or PI (138–143). Based on rates of virological failure, the use of DTG as a monotherapy for PLHIV is contra-indicated, whereas the evaluation of dual therapy options is ongoing (143) (reviewed in Ref. 144). Current guidelines recommend the use of DTG, BIC, or RAL with two NRTIs for most PLHIV (9, 10).

The rates at which INSTIs dissociate from IN-viral DNA complexes in vitro have informed the mechanisms of drug action and drug resistance. Consistent with its comparatively high resistance barrier, the dissociative half-life of DTG, 71 h, was significantly longer than the corresponding RAL and EVG values of about 9 and 3 h, respectively (145). Although analyses of mutant IN–viral complexes failed to identify a direct correlation between drug dissociation half-life and antiviral potency and resistance, HIV-1 was generally sensitive to INSTIs when compound dissociative half-life was greater than 4 h and resistant to inhibition when half-lives were less than 1 h (145). Thus, INSTI dissociative half-life is a useful predictor of drug potency and drug resistance. Whereas some IN amino acid substitutions, such as Y143R/K/C, increase dissociation by altering a direct IN-INSTI contact (35), changes such as Q148H/G140S seemingly act indirectly by altering the conformation of the IN active-site region (38).

Alterations of viral DNA sequence, especially the terminal deoxyadenylate residue, also alter INSTI dissociative half-life (91), although to date, LTR sequence changes have not been implicated in INSTI resistance. Reverse transcription initiates with minus-strand DNA synthesis via a co-packaged host tRNAlys3 primer that engages the primer–binding site near the 5’ end of the viral RNA genome (see Ref. 4 for review). Synthesis of the plus-strand of retroviral DNA is primed via an oligonucleotide derived from the 3’ poly purine (PPT) tract. In the RNA genome, the 3’ PPT abuts the U3 sequence that will form the upstream viral DNA terminus after reverse transcription. Selection of DTG resistance in cell culture has revealed changes in the HIV-1 3’ PPT, which was unexpected because this sequence abuts the downstream LTR distal from the viral DNA termini (Fig. 2) (146). One possible explanation is that alterations lead to misprocessing of the PPT during reverse transcription and accordingly extend the U3 DNA terminus, which would be a suboptimal sequence for IN binding (147). However, sequencing of 2-LTR circles, which form at low frequency in the cell nucleus via DNA ligation and thus provide a snapshot of viral DNA end sequences, failed to identify the hypothesized PPT extension (148). PPT mutations have been recorded in one patient who received DTG monotherapy (149), indicating that such changes may very well be clinically relevant. Additional work is required to more fully document the frequency of PPT changes in patients that fail DTG therapy as well as how such changes engender drug resistance.

Other changes outside of the IN coding region, including the HIV-1 env gene, can confer resistance to DTG (150). HIV-1 can infect cells through fusing directly with the cellular plasma membrane or through the virological synapse that forms between an infected cell and an uninfected cell (151) (reviewed in Ref. 152). The env mutations effectively increased the multiplicity of HIV-1 infection by significantly increasing the efficiency of cell-to-cell infection (150). This mechanism of drug resistance, which is indirect because it is highly unlikely to influence the dissociative half-life of DTG from the HIV-1 intasome, is reminiscent of prior reports that HIV-1 infection through the virological synapse reduced the efficacy of certain cART compounds (153, 154). Additional work is required to determine whether changes in HIV-1 env can confer resistance to DTG in the clinical setting (150).

The investigational second-generation INSTI cabotegravir (CAB) formulated as a crystalline nanoparticle conferred long-acting (LA) protection against challenge by chimeric simian-HIV (SHIV) in the macaque model of HIV/AIDS (155–157). LA-CAB administered as a monotherapy or in combination with LA-rilpivirine, which is a long-acting NNRTI, is being evaluated as a pre-exposure prophylaxis (PrEP) to prevent HIV-1 infection (reviewed in Ref. 158). One of the biggest factors contributing to the emergence of anti-HIV drug resistance is dosing protocol compliance, which for oral cART formulations is one to several pills daily. LA regimens largely obfuscate the need for end-user dose monitoring, which could increase compliance, although at the same time such regimens require regular injections to maintain plasma trough concentrations above values required to inhibit HIV–1 replication. Macaques that were positive for SHIV RNA but seronegative at the time of infection could develop resistance to LA-CAB, and the associated IN changes conferred potent cross-resistance to all licensed INSTIs (159). Such observations highlight the need to carefully monitor patients to avoid initiating PrEP during unrecognized acute HIV–1 infection. CAB in cell culture is marginally less effective at inhibiting infection by certain INSTI-resistant viruses than is either DTG or BIC (160), and both DTG (161) and BIC (162) have been formulated as LA compounds. Future research will evaluate the efficacy of LA INSTIs to treat at risk patients with PrEP as well as PLHIV.
ALLINIs

Despite relatively high barriers, second-generation INSTIs do select for resistance (149, 159, 163). As exemplified by the clinical successes of the NRTIs and NNRTIs, it would accordingly be highly beneficial to have additional drug classes that inhibit IN activity through novel mechanisms of action.

 Whereas a number of different types of IN-targeting molecules have been described in the literature, the class of compounds collectively known as ALLINIs has advanced the furthest. Predecessor compounds of potent ALLINIs were discovered via two different means, including a high-throughput screen for inhibitors of IN 3’ processing activity (164–166) and structure-guided modeling of the amino acid contacts that mediate the interaction of HIV-1 IN with the host integration targeting cofactor lens epithelium-derived growth factor (LEDGF)/p75 (167). In addition to ALLINI (168), such compounds have been referred to as LEDGIN for LEDGF-interaction site (167), NCINI for noncatalytic site IN inhibitor (165, 169), IN-LAI for IN-LEDGF allosteric inhibitor (170), and MINI for multimeric IN inhibitor (171).

Although HIV-1 in large part integrates throughout the human genome (46), it does so in nonrandom fashion, on average favoring active genes that reside within relatively gene-dense regions of chromosomes (172). This targeting preference is largely dictated through specific interactions of two PIC-associated proteins, IN and capsid, with respective host factors LEDGF/p75 and cleavage and polyadenylation specificity factor 6 (173, 174) (for a recent review, see Ref. 50). LEDGF/p75 is a chromosome-associated (59, 175, 176) transcriptional co-activator (177) that harbors two globular domains, an N-terminal PWPP (for Pro-Trp-Trp-Pro) chromatin reader with affinity for histone 3 Lys-36 trimethylation (178–180), and a downstream region that was termed the IN-binding domain (IBD) because it mediated the binding of LEDGF/p75 to HIV-1 IN in vitro (181). The LEDGF/p75 IBD is a PHAT domain (for pseudo-HEAT repeat analogous topology) composed of two helix-hairpin-helix HEAT repeats (182) (Fig. 6A, left). The IN CCD dimerizes via an extensive interface with DDE catalytic triads positioned at distal apices (183). LEDGF/p75 hotspot interaction residues Ile-365, Asp-366, and Phe-406 within the IBD hairpins engage both IN monomers at the CCD dimer interface (182, 184). Whereas Asp-366 hydrogen-bonds with the backbone amides of residues Glu-170 and His-171 within one IN monomer, Ile-365 occupies a hydrophobic pocket composed of IN residues from both IN monomers (Fig. 6A, right). Electro-positive residues within IBD α1 make additional contacts with electronegative residues within the HIV IN NTD (185). HIV-1 IN subunits undergo dynamic exchange in solution (61), and LEDGF/p75 binding accordingly stabilized HIV-1 IN dimers and tetramers (40, 61, 186, 187) and significantly stimulated IN catalytic activities in vitro (40, 59, 61, 185, 188). The LEDGF/p75-IN interaction is specific to the lentivirus genus of Retroviridae (189–191).

Cellular depletion of LEDGF/p75 predominantly limits HIV-1 infection by reducing the level of integrated viral DNA (192–197). Expression of fusion proteins in susceptible target cells comprised of GFP and the LEDGF/p75 IBD also inhibited integration (192, 198), an effect that was exacerbated significantly by RNAi-mediated knockdown of LEDGF/p75 expression (199). Such observations highlighted the antiviral potential of small molecules designed to inhibit the interaction between HIV-1 IN and LEDGF/p75. Although inhibition of IN-LEDGF/p75 binding was initially espoused as the antiviral mechanism of action (167), there has since been little evidence to suggest that inhibition of the protein–protein interaction significantly contributes to ALLINI potency.

ALLINI compounds are built around heterocyclic cores, such as pyridine (171, 200), thiophene (201), quinoline (165, 167, 168, 170, 197, 202–204), isoquinoline (205), thienopyridine (167, 206) (Fig. 4B), or naphthyridine (207); additional chemotypes have been described in the patent literature (reviewed in Ref. 208). Potent compounds contain a 2-carbon arm harboring t-butoxy and carboxylic acid that is commonly connected to the ring two positions from the heteroatom (Fig. 4B; also see Fig. 6, B and C). As espoused in the initial LEDGIN paper (167), the binding modes of these compounds to HIV-1 IN in large part mimic LEDGF/p75 binding. Co-crystal structures with the HIV-1 IN CCD dimer revealed that ALLINI carboxylic acids mimic the carboxylate side chain of LEDGF/p75 residue Asp-366 by making similar contacts with the backbone amides of IN residues Glu-170 and His-171 (Fig. 6, cyan IN monomer). The t-butoxy moiety additionally interacts with IN residue Thr-174 from this same monomer. ALLINI central rings mimic LEDGF/p75 residue Ile-365 by occupying a hydrophobic pocket composed of residues from both IN monomers. The benzimidazole moiety in pyridine ALLINI KF116 additionally interacts with IN residue Thr-125 of the green IN monomer (Fig. 6C). Similar binding modes can explain why quinoline ALLINIs effectively inhibited the LEDGF/p75-IN interaction in vitro (167, 170, 202, 204, 206). By contrast, pyridine (171) and isoquinoline (205) ALLINIs are comparatively weak inhibitors of the virus–host interaction.

ALLINIs can inhibit HIV-1 IN 3’ processing and strand transfer activities in vitro in a LEDGF/p75-independent manner (168, 170, 202, 205, 206). Accordingly, quinoline ALLINIs inhibited HIV-1 infection at the integration step when the compounds were added to susceptible target cells at the time of virus infection (12, 167, 170, 202). However, inhibition of integration is arguably a side effect of ALLINI antiviral potency (13, 169, 171), as the compounds display much greater potencies during the late phase of HIV-1 replication (12, 169–171, 204, 209, 210). Retroviral RNP complexes appear electron-dense in negatively stained thin sections due to the comparative inability of electrons to pass through these structures. The underlying basis for ALLINI antiviral activity is HIV hyperm팀ointedization (12, 169–171, 204, 209–214), which inhibits IN binding to RNA in virions (63) and elicits the formation of eccentric HIV-1 particles with viral RNP complexes situated outside of comparatively electron-translucent and often deformed capsid shells (12, 13, 169–171, 201, 209, 210, 212, 215). The morphology defect is reminiscent of what is seen via a variety of mutations in the IN region of HIV-1 pol that yield so-called class II IN mutant viruses (216) (reviewed in Refs. 217 and 218). Such eccentric viral particles are noninfectious due to their inability to promote reverse transcription in target cells (12, 13, 169–
Both IN and viral RNA are rapidly degraded after cell entry, likely due to their exposure to the cell cytoplasm outside the confines of the protective capsid shell (214, 219). ALLINI potency during the late phase of HIV-1 replication is independent of cellular LEDGF/p75 content (12, 169, 220). Remarkably, LEDGF/p75 depletion significantly increased the potency at which the quinoline ALLINI BI-D inhibited HIV-1 integration (12, 197). Instead of being the antiviral target, engagement of IN by LEDGF/p75 during the early phase of HIV-1 infection protects the virus from the inhibitory action of the compounds (221). Accordingly, quinoline and thienopyridine ALLINIs can significantly diminish the extent of HIV-1 integration into genes in LEDGF/p75-expressing cells (171, 204, 221, 222).

Cells that comprise the latent HIV-1 reservoir show variable growth characteristics, with integrations near growth-promoting genes linked in some cases to cellular proliferation (223–225) (reviewed in Ref. 226). Because LEDGF/p75 depletion results in global shifts of HIV-1 integration sites toward gene 5’ end regions (173, 195, 227), treating patients with quinoline ALLINIs could cause unwanted side effects from promoter-proximal integration if growth promotion was sufficiently up-regulated to seed tumorigenesis. However, preliminary work in this area has revealed that HIV-1 proviruses formed in the absence of LEDGF/p75 are transcriptionally repressed. The use of HIV-1 reporter vectors that express two different fluorophores—one from the LTR and another from a constitutively active internal promoter—indicated that LEDGF/p75 depletion or treatment with thienopyridine ALLINI CX014442 specifically decreased LTR activity and accordingly increased the proportion of latent proviruses that form during the early phase of HIV-1 infection in cell culture (222). Moreover, such provi-

Figure 6. ALLINI mimicry of LEDGF/p75 binding to the HIV-1 IN CCD dimer. A, solution structure of the LEDGF/p75 IBD (left; PDB code 1Z9E) and IBD-CCD co-crystal structure (right; PDB code 2B4J) highlight the locations of hotspot-interacting residues Ile-365 and Asp-366 in the hairpin that connects α-helices 1 and 2 and Phe-406 in the α4-α5 hairpin (left). Whereas Asp-366 interacts with the backbone amide groups of IN residues Glu-170 and His-171 of the cyan IN monomer (dashed lines), Ile-365 occupies a hydrophobic pocket composed of IN residues from each monomer (i.e. Trp-132 of the green IN monomer and Met-178 of the cyan monomer; right). Other colorings denote atoms of interacting amino acid residues: nitrogen (blue), sulfur (yellow), and oxygen (red). B, quinoline ALLINI BI-224436 (left, chemical diagram) bound to the IN CCD dimer (right, PDB code 6NUJ). The interactions between the compound carboxylic acid and backbone amides within the IN cyan monomer are analogous to those shown in A for LEDGF/p75 residue Asp-366. Thr-174 of the IN cyan monomer additionally interacts with the τ-butoxy moiety of the drug. Other labeling is the same as in A. C, binding of pyridine ALLINI KF116 (left, chemical structure) to the IN CCD dimer (right, PDB code 4O55). This view, rotated down ~90° from A and B, is shown to accentuate the drug-binding pocket. In addition to the contacts described in B, Thr-125 of the green IN monomer interacts with the benzimidazole moiety of KF116. Other labeling is as defined in A and B.
ruses were refractory to transcriptional activation by latency reversal agents (LRAs) (222). Such observations have prompted the notion that quinoline or thienopyridine ALLINIs could be used as part of PrEP regimens to limit the size of the latent viral reservoir that forms during the acute phase of HIV-1 infection (228). Curiously, LEDGF/p75 can repress the transcription of established HIV-1 proviruses (229). The mechanistic connection between the transcriptional competency of newly formed HIV-1 proviruses and IN-LEDGF/p75 binding is currently unclear. It should be informative to fine map the positions of these proviruses as well as their responses to LRA treatment.

Both an X-ray crystal structure (212) and molecular modeling (213, 214) have yielded clues as to the nature of ALLINI-induced IN hypermultimerization. Quinoline ALLINIs bound at the LEDGF/p75-binding pocket of an IN dimer engaged the CTD of a separate IN dimer, thereby templating the polymerization of IN dimers through successive interdimeric CTD-ALLINI-CCD bridge contacts (212, 213). Whereas quinoline ALLINIs similarly hypermultimerized IN dimers and tetramers, the pyridine ALLINI KF116 specifically multimerized IN tetramers (214). Modeling revealed that hypermultimerization in this case occurred through successive CTD-ALLINI-CCD bridge contacts whose formation specifically required IN tetramers (214). KF116 potency tracked hand-in-hand with HIV-1 IN tetramerization, indicating that the tetramer is the predominant form of IN in HIV-1 virions (214).

Cell-free virions are recalcitrant to ALLINI treatment, revealing that inhibition requires HIV-1 exposure to ALLINIs in the confines of virus-producing cells (12, 169, 170). Thienopyridine ALLINI CX05045 enhanced the multimerization of purified HIV-1 Pol protein in vitro, indicating that the antiviral target during HIV-1 infection could be Gag-Pol (209). Genetic experiments, however, revealed that this need not be the case. Although usually incorporated into HIV-1 particles via Gag-Pol, IN can be supplied in trans as a fusion protein with the accessory protein Vpr (230). HIV-1 harboring IN expressed solely from Vpr-IN remained fully sensitive to ALLINI inhibition, revealing that Gag-Pol need not be the initial site of ALLINI engagement (12, 13). ALLINI-mediated multimerization of Pol protein in vitro minimally suggests that the CCD dimer interface within IN is present in Pol. Additional work is required to ascertain whether ALLINIs may first gain access to IN via engaging Gag-Pol under normal infection conditions. Conceivably, initial engagement via Gag-Pol or IN could depend on the type of compound, as the pyridine ALLINI KF116 specifically targeted tetrameric IN (214), and it is unknown whether IN could tetramerize in the context of Gag-Pol.

Similar to the NNRTIs, which engage an allosteric binding pocket on RT (reviewed in Ref. 231), ALLINIs are specific for HIV-1 and do not inhibit closely related primate lentiviruses such as HIV-2 or simian immunodeficiency virus from rhesus macaques (167, 201, 209). Eccentric HIV-1 particles produced by exposure to the thiophene ALLINI MUT-A displayed immunoreactivity characteristics similar to mock-treated virions, indicating a potential novel avenue for chemically inactivated immunogens as vaccine candidates (201). Given the specificity of ALLINIs for HIV-1 IN, intensive safety evaluations necessitated by such approaches will likely require chimeric SHIV strains that carry HIV-1 IN (232).

The role of IN in HIV-1 particle morphogenesis is an ongoing area of investigation. IN, as a free protein or as part of Gag-Pol, could nucleate the formation of the capsid shell around the RNP (13). Disruption of IN–RNA binding (63) and/or IN–IN dynamics through mutations or ALLINIs would then yield non-infectious particles with eccentric electron density. Such models invoke IN as a molecular tether or communicator between the RNP and the capsid; although IN can directly interact with different components of the RNP, including RT (233–237) and genomic RNA (63), interactions with the virus capsid protein have not been reported. ALLINIs are important compounds with which to further probe the role of IN in HIV-1 particle morphogenesis.

**HIV-1 resistance to ALLINI compounds**

Resistance against quinoline and thienopyridine ALLINIs is readily selected in cell culture, with most changes mapping to IN residues in the vicinity of the LEDGF/p75-binding pocket (165, 167, 201–203, 212). Whereas some of these, such as Thr-174, are invariant among circulating HIV-1 strains, others, such as Ala-124 and Ala-125, are highly polymorphic in nature (238–241) (reviewed in Ref. 242). Because Asn is often found at position 124 and A124D conferred significant resistance to the quinoline ALLINI BI-D (165), recent studies have tested antiviral activities of compounds against HIV-1 strains harboring representative 124/125 polymorphisms, such as Thr/Thr, Thr/Ala, Ala/Thr, Ala/Ala, Asn/Thr, and Asn/Ala (200, 207, 215). Whereas Asn-124 and Ala-125 each conferred ~50-fold resistance to pyridine ALLINI compound 20 (200) and thiophene ALLINI MUT-A (215), respectively, naphthyridine ALLINI compound 23 remained active against such strains (207). Counterscreening against representative polymorphic strains such as these, as well as viruses containing changes such as A128T and T174I that are commonly selected during virus passage (165, 167, 171, 201–203, 212), is important to the ongoing development of the ALLINI drug class.

Similar to INSTI resistance (35, 38), some changes in IN that confer ALLINI resistance, such as T174I, alter a direct binding contact (Fig. 6, B and C), whereas others work via less direct mechanisms. The A128T change in IN conferred significant resistance to the hypermultimerization activities of quinoline ALLINIs without affecting the binding of LEDGF/p75 to the mutant IN protein (211). IN CCD co-crystal structures revealed that the bulky Thr substituent shifted the position of ALLINI binding, lowering its propensity to hypermultimerize the mutant IN (211). On the flip side, thiophene ALLINI MUT-A efficiently inhibited LEDGF/p75 binding to Ala-125–containing IN yet in large part lost the ability to hypermultimerize this polymorphic variant. In this case, downstream interactions of the CCD-engaged ALLINI with the CTD of another IN oligomer seemed to underlie the loss of hypermultimerization activity (215). Thus, resistance to ALLINIs can be instilled by loss of direct IN binding contact, a shift in compound position within the CCD-binding pocket, or inability of bound compound to mediate downstream interactions.
Development of resistance to the pyridine ALLINI KF116 required successive changes in IN that initiated with T124N, followed by T174I, and culminated with T124N/V165I/T174I (171). Whereas recombinant T124N virus was, as expected, similarly infectious as the WT, the infectivity of T124N/T174I was reduced almost 1,000-fold, and T124N/T174I virions failed to mature due to processing defects in both Gag and Gag-Pol precursor proteins (243). These data highlight how a single amino acid substitution in IN can exert catastrophic consequences on the late stages of HIV-1 replication. Remarkably, the added V165I change restored polyprotein processing and boosted infectivity about 120-fold, to 17% of the level of the WT (243). Because resistance required the virus to pass through a nearly noninfectious mutational bottleneck, KF116 harbors a higher genetic barrier to resistance compared with predecessor quinoline ALLINIs (171, 243). Assessment of genetic resistance barrier is another important consideration in the development of clinical ALLINI compounds.

Antiretroviral compounds display class-specific slopes in their dose–response curves. For example, whereas INSTIs and NRTIs display slopes close to 1, NNRTIs and PIs display steeper slopes of ~1.7 and from 1.8 to 4.5, respectively (244). Slope is related to the Hill coefficient, which is a measure of the intramolecular cooperativity of ligand binding to a multivalent receptor. Although ~120 molecules of IN enter a susceptible target cell, only the two that engage the ends of HIV-1 DNA within the confines of a single intasome are sensitive to INSTI action. Likewise, bystander RT molecules that are not actively engaged in DNA synthesis are unseen by NRTI compounds. The limited number of enzyme–substrate targets per replication cycle accounts for the comparatively shallow slopes of INSTI and NRTI dose–response curves (244). By contrast, NNRTIs and PIs target apoenzymes. Reasons for steeper dose–response curve slopes in these cases include the requirement to inhibit the full complement of viral enzymes, which in this sense likens the enzyme population to a multivalent receptor (244), or the inhibition of multiple steps within the viral replication cycle (245). ALLINIs like PIs display comparatively steep dose–response curve slopes (n = 4) (168, 203). Because eccentric HIV-1 particles made in the presence of ALLINIs are defective for reverse transcription, the compounds potently inhibit minimally two steps of the viral replication cycle, which could account for the steep slopes. Determining ALLINI-to-IN stoichiometries required to inhibit HIV-1 replication would be expected to further inform the cooperative nature of drug action.

**Other IN functionalities**

IN has been proposed to play additional roles in the HIV-1 lifecycle, including virus particle uncoating after cell entry (246) and PIC nuclear import (247, 248). IN accordingly has been shown to interact with numerous karyopherin (KPN) nuclear transport receptors, including KPN2A/importin α (247, 249–251), KPNB1/importin β1 (249, 251), KPNB2/transportin-1 (TNPO1) (249), KPN4/importin α3 (252, 253), importin 7 (249, 254), Ran-binding protein (RANBP) 9/importin 9 (255), RANBP4/importin 4 (256), and TNPO3 (257). Genetic mapping experiments at the same time have highlighted the capsid protein as the key mediator of HIV-1 PIC nuclear import (258) (reviewed in Ref. 3), and numerous follow-up studies have questioned purported roles for IN nuclear localization signals (259–262) and/or IN–host factor interactions (263–267) in HIV-1 nuclear import. Compounds that inhibited IN binding to TNPO3 (268) or KPN2A/KPNB1 (269) in vitro displayed comparatively weak antiviral activities of less than 50% inhibition at 100 μM and 50% inhibition at ~50–100 μM, respectively. To establish IN–KPN interactions as bona fide antiviral targets, it will be important to show that resistance to compounds with 10–100-fold greater potencies maps to the IN region of HIV-1 pol.

Novel tert-butylsulphonamide (t-BSF) compound 1 inhibited the late phase of HIV-1 infection 6-fold more potently than the early phase, indicating bona fide ALLINI activity (270). Whereas the T174I substitution in the LEDGF/p75 IBD binding pocket conferred about 500-fold resistance to a control quinoline ALLINI compound, the mutant virus was 5-fold more sensitive to inhibition by t-BSF ALLINI compound 1, indicating engagement of the IN CCD dimer at a location other than the LEDGF/p75-binding pocket (270). The binding of a predecessor ALLINI compound, which inhibited IN subunit exchange in solution, mapped to the CCD dimer interface adjacent to the LEDGF/p75-binding pocket (271). These studies established that regions of the CCD dimer interface outside of the LEDGF/p75-binding pocket are potential targets for novel ALLINI development. Structural determination of the IN region within Pol or the tetrameric form of IN within virions (214) may reveal additional IN-IN interfaces for the development of novel ALLINI compounds.

**Conclusions**

IN inhibitors have come a long way since the early days when some of the most notable individuals in the field felt the goal of clinical IN inhibition was unattainable. The impressive potencies and resistance barriers of second-generation INSTIs have prompted worldwide rollouts, although safety profiles for pregnant women at the time of conception require careful monitoring and comprehensive follow-up. The assessment of second-generation INSTI-containing dual therapy regimens is ongoing for both oral administration for PLHIV and LA formulations for PrEP. The odds-on bet is that second-generation INSTIs will be a mainstay part of cART formulations for the foreseeable future.

The elucidation of retroviral intasome structures by X-ray crystallography and single-particle cryo-EM over the past decade has provided unprecedented insight into the mechanism of retroviral integration. The initial PFV intasome structures additionally provided important insight into the mechanisms of INSTI action, and ongoing cryo-EM work with HIV-1 and related primate lentiviral intasomes is expected to further advance our understanding of drug action and drug resistance mechanisms. Such high-resolution structures should critically inform the future development of these drugs to deal with resistance mutations prevalent from prior failure to first-generation INSTI-containing regimens as well as de novo resistance that will inevitably arise from global second-generation INSTI rollouts.
ALLINIs provide a clear example of how drugs against a viral enzyme can primarily inhibit virus replication at a step that is distinct from where catalytic function transpires. ALLINIs in large part recapitulate the class II HIV-1 IN mutant phenotype, revealing a remarkable example of pharmacological mimicry of biological phenotype. ALLINI and INSTI potencies are additive/synergistic and ALLINIs retain their potency in the face of clinically relevant INSTI resistance mutations (167, 170, 203). Thus, ALLINIs have the potential to fill the needed role of a second clinical class of anti-IN compounds with novel mechanism of action. Ongoing work to improve bioavailable chemotypes with broad antiviral activity against polymorphic HIV-1 variants (207) should further advance the evaluation of this promising drug class.

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