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Virus Maturation as a Novel HIV-1 Therapeutic Target

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Abstract

Development of novel therapeutic targets against HIV-1 is a high research priority due to the serious clinical consequences associated with acquisition of resistance to current antiretroviral drugs. The HIV-1 structural protein Gag represents a potential novel therapeutic target as it plays a central role in virus particle production, yet is not targeted by any of the currently approved antiretroviral drugs. The Gag polyprotein precursor multimerizes to form immature particles that bud from the infected cell. Concomitant with virus release, the Gag precursor undergoes proteolytic processing by the viral protease to generate the mature Gag proteins, which include capsid (CA). Once liberated from the Gag polyprotein precursor, CA molecules interact to reassemble into a condensed conical core, which organizes the viral RNA genome and several viral proteins to facilitate virus replication in the next round of infection. Correct Gag proteolytic processing and core assembly are therefore essential for virus infectivity. In this review, we discuss novel strategies to inhibit maturation by targeting proteolytic cleavage sites in Gag or CA-CA interactions required for core formation. The identification and development of lead maturation inhibitors are highlighted.

1. Background

As the causative agent of the global AIDS epidemic, the impact of HIV-1 on humanity has been immense. Approximately 33 million people are estimated to be living with HIV-1, and since the beginning of the epidemic at least 25 million people have died of HIV-1-related causes (1). There is currently no effective vaccine or cure and the only option for the treatment of HIV-1 infection is long-term suppression of viral replication with antiretroviral drugs.

To date, the U.S. Food and Drug Administration (FDA) has approved for clinical use more than twenty different drugs belonging to six mechanistic classes. These drugs have greatly increased patient survival. However, therapeutic regimes referred to as highly active antiretroviral therapy (HAART), are often complex, as a combination of different drugs must be administered (2,3). Multi-drug HAART regimens are required to avoid the rapid emergence of drug-resistant HIV-1 variants (4). The virus population within an HIV-1-infected patient exists as a diverse but related viral swarm, known as a quasi-species (4). The high degree of genetic diversity is a consequence of a rapid rate of viral replication combined with the error-prone nature of the viral polymerase [reverse transcriptase (RT)], which results in nucleotide substitutions, insertions and/or deletions (4–6). High rates of genetic recombination also contribute to the high degree of genetic diversity (7,8). Potential drug-resistant variants present within the viral quasi-species emerge when the viral

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population is placed under positive selection pressure by antiretroviral drugs (4). Emergence of drug resistance occurs very rapidly during drug mono-therapy but HAART can successfully suppress viral replication for years at a time (2,3,9–11). Despite the positive impact of HAART on patient survival, drug resistance can still emerge even in the face of this multi-drug therapy (2,3,9–13).

The emergence of multi-drug-resistant (MDR) HIV-1 isolates has serious clinical consequences and must be suppressed with new drug regimes, known as salvage therapy (10–12,14). Current guidelines recommend that salvage therapy includes at least two, and preferably three, fully active drugs (15). Typically, first-line therapies combine three to four drugs targeting the viral enzymes RT and protease (PR) (15). RT inhibitors are classified into two mechanistic categories: the nucleoside reverse transcriptase inhibitors (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs) (13). One option for salvage therapy is to administer different combinations of drugs from the same mechanistic class that remain active against the resistant isolates (15). However, the options for this approach are often limited, as resistance mutations frequently confer broad cross-resistance to different drugs in the same class (15). Alternative therapeutic strategies have recently become available with the development of fusion, entry, and integrase (IN) inhibitors (14,16–20). However, resistance to all three new drug classes has already been reported both *in vitro* and *in vivo* (14,18–21). Sustained successful treatment of HIV-1-infected patients with antiretroviral drugs will therefore require the continued development of new and improved drugs with novel targets and mechanisms of action.

The HIV Gag polyprotein precursor ($Pr55^{Gag}$), which is composed of four protein domains - matrix (MA), capsid (CA), nucleocapsid (NC) and p6 - and two spacer peptides, SP1 and SP2 (Fig. 1A), represents a novel therapeutic target as it plays a central role in infectious virus particle production yet is not targeted by any of the currently approved antiretroviral drugs. HIV-1 particle production has been extensively reviewed (22–28). Briefly, Gag drives particle production via Gag-Gag interactions, mediated primarily via CA and SP1, to form the structural shell of the assembling immature particle (Fig. 1B). In most cell types, assembly occurs at the plasma membrane and the MA domain of Gag mediates membrane binding. During assembly, viral genomic RNA, the Env glycoprotein complex and the Gag-Pol precursor protein ($Pr160^{Gag\text{-}Pol}$) are incorporated into the assembling particle. Gag-Pol encodes the viral enzymes PR, RT and IN and is translated via a −1 frameshift. Gag is actively involved in the recruitment of these components into the assembling particle. More specifically, the NC domain mediates viral RNA encapsidation. MA is thought to be responsible for active incorporation of the Env glycoprotein complex, and Gag-Gag interactions mediate Gag-Pol incorporation. Assembly is completed by budding of the immature particle from the cell. Budding is catalyzed by components of the cellular endosomal sorting machinery, including the ESCRT-1 complex (for endosomal sorting complex required for transport) and associated factors, which are recruited to the site of assembly and release primarily by the P(T/S)AP late domain in p6.

Concomitant with particle release, the virally encoded PR cleaves Gag into the four mature protein domains; MA, CA, NC and p6 and the two spacer peptides, SP1 and SP2 (Fig. 1A). Gag-Pol is also cleaved by PR, liberating the viral enzymes PR, RT and IN. Gag proteolytic processing induces a morphological rearrangement within the particle, known as maturation. Maturation converts the immature, donut-shaped particle to the mature virion, which contains a condensed conical core (Fig. 1B) composed of a CA shell surrounding the viral RNA genome in a complex with NC and the viral enzymes RT and IN. Maturation prepares the virus for infection of a new cell and is absolutely essential for particle infectivity. This review discusses novel strategies to inhibit HIV-1 maturation by targeting proteolytic cleavage sites in Gag or CA-CA interactions required for mature core formation, together

with current progress on the identification and development of novel molecules that inhibit HIV-1 maturation.

2. Targeting proteolytic cleavage sites in Gag

2.1 Introduction

Proteolytic processing of Gag follows a sequential cascade of events that is kinetically controlled by the differential rate of processing at each of the five cleavage sites in Gag (Fig. 1A) (22,29–34). The first processing event cleaves Gag into two fragments, MA-CA-SP1 and NC-SP2-p6. Subsequent cleavage reactions occur at the MA-CA and SP2-p6 junctions. Finally, the CA and NC domains are liberated by cleavage of the CA-SP1 and NC-SP2 intermediates. Accurate proteolytic processing of Gag is essential for maturation and infectivity; disrupting cleavage at individual sites or altering the order of cleavage results in aberrant particles that have significantly reduced infectivity (33–40). Disruption of Gag proteolytic processing therefore represents an attractive therapeutic target for inhibiting HIV-1.

Two different approaches have been used to inhibit Gag proteolytic processing; one targets the PR enzyme itself, the other targets the Gag substrate. PR inhibitors (PIs) competitively inhibit PR enzymatic activity thereby preventing processing at all cleavage sites in the Gag and Gag-Pol polyprotein precursors. Multiple PIs have been developed and successfully used in the clinic; these compounds have been reviewed elsewhere (9,41–44). The second approach specifically targets individual Gag cleavage sites (39,40). Inhibitors that act by this second mechanism have been classified as maturation inhibitors, and represent a new class of potential antiretroviral drugs (18,45,46).

2.2 Bevirimat: Identification and mechanism of action

2.2.1 Discovery—The first drug candidate in this new class is 3-O-(3’,3’-dimethylsuccinyl)betulinic acid, variously known as bevirimat, BVM, PA-457, DSB and MPC-4326 (Fig. 1C) (18,45,46). A natural-products screen identified betulinic acid as a weak inhibitor of HIV-1 replication (47). Bevirimat is a chemical derivative of betulinic acid that potently inhibits HIV-1 replication with an *in vitro* IC_{50} of ~10 nM (39,40). Importantly for a new antiretroviral drug candidate, bevirimat potently inhibits both wild-type (WT) HIV-1 and viral isolates that are resistant to currently approved RT, PR and fusion inhibitors (39,40,46,48).

2.2.2 Mechanism of action—Bevirimat retains activity against drug-resistant HIV-1 isolates because of its novel mechanism of action, which involves specifically blocking the cleavage of SP1 from the C-terminus of CA (Fig. 1A) (39,40). Disrupting CA-SP1 cleavage, either with bevirimat treatment or by mutation of the CA-SP1 cleavage site, results in the formation of virions that exhibit an aberrant morphology typified by an acentric core and an electron-dense layer of Gag inside the viral membrane (Fig 1B) (34,39). Under certain circumstances bevirimat may also interfere with virus assembly and release; however this phenomenon occurs only at very high inhibitor concentrations that are not likely to be clinically relevant (49).

Several independent lines of evidence support the hypothesis that the CA-SP1 junction in Gag is the primary molecular target of bevirimat. Single-amino-acid substitutions that confer resistance to this compound all map to the CA-SP1 junction and not elsewhere in Gag or in PR (Fig. 2A) (39,40,48,50–52). Variability in the amino acid sequence at the CA-SP1 junction between HIV-1, HIV-2, and simian immunodeficiency virus from rhesus macaques (SIVmac) causes HIV-2 and SIVmac to be intrinsically resistant to bevirimat (Fig 2B) (40).

Swapping residues between the CA-SP1 junctions of HIV-1 and SIVmac results in the exchange of bevirimat sensitivity between these two viruses (53). Notably, some sites of sequence divergence between HIV-1 and SIVmac occur at residues to which bevirimat-resistance maps *in vitro* (Fig. 2B) (40,50).

Mutations that confer resistance to bevirimat cluster at or near the CA-SP1 cleavage site. Of the mutations acquired *in vitro*, three were identified within the C-terminal-most residues of CA (CA-H226Y, CA-L231F and CA-L231M) and three at the first and third residues of SP1 (SP1-A1V, SP1-A3T and SP1-A3V) (Fig 2A) (39,40,50). The SP1-A1V mutation arose most frequently (48,50), likely due to its robust replication even at high bevirimat concentrations (48,50,52). The CA mutations (CA-H226Y, CA-L231F and CA-L231M) did not impose a significant virus replication defect, although varying degrees of partial resistance are associated with these changes (48,50,52). Substitutions at SP1 residue 3 (SP1-A3V and SP1-A3T) imposed a significant replication defect in both the presence and absence of bevirimat (48,50). Delayed replication of the SP1-A3V mutant was typically accompanied by acquisition of second-site changes at relatively non-conserved residues immediately outside the CA-SP1 cleavage site (50). Interestingly, SP1-A3V replication and virion maturation were enhanced in the presence of bevirimat (50). The different phenotypes associated with this panel of bevirimat-resistance mutations suggest the existence of multiple mechanisms by which HIV-1 can evolve resistance to this compound. A straightforward mechanism of resistance would involve the mutation simply preventing the compound from binding to Gag. Consistent with this mechanism, a reduction in bevirimat binding was observed for several bevirimat-resistance mutations including CA-H226Y, CA-L231F, CA-L231M and SP1-A1V (52). Interestingly, the CA-L231M and SP1-A1M mutants retained some capacity to bind bevirimat despite exhibiting resistance (52). Additionally, the observed drug dependence associated with the SP1 residue 3 substitutions (50) suggests that they confer resistance via an alternative mechanism that does not involve a simple block to compound binding.

2.2.3 Gag binding—Despite the evidence establishing the CA-SP1 junction as the molecular target of bevirimat, the binding site of this compound remains undefined. Several pieces of data, however, suggest that bevirimat interacts with a binding site that forms during virus assembly. First, bevirimat does not inhibit CA-SP1 processing in the context of monomeric Gag in solution (39) but instead requires immature virus assembly for its activity (39,54,55). Second, bevirimat binds to immature but not mature HIV-1 particles, suggesting that Gag processing leads to disruption of the bevirimat-binding site (54). Finally, as mentioned above, bevirimat incorporation into virus particles is reduced to some extent by bevirimat-resistance mutations, linking the antiviral activity of bevirimat to HIV-1 particle association (52,54).

A detailed characterization of the putative bevirimat binding pocket has been hindered by the lack of high-resolution structural data for the CA-SP1 junction. This region of Gag is disordered in X-ray crystallographic analyses, probably because of the intrinsic flexibility of this protein sequence (56–58). However, the region of Gag spanning the CA-SP1 junction has been predicted to be α-helical (37,58–60). Modeling of NMR structures derived from CA-SP1 peptides suggested the formation of dimers or trimers (59). More recently, a cryo-electron tomography study led to the proposal that the CA-SP1 junction forms a six-helix bundle within immature Gag particles (60). A working hypothesis for bevirimat activity is that the compound inhibits CA-SP1 cleavage by directly blocking access of PR to the CA-SP1 cleavage site. Alternatively, bevirimat could bind at or near the CA-SP1 junction and alter the conformation, exposure, or flexibility of this region such that it becomes less efficiently cleaved by PR. Further structure-function analysis of the CA-SP1 region of Gag will be required to more fully understand the mechanism of action of bevirimat; such

information may also provide a rational basis for the design of additional compounds that target this cleavage site.

2.3 Bevirimat: Clinical development

2.3.1 Introduction—The *in vitro* potency and novel mechanism of action of bevirimat encouraged its development as an antiretroviral drug. Initial support for efficacy *in vivo* was obtained in the severe combined immunodeficiency (SCID)-hu Thy/Liv BALBc mouse model (61). Promising pharmacological and safety profiles in animal models and in phase I clinical trails in humans led to the testing of bevirimat in HIV-1-infected patients (62–67). Initial phase II clinical studies in HIV-1-infected patients demonstrated statistically significant, dose-dependent reductions in viral loads (65). However, further phase II studies revealed that not all bevirimat-treated patients exhibited significant viral load reductions, despite optimal inhibitor concentrations in the plasma (68). In a 14-day functional monotherapy phase IIb trial, 55% of the 44 patients receiving bevirimat were defined as non-responders with viral load reductions < 0.5 log (68).

2.3.2 Virological determinants of bevirimat susceptibility—Studies to understand the determinants of variable clinical response to bevirimat *in vivo* are currently underway. As >75% of those patients who did not respond to bevirimat had inhibitor plasma concentrations that appeared to be adequate (plasma bevirimat trough > 20μg/ml) it was hypothesized that virological parameters may be responsible for the observed variable clinical outcome (68–72). Indeed, failure to respond to bevirimat was found to correlate with the presence of virus isolates containing polymorphisms at SP1 residues 6, 7 and 8 but not the amino acid positions to which bevirimat resistance mapped *in vitro* (68–72). Among different HIV-1 isolates, the residues to which in vitro bevirimat-resistance mutations map are all highly conserved; however, polymorphisms occur in less-conserved SP1 residues 6, 7 and 8 (Fig 2B) (50,68–74). The degree of conservation of residues in this region of Gag appears to be independent of antiretroviral treatment in HIV-1-infected patients (73,74). Studies are underway to understand the relationship between these polymorphisms and HIV-1 susceptibility to bevirimat. The results of these studies should help to identify patients that are most likely to respond to bevirimat treatment and could have implications for the durability of patient response to this maturation inhibitor.

2.3.3 Impact of PR mutations on bevirmat resistance—The fact that new antiretroviral drug candidates such as bevirimat will likely be used in PI-experienced patients raises interesting and clinically significant questions about the emergence of resistance to bevirimat in the context of viral isolates encoding PI-resistant (PIR) PR enzymes. A recent study investigated this issue by combining the bevirimat-resistance mutations with a PIR PR and selecting for bevirimat resistance in the context of either the WT or PIR PR (48). The PIR PR used for this analysis contains five mutations that confer high-level resistance to multiple PIs (75,76). Combination of most of the bevirimat-resistance mutations with this PIR PR resulted in additive defects in virus replication capacity, particularly in the presence of bevirimat. The SP1-A1V substitution was the exception, as it did not significantly impair virus replication independent of bevirimat treatment or the PR context (WT or PIR). Consistent with these observations, emergence of bevirimat resistance was delayed in the presence of the PIR PR. Perhaps not surprisingly when resistance did emerge it was via acquisition of SP1-A1V. These results suggest that resistance to bevirimat may be less likely to emerge in patients that have failed PIs relative to patients that are PI-naïve.

2.3.4 Acquisition of bevirimat resistance in vivo—The *in vitro* bevirimat resistance studies led to the prediction that the SP1-A1V substitution is most likely to arise *in vivo*.

However, SP1 residue A1, along with the other residues to which bevirimat resistance maps *in vitro*, are highly conserved amongst HIV-1 isolates (50). It might therefore be anticipated that a fitness cost would be associated with replication of these viruses *in vivo*. However, we observe that SP1-A1V can replicate efficiently in primary lymphocytes and macrophages (Adamson, Ablan, and Freed, unpublished data) and in SCID-hu Thy/Liv mice (61). Furthermore, the SP1-A1V mutation has been observed in virus from two of 46 patients participating in bevirimat phase IIb clinical studies (K. Salzwedel, unpublished data). The CA-L231M substitution has been reported in the context of one PI-experienced but bevirimat-naïve patient sample (77) and the SP1-A3T mutation exists in one viral isolate listed in the Los Alamos HIV sequence database (http://www.hiv.lanl.gov) (46). However, the variable clinical responses to bevirimat in the phase II clinical trial discussed above were not associated with the bevirimat-resistance mutations identified *in vitro*. Ongoing clinical trails will ultimately reveal the types of resistance mutations that will arise in patients who respond effectively to bevirimat.

3. Targeting CA-CA interactions required for assembly of the viral core

3.1 Structure and assembly of the CA core

3.1.1 Introduction—The mature CA protein is produced as a late product in the Gag processing cascade (32–34). The newly liberated CA molecules reassemble to form a condensed core containing the viral RNA in complex with NC and the viral enzymes RT and IN. The function of the core is to facilitate reverse transcription of the viral RNA genome following infection of a new target cell. Correct core formation and stability are therefore essential for virus infectivity (34,39,78–84). Disrupting CA-CA interactions required for core formation thus represents a potential therapeutic strategy for inhibiting HIV-1.

3.1.2 Core morphology and structure—Much of the early progress in understanding the morphology and organization of HIV-1 cores was made possible by the observation that purified CA could assemble *in vitro* to form long tubes (79,85,86). The individual CA molecules in these tubes are organized in a manner that is very similar to the organization of CA in mature viral cores (87,88). The *in vitro* assembly systems therefore offer a tractable approach to testing for CA-based inhibitors of virion maturation (89,90).

Examining the overall organization of CA in *in vitro*-assembled tubes and viral cores has provided significant insights into the nature of the CA-CA interactions required for core formation (Fig. 3A–C) (88). HIV-1 cores are typically cone-shaped, although a diverse range of related structures have been described (91–94). Non-lentiviral cores can adopt an even wider range of shapes, including spheres and cylinders (95, 96). The diversity of core structures suggests that cores are assembled via related but non-stringent design principles. These geometric principles are based on a fullerene cone model in which the core is assembled from a curved hexagonal CA lattice (Fig. 3A–C) (84, 87, 88, 97). The overall shape and closure of the core is thought to be regulated by the inclusion and positioning of pentamers within the hexameric lattice (Fig. 3C) (84, 87, 88), a hypothesis supported by imaging studies of authentic mature retroviral particles, including those of HIV-1 (92–94, 96).

3.1.3 CA interactions required for core formation—CA is composed of two structurally independent and largely helical folded domains, known as the CA N-terminal (CA_{NTD}) and C-terminal (CA_{CTD}) domains. CA_{NTD} and CA_{CTD} are separated by a short, flexible interdomain linker (Fig. 4A) (28,56,57,98–101). CA_{NTD} is composed of an N-terminal β-hairpin plus seven helices (helix 1–7) and an extended loop that binds the cellular protein cyclophilin A (98). The N-terminal β-hairpin forms upon refolding of the N-terminus of the CA_{NTD} after PR-mediated processing at the MA/CA junction (79,86). CA_{CTD} is

composed primarily of four helices (helix 8–11). The hexameric CA lattice is constructed from rings of six CA_{NTD} domains connected to the neighboring hexameric rings via CA_{CTD} dimer interactions (Fig. 3A) (88,97). Docking of high-resolution CA structures into a hexameric lattice map, created from cryoEM reconstructions of 2D crystals of CA hexagonal arrays, led to the identification of three major CA-CA interfaces required for core formation (88,97). These interfaces facilitate intrahexamer CA_{NTD}-CA_{NTD}, intrahexamer CA_{NTD}-CA_{CTD} and interhexamer CA_{CTD}-CA_{CTD} interactions (97).

A variety of genetic, biochemical, and biophysical studies have defined CA-CA interacting interfaces. Extensive mutational analyses have demonstrated an important role for the CA_{NTD} in core formation (78–81,83,102,103). Mutations that disrupt core formation primarily map to CA_{NTD} helices 1–3 (83,84). In addition, deuterium exchange experiments demonstrated that helices 1–3 contribute to important protein-protein interactions in both mature and immature virions (104,105). These studies support a model in which helices 1–3 form an intrahexamer CA_{NTD}-CA_{NTD} interface, which associates as an 18-helix bundle at the center of the hexameric ring (88,97). This structure matches the hexamer formed by the CA_{NTD} of the distantly related retrovirus MLV (106).

CA_{CTD}-CA_{CTD} intermolecular interactions occur via dimers that connect neighboring hexamers (88,97). The capacity of CA_{CTD} to dimerize has been demonstrated both in solution (56,107,108) and in high-resolution structures (56,57). Dimer-disrupting mutations at CA residues W184 and M185 significantly reduce virus particle production (56,83), demonstrating that the CA_{CTD}-CA_{CTD} dimerization interface contributes to Gag-Gag interactions that are required for HIV-1 particle assembly (83,109). Structural studies mapped the dimer interface to CA_{CTD} helix 9, which includes residues W184 and M185 (56,57). These studies suggest that parallel packing of helix 9 from two CA_{CTD} monomers forms the dimer (56,57). This arrangement is consistent with the CA docking model of the CA_{CTD}-CA_{CTD} interhexamer interface (97).

The third CA-CA interface is formed by intermolecular interactions between the CA_{NTD} and CA_{CTD} of different CA molecules within the same hexamer. This interaction was first implicated by cross-linking assays and further established both *in vitro* and in bona fide VLPs by deuterium exchange experiments (104,105). These studies mapped the interactions to CA_{NTD} helix 4 and CA_{CTD} helices 8 and 9. The CA docking model proposes that the amino-terminal end of helix 4 of CA_{NTD} inserts into a grove in the CA_{CTD}, forming contacts with helices 8 and 9 on one side of the groove and a loop connecting helices 10 and 11 on the other side (97). The interface may also extend to involve helices 7 and the loop between helices 3 and 4 at the bottom of the CA_{NTD} (97). Mutation K70A in helix 4 has been shown to diminish core formation (84). Mutations in helices 4–6 impair particle production (83), suggesting that this region of CA participates in protein-protein interactions involved in both immature and mature assembly events.

Functional surfaces that facilitate protein-protein interactions represent possible therapeutic targets; disrupting the interactions between the CA-CA interfaces described above and therefore represents a potential therapeutic strategy to inhibit HIV-1 replication. Identification and development of inhibitors that target protein-protein interfaces is challenging as the interacting surfaces may be flat and cover relatively large areas (110). Successful drugs are more typically small molecules that bind defined pockets. The CA_{NTD}-CA_{CTD} intermolecular interface may act as “hot-spot” for inhibitors of CA-CA interactions as two independent inhibitor screens identified molecules that appear to target pockets within or adjacent to this interface in the mature CA lattice (28).

3.2 CAP-1: Identification and mechanism of action

The first major strategy employed to identify CA-targeted inhibitors used an *in silico* approach to identify compounds that bound clefts on the CA surface followed by binding studies using NMR titration spectroscopy (111). This approach identified one lead compound, CAP-1 (N-(3-chloro-4-methylphenyl)-N'-{2-[({5-[(dimethylamino)-methyl]-2-furyl}-methyl)-sul-fanyl]ethyl}urea). HIV-1 particles produced from cells treated with CAP-1 exhibited size heterogeneity and more importantly did not contain conical core structures (111). The primary mechanism of action of this compound thus appears to involve inhibition of core formation during maturation, although it may also interfere with Gag-Gag interactions during immature particle assembly (111). In agreement with this model, initial binding studies showed that CAP-1 interacts with the C-terminal end of CA_{NTD} in both processed CA (Fig. 4A and B) and precursor Gag (111). High-resolution structures of CA_{NTD} in complex with CAP-1 reveal that inhibitor binding induces a conformational change in which residue F32 of CA_{NTD} is displaced from its buried position in the protein core to create a hydrophobic cavity that serves as the CAP-1 binding site (Fig 4B) (112). The aromatic ring of CAP-1 is sequestered within the hydrophobic pocket vacated by F32. The position of the CAP-1 binding site and F32 suggests that CAP-1 binding inhibits the formation of the CA_{NTD}-CA_{CTD} interface. NMR studies demonstrated that residues in helices 1, 2, 4 and 7 that appear to form part of this interface exhibit the most significant chemical shift changes upon CAP-1 binding (111,112). Fitting CAP-1 onto the CA lattice model is consistent with this CAP-1 mechanism of action (28,97,112).

3.3 CAI/NYAD-1: Identification and mechanism of action

A second strategy to identify CA-targeted inhibitors used *in vitro* assembly assays to facilitate systematic high-throughput screening of a phage display random peptide library (89). This approach identified the 12-mer peptide, CAI (capsid assembly inhibitor), which inhibits HIV-1 immature and mature assembly *in vitro* (89,113). Interestingly CAI binding dismantles in vitro preassembled tubes, suggesting that CAI binding also affects core stability (113). Assessment of the effect of CAI in cells was not possible due to its cell impermeability (89). However, modifying CAI by using the technique of "hydrocarbon stapling" generated cell-penetrating versions of CAI, termed NYAD-1 and NYAD-13, that inhibited a range of HIV-1 isolates by disrupting immature and mature assembly both *in vitro* and in cells (90). CAI binding was initially mapped to the CA_{CTD} (Fig. 4A and C) (89). A solution NMR structure of CAI bound to CA_{CTD} demonstrates that CAI has an α-helical conformation that binds to a hydrophobic groove between CA_{CTD} helices 8, 9 and 11 to create a globular 5-helix bundle with an altered CA_{CTD} protein conformation (Fig. 4C) (114). Mutational analysis of CA_{CTD} residues that mediate strong interactions with CAI have helped to determine the inhibitor's mechanism of action (115). The data suggest that CAI exerts its action in mature CA assembly in two ways, first by blocking the CA_{NTD}-CA_{CTD} interaction in mature core assembly by competing for the natural binding region in the CA_{NTD}. The second effect of CAI is to alter the CA_{CTD}-CA_{CTD} dimeric interaction that is important for connecting the hexameric lattice (114,115). Structural analysis of the cyclical cell-penetrating peptides (NYAD-1 and 13) suggests that they bind CA_{CTD} in a similar manner to CAI and therefore presumably act by the same mechanism (90,116). Both NYAD-1 and 13 bind CA with approximately 10-fold greater affinity than CAI; this is likely due to their enhanced α-helicity, which is required for binding (90,116). Another peptide-based approach recently identified the modified amino acid alpha-hydroxy-glycineamide (α-HGA) as an inhibitor of HIV-1 replication and CA assembly *in vitro* (117). The compound potentially acts by binding the linker region between CA_{NTD} and CA_{CTD}, although the mechanism of action remains to be defined (117).

The discovery of CAP-1 and CAI resulted from systematic molecular approaches to identify molecules that can inhibit CA and/or Gag interactions required for HIV-1 assembly events and hence HIV-1 infectivity. Although they are mechanistically interesting leads, CAP-1 and CAI are not drug candidates in part because of their weak binding affinity for CA (89,111). CAI has the additional problem of cell impermeability (89). Despite these limitations, knowledge about these inhibitors and their molecular targets can potentially be exploited for the rational design of more effective maturation inhibitors. For example, as mentioned above, structure-based rational design was used to convert CAI into the cell-penetrating peptides NYAD-1 and 13 (90,116).

4. Summary

The frequent emergence of drug-resistant HIV-1 isolates is driving research to identify new inhibitors directed against novel therapeutic targets, including the HIV-1 Gag protein, which is currently not targeted by any clinically approved antiretroviral drugs. Here we review the current status of HIV-1 maturation inhibitor development. Two general strategies have been used to block HIV-1 maturation. The first strategy targets proteolytic processing sites in Gag, as correct processing of this polyprotein precursor is essential for infectivity. This approach has been validated by the identification of bevirimat, a small molecule that specifically inhibits the cleavage of SP1 from the C-terminus of CA. The development of bevirimat as an antiviral drug is currently ongoing; although the compound demonstrates clinical efficacy, variable clinical responses have been associated with baseline polymorphisms in SP1. The second strategy targets CA-CA interactions that are required for core formation within the mature particle. Correct core formation and stability are essential for infectivity. Three major CA-CA interaction interfaces have been identified, all of which represent possible targets for disrupting core formation. Inhibitor screens have identified two lead molecules, CAP-1 and CAI, which primarily function by disrupting the CA_{NTD}-CA_{CTD} interface. CAI has been modified further to generate the cyclic peptides NYAD-1 and NYAD-13. These peptides are cell-permeable and display improved binding affinity to CA relative to CAI. Although these CA-targeted compounds are not suitable candidates for clinical development, knowledge of their mechanisms of action opens the way for rational design of future maturation inhibitors.

5. Expert opinion

HIV-1 maturation inhibitors – defined as compounds that target the substrate of the viral PR rather than the enzyme itself – constitute a promising new class of antiretroviral therapeutics, the potential of which is only beginning to be explored. As detailed in the main body of this article, two general approaches can be used to disrupt virus maturation by targeting the viral Gag proteins. The first approach involves disrupting specific cleavage events in the Gag processing cascade; the second entails interfering with the ability of the CA protein to refold and reassemble into the conical cores that are essential for virus infectivity. The maturation inhibitor bevirimat functions by the first mechanism; it prevents the cleavage of the Gag processing intermediate (CA-SP1) to mature CA. The initial clinical data with bevirimat are promising. However, the lack of response in approximately 50% of treated patients is a vexing problem that must be understood and addressed. The region of Gag immediately surrounding the CA-SP1 cleavage site is highly conserved across all clades of HIV-1. Sequences downstream in SP1 are much more polymorphic, and it appears that this region influences HIV-1 sensitivity to the compound. However, a rapid and cost-effective genotyping assay that would allow patient populations to be screened for these polymorphisms prior to the initiation of therapy is readily available (118). Still to be resolved is why resistance mutations mapping to these polymorphic positions in Gag have not been observed *in vitro*.

Because processing at each of the cleavage sites in Gag is essential for virion maturation, in principle each of the cleavage sites constitutes a potential target for novel antiretrovirals. As discussed in the main text, currently available data suggest that the binding site for bevirimat is generated during VLP assembly. Hence, it is likely that compounds blocking Gag processing at other sites will also recognize targets present on higher-order Gag complexes but perhaps not existing on monomeric Gag. Structural information on individual Gag domains may therefore be of little use in developing such inhibitors; progress in this area will benefit from the development of effective high-throughput screens for inhibitors of Gag processing at individual sites. Less target-oriented approaches could also be devised that simply screen for inhibitors of maturation and thus cast a wide net for compounds that disrupt virus maturation by blocking Gag processing or by binding to CA.

Although the clinical development of CA-based maturation inhibitors lags well behind that of bevirimat, CA remains an exciting and under-developed antiretroviral target. Given the complexity of the refolding and reassembly reactions that CA undergoes after its liberation from the Gag polyprotein precursor, and the diversity of intra- and inter-molecular CA contacts that are required for core condensation, one can envision a number of potential targets on the CA protein that might be suitable sites for small-molecule binding. The discovery of such molecules will require effective screens and successful resolution of the standard problems of cell permeability, toxicity, and specificity.

While this review focuses on Gag processing and CA core formation as targets for maturation inhibitors, other regions of Gag such as MA, NC and p6 could also be targeted by antiretroviral compounds. In particular, compounds that react with the NC Zn fingers display antiviral activity in vitro (119) and in murine systems (120) and budding inhibitors that target interactions between p6 and the ESCRT machinery could be developed as budding inhibitors (121,122)

The most effective targets are likely to be functionally critical pockets that are highly conserved between viral isolates. Although highly conserved targets are more difficult for the virus to modify in developing drug resistance, HIV-1 has proven to be an extremely adaptable adversary; resistance to Gag-targeted compounds is therefore inevitable. To provide durable activity, maturation inhibitors will need to be administered in combination with other antiretroviral therapeutics. Despite the many challenges facing all new classes of anti-HIV drugs, the development of maturation inhibitors is likely to be an exciting and productive area of research in the future.

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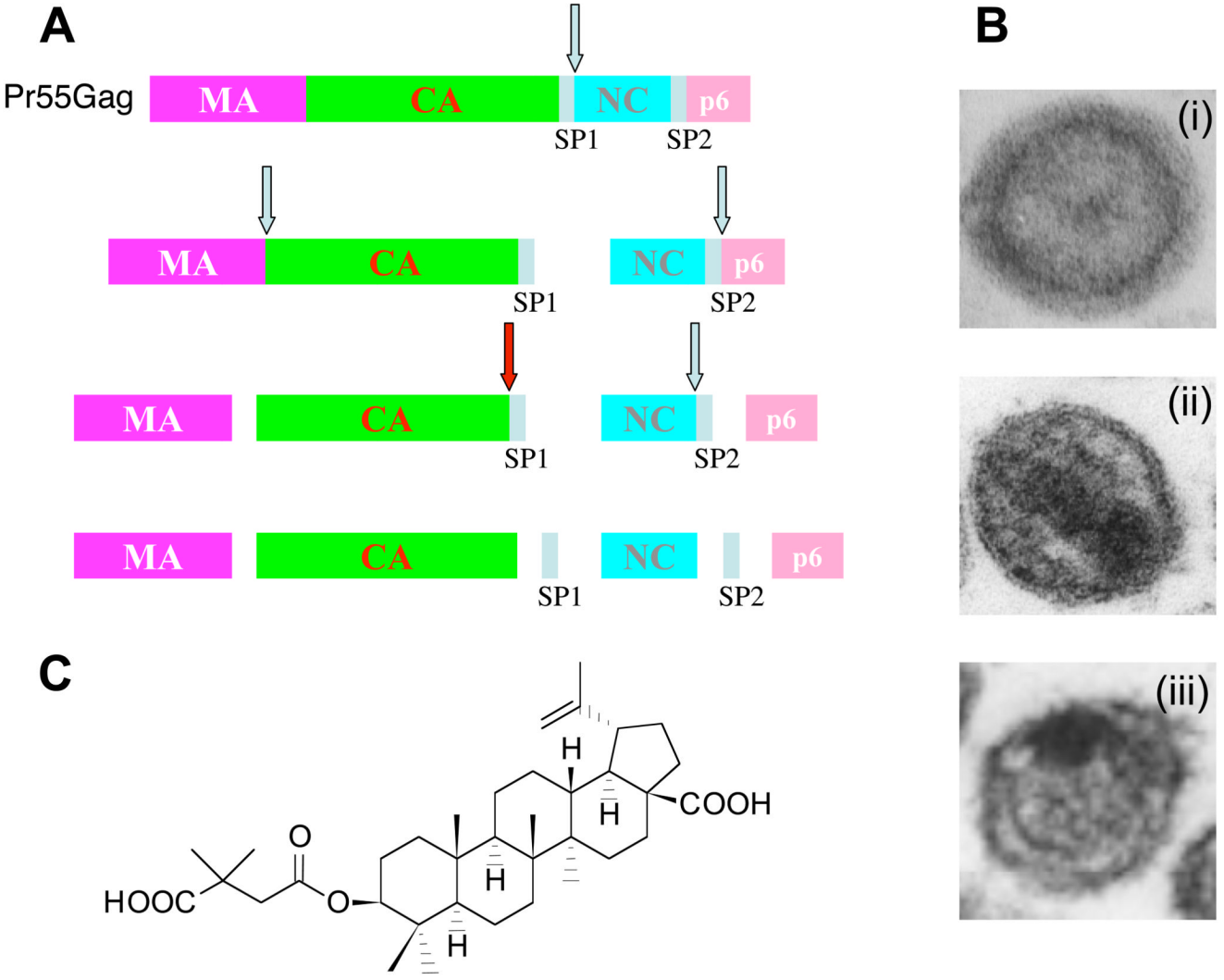
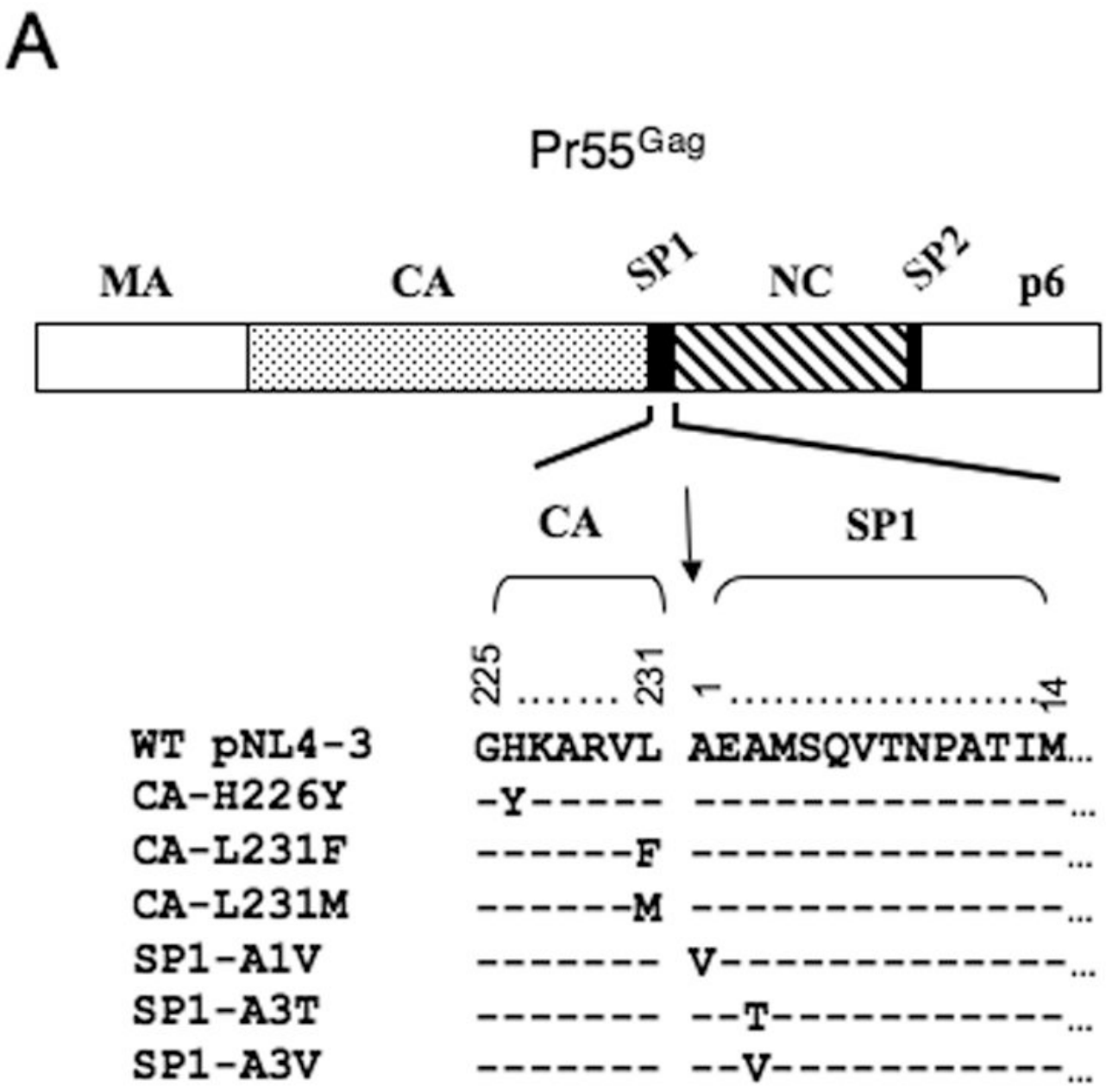


Figure 1.

(A) The HIV-1 Gag proteolytic processing cascade. The Gag polyprotein is shown at the top with the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domains and two spacer peptides SP1 and SP2 indicated. The order of processing events is depicted by the flow diagram, with arrows indicating PR cleavage sites. The red arrow denotes the cleavage event blocked by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (bevirimat, PA-457, DSB, MPC-4326). (B) Immature HIV-1 particle showing donut-like morphology (i); mature HIV-1 virion with condensed CA conical core (ii); HIV-1 virion produced in the presence of bevirimat, showing aberrant core and crescent of electron density inside the viral lipid bilayer (iii). (C) Chemical structure of bevirimat. Reprinted from Drugs Discovery Today, Vol 13 (9/10), Catherine S Adamson and Eric O Freed, Recent progress on antiretrovirals – lessons from resistance, pages 424–432, Copyright (2008), with permission from Elsevier (18).



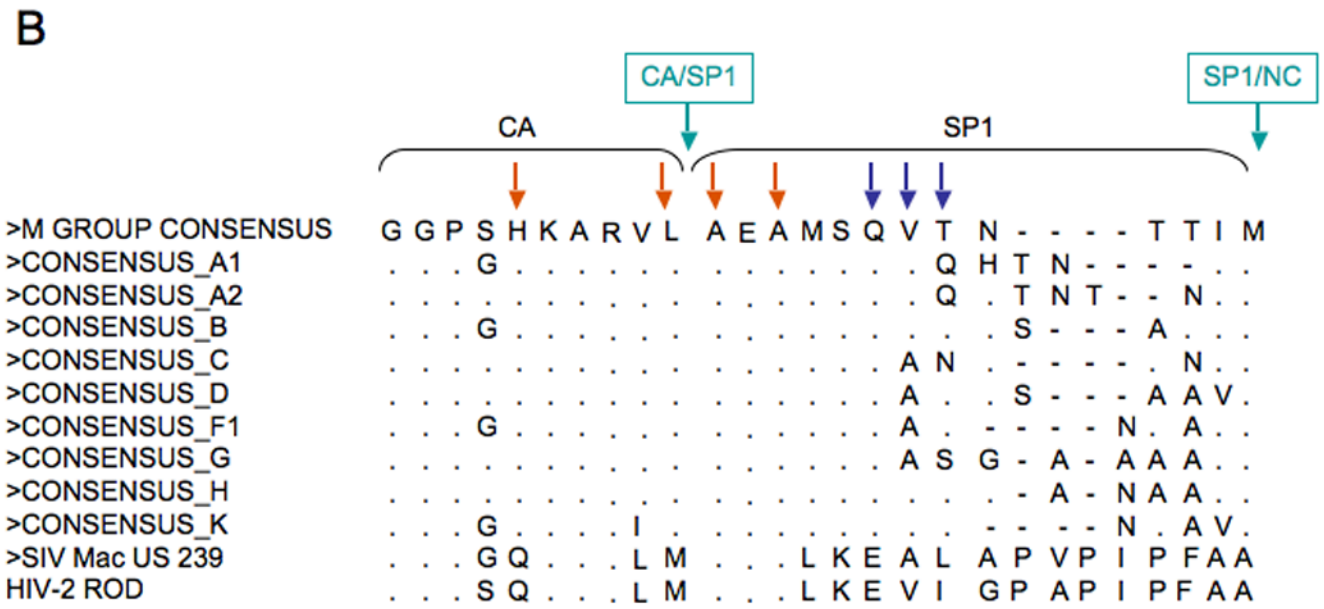


Figure 2. Location of bevirimat-resistance mutations

(A) Gag is represented at the top, with MA, CA, SP1, NC, SP2 and p6 indicated. The alignment shows a panel of six single-amino-acid substitutions that independently confer bevirimat resistance. The mutations, which were selected in vitro (Adamson 2006), cluster at or near the CA-SP1 cleavage site (arrow). (B) Amino acid sequence alignment across the CA-SP1 boundary region. The alignment was constructed from the 2004 Los Alamos HIV-1 sequence database group M consensus sequences, HIV-2 ROD and SIVmac239 (123) http://www.hiv.lanl.gov. Green arrows indicate PR cleavage sites, red arrows indicate amino acid positions to which bevirimat resistance maps *in vitro* and blue arrows indicate polymorphic amino acid positions correlated with variable clinical responses to bevirimat in HIV-1 infected patients. Adapted and Reprinted from Journal of Virology, Copyright (2006), vol 80 (22), pages 10957–10971 DOI:10.1128/JVI.01369-06 and reproduced/ amended with permission from American Society for Microbiology (50).

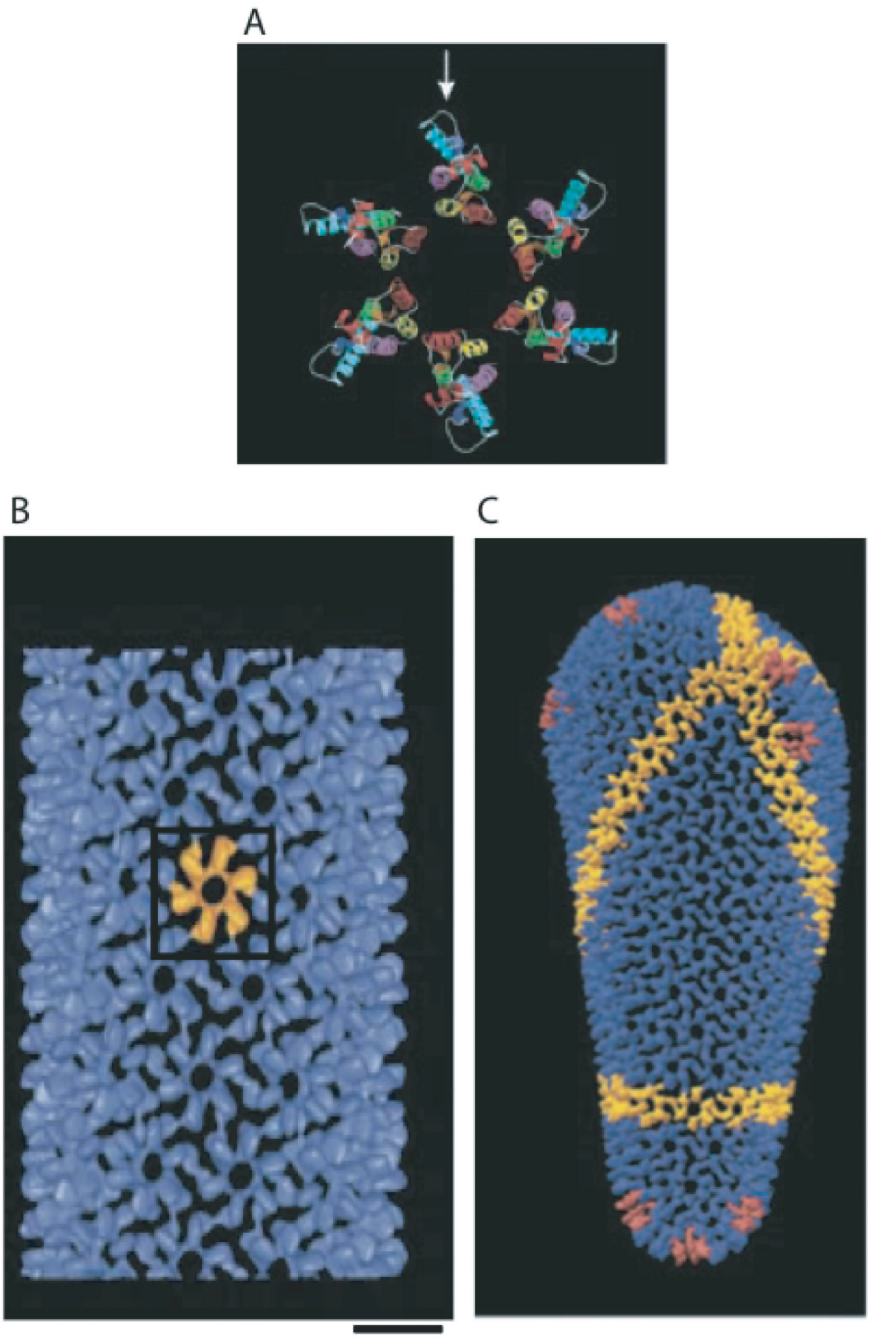


Figure 3.

Image reconstructions of helical assemblies of HIV-1 CA. (A) Molecular model of the hexameric ring formed by CA_{NTD}. Structural features: Cyclophilin A-binding loop (arrow), β-hairpin (orange), helix 1 (red), helix 2 (yellow), helix 3 (green), helix 4 (cyan), helix 5 (dark blue), helix 6 (red), helix 7 (pink). (B) Exterior view of the assembled tube structure showing a single hexamer (yellow) and the hexagonal CA lattice (blue). Scale bar = 100 Å (C) Model of an HIV-1 conical core. A continuous line of hexamers is highlighted in yellow and pentamers are shown in pink. Adapted with permission from Macmillam Publishers Ltd: [Nature], Vol 407, Su Li, Christopher P. Hill, Wesley I. Sundquist & John T. Finch. Image reconstructions of helical assemblies of the HIV-1 CA protein, pages 409–413, Copyright

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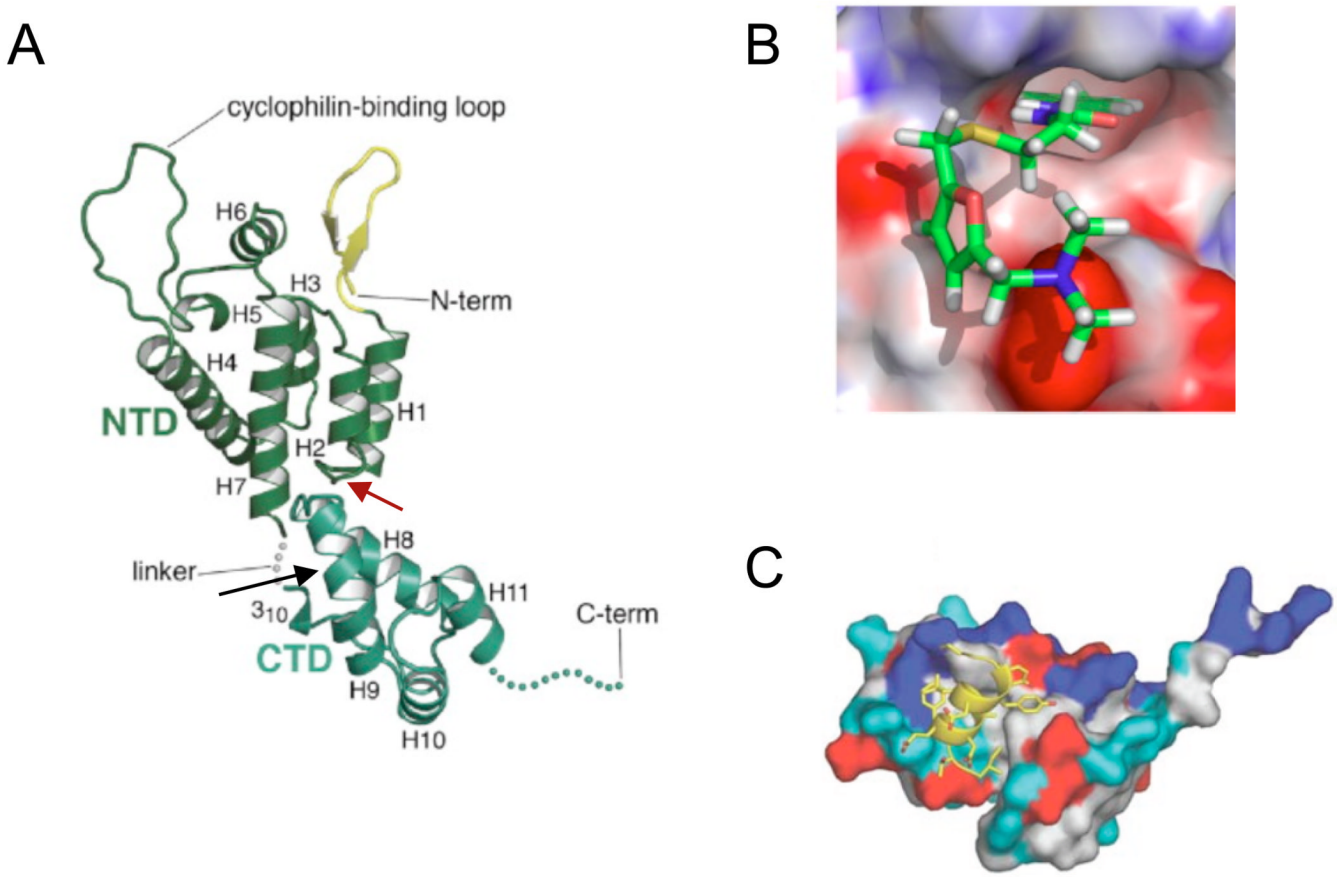

Figure 4. Binding sites of CA-based maturation inhibitors

(A) Structure of monomeric CA with the CA_{NTD} (green) and CA_{CTD} (blue) depicted and separated by the flexible linker region. The C-terminus is indicated as a dashed line to represent its disordered nature in crystal structures. Secondary structural features are labeled: N-terminal β-hairpin (yellow), cyclophilin loop and helices 1– 11 (H1-11). The site of binding of CAP1 is shown with a red arrow; the binding site of CAI/NYAD-1/NYAD-13 is indicated by a black arrow. Reprinted from Current Opinion in Structural Biology, vol 18. Barbie K Ganser-Pornillos, Mark Yeager and Wesley I Sundquist, The structural biology of HIV assembly, pages 203–217, Copyright (2008), with permission from Elsevier (28). (B) Electrostatic representation of CAP-1 bound to CA_{NTD}. The aromatic ring of CAP-1 is shown inserted into the pocket vacated by Phe32 upon CAP-1 binding. Reprinted from Journal of Molecular Biology, Vol 378, Brian N. Kelly, Sampson Kyere, Isaac Kinde, Chun Tang, Bruce R. Howard, Howard Robinson, Wesley I. Sundquist, Michael F. Summers and Christopher P. Hill, Structure of the antiviral assemble inhibitor CAP-1 complex with the HIV-1 CA Protein, pages 355–366, Copyright (2007) with permission from Elsevier (112). (C) Electrostatic representation of CAI bound to CA_{CTD}. CAI in a helical conformation is shown bound to the hydrophobic groove (white). Reprinted with permission from Ternois et al., 2005 (114).