



## REVIEW

# New Approaches for Antiviral Targeting of HIV Assembly

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The pressing need to develop antivirals active against resistant strains of HIV-1 has led to efforts to target steps in the virus life cycle other than reverse transcription and Gag proteolysis. Among those steps are entry, integration, and assembly and/or maturation. Advances in understanding the structural biology of both the immature and the mature forms of the HIV capsid have made it possible to design or discover small molecules and peptides that interfere with both assembly and maturation. Here, we review the current state of the art in assembly and maturation inhibitors.

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## Introduction

Roughly 20 years ago, Salunke *et al.* observed that under a particular set of solution conditions, the subunits of polyoma virus capsid protein assembled into octahedra.<sup>1</sup> They suggested that “If, in fact, the variant octahedral structures occur naturally, they could be stabilized by a tetrameric reagent designed to bind selectively to the four unoccupied VP, bonding sites facing each fourfold axis. Such a reagent might interfere with virion assembly without impeding normal cell functions. Thus, further studies on conditions for stabilizing octahedral assemblies of papovavirus capsomeres could lead to applications for blocking assembly of infectious papillomaviruses.” Two years later, Teschke *et al.* demonstrated that the small hydrophobic molecule bis-ANS could block *in vitro* assembly of bacteriophage P22 through binding to the capsid protein with a micromolar  $K_d$ .<sup>2</sup> The compound did not appear to significantly alter the protein conformation, and it was therefore suggested that binding at intersubunit interfaces directly

inhibited assembly. Subsequent studies suggested that the mechanism was actually the promotion of subunit association into assembly inactive dimers.<sup>3</sup> The recognition that small-molecule inhibition of capsid assembly was possible led to mathematical modeling of the process.<sup>4</sup> A key insight was the recognition that it was not necessary to completely prevent subunit–subunit association. Modeling suggested that it was sufficient and perhaps preferable to misdirect the assembly pathway, resulting in the accumulation of nonviable aberrant forms of capsids that would act as “sinks” for large numbers of subunits. Viewed in this way, viral capsids represent a single target containing hundreds to thousands of identical potentially inhibitory binding sites. Substantial progress has been made over the past 10 years in applying these concepts to targeting hepatitis B assembly.<sup>5–7</sup>

Despite the success of HAART (highly active antiretroviral therapy) in treating HIV-1 infections, the emergence of resistance drives a pressing need to develop new antivirals. Cross-resistance, a phenomenon in which development of resistance to one particular therapeutic concurrently results in development of resistance to other agents in that class, suggests the need for not only novel compounds but also compounds active against novel targets. While the capsids of polyoma, bacteriophage P22, and hepatitis are icosahedral, the fullerene core of the

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Abbreviations used: CTD, C-terminal domain; NTD, N-terminal domain; EM, electron microscopy.

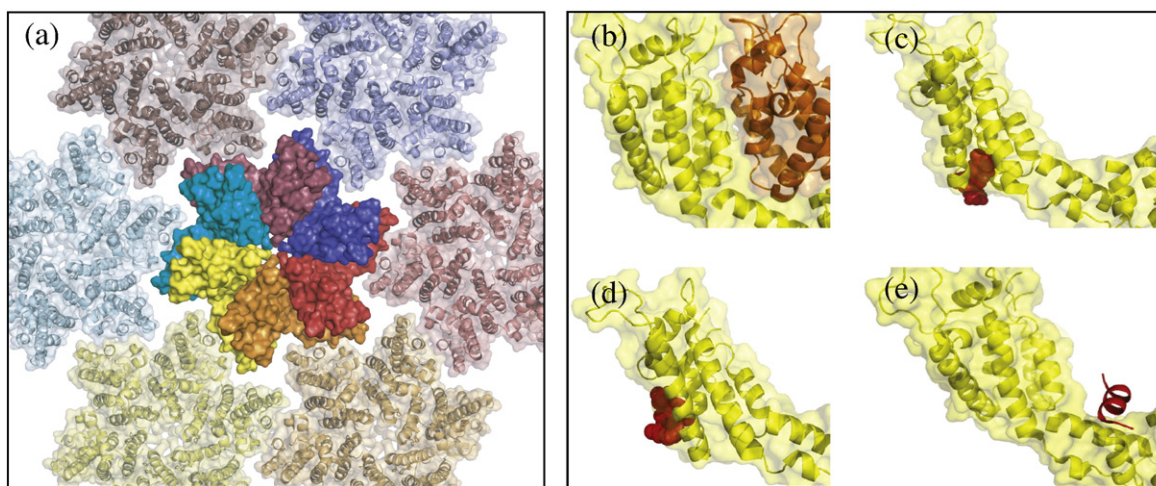
mature HIV particle and the striking rearrangement of subunit interactions during the transition from immature to mature lattice suggest the possibility of identifying compounds that target either or both immature assembly and maturation. Central to this strategy is the fact that mutational studies indicate that not only Gag cleavage but also proper core formation is required for infectivity.<sup>8</sup> Malformed cores appear to be defective at the stage of reverse transcription,<sup>9,10</sup> and, in fact, mutations as subtle as those that increase or decrease core stability result in a loss of infectivity.<sup>11</sup> Thus, it seems reasonable that compounds that alter the assembly pathway or the stability of the viral core would exhibit antiviral effects.

### Targeting the N-terminal Domain of HIV Capsid Protein

In proof-of-concept experiments, the entire C-terminal domain (CTD) of CA or even simply, a peptide derived from helix nine, the helix driving CTD dimerization, was shown to inhibit polymerization of CA into helical tubes<sup>12</sup> (M. G. Mateau, personal communication). However, the first small-molecule inhibitors of HIV assembly were the compounds CAP-1 and CAP-2 reported by Tang *et al.*<sup>13</sup> Their

discovery was based on a computational screen of public domain compounds for molecules that could bind to clefts on the N-terminal domain (NTD) of CA (CA<sup>N</sup>). NMR titration experiments determined that CAP-1 bound to CA<sup>N</sup> with a  $K_d$  of 1 mM and CAP-2 with a  $K_d$  of 52  $\mu$ M. Mapping of the binding site by NMR perturbation experiments indicated that both compounds bound at the same site, the apex of a helical bundle composed of helices 1, 2, 3, 4, and 7 (Fig. 1c). This region has been demonstrated to be involved in an intersubunit CA NTD–CTD interaction unique to the mature lattice (Fig. 1b), suggesting that CAP compound binding might act to block the formation of this interaction.<sup>14–16</sup> The structure of CA<sup>N</sup> crystallized in the presence of CAP-1 indicated that CAP-1 binding induces a conformational rearrangement in CA<sup>N</sup> that resulted in the formation of a hydrophobic docking pocket.<sup>17</sup> Despite the fact that the CAP-1 compound itself was not seen in the crystal structure, computational docking experiments generated a docking model consistent with all X-ray and NMR data in which CAP-1 docking forces the exposure of a buried phenylalanine group (Phe32). The energetic penalty of exposing a hydrophobic Phe residue might be offset by relief of a strained main-chain conformation seen in the unliganded CA<sup>N</sup>.

Both CAP-1 and CAP-2 block *in vitro* assembly of CA into helical tubes that have been demonstrated



**Fig. 1.** Intersubunit interactions and inhibitor binding sites in HIV-1 capsid protein. (a) Structure of the mature CA hexamer. One full hexamer is shown in space filling form, and six surrounding hexamers are shown as ribbon diagrams. The individual subunits of the full hexamer are displayed with different colors. Note how the CTD of one subunit sits underneath the NTD of the adjacent (counterclockwise) subunit. (b) Structure of two interacting CA subunits extracted from the mature HIV-1 hexamer. The NTD of both subunits is pictured. The CTD of the yellow subunit contacts the NTD of the adjacent (orange) subunit. This NTD–CTD interaction is unique to the mature form. (c) CAP-1 (red) is a small organic compound that binds to the NTD of HIV-1 CA and prevents assembly of the mature lattice, presumably by interfering with the NTD–CTD interaction. (d) PF-74 (red) is a small organic compound that binds to the NTD of HIV-1 CA and destabilizes cores. (e) CAP-1 (red) is a small  $\alpha$ -helical peptide that binds to the CA CTD and prevents assembly of both the immature and mature forms. The compounds were docked into subunits extracted from the hexamer crystal structure (PDB code 3MGE) by alignment of overlapping regions using domain/compound co-crystal structures (PDB code 2JPR for CAP-1, 2XDE for PF-74, and 2BU0 for CAI) and hence do not reflect conformational changes induced by compound docking. The viewpoint varies slightly from panel to panel to best illustrate the relevant interaction.

to recapitulate the intersubunit interactions seen in the mature virus.<sup>14,15</sup> CAP-2 proved to be cytotoxic, but 100  $\mu\text{M}$  concentrations of CAP-1 were nontoxic and resulted in greater than 95% inhibition of virus replication in cell culture.<sup>13</sup> Mechanism of action studies demonstrated that CAP-1 treatment did not reduce the amount of virus produced or the incorporation of envelope glycoprotein (Env). CAP-1 itself was not virucidal. Morphological analysis indicated that virions produced in the presence of CAP-1 were more heterogeneous in size than those produced in its absence and displayed aberrant core morphology. Together, these results indicate that CAP-1 functions to block the subunit–subunit interactions formed during maturation. While no direct measurements of the number of CAP-1 molecules bound per virion have been made, on the basis of the measured CAP-1–CA<sup>N</sup> affinity, it is estimated that as few as ~25 molecules of CAP-1 per particle are sufficient to inhibit the formation of a functional core particle, an observation consistent with the concept of considering the capsid as a single target with multiple binding sites.

These findings paved the way for subsequent efforts to screen for small-molecule inhibitors of HIV-1 assembly and/or maturation. A screen of 10,000 compounds using an *in vitro* mature lattice assembly assay led to the identification of approximately 100 inhibitors of assembly with an average IC<sub>50</sub> of 10  $\mu\text{M}$ .<sup>18</sup> Of these 100 compounds, 6 displayed favorable selectivity indices in cell culture, and mechanism of action studies suggested a subset selectively blocked maturation (P.E.P., unpublished results).

Larger-scale screens have been carried out in the pharmaceutical industry. Boehringer-Ingelheim recently reported screens using an *in vitro* assembly system that resulted in the identification of several classes of small molecules with antiviral activity. Optimization of two of these compounds led to compounds with an EC<sub>50</sub> <100 nM and low cytotoxicity. These compounds bound the CA<sup>N</sup> and induced the formation of a binding pocket that overlapped the binding pocket for CAP-1. Electron microscopy (EM) studies of virus produced under treatment conditions showed dramatically altered core morphology. Although these observations are consistent with their acting to block subunit–subunit interactions required for maturation, mechanistic studies indicated that some of the compounds had a dual mode of action, acting as both capsid assembly inhibitors and non-nucleoside inhibitors of reverse transcriptase. However, it was possible to drive optimization of these compounds toward assembly inhibition as the single mode of action.<sup>19</sup> Passage of virus in the presence of these inhibitors led to the appearance of escape mutants; the mutations were primarily localized to the NTD

near the binding pocket, although some CTD escape mutations were also seen.

In a search for novel antiviral inhibitors of HIV replication, Pfizer developed and employed a cell-based screen that required completion of the entire viral replication cycle to screen 10<sup>6</sup> compounds.<sup>20</sup> Among the compounds with antiviral activity discovered was a series of compounds that appear to interfere with both assembly and uncoating. The preferred compound, known as PF-74, bound CA with a  $K_d$  of 3  $\mu\text{M}$  and had an EC<sub>50</sub> of 0.6  $\mu\text{M}$  and a therapeutic index of 121 when tested against HIV NL4-3 in MT-2 cells.<sup>21</sup> The compound was co-crystallized with CA<sup>N</sup> and, unlike CAP-1, did not induce a conformational change in the protein. Rather, it bound in a preformed pocket defined by helices 3, 4, 5, and 7 with the indole group protruding into the NTD–CTD intersubunit interface, a location in which it might act to prevent mature core formation (Fig 1d). Morphological analysis of particles produced in the presence of PF-74 bear this out, as they appear highly heterogeneous and lack the central conical core.<sup>21</sup> Selection of escape mutants resulted in strains carrying mutations in or near the compound binding pocket. Multiple mutations were required to reach a high level of resistance to the compound. Interestingly, there was no obvious decrease in fitness associated with the escape mutations.

In addition to acting late in the replication cycle to block assembly, PF-74 was able to act early by altering post-entry uncoating. It is well documented that the stability of the core is a key determinant of infectivity. Cores that are either too stable or too unstable show reduced infectivity. Single-cycle infectivity experiments suggested that PF-74 was capable of acting early during infection, and this was borne out when virus that was incubated with PF-74 was shown to be capable of entry but incapable of undergoing reverse transcription. Experiments to address the mechanism of inhibition took advantage of both *in vitro* and *in vivo* assays of core stability.<sup>22</sup> PF-74 bound to preformed mature HIV particles but as expected was incapable of binding to the escape mutant. Cores purified from PF-74-treated particles displayed a dose-dependent decrease in stability when analyzed by sucrose gradient sedimentation, whereas the cores derived from the escape mutant were unaffected by compound treatment. Similar results were found when naked cores (rather than viral particles) were treated with PF-74. To ascertain whether PF-74 destabilized cores *in vivo*, VSV-G pseudotyped particles were used to infect cells in the presence or absence of PF-74. Four hours after infection, the cells were lysed and the fraction of soluble and pelletable CA was determined. Once again, treatment with PF-74 increased the extent of dissociation of wild-type cores but

had no effect on the escape mutants. Finally, the effect of PF-74 on CA mutants known to result in hyperstable or destabilized cores was tested. Hyperstabilized cores were insensitive to PF-74, while intrinsically destabilized cores proved more sensitive. Together, these studies provide compelling evidence that PF-74 interferes with protein–protein interactions in the mature capsid and that this interference can result in failed maturation or premature post-entry core dissociation.

## Targeting the CTD of HIV Capsid Protein

While the compounds described above bind to the NTD and inhibit the assembly or the stability of the mature capsid lattice, a peptide that inhibits the assembly of both the immature and the mature form has been identified using phage display techniques. Sticht *et al.* panned random 12-mer phage display libraries against the entire CA molecule or a protein comprising the CTD, SP1, and NC regions of Gag (C-CANC).<sup>23</sup> They were able to identify 16 different peptides capable of binding to both CA and C-CANC. These 16 peptides could be classified into four groups based on sequence similarity, with one group being dominant. Several synthetic peptides based on the discovered sequence were tested for binding, and one peptide, called CAI, was selected for further study on the basis of its favorable solubility profile. The authors demonstrated the ability of a fivefold molar excess of CAI to inhibit the *in vitro* assembly of a Gag-derived protein lacking p6 and residues 16–99 of MA ( $\Delta$ MACANCSP2), which assembles into immature like particles. In addition to inhibiting immature assembly, the peptide was also capable of effectively inhibiting the assembly of CANC or CA only into tubes that reflect the mature capsid lattice, thereby displaying dual function. The interaction of CAI with CA<sup>C</sup> was mapped by deletion analysis, NMR experiments, and, ultimately, a co-crystal structure.<sup>23,24</sup> The CAI peptide adopts an  $\alpha$ -helical conformation and inserts in a groove between helices 1, 2, and 4 of the CTD (Fig. 1e). Binding is mediated primarily by hydrophobic interactions, and binding of the peptide induces a conformational change in the N-terminus of helix 2 that alters the CTD–CTD dimer interface in a manner that roughly halves the buried surface area. However, NMR rotational correlation time measurements indicated that CAI binding does not disrupt CA<sup>C</sup> dimerization, leading to the suggestion that altered flexibility in the CTD might be the underlying cause of the assembly deficit. In a series of experiments whose outcome was surprising, using the co-crystal structure as a guide, a series of alanine substitutions were introduced into the CAI binding site on CA.<sup>25</sup> As expected, the mutations increased the  $K_d$  from

$\sim 1.6 \mu\text{M}$  as seen for the wild type to values as high as  $\sim 65 \mu\text{M}$ . While the mutations had no effect on the *in vitro* assembly of immature particles, most of them prevented the *in vitro* assembly of mature particles. When the mutations were introduced into an infectious clone, similar results were seen. By and large, the mutant viruses budded similar amounts of properly processed CA. However, mutations at two residues (Y169A and L211S) rendered the viruses noninfectious, and thin-section EM analysis revealed that the cores had failed to mature properly. The surprising finding was that the structure of the mutant CA in the absence of CAI was similar to the structure of the wild-type protein with CAI bound. The implication of this finding for their mechanism of inhibition is that CAI does not only inhibit maturation exclusively through steric occlusion, but may also function to lock the CTD in a conformation incompatible with maturation. In the case of inhibition of assembly of the immature form, the mechanism is apparently more subtle, as the mutations did not prevent immature core formation.

The CAI peptide itself was not capable of cell entry, and therefore its antiviral efficacy could not be directly tested. However, using the structure of the co-crystal as a guide made it possible to take a structure-based design approach to stabilize the  $\alpha$ -helical peptide and convert it to a form that could penetrate cells using an approach known as hydrocarbon stapling.<sup>26,27</sup> The resulting molecule was named NYAD-1. Fluorescently labeled NYAD-1 was capable of entering cells as determined by both FACS (fluorescence-activated cell sorting) analysis and confocal microscopy. Confocal microscopy demonstrated colocalization of NYAD-1 with Gag in transit to the plasma membrane.

When cells were treated with NYAD-1 and transiently transfected with HIV-1, the yield of virus was reduced and an intracellular buildup of Gag was detected. EM analysis indicated that the production of both immature and mature virus was inhibited in NYAD-1-treated cells, results consistent with the *in vitro* findings. In experiments in which virus was incubated with NYAD-1 and then used to infect cells, entry was not affected, but infectivity was reduced and the virus morphology was perturbed. These results suggest that NYAD-1 might also act as an early post-entry inhibitor.<sup>27</sup> An important consideration for any HIV antiviral is whether it is capable of broad-spectrum antiviral activity. NYAD-1 showed antiviral activity against multiple laboratory-adapted strains, a diverse collection of clinical isolates, and two RT-resistant HIV strains.

While the studies with CAI and NYAD-1 clearly demonstrated the therapeutic potential of targeting the hydrophobic pocket formed by helices 1, 2, and 4, small-molecule inhibitors are likely to represent more promising lead compounds than stapled

peptides. Toward this end, Curreli *et al.* performed a high-throughput virtual docking screen for compounds binding in the pocket and selected the 200 top-scoring compounds for further analysis from 100,000 compounds analyzed.<sup>28</sup> Based on visual analysis, they selected and purchased 8 compounds for testing, 2 of which showed antiviral activity and were deemed lead compounds. These compounds prevented mature but not immature assembly *in vitro*. When tested in cell culture, low micromolar concentrations of the compounds effectively suppressed the release of a broad range of laboratory-adapted and primary isolates of HIV, and the released particles were less infectious. These studies validate both the NTD and CTD of HIV-1 capsid protein as potential antiviral targets.

### Targeting the CA–SP1 Cleavage Site

A natural products screen identified betulinic acid as a weak inhibitor of viral replication,<sup>29</sup> and subsequent activity-based modification led to a more effective molecule called, alternatively, bevirimat, DSB, or PA-457. Bevirimat inhibits the *in vitro* replication of a variety of HIV-1 strains including those resistant to protease or reverse transcriptase inhibitors and does so with IC<sub>50</sub> in the nanomolar range. It is specific for HIV-1 and displays no activity against HIV-2 or SIV.

Bevirimat inhibition of viral replication occurs by a unique mechanism. In contrast to the protease inhibitors, which inhibit all Gag processing, bevirimat inhibits only the final cleavage event in Gag processing, cleavage at the CA–SP1 junction. Mutational studies have demonstrated that this cleavage event is necessary for maturation, and therefore for viral replication. Tomographic reconstructions of the immature virus suggest that the CA–SP1 of Gag forms a helical bundle that serves to anchor the CTD below the plane of the NTD. Maturation requires the upward translation of this region to form the NTD–CTD interactions that stabilize the mature hexameric lattice (see article by Yeager in this issue). Cryo-electron microscopy studies indicate that in the absence of this cleavage event, immature cores appear to have been disassembled but mature cores cannot be formed.<sup>30,31</sup> Hydrogen/deuterium exchange studies on virus like particles that do not undergo CA–SP1 cleavage suggest that extended order is lost but most of the local intersubunit interactions remain intact.<sup>32</sup> Bevirimat-treated particles, in contrast to the CA–SP1 cleavage mutants, appear to be incapable of disassembly and immature appearance. This observation suggests that bevirimat not only prevents CA–SP1 cleavage but also may stabilize the immature lattice.<sup>33</sup>

Passage of HIV-1 in the presence of increasing amounts of bevirimat led to the selection of escape

mutations that mapped to the CA–SP1 junction.<sup>34–37</sup> Three of the mutations mapped at the C-terminus of CA, and three resistance mutations occurred in SP1, one at the first residue and two at the third residue, suggesting that this region might correspond to the bevirimat binding site. Further support for this model is the fact that binding studies using radiolabeled bevirimat showed that it bound to the immature core but was incapable of binding to either monomeric protein or the mature core, suggesting that the binding site required subunit multimerization and was destroyed by CA–SP1 cleavage. Biochemical studies using labeled bevirimat indicated that it bound less well or not at all to strains carrying the CA–SP1 junction bevirimat escape mutations. Finally, moving the HIV-1 CA–SP1 region into HIV-2 rendered it susceptible to bevirimat inhibition.<sup>38</sup> While this evidence strongly supports the hypothesis that bevirimat binds to the CA–SP1 region, the inability to obtain a high-resolution structure of the immature virus with or without bevirimat bound limits the depth of mechanistic understanding or our ability to rationally design small molecules that target this presumptive binding site.

Bevirimat is the only assembly or maturation inhibitor to undergo clinical testing and development to date. The compound showed promise in a mouse model, and promising phase I clinical trial data led to testing in HIV-infected individuals.<sup>39–43</sup> These phase II trials produced mixed results. Some patients responded well, displaying an average of 1.26 log decrease in viral load. Others displayed no significant decrease in viral load and were classified as nonresponders. Genotypic analysis suggested that poor response was associated with baseline polymorphisms in residues 6–8 of SP1, and these observations were borne out during *in vitro* testing of bevirimat susceptibility using genotyped patient isolates.<sup>44</sup> It is noteworthy that this is not the region where escape mutations occurred during *in vitro* selection experiments.

### Future Prospects

Despite the success of current antiretroviral therapies, selection toward resistance remains a significant problem and drives the need for new antivirals that are ideally directed toward new targets. The complexity and delicate balance of HIV-1 viral protein interactions with both viral and host proteins suggest that these interactions could make attractive antiviral targets, and agents that stabilize or destabilize them might prove effective. While protein–protein interactions have long been considered unattractive targets, the recognition of intersubunit “hot spots” responsible for the bulk of the binding energy and some recent successes

suggest that this approach is tractable.<sup>45–47</sup> Recent advances in understanding the structural biology of HIV coupled with the promising results presented in this review suggest that it will be possible to identify therapeutically useful compounds that interfere with HIV-1 assembly and maturation.

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