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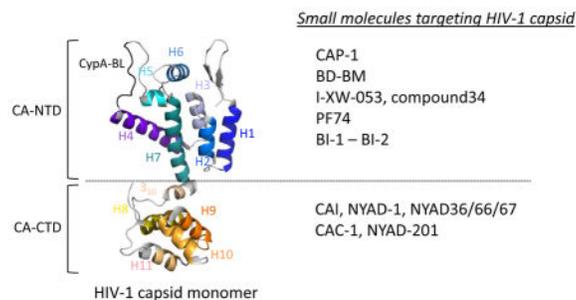
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Abstract

The infectious human immunodeficiency virus (HIV) particle is characterized by a conical capsid that encloses the viral RNA genome. The capsid is essential for HIV-1 replication and plays crucial roles in both early and late stages of the viral life cycle. Early on, upon fusion of the viral and cellular membranes, the viral capsid is released into the host cell cytoplasm and dissociates in a process known as uncoating, tightly associated with the reverse transcription of the viral genome. During the late stages of viral replication, the Gag polyprotein, precursor of the capsid protein, assemble at the plasma membrane to form immature non-infectious viral particles. After a maturation step by the viral protease, the capsid assembles to form a fullerene-like conical shape characteristic of the mature infectious particle. Mutations affecting the uncoating process, or capsid assembly and maturation, have been shown to hamper viral infectivity. The key role of capsid in viral replication and the absence of approved drugs against this protein have promoted the development of antiretrovirals. Screening based on the inhibition of capsid assembly and virtual screening for molecules binding to the capsid have successfully identified a number of potential small molecule compounds. Unfortunately, none of these molecules is currently used in the clinic. Here we review the discovery and the mechanism of action of the small molecules and peptides identified as capsid inhibitors, and discuss their therapeutic potential.

Graphical Abstract



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Keywords

HIV-1; capsid; inhibitors; small molecules; assembly and maturation; uncoating

1- Introduction

Worldwide the human immunodeficiency virus (HIV-1) affects more than 35 million people. Over the past decades, antiretroviral (ARV) therapy has made remarkable strides improving the quality-of-life of infected individuals. However, viral resistance to therapy continues to develop, highlighting the need for novel classes of ARVs directed at alternative targets. From the currently 28 FDA-approved drugs used in ARV therapy, none of them directly inhibits viral capsid stability [1]. Given that the proper capsid assembly and disassembly so finely regulates HIV-1 replication, it represents an emerging and very attractive target for drug development.

HIV-1 capsid plays a critical role in both early and late stages of the viral replication cycle. Early on, soon after viral entry and fusion of the viral and target cell membranes, the capsid core is released into the cytoplasm and disassembles by a process referred to as uncoating. Although uncoating is not completely understood, it is tightly connected with reverse transcription of the viral genome and subsequently with the nuclear import of viral DNA [2–4]. Functional studies of HIV-1 variants by site-directed mutagenesis revealed that capsid stability is essential for efficient reverse transcription and infectivity [5–7]. Moreover, cellular restriction factors, such as Tripartite motif 5-alpha (TRIM5 α), which promotes a rapid and premature disassembly of viral capsids [8–11], and the interferon-inducible MxB protein, which prevents uncoating by stabilizing the HIV-1 core during infection [12–15], have been shown to severely impair HIV-1 replication. These two restriction factors reinforce the notion that targeting the capsid protein can result in potent blocks to viral replication. During the late stages of the viral life cycle, capsid assembly and maturation are essential for the formation of infectious viral particles. Mutagenesis studies have confirmed that the proper assembly and morphogenesis of viral particles are essential for HIV-1 infectivity [7, 16–20].

The viral capsid is synthesized from the precursor Gag polyprotein, which is composed of four independently folded structural domains separated by flexible regions (matrix, capsid, nucleocapsid and p6) and two spacer peptides (SP1 and SP2) (Fig 1A). These proteins play different roles: the N-terminus, corresponding to the matrix domain (MA), facilitates the binding of Gag to the plasma membrane and recruits the viral Env protein. The central domain corresponds to the capsid (CA). This domain mediates Gag-Gag interaction and CA-CA interaction to form the immature and mature lattices, respectively. The third domain, the nucleocapsid domain (NC), is composed of two zinc fingers, which bind to the viral RNA genome during assembly. Finally, the C-terminus domain, corresponding to the p6 region, contains two short sequence motifs (termed “late assembly domain”), that bind to TSG101 and ALIX proteins, members of the cellular ESCRT machinery essential for the budding process. The two spacer peptides, SP1 and SP2, located between CA and NC domains for SP1, and NC and p6 domains for SP2, help regulate the conformational changes that occur during viral maturation (reviewed in [21]). Upon budding, the viral particles released from

the host cell are immature and characterized by a layer of unprocessed Gag polyproteins beneath the plasma membrane (Fig 1B). Concomitantly with the budding, the Gag precursor undergoes a maturation process by the viral protease. The separation of the different domains of Gag triggers structural rearrangements, followed by CA protein oligomerization to form infectious viral particles characterized by a mature fullerene-like conical capsid (Fig 1C). The CA protein is composed of two independently folded domains connected by a flexible linker: a N-terminal domain (CA-NTD; residue 1-145) and a C-terminal domain (CA-CTD; residues 151-231). Both domains have predominantly a α -helical structure with CA-NTD composed of 7 α -helices (CA helices 1 – 7), and CA-CTD of a short 3_{10} -helix and 4 α -helices (CA helices 8 - 11) (Fig 1D).

The structures of the full-length capsid [22–26], NTD [27] and CTD [28–30] domains, as well as the immature [31–33] and mature [24, 25, 34, 35] hexameric capsid have been studied by crystallography, cryo-electron microscopy or nuclear magnetic resonance (NMR). Although the immature and mature lattices are formed of hexamers (and in the mature capsid, hexamers and pentamers), several different interactions are required between the different CA subunits to allow proper folding. The immature virions contain approximately 2,500 copies of the Gag protein, which assemble into a spherical shell immediately beneath the viral envelope. The Gag molecules are extended and oriented radially, with the N-terminal MA domain bound to the inner membrane leaflet and the C-terminal p6 domain facing the interior of the particle. The Gag-Gag lattice interactions in the immature virion are mediated primarily by CA and SP1. The immature lattice is formed by a layer of CA-NTD hexameric ring surrounding a CA-CTD hexameric ring, stabilized by a six-helix bundle of SP1 peptide. Hexamers are linked one another by CA-NTD/CA-NTD and CA-CTD/CA-CTD interactions [31–33, 36] (Fig 1E). After the maturation process by the viral protease, Gag polyprotein is cleaved and the capsid assemble to form a fullerene-like conical shape. The mature capsid is composed of approximately 1,500 capsid monomers, organized in 250 hexamers, with 12 pentamers incorporated to allow closing of the structure. Hexamers are formed by both CA-NTD/CA-NTD and CA-NTD/CA-CTD interactions between adjacent CA around a 6-fold axis, and the hexameric lattice is formed by CA-CTD/CA-CTD interaction at the 2- and 3-fold axes [24, 25, 34, 35] (Fig 1F). In solution, HIV-1 CA can dimerize with a dissociation constant (K_d) of 18 μ M. This weaker interaction between CA dimers may reflect the ability of both the immature and mature capsid lattice to establish interactions with different protein surfaces during the uncoating and assembly processes. This dimerization is dependent on residues in helix 9 of the CA-CTD, Trp184 and Met185 [29]. Mutation of these residues to alanine interferes with CA assembly *in vitro* and abolishes viral infectivity, confirming that this interface is essential for efficient assembly of both the mature and immature capsid lattice [26, 29, 37, 38].

The importance of the viral capsid as an attractive target for drug development was reinforced over the past years by the identification of several capsid inhibitors. So far, only one of these molecules, Bevirimat, went into phase II clinical trials. Bevirimat is a maturation inhibitor that inhibits the final step of Gag processing, resulting in the production of immature non-infectious viral particles. Even if a natural viral polymorphism allows natural resistance to this small molecule, a significant decrease in viral load was observed in

Bevirimat treated HIV-1 infected patients [39]. This clinical trial demonstrated the usefulness of capsid inhibitors in ARV therapy. This review will focus on the discovery and mechanism of action of small molecules and peptides identified as capsid inhibitors, and discuss their therapeutic potential.

2. Small molecules and peptides capsid inhibitors

2.1. Small molecules targeting CA-NTD (Table 1)

2.1.1. CAP-1—CAP-1 and CAP-2 are the first small molecules identified as inhibitors of HIV-1 capsid. These compounds were identified from a computational screen of chemical libraries for molecules that could bind to clefts on the CA-NTD domain [40]. While CAP-2 was toxic to cells, CAP-1 was shown to reduce by 95% HIV-1 replication at a concentration of 100 μ M in U1 cells. CAP-1 targets a late-phase viral event by inhibiting CA-CA interactions during virus assembly and maturation [40]. ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) NMR analysis revealed that CAP-1 binds with modest affinity to CA-NTD, with a dissociation constant (K_d) around 800 μ M. X-ray crystallography and NMR spectroscopy data revealed that binding of CAP-1 induces conformational rearrangements leading to the formation of a deep hydrophobic cavity that serves as the ligand binding site. This hydrophobic pocket is formed by helices 1, 2, 3, 4 and 7 at the base of the CA-NTD domain. Residues that exhibited the greatest chemical shift changes appear to form part of the CA-NTD/CA-CTD interface. Thus, CAP-1 seems to inhibit mature capsid assembly by inhibiting intersubunit CA-NTD/CA-CTD interaction [40, 41]. A recent study from Fricke *et al.*, revealed that CAP-1 does not affect the stability of HIV-1 preassembled CA-NC complex *in vitro*, suggesting that once the hexameric lattice is formed, CAP-1 is unable to bind CA [42].

2.1.2. Benzodiazepines and benzimidazoles series compounds

The BD (benzodiazepines – BD1-BD4) and BM (benzimidazoles – BM1-BM5) inhibitor series target a binding site on HIV-1 CA similar to CAP-1. These inhibitors were identified from screening a Boehringer Ingelheim corporate compound collection, using an *in vitro* capsid fluorescence assembly assay based on the association of CA-NC subunits on immobilized oligonucleotides [43]. The most potent inhibitor of each series, BD1 and BM1, displayed an EC_{50} of 70 ± 30 nM ($\text{CC}_{50} > 28$ μ M) and 62 ± 23 nM ($\text{CC}_{50} = 20$ μ M), respectively. The binding affinity of compounds BD2, BM2 and BM3 with CA-NTD was determined by isothermal titration calorimetry (ITC), and revealed a K_d between 87 and 690 nM. NMR spectroscopy and X-ray crystallography studies of compounds BD3 and BM4 in complex with recombinant CA-NTD showed that both series of inhibitors bind to CA-NTD. This association induces the formation of a pocket that strongly overlaps with the binding site for the previously reported CAP-1 inhibitor, although there are differences in the interaction between these compounds and the protein. Virus passaged in the presence of two representative compounds from each series selected resistance mutations that map to highly conserved residues surrounding the inhibitor binding pocket, but also the CA-CTD domain, indicating that these compounds may interfere with the formation of CA-NTD/CA-CTD interface. Although both compounds bind to the same binding site and act at the late stage of the viral life cycle, they display distinct activities on capsid assembly and maturation. BD

compounds have been shown to inhibit Gag assembly and prevent virion release, while BM compounds allow virus budding, but inhibit the formation of the mature capsid [43].

Using the above mentioned capsid assembly assay, the Boehringer Ingelheim group identified other capsid inhibitors. A Benzodiazepine hit, 1,5-dihydrobenzo[*b*] [1,4]diazepine-2,4-dione, has been shown to inhibit capsid assembly *in vitro* with an IC₅₀ of 1.4 μM and inhibits HIV-1 replication with an EC₅₀ of 17 μM. NMR spectroscopy and X-ray crystallography data revealed that this compound binds to the CA-NTD domain [44]. Two benzimidazole compounds have also been identified. The first compound, 5-(5-furan-2-ylpyrazol-1-yl)-1*H*-benzimidazole, displays an IC₅₀ of 6 μM in capsid disassembly assays but has no detectable antiviral activity. Optimization of this compound improved its inhibitory activity to an EC₅₀ of 27 nM. The X-ray crystallography structure with one of the derivative compound revealed that this inhibitor induces the formation of a pocket at the base of the CA-NTD domain. However, this series of capsid inhibitor has been halted due to poor metabolic stability and pharmacokinetic profile [45]. The second benzimidazole compound identified, named compound 1, displays an IC₅₀ of 6.1 μM in capsid assembly assays and a binding affinity to CA-NTD of 43 ± 2.9 μM. Optimization of this compound has led to an improvement of capsid assembly activity (IC₅₀ = 0.35 μM), binding affinity (K_d = 0.5 μM as measured by ITC) and antiviral activity (EC₅₀ = 0.95 μM) [46]. Using NMR and X-ray crystallography analyses, it has been shown that this compound series binds to a novel binding site, at the top of the helical bundle of CA-NTD, well removed from the two previously reported binding sites of CAP-1 and PF74 [46, 47]. Although this novel site is located in proximity to the cyclophilin A binding site, the binding of the benzimidazole compound does not affect the association of cyclophilin A to CA-NTD. However, even if these compounds demonstrated promising results in capsid assembly assay and inhibition of HIV-1 replication, they were found to have a weaker or no activity in capsid assembly assays *in vitro* on CA-NC proteins bearing naturally occurring CA-NTD polymorphisms found in clinical isolates [46]. For this reason, optimization of this compound series was stopped.

2.1.3. CK026, I-XW-053 and compound 34—CK026 inhibitor was identified from a virtual screen of 3 million small molecules using the hybrid structure-based screening method, which employs both structural and biochemical information, to design inhibitors of HIV-1 CA-NTD/CA-NTD interface [48, 49]. CK026 inhibits HIV-1 replication with an EC₅₀ of 33.3 ± 0.31 μM in single-round infection and 89 ± 3.2 μM in multiple-round infection, but displays no activity in peripheral blood mononuclear cells (PBMCs). However, an optimized compound, I-XW-053, inhibits HIV replication in PBMCs on a panel of non-adapted virus of different subtypes (A, B, C, D, E, F, G and a group O clinical isolate) with an EC₅₀ ranging between 9.03 and 100 μM (CC₅₀ > 100 μM). The antiviral activity of this compound is specific to HIV-1, as I-XW-053 has no impact on SIVmac239 and other viruses such as Dengue virus, respiratory syncytial virus (RSV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), H1N1 influenza virus and Chikungunya virus. The interaction of I-XW-053 with CA has been monitored by surface plasmon resonance (SPR) and ITC with a K_d of 66.3 ± 4.8 μM and 85 μM, respectively. Mutational analysis based on a docking model of I-XW-053 with monomeric CA, indicated that I-XW-053

interacts through a novel binding site located at the CA-NTD/CA-NTD interface, by interacting with residues I37 in helix 2 and R173 in helix 8. This compound has been shown to act during early viral events by drastically decreasing late reverse transcription products [48]. Although the impact of this compound on HIV-1 capsid core stability (uncoating) has not been studied in infected cells, Fricke *et al.*, showed that this compound had no impact on the stability of HIV-1 CA-NC complexes [42]. Given that I-XW-053 displays a high EC₅₀ (up to 100 μM), analogs have been developed through chemical optimization [50]. From over 56 analogues tested, compound 34 presents 11-fold improvement in antiviral potency compared to the parental compound I-XW-053 with an EC₅₀ of 14.2 ± 1.7 μM (CC₅₀ > 100 μM) and a K_d of 11.8 ± 4.7 μM. Molecular docking suggested that I-XW-053 and compound 34 both bind to the CA-NTD/CA-NTD interface region but with different binding modes [50].

2.1.4. PF-3450074—PF-3450074 (PF74) is the most studied antiviral capsid inhibitor, although its mechanism of action is not yet fully understood. PF74 was identified through a high-throughput antiviral screen analyzing encompassing the full HIV-1 replication cycle [51, 52]. It displays a potent broad-spectrum antiviral activity in PBMCs against HIV-1 laboratory strains and clinical isolates of different subtypes (A, B, C, D and E) with an EC₅₀ between 80 and 640 nM (CC₅₀ > 10 μM) [52], and blocks HIV-1 replication in macrophages [53]. The binding affinity was determined by ITC with the full-length CA (K_d = 2.79 μM) and the CA-NTD domain (K_d = 2.24 μM) [52]. This compound has been shown to be active against HIV-2 (in a micromolar range) [52] and SIV [54–56], but not against other retroviruses such as bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), N-tropic murine leukemia virus (N-MLV), B-tropic murine leukemia virus (B-MLV) and Moloney murine leukemia virus (Mo-MLV) [54]. The antiviral target was investigated by *in vitro* resistance selection, and identified the CA-NTD domain as the target of PF74 in infected cells. This small molecule has been shown to interfere with both early and late events in the viral replication cycle. PF74 displays an inhibitory activity in single-round infection [52], inhibits the accumulation of total viral cDNA produced, late reverse-transcription products, 2-LTR circles (a nuclear episomal form of the viral cDNA), and integrated provirus [52, 54, 56], indicating that PF74 inhibitor targets an early step in the virus replication cycle, prior to reverse transcription. PF74 also inhibits late events of HIV-1 replication by disrupting the formation of native-like particles [52].

The impact of PF74 on the viral uncoating process has been investigated. However, depending of the methods used conflicting results have been obtained. In some reports, PF74 decreases capsid stability, inducing premature uncoating of the viral capsid core. This was shown using cell-free core systems [56], in HIV-1 infected cells by fate-of-capsid assay (assay for capsid stability, measures relative levels of soluble vs. pelletable capsid in the cytoplasm of infected cells) [54, 56, 57], and by cell-imaging for the detection of intracellular viral RNA during infection [58]. Other reports, using cell-imaging of Gag-iGFP and mcherry-VPR pseudoviruses, found that PF74 does not affect the integrity of the viral capsid in infected cells [59]. This results was confirmed by cyclosporine (CsA) washout assay in which PF74 had no impact on the capsid uncoating process but rather bound to a

subset of CA which remains associated with the viral complex after uncoating [59]. Unexpectedly, PF74 has been shown to stabilize preassembled CA-NC tubes that are composed of hexamers [42, 57, 60] and to increase the rate of CA assembly *in vitro* [52, 60]. Recent studies revealed that PF74 preferentially binds to the assembled (hexameric) form of CA when compared to the monomeric form ($K_d = 120$ nM and 2.7 μ M, respectively) [57, 60, 61]. The crystal structure of hexameric CA in complex with PF74 showed that PF74 binds to residues in both CA-NTD and CA-CTD domains, in a preformed pocket encompassing the CA-NTD of one CA monomer and the CA-CTD of another, suggesting that the principal target of PF74 is the assembled capsid [34, 61]. PF74 stabilized the CA-NTD/CA-CTD intersubunit interactions, resulting in a more stable polymerized CA structure not easily disassembled [60]. Derivatives of PF74 have been designed and tested for binding affinity to CA hexamers and inhibitory activity in single-round infection assay. None of the analogs display a better binding affinity ($K_d = 5.5 - 125$ μ M) compared to PF74 ($K_d = 120$ nM), and all present a weaker ability to inhibit HIV-1 infection [61].

One proposed mechanism of action of PF74 may be by interference with host factors necessary for proper uncoating and nuclear import of the preintegration complex (PIC). Several host factors, such as cyclophilin A, the cleavage and polyadenylation specific factor 6 (CPSF6), nuclear import/pore proteins TNPO3, NUP153 and NUP358, have been shown to regulate capsid uncoating and nuclear entry processes [62–64]. Competition assays and crystal structure studies have shown PF74 to share the same binding site as CPSF6 and NUP153 host factors on the viral capsid [55, 57, 61, 65, 66]. Furthermore, a recent study demonstrated that PF74 displays a bimodal mechanism of action: at lower concentrations (< 2 μ M) it competes with binding of the host factor CPSF6 affecting nuclear entry, while at higher concentrations, PF74 abrogates reverse-transcription [67]. Price *et al.* also observed that, at low drug concentrations, the inhibition by PF74 is not associated with any block to reverse transcription while, at higher concentrations (around 10 μ M), the inhibition of infection occurs with the inhibition of reverse transcription [61]

2.1.5. Pyrrolopyrazolones: BI-1 and BI-2—A novel class of compounds targeting CA-NTD was identified from a screen for inhibitors of postentry events using a single-round infection cell-based assay [68]. BI-1, a representative of pyrrolopyrazolones, and its analog BI-2, block HIV-1 replication in multiple-round infection, with an $EC_{50} = 7.5 \pm 2.1$ μ M for BI-1 and 1.4 ± 0.66 μ M for BI-2, and display a CC_{50} superior to 91 μ M and 76 μ M, respectively. BI-2 has been shown to potently block HIV-1 and SIVmac but not HIV-2_{ROD}, BIV, FIV, EIAV, N-MLV, M-MLV and Mo-MLV [54]. Virus passaging in presence of BI-2 and resistance selection revealed mutations within the CA-NTD domain, implying CA as target of BI compounds. ITC and NMR analyses confirmed that BI compounds directly bind to CA-NTD with a K_d of 20 μ M for BI-1 and 3 μ M for BI-2. BI compounds inhibit single-round infection ($EC_{50} = 8.2 \pm 2.8$ μ M (BI-1) and 1.8 ± 0.34 μ M (BI-2)), do not alter the production of infectious virion from 293T producer cells [68], and do not affect late reverse transcription products. However, they reduce the levels of 2-LTR circles [54, 68], demonstrating that this class of compounds acts at early postentry stages of viral replication targeting a step following reverse transcription but prior to nuclear import of the preintegration complex. Like PF74, BI-2 has been shown to destabilize the HIV-1 core

during infection by fate-of-capsid experiments [54], to stimulate CA-NC assembly [68], and to stabilize preassembled CA-NC complexes [42, 54, 68]. The crystal structure of CA-NTD with BI-1 revealed that the inhibitor binds in a preformed binding pocket formed by residues from helices 3, 4, 5 and 7, same as PF74 binding site. However, these two compounds differ in their interaction with the hexameric capsid. The cocrystal structure of capsid hexamer with BI-2 showed that BI-2 binds similarly to hexamers and monomers of capsid. This was confirmed by ITC, with BI-2 binding hexamers with similar affinity to monomers ($K_d = 2.8 \mu\text{M}$ and $1.2 \mu\text{M}$, respectively). However, unlike PF74, BI-2 does not make interaction across adjacent monomers in the context of the hexameric capsid [61]. Nevertheless, just as PF74, BI-2 shares CPSF6 and NUP153 binding site, and has also been shown to prevent the binding of these two host cofactors to HIV-1 core [54, 61].

Taken together, PF74 and BI-2 seem to act similarly: (i) both compounds destabilize HIV-1 capsid core in fate-of-capsid experiments, (ii) they both increase CA-NC assembly and stabilize preassembled CA-NC complexes, and (iii) they both share a binding site with cellular host cofactors CPSF6 and NUP153, and prevent their binding to the HIV-1 core. However, PF74 and BI-2 also differ in different ways: (i) PF74 is active during both early and late stages of viral replication, while BI compounds are active only during early postentry events, (ii) PF74 acts at a step before reverse transcription while BI compounds have no impact on reverse transcription but act prior to nuclear import of the PIC (note that at low concentrations PF74 has been shown to block infection independently from reverse transcription), (iii) although BI-2 and PF74 share the same binding site on CA-NTD, in the context of hexameric capsid, BI-2 does not interact across the CA-NTD/CA-CTD interface. Different affinities for capsid between these two compounds may explain these results [61], or suggest that PF74 and BI-2 display a different mechanism of action on HIV-1 replication.

2.2. Small molecules and peptides targeting CA-CTD (Table 2)

2.2.1. CAI peptide and derivatives, NYAD-1 and NYAD-36/66/67 peptides—The capsid assembly inhibitor (CAI) is a 12-residue peptide (ITFEDLLDYGP), discovered through a phage display screen against the full-length CA or a protein comprising the CA-CTD, SP1 and NC regions of Gag. CAI efficiently abrogates both immature and mature assembly *in vitro* as shown by electronic microscopy studies; unfortunately it does not penetrate cells [69]. NMR analysis of CAI with CA-CTD bearing the mutation W184A and M185A (key residues involved in the CA-CTD dimerization) revealed that CAI binds with a K_d of $15 \pm 7.2 \mu\text{M}$, and interacts with residues located in helices 8, 9 and 11 of CA-CTD, with the most perturbation shift observed for residues 169 to 191 (helices 8 and 9) [69]. An X-ray crystal structure of CAI in complex with CA-CTD dimer revealed that CAI inserts as an amphipatic α -helix into a conserved hydrophobic groove of CA-CTD, resulting in the formation of a five-helix bundle which induces conformational rearrangements of the quaternary structure of the dimer [70]. Moreover, in the context of full CA, CAI peptide has been shown to bind in the same groove occupied by helix4 from CA-NTD, suggesting that CAI inhibits the CA-NTD/CA-CTD interface [23]. A series of alanine substitutions of residues mediating strong interaction with CAI have been introduced in CA, and the results suggested an indirect (allosteric) effect of CAI on the assembly of the immature particle, but a direct binding activity in the conserved CTD binding pocket during mature assembly [71].

Studies from Barklis *et al.*, and Fricke *et al.*, have shown that CAI not only inhibits assembly but also decrease stability of CA hexameric tubes [42, 72]. The binding of CAI to CTD is thought to weaken the hexamers, impair assembly and destabilize the assembled cores. Therefore, CAI would act during capsid assembly and uncoating.

Due to the lack of cell permeability of CAI, its antiviral efficiency has not been tested. Using a structure-based rational design approach, Zhang *et al.* stabilized the α -helical structure of CAI by hydrocarbon stapling, converting it into a cell-penetrating peptide [73]. The resulting peptide, NYAD-1 (*i,i* +4 stapled peptide, ITFXDLLXYYGP, where X is a nonstandard amino acid (*S*)-2-(2'-pentenyl)alanine, which functions as a bridge), has been shown to efficiently penetrate cells, and to display a broad spectrum anti-viral activity. It acts on a panel of laboratory-adapted and primary isolates of different subtypes (A, B, C, D, E, F G and group O) with an EC₅₀ between $4.29 \pm 0.42 \mu\text{M}$ and $21.60 \pm 3.04 \mu\text{M}$ in MT2 cells or PBMCs. NMR spectroscopy studies on monomeric CA-CTD W184A/M185A revealed that NYAD-1 maps to the same binding site as CAI, in helices 8, 9 and 11 of CA-CTD. However, the low solubility of the peptide interfered with a reliable estimate of the binding affinity by NMR. The binding of a high soluble NYAD-1 analogue, NYAD-13 (the C-terminal proline was replaced by 3 lysines), was about $1 \mu\text{M}$, versus $15 \mu\text{M}$ for CAI. As expected the binding site of NYAD-13 to the CTD monomer is the same as NYAD-1 and CAI [74]. Like CAI, NYAD-1 has been shown to efficiently disrupt the *in vitro* assembly of both immature and mature like particles in electronic microscopy analysis, and to inhibit the formation of spherical and cone-shaped particles in Gag- and GagPol-expressing 293T cells, respectively. These data confirmed that NYAD-1 targets Gag and impairs proper particle assembly and maturation in Gag-expressing cells. Moreover, NYAD-1 has been shown to inhibit virus release in a dose-dependent manner. In addition to these effects on late events, NYAD-1 inhibits early stages of HIV-1 replication, as shown by single-round infection and time-of-addition drug assays [73]. Therefore, NYAD-1 is a dual-acting peptide inhibitor possibly acting both in early (uncoating) and late events of the HIV-1 life cycle.

With the objective of enhancing binding and antiviral activity of CAI peptide, Zhang *et al.* developed a new *i,i* +7 series of stapled peptides. Such as for NYAD-1, this approach converts linear and non-penetrating peptides into α -helical and cell-penetrating peptides [75]. These peptides contain a R8 [(R)-2-(7'-octenyl)alanine] and a S5 [(S)-2-(4'-pentenyl)alanine] residues that functions as a bridge. The antiviral activity of the 16 resulting peptides was tested and revealed that NYAD-36, NYAD-66 and NYAD-67 were the most potent against HIV-1 IIIB with EC₅₀ of $1.5 \pm 0.7 \mu\text{M}$ (CC₅₀ > $189.4 \mu\text{M}$), $3.94 \pm 0.32 \mu\text{M}$ (CC₅₀ > $115 \mu\text{M}$) and $3.88 \pm 0.3 \mu\text{M}$ (CC₅₀ > $107.4 \mu\text{M}$), respectively. The binding affinity of these peptides to CA-CTD monomeric mutant W184A/M185A was determined by ITC and revealed a K_d of NYAD-36, NYAD-66 and NYAD-67 of $10.12 \pm 1.4 \mu\text{M}$, $3.60 \pm 0.16 \mu\text{M}$ and $2.63 \pm 0.22 \mu\text{M}$, respectively. NMR spectroscopy analysis confirmed a tight binding of these peptides to monomeric CA W184A/M185A, with spectral changes consistent with those observed between NYAD-1 and the isolated CA-CTD domain. Like CAI and NYAD-1 peptides, the 3 stapled peptides disrupted the formation of mature-like particles as shown by electronic microscopy and turbidity assays. In cell-based assays, even if cell penetration of these peptides was less efficient than observed with the *i,i* +4 peptides,

NYAD-36/66/67 peptides impaired Gag processing and diminished particle infectivity. To identify the binding target of these peptides, viral mutants were selected in presence of NYAD-36 and, unexpectedly, identified 2 mutations in Env gp120 (V120Q in C1 region, and A327P at the base of the V3 loop), but none in the CA region. These 2 mutations have been shown to confer a marked degree of resistance to the 3 peptides in single-round infection assay. Moreover, these 3 peptides have been shown to bind directly to full-length YU2 gp120 with a K_d of 1.7 μ M, 2.6 μ M and 1.3 μ M, respectively. The deletion of V3 on YU2 gp120 impaired the binding of NYAD36 and NYAD-67 by more than 5-fold. Therefore, NYAD-36, NYAD-66 and NYAD-67 display dual activity by binding to capsid and blocking Gag processing, and also by blocking virus entry in a V3-loop-dependent manner.

2.2.2. CAC-1 peptide and derivative, NYAD-201/202 peptides—CA-CTD/CA-CTD interactions play an essential role in HIV-1 capsid assembly. It has been shown that the CTD domain alone is able to efficiently inhibit the assembly of mature capsid *in vitro* [37]. With the goal of disrupting CA-CTD/CA-CTD dimerization, Garzon *et al.* designed a 20-mer peptide, CAC1, that represents the sequence of helix 9 plus 3 flanking residues at each site (EQASQEVKNWMTETLLVQNA) [76]. CAC1 is able to form a complex with the entire CA-CTD domain and to promote dissociation of the CA-CTD dimer. The binding affinity has been assessed by fluorescence spectroscopy, affinity chromatography and ITC, and revealed an average K_d of $50 \pm 25 \mu$ M. However, CAC-1 has shown tendency to aggregate. In order to improve CAC-1 solubility and affinity for CA-CTD, two derivative peptides of CAC1 have been designed: CAC1-C and CAC1-M. These 2 peptides bind CA-CTD with binding affinities lower than CAC1, with a K_d of $19 \pm 8 \mu$ M and $8 \pm 1 \mu$ M for CAC1-C and CAC1-M, respectively. NMR spectroscopy on monomeric CA-CTD mutant W184A revealed that CAC1 and CAC1-derivative peptides bind essentially in the same region of the CA-CTD overlapping extensively with helix 9 of the CA-CTD dimerization interface [77]. Both peptides are able to efficiently inhibit *in vitro* assembly of the mature capsid.

Another series of peptides mimicking the sequences of helices 2 and 3 (involved in CA-NTD/CA-NTD interfaces in the mature capsid) and helices 4 and 8 (involved in CA-NTD/CA-CTD interfaces) were designed to inhibit CA-NTD/CA-NTD or CA-NTD/CA-CTD interfaces. Only a peptide named H8 inhibited capsid assembly. In order to assess their antiviral activity, CAC1, CAC1-M and H8 were transported into cells using a cell-penetrating peptide. Both peptides showed poor antiviral activity, but when used in combination with other CA-binding peptides (CAC1/CAC1M+H8; CAC1/CAC1M+CAI; CAC1/CAC1M+H8+CAI), they additively inhibited HIV-1 infection [77].

In order to stabilize CAC1 peptide, a stapled version of CAC1 was designed by Zhang *et al.* [78]. The resulting peptides, NYAD-201 and NYAD-202, have been shown to bind competitively to CA-CTD monomer and inhibit dimerization. In assembly assays *in vitro*, the peptides inhibited HIV-1 mature-like virus particle formation and specifically inhibited particle production in cell-based assays. NYAD-201 and NYAD-202 also showed potent antiviral activity against a large panel of laboratory-adapted and primary isolates, including viral strains resistant to inhibitors of reverse transcriptase and protease, with an EC_{50} between $1.58 \pm 0.57 \mu$ M and $9.88 \pm 0.3 \mu$ M for NYAD-201 and $2.23 \pm 0.44 \mu$ M and $6.34 \pm$

2.22 μM for NYAD-202 [78]. Moreover, in addition to inhibiting virus production, NYAD-201 elicited additional inhibitory effects on virus replication not directly linked to their ability to bind CA-CTD, but in an Env-dependent manner.

2.2.3. Taurocholic acid and glycodeoxycholate—A study by Lampel *et al.* recently described a fluorescent-based method, bimolecular fluorescence complementation (BiFC) for the detection and visualization of CA-CTD dimerization. Using this method, they identified taurocholic acid (TA) as a potent inhibitor of *in vitro* CA assembly. A derivative of TA, glycodeoxycholate (GDC), displays a better inhibition in *in vitro* CA assembly assays, and inhibits HIV-1 replication with an EC_{50} of $3.12 \pm 0.37 \mu\text{M}$ ($\text{CC}_{50} > 200 \mu\text{M}$). Although this inhibitor was found in a CTD dimerization screen, unexpectedly, GDC has been shown to bind both CA-CTD and CA-NTD by NMR analysis. GDC inhibits HIV-1 replication by interfering with viral assembly [79].

2.2.4. Small compounds of diverse classes—A docking-based virtual screen was conducted to identify small molecules binding to the hydrophobic cavity of CA-CTD, the same binding site as CAI peptide [80]. These efforts lead to the identification of diverse classes of inhibitors displaying antiviral activity against HIV-1_{IIIIB} with an EC_{50} of 1.06 μM (compound 6) and 6.69 μM (compound 50). These two compounds inhibit laboratory-adapted viruses belonging to subtype B in MT-2 cells, as well as a panel of primary isolates in PMBCs with an EC_{50} ranging between $1.06 \pm 0.05 \mu\text{M}$ and $6.17 \pm 0.5 \mu\text{M}$ for compound 6 ($\text{CC}_{50} > 61.45 \mu\text{M}$ in PBMC), and between $1.12 \pm 0.2 \mu\text{M}$ and $10.95 \pm 2.51 \mu\text{M}$ for compound 50 ($\text{CC}_{50} > 64 \mu\text{M}$ in PBMC). Compounds 6 and 50 efficiently block the formation of mature-like particles *in vitro* as shown by electronic microscopy, and interfere with viral assembly and/or maturation in cell-based assays [80].

2.3. Small molecule targeting CA-SP1: Bevirimat

Bevirimat (BVM), also known as PA-457, DSB and PMC-4326, is the only capsid inhibitor tested in a clinical trial phase II. It's a betulinic acid derivative, a plant-derived natural product, which had been originally identified as a weak inhibition of HIV-1 replication [81]. BMV is an HIV-1 maturation inhibitor with a mode and site of action that is distinct from other capsid inhibitors. It inhibits HIV-1 replication by interfering with the final CA-SP1 cleavage step of Gag processing, resulting in the accumulation of p25 and immature non-infectious viral cores [82–85]. BMV has been shown to bind to the CA-SP1 junction, which prevents the protease enzyme from cleaving CA-SP1 and stabilizes the immature Gag lattice [86, 87]. Bevirimat displays an antiviral activity on both clinical HIV-1 isolates in PBMCs (EC_{50} between 4.5 and 20.1 nM) and drug-resistant virus isolates (NNRTI, PI and NRTI; EC_{50} between 2.7 and 13.3 nM). With an average CC_{50} of 25 μM , the therapeutic index of BMV is $> 2,500$ [84]. It is specific to HIV-1, as BMV does not inhibit the replication of HIV-2 and SIVmac251 [84, 85]. The selection of virus resistant to BMV *in vitro* identified mutations that map to the CA-SP1 cleavage site, with the last residue of capsid (L363) and the first residue of SP1 (A364) being crucial to BMV activity [84, 85, 88, 89].

Berivimat is the only capsid inhibitor that was clinically tested. BMV presented promising pharmacological and safety profiles in animal model and phase I clinical trials prompting

phase II trials in HIV-1 infected patients [90]. In phase II BMV caused a significant viral load reduction [39, 90]. However, the trials also revealed a high baseline drug resistance to BMV. A significant fraction (40–50%) of treated patients had pre-existing resistant viruses that had significantly reduced sensitivity to BMV. The examination of patient-derived virus revealed that BMV resistance was linked to naturally occurring polymorphisms within SP1 downstream of CA-SP1 cleavage site, in the “QVT motif” comprising SP1 residues 6, 7 and 8 [91, 92]. *In vitro* assays confirmed that SP1 polymorphisms, particularly SP1 V7A, reduced sensitivity of HIV-1 to BMV [88, 93]. This lack of response in a significant percentage of treated patients promoted the development of BMV analogs. The modification of C-28 side-chain improved antiviral activity compared to the parental compound and inhibited HIV-1 strains carrying the BMV V7A mutation [94–96].

3. Conclusions

Despite the success of antiretroviral therapy, the emergence of viral resistance highlights the need of novel drugs with alternative mechanisms of action. The development of inhibitors of protein-protein interactions in drug discovery was traditionally thought to be complicated, because the interactions involved large protein surfaces with less defined binding pockets than those present in tradition targets of most current drugs (G-protein coupled receptors, ion channels, and enzymes) [97]. However, in the last two decades protein-protein interaction inhibitors have proven quite effective, probably because protein-protein interactions have hot-spots, which are relatively small parts of the interface that are essential for high affinity binding. Besides, protein-protein interactions offer many attractive therapeutic opportunities as they play key roles in a large range of biological processes. Given that capsid is essential for a productive infection, and shows a high degree of conservation, presenting 70% of sequence homology between isolates [98], interactions between the capsid subunits emerged as a very attractive target for drug development. The multiple interfaces within the capsid lattice, CA-NTD/CA-NTD, CA-NTD/CA-CTD and CA-CTD/CA-CTD, essential for the architecture of the mature capsid, offers a wide range of target opportunities. Besides, as the hexamers and pentamers need to be precisely packaged in the lattice, small molecule interference in some interfaces may affect the capsid structure as a whole, reducing the occurrence of resistant mutations. Even if a natural polymorphism conferring resistance to Bevirimat compromised the effectiveness of its phase II trial, this compound demonstrated the usefulness of capsid inhibitors in ARV therapy. Analogs of Bevirimat and the small molecule PF74 look very promising as inhibitors of HIV-1 replication. In 2014, at least three patents (WO2114/110296, WO2114/110297 and WO2014/110298) have been proposed for chemical optimization of PF74 for use in human clinical trials. The combination of such small molecules, targeting different binding sites on the capsid, may improve the overall antiviral activity and represent a potential strategy for clinical trials. There is a need for continuous development of small molecules and peptides targeting the capsid as this viral protein is an excellent target to harness viral replication, and the non-human primate resistance to HIV infection mediated by TRIM5 α has clearly proven this point [10].

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List of abbreviation

ARV	antiretroviral
CA	capsid
CA-CTD	C-terminal domain of capsid
CA-NTD	N-terminal domain of capsid
CC₅₀	Half maximal cytotoxic concentration
HIV-1	Human immunodeficiency virus type 1
K_d	constant dissociation
MA	Matrix
NC	nucleocapsid
NMR	Nuclear magnetic resonance
ITC	isothermal titration calorimetry
SP1	spacer peptide 1
SP2	spacer peptide 2
SPR	surface plasmon resonance
EC₅₀	Half maximal effective concentration

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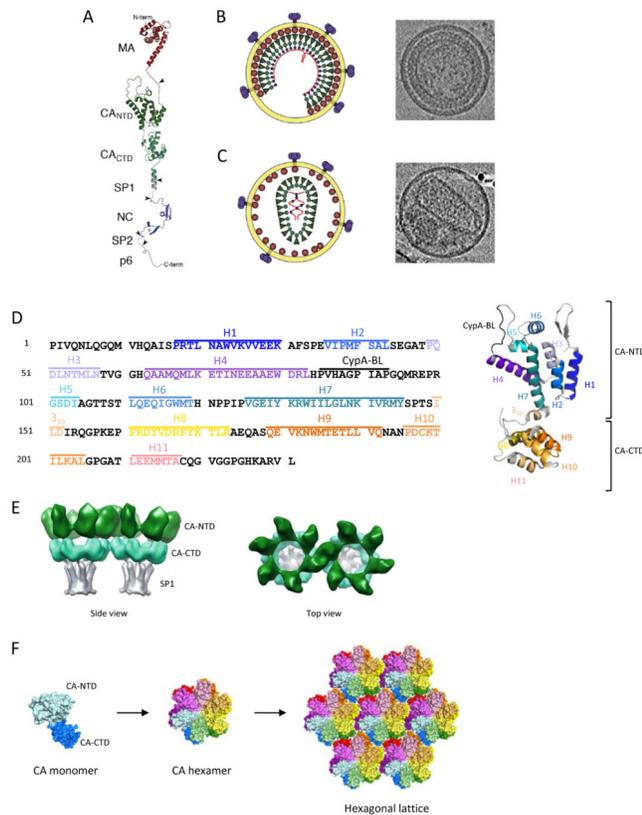
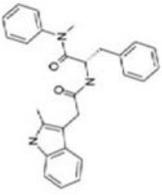
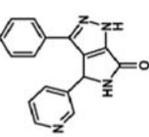
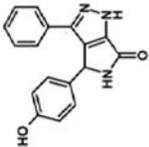


Figure 1. Organization and structure of immature and mature HIV-1 virions

(A) Schematic secondary structure of HIV-1 Gag polyprotein. Individual domains are represented in different colors. Protease cleavage sites are indicated by the arrowheads. (B) Schematic model and electron cryotomography of immature HIV-1 particle. (C) Schematic model and electron cryotomography of mature HIV-1 particle. (D) Sequence and secondary structure of mature capsid (PDB 2LF4; [26]). The different helices are represented in different colors, in blue and purple for CA-NTD helices, and orange and yellow for CA-CTD helices. (E) The immature Gag lattice. Side view and top view of a low-resolution model of interaction between two Gag hexamers. The CA-NTD, CA-CTD and SP1 domains are represented with different colors. (F) The mature CA lattice. Crystal structure of a monomeric CA, hexamer and the hexagonal lattice. (Fig A–C: from Ganser-Pornillos *et al.*, 2008 [99]; Fig E: from Ganser-Pornillos *et al.*, 2012 [100]; Fig F: adapted from Gres *et al.*, 2015 [34], with permission from Bentham Science Publishers).

Compound	Structure	Screen	EC ₅₀	CC ₅₀	K _d	Binding site	HIV-1 life cycle step	Ref.
(4) PF74		HTS antiviral assay	80 – 640 nM (PBMCs)	> 10 μM (PBMCs)	monomer: 2.7 μM hexamer: 120 nM	CA-NTD CA-CTD	early/late	[52]
(5) BI-1		Single-round infection	7.5 ± 2.1 μM	> 91 μM	20 μM	CA-NTD	early	[68]
(5) BI-2		Optimization of BI-1	1.4 ± 0.66 μM	> 76 μM	monomer: 1.2 μM hexamer: 2.8 μM	CA-NTD	early	[68]

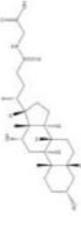
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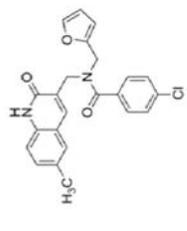
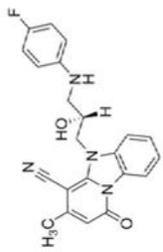
The number of the capsid inhibitor refers to the section in the text.

Early event of the HIV-1 life cycle include steps prior to integration of the viral genome into the host cell. Small molecules acting on early event display an antiviral activity in single-round infection, decreased the amount of reverse transcription product and/or integrated proviruses, or directly impact the uncoating process. Late events encompass the capsid assembly and maturation steps.

Table 2

Small molecules and peptides targeting CA-CTD.

Compound	Structure / peptide sequence	Screen	EC ₅₀	CC ₅₀	K _d	Binding site	HIV-1 life cycle step	Ref.
(1) CAI	ITFEDLLDYYGP	Phage display	N.D.	N.D.	15 ± 7.2 μM	CA-CTD	late	[69]
(1) NYAD-1	ITF-X-DLL-X-YYGP X: (S)-2-(2'-pentenyl)alanine	<i>i,i</i> +4 stapled peptide of CAI	4.29 – 21.6 μM (PBMCs)	N.D.	≈ 1 μM (overlap CAI site)	CA-CTD	early/late	[73]
(1) NYAD-36	ISF-R8-ELLDDYY-S5-ESGS R8: (R)-2-(7'-octenyl)alanine S5: (S)-2-(4'-pentenyl)alanine	<i>i,i</i> +7 stapled peptide of CAI	1.5 ± 0.7 μM	> 189.4 μM	10.12 ± 1.4 μM (overlap CAI site)	CA-CTD	early/late	[35, 75]
(1) NYAD-66	ISF-R8-ELLDDYY-S5-ED R8: (R)-2-(7'-octenyl)alanine S5: (S)-2-(4'-pentenyl)alanine	<i>i,i</i> +7 stapled peptide of CAI	3.94 ± 0.32 μM	> 115 μM	3.60 ± 0.16 μM (overlap CAI site)	CA-CTD	early/late	[75]
(1) NYAD-67	ISF-R8-EWLQAY-S5-EDE R8: (R)-2-(7'-octenyl)alanine S5: (S)-2-(4'-pentenyl)alanine	<i>i,i</i> +7 stapled peptide of CAI	3.88 ± 0.3 μM	> 107.4 μM	2.64 ± 0.22 μM (overlap CAI site)	CA-CTD	early/late	[75]
(2) CAC1	EQASQEVKNWMTETLLVQNA	Mimic helix 9	N.D.	N.D.	50 ± 25 μM	CA-CTD(helix 9)	late	[76]
(2) CAC1-C	ESASSSVKAWMTETLLVQNA	Derivative of CAC1	≈ 25% inhibition	N.D.	19 ± 8 μM	CA-CTD	late	[77]
(2) CAC1-M	SESAASSVKAWMTETLLVANTSS	Derivative of CAC1	≈ 25% inhibition	N.D.	8 ± 1 μM	CA-CTD	late	[77]
(2) NYAD-201	AQEVK-X-WMT-X-TLLVA X: (S)-2-(2'-pentenyl)alanine	<i>i,i</i> +4 stapled peptide of CAC1	1.58 – 9.88 μM	N.D.	N.D.	CA-CTD	late	[78]
(3) GDC		bimolecular fluorescence complementation	3.12 ± 0.37 μM	> 200 μM	N.D.	CA-CTD CA-NTD	late	[80]

Compound	Structure / peptide sequence	Screen	EC ₅₀	CC ₅₀	K _d	Binding site	HIV-1 life cycle step	Ref.
(4) compound 6		Virtual screen	1.6 – 6.17 μM (PBMCs)	> 61.45 μM (PBMCs)	N.D.	CA-NTD	late	[80]
(4) compound 50		Virtual screen	1.12 – 10.95 μM (PBMCs)	> 64 μM (PBMCs)	N.D.	CA-NTD	late	[80]

N.D.: not determined

The number of the capsid inhibitor refers to the section in the text.

Early event of the HIV-1 life cycle include steps prior to integration of the viral genome into the host cell. Small molecules acting on early event display an antiviral activity in single-round infection, decreased the amount of reverse transcription product and/or integrated proviruses, or directly impact the uncoating process. Late events encompass the capsid assembly and maturation steps.