Viral structural proteins as targets for antivirals

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Abstract

Viral structural proteins are emerging as effective targets for new antivirals. In a viral lifecycle, the capsid must assemble, disassemble, and respond to receptors, all at the right time and place. These reactions work within a narrow range of conditions, making them susceptible to small molecule interference. In at least three specific viruses, this approach has had met with preliminary success. In rhinovirus and poliovirus, compounds like pleconaril bind capsid and block RNA release. Bevirimat binds to Gag protein in HIV, inhibiting maturation. In Hepatitis B virus, core protein allosteric modulators (CpAMs) promote spontaneous assembly of capsid protein leading to empty and aberrant particles. Despite the biological diversity between viruses and the chemical diversity between antiviral molecules, we observe common features in these antivirals' mechanisms of action. These approaches work by stabilizing protein-protein interactions.

Introduction

One advantage of targeting viral structural proteins is that they are unique to the pathogen and generally have no human homologs. Another advantage of structural proteins is their propensity to form oligomers, leading to a dominant negative effect: a drug does not necessarily need to act on every viral protein subunit to disrupt the oligomer's function (••1). The challenge of targeting viral structural proteins is that they are not enzymes, where the substrate provides a straightforward starting point for transition state analogs. The regions of functionality for structural proteins are often macromolecular interfaces, and small molecules which bind to these interfaces must be identified through naïve screens. A further challenge in targeting a molecular interface is allostery, where the site of drug binding and the site of its effect can be spatially distinct. However, by understanding the requirements for a structural protein to function properly, we concomitantly identify the targets for antiviral effectors.

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Physical Chemistry of Capsid Assembly: A Qualitative Summary

The four critical lessons to be learned from modelling capsid assembly are (••2–6):

i. Subunits are multivalent. They may interact one subunit at a time to build capsid shells, or hierarchically first making small oligomers. Multivalency gives them avidity.

ii. The pairwise association energy between any two subunits during the assembly reaction is weak, but summing the energy of a large number of contacts results in a particle that can be very stable (7).

iii. Assembly reactions follow a steep downhill energy gradient. More subunits mean more contacts. Like a car rolling downhill, such reactions are very difficult to stop.

iv. Assembly and disassembly are not always mirror images. Capsid closure and postassembly conformational maturation can both contribute to capsids that are much more stable than might be expected (8).

In the simplest model, an assembly reaction consists of subunits adding one at a time to a starting nucleus. To model this reaction, one need only consider the geometry of subunits, pairwise per-contact association energy, and on-rate (••2–6). Overly strong association energy can lead to kinetic traps where the reaction runs out of subunits before completing the maximum possible number of capsids or, worse, any defects that are accidentally incorporated get cemented in place by subsequent additions (9, ••10). Weak association energy allows the reversibility needed to repair defects and escape dead-end kinetic traps.

Nucleation factors are also important to consider (11, 12). Nucleation regulates the number of assembling particles, thus decreasing the potential for kinetic traps. Nucleation may also direct the path of assembly, affecting the size and shape of the resulting particle. The viral genome is often involved in nucleation, and the role(s) of specific sites on the genome may direct assembly and genome organization (6, 13, ••14)

Though capsids assemble based on weak interactions, the end products may be remarkably stable (6, 8). Some of this stability arises because the first subunit to leave a capsid must break many contacts simultaneously, creating a kinetic and thermodynamic barrier to dissociation. Capsid stability may also arise from post-assembly conformational and chemical changes (e.g. proteolytic maturation, see below) (15).

Enteroviruses and Capsid Stabilization

The enterovirus genus of the picornavirus family includes rhinovirus, poliovirus, and Coxsackie virus. They are small unenveloped RNA viruses with pseudo-T=3, or P=3, symmetry (16). The immature capsid is comprised of trimers of Vp0, Vp1, and Vp3 subunits, initially part of a polyprotein. These trimers assemble into stable pentamers that can reversibly assemble to empty capsids (17) or package RNA. Assembled capsids autoproteolytically mature by cleavage of Vp0 to Vp2 and the ~70-residue Vp4 peptide. In response to receptor binding or to heat, mature picornaviruses undergo a conformational change to release viral RNA and Vp4, a reaction termed "uncoating" though the capsid may

remain largely intact. Unless stimulated to uncoat, mature enteroviruses can be remarkably stable.

The "WIN" series of compounds (after Sterling-Winthrop) contributed to the development of pleconaril and pirodavir and were found to inhibit the uncoating reaction of rhinovirus (18, 19). They show the same effects in many, but not all enteroviruses. Structurally first observed in 1986 (•20), these stabilizing compounds fill a long, tubular pocket that is entirely within Vp1, distal to sites of pentamer-pentamer interaction (Figure 1). To examine mechanism, the temperature dependence of poliovirus uncoating was determined in the presence and absence of WIN compounds (••21). Via an Arrhenius analysis, it was determined that the entropy of the complex decreased, increasing the barrier to uncoating. Consistent with this result, molecular dynamics showed that WIN compounds could decrease the volume filled by rhinovirus capsid proteins and alter concerted movements of the capsid proteins (22). Similarly, it was observed that WIN compounds suppress transient exposure of Vp4 outside the capsid, an indicator of virus "breathing" (23). Though it was an effective antiviral, pleconaril did not ultimately gain FDA approval because of off-target effects. Furthermore, not all enteroviruses possess a pocket accessible for this class of drugs (24).

More recently a new class of uncoating inhibitor was reported for Coxsackie virus (25). These molecules, exemplified by "compound 17" (CP17), bind at the junction of three subunits, two VP1s and a VP3. This pocket is distal to both the WIN pocket and the interaction between pentameric subunits that forms during capsid assembly (Figure 1). Yet, like the WIN compounds, CP17 inhibits uncoating. Because the interface between proteins is a geometric necessity, the structure of the pocket is well-conserved. Thus, such molecules are likely to show activity across many enteroviruses. In summary, stabilization of enterovirus capsids interferes with uncoating and RNA release. A likely mechanism is that they damp capsid dynamics, increasing the energetic barrier to uncoating.

CpAMs: Targeting Hepatitis B Virus Capsid Assembly

Despite an effective vaccine, Hepatitis B virus (HBV) remains a global health problem, particularly in Asia and Africa where chronic infection is underdiagnosed (26). HBV is a small enveloped virion with an icosahedral core containing a circular viral genome of partially double stranded DNA. Because the genome is not completely double stranded, it has been named relaxed circular DNA (rcDNA). After cell entry, the rcDNA genome is deposited in the nucleus where it is repaired by host enzymes to yield a covalently closed circular DNA chromosome. Viral RNA transcripts are then exported to the cytoplasm, which include a pre-genomic RNA (pgRNA). Core protein dimers (Cp) assemble around a pgRNA-reverse transcriptase complex, usually forming a T=4 icosahedral capsid. In addition to the genome-filled particles, a large fraction of newly assembled capsids in infected cells are empty. Reverse transcription of pgRNA to rcDNA occurs inside the capsid and the mature particles are notably fragile, partly due to the fact that DNA is a stiff polymer compared to the 25nm inner diameter of an HBV capsid (27, 28). Arguably, HBV Cp plays roles in almost every step of the viral lifecycle (29), and Cp has become an important target of HBV-specific antivirals. Variants of capsid-specific molecules have a proven capability

to suppress virus replication in cell culture, and more than ten are currently undergoing clinical trials (30). We prefer to call these molecules core protein allosteric modulators (CpAMs); other names include assembly agonists, capsid assembly modulators (CAMs), capsid assembly affectors (CAEs), and core protein inhibitors (CIs).

Initially, phenylpropenamides (PPAs) were found to reduce HBV replication in cell culture (31, 32) and led to accumulation of empty particles by somehow inhibiting RNA packaging (33). Soon after, heteroaryldihydropyrimidines (HAPs) were described in the literature, where the authors established that their mechanism of action involved Cp and led to reduced viral DNA and reduced intracellular Cp levels (34, 35). Much later, sulfamoylbenzamides (SBAs) emerged, which also reduced cytoplasmic pgRNA capsids, but did so without significantly decreasing total capsid protein (36).

Efforts to identify the target of HAP activity quickly converged on the process of capsid assembly (37). Mechanistically, rather than inhibit assembly, CpAMs stimulate assembly, increasing both the rate and extent of assembly reactions with purified core protein (38). Super-stoichiometric concentrations of HAP (more HAP molecules than Cp sites) caused Cp to assemble into larger, flatter structures with distinctly hexameric features (Figure 2) (38, 39). The shape of these assemblies appears to be tunable, forming the larger flatter structures when assembling slowly, and more spherical structures when assembling quickly (•40). A cryo-EM study of HAP assembly products was able to selectively reconstruct tubular assemblies, and while the HAP was not resolved, the protein subunits exhibited a striking pattern of regular repeating hexamers.(41)

An important component to this mechanism is that CpAMs strengthen the association energy between subunits, often on the order of −1kcal/mol per-contact (42). This modest energy shift is amplified as each Cp dimer is tetravalent, and because assembly is already a steep energy gradient.

Crystal structures of core protein bound to CpAMs used pre-assembled capsids, co-crystalized with a HAP (43, 44) or a PPA (45). All three CpAM-containing capsids expanded by about 5% compared to ligand-free capsids, effectively an allosteric response to CpAM binding. For both chemotypes, the molecules bound to two of the four quasi-equivalent protein interfaces of the hexameric capsomers. The molecules bind in a pocket formed at the junction of two Cp dimers (Figure 2b, c). The dimers must be oligomerized to form a complete pocket, suggesting that subunits associate spontaneously in the absence of a CpAM, which can then insert into the pocket and raise the energy barrier for dissociation.

Capsid-CpAM structures have provided a clear view of the CpAM binding site(s) and the effect of CpAMs on quaternary structure, however capsids are not convenient for crystallographic studies. Dimers with a point mutation (Y132A) that removes a tyrosine from the dimer-dimer interface and renders the protein assembly-incompetent (46) will co-crystalize with CpAMs, packing as a planar lattice of hexamers with a CpAM bound at every inter-subunit interface (47). Using Y132A, structures have been solved of at least three HAPs (47–49), a sulfamoylbenzamide (49), and the antifungal derived CpAM Ciclopirox (50). All molecules bind at the inter-subunit interface, but each has a distinct binding pose.

With assembly-active dimer, each of these molecules drives Cp assembly and therefore strengthens Cp-Cp interaction.

The effects of some CpAMs on capsid structure imply that the molecules not only interfere with the assembly process but can also disrupt intact capsids. This could have the effect of blocking deposition of viral DNA in the nucleus (51). We envision a single antiviral with antiviral capability at opposite ends of the viral lifecycle (release of nucleic acid to establish infection, packaging of nucleic acid during assembly). Evidence for structural disruption includes the capsid crystal structures, where drug binding causes faceting, becoming flatter at hexamers (where the molecules bind), and becoming more highly curved at the 5-fold vertices. Faceting occurs due to concerted changes in quaternary structure, so it was likely constrained due to the inter-dimer crosslinks that were necessary to get diffraction quality crystals. Using cryo-EM to visualize capsids with a bound fluorescently-labeled HAP CpAM, un-crosslinked capsids were irregular, with elliptical and asymmetrically faceted capsids visible (42). The mechanisms for disruption of preformed capsids were investigated further with another CpAM, a dibenzothiazapine (DBT), which both drives assembly and paradoxically causes pre-formed capsids to ultimately disassemble (••52). HAPs will also cause capsids to become strained, eventually rupturing and reassembling into larger flatter structures. These observations led to a model where CpAMs induce local deformations that propagate to global capsid strain, leading to eventual rupture (Figure 2d). The energetic cost of adopting a strained state, an unfavorable quaternary structure, is paid for at every protein interface where the binding energy of a CpAM strengthens the interaction. Essentially, capsid strain is an emergent property of local changes to the subunit interfaces.

HIV: successfully targeting a non-icosahedral virus

Human immunodeficiency virus (HIV) capsid assembly is a multistep process that starts with the Gag polyprotein (53). Starting from the N-terminus, most Gag polyproteins are comprised of matrix, capsid (CA), spacer peptide 1 (SP1), nucleocapsid, spacer peptide 2, and p6 domains. About 5% of HIV Gag translation products incorporate a ribosomal frameshift that appends the protease, polymerase, and integrase domains. Formation of a new virion starts with Gag localized to the plasma membrane bound to viral RNA (53). In nascent virions, Gag is arranged in a hexagonal lattice with numerous flaws, forming a partially complete spherical shell (54). Consistent with other examples of virus capsid proteins, Gag-Gag interaction (largely mediated by CA-CA association) is weak (55). Hexameric and pentameric capsomers are formed by CA N-terminal domains (CA-NTDs) which are supported by interdigitating C-terminal domains (CA-CTDs). Capsomers are crosslinked by dimerization of CA-CTDs. During or after budding, the viral protease becomes active and processes Gag into individual proteins. In the mature virus, about 1500–2000 CA proteins reorganize into a Fullerene cone, an arrangement of CA hexamers and pentamers (56, •57). After mature virus infects a new host cell, the fullerene cone is released, and its dissociation appears to play important roles in the transport and reverse transcription of the viral genome. Both the maturation and ultimate capsid dissociation steps have been targets for antiviral intervention.

Gag maturation begins with an ordered series of cleavages by the HIV protease (53). Typical transition state analogs are effective protease inhibitors. Maturation inhibitors, however, bind Gag, not the protease. Maturation inhibitors like the betulinic acid derivative Beviramat (BVM) or PF46396 specifically inhibit cleavage of the CA-SP1 peptide bond (58). Normal HIV assembly is sensitive to mutations near the CA-SP1 junction (59), implying that the mobility and structure(s) of this junction are restricted (60, 61). Remarkably, some PF46396-resistant mutants are drug-dependent for normal assembly (58, 62). The structure of a CA-CTD-SP1 hexamer with a bound BVM was determined to 2.9Å using micro-electron diffraction (Figure 3) (••63). The proteins are arranged with approximate 6-fold symmetry with a single BVM filling a pore along the oligomer axis. It is presumed that PF46396 binds near or at the BVM site (58). The CA-SP1 junction is alpha helical and located near the hexamer axis; its conformation is unlike the extended conformation of a CA-SP1 peptide bound to the protease, indicating that the sequence is cleaved in an unfolded state (64). Because BVM can support Gag assembly and stabilize Gag polymers (65), we can deduce that the small molecule is stabilizing the helical conformation. Of course, it may also act to block protease access to the scissile bond. Without proteolytic cleavage, the Gag polymer remains trapped as a low energy intermediate in HIV assembly. Maturation inhibitors buttress this stable state.

At the other end of the viral lifecycle, uncoating is temporally linked to the processes of reverse transcription and nuclear entry, making it a critical step in initiating infection (53). Uncoating inhibitors stabilize interactions between CA subunits. The molecule PF74 is a much-studied tool compound that informs us of the mechanisms of more recently developed molecules (•66). Like DBT1 with HBV, PF74 has the effect of inducing dissolution of HIV capsid complexes (67). In the simplest case, from a thermodynamic perspective, this would require PF74 bind CA monomer more tightly than capsid: PF74 binds CA monomer with a K_D of ~4000 nM and hexamers with a K_D of 262nM, indicating that it stabilizes CA polymers (••68). Historically, efforts to identify molecules that inhibit assembly, binding monomer more tightly than oligomer, has led to candidates that only worked at very high concentrations and were, in the end, unsuccessful (69, 70). Structurally, it makes sense that PF74 binds more tightly to oligomer: PF74 binds an exposed pocket in the CA-NTD that is partially capped by the CTD from an adjacent monomer (••68) (Figure 3). The site is apparently modified by formation of a complex of hexamers: PF74 stabilizes cylinders of CA but induces dissociation of isolated viral cores. By analogy to studies with HBV and DBT1, we speculate that PF74 may stabilize local hexagonal conformations that lead to ruptures in an irregular conical HIV capsid. Global strain arises because virus capsids are an oligomeric network, where a conformational change at one point must be accommodated throughout the shell.

Conclusion

Capsid-directed antivirals remain in the early stages of development and will require tailoring the medicinal chemistry to each specific protein target. Nevertheless, core principles can be identified which generalize across viruses and across specific chemistries. Capsid-directed antivirals strengthen the association energy between subunits with the following implications:

a.   assembly-directed drugs bind to oligomeric complexes tighter than to single subunits

b.   dissociation of subunits becomes locally unfavorable

c.   the rate and extent of assembly increases

d.   free subunit is depleted

e.   incomplete and defective particles are kinetically trapped

f.   protein dynamics are modulated

In many cases, structural protein-directed molecules modify the preferred orientation of adjacent subunits:

a.   assembly reactions can proceed off-path

b.   reaction products include heterogenous mis-assemblies

c.   pre-formed capsids experience a global strain

d.   strained capsids may rupture

By preferentially binding subunit oligomer, assembly reactions are driven forward. A hypothetical capsid assembly inhibitor would have a much more demanding task: binding protein subunit very tightly, and with nearly equivalent stoichiometry. In contrast, a modest increase of the subunit interaction energy can be leveraged into a dramatic change in the reaction products, as seen with CpAMs driving HBV assembly to yield empty and deformed capsids. The proposed mechanism of picornavirus uncoating inhibitors and HIV maturation inhibitors also share this theme: picornavirus capsids are over-stabilized, preventing uncoating; HIV CA hexamers are over-stabilized at the expense of Gag cleavage and subsequent maturation.

A further consequence of using small molecules to strengthen the interface between two protein subunits is that the relative orientations of subunits are likely to be modified. This could be as simple as extra atoms forming a wedge, or it could be a more dynamic or allosteric shift in the conformational ensemble between two subunits. Finally, a combination of the two effects (increased local association energy and modified preferred orientation) can lead to circumstances where a capsid becomes globally strained and ruptures, as observed with DBT1 in HBV and PF74 in HIV.

Antiviral molecules directed against structural proteins facilitate action at a distance. They are assembly agonists, maturation inhibitors, modulators of capsid dynamics, inhibitors of nucleic acid release, and destabilizers of large complexes. In all cases evolution has optimized the structural protein interactions to the requirements of the viral lifecycle. Targeted modification of these interactions is disruptive to the virus. These principles provide a framework with which to approach further antiviral research.

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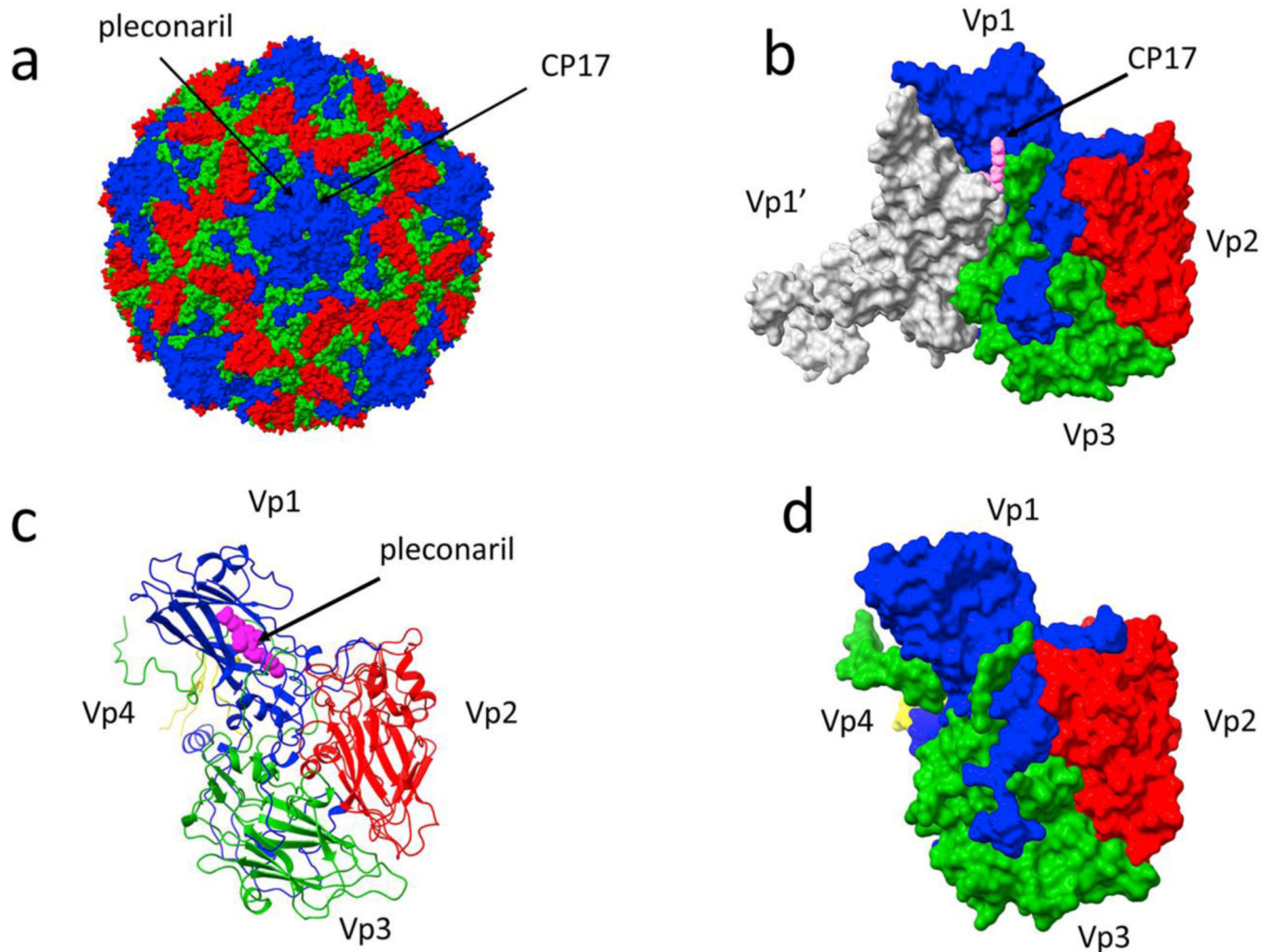
Figure 1. Antiviral binding sites on a picornavirus capsid.
(a) A coxsackie virus capsid (1cov), with the viral structural proteins Vp1–3 colored blue, red, and green, respectively; Vp4 is inside the capsid and not visible in this image. Two distinct sites for capsid-directed antiviral compounds are denoted with arrows. (b) The binding site for compound 17 (CP17, pink), from Abdelnabi et al. 2019 (6gzv), is at an interface of a Vp3 and the two adjacent Vp1 molecules (one shown in white). The view is from the outside of the capsid. (c) In contrast, the binding location for pleconaril (magenta), binding the same site as a WIN compound, only contacts a single copy of Vp1 and is not located at an intermolecular interface (1ncr). (d) A surface representation of the same complex as (c) emphasizes that pleconaril is completely enclosed by Vp1.
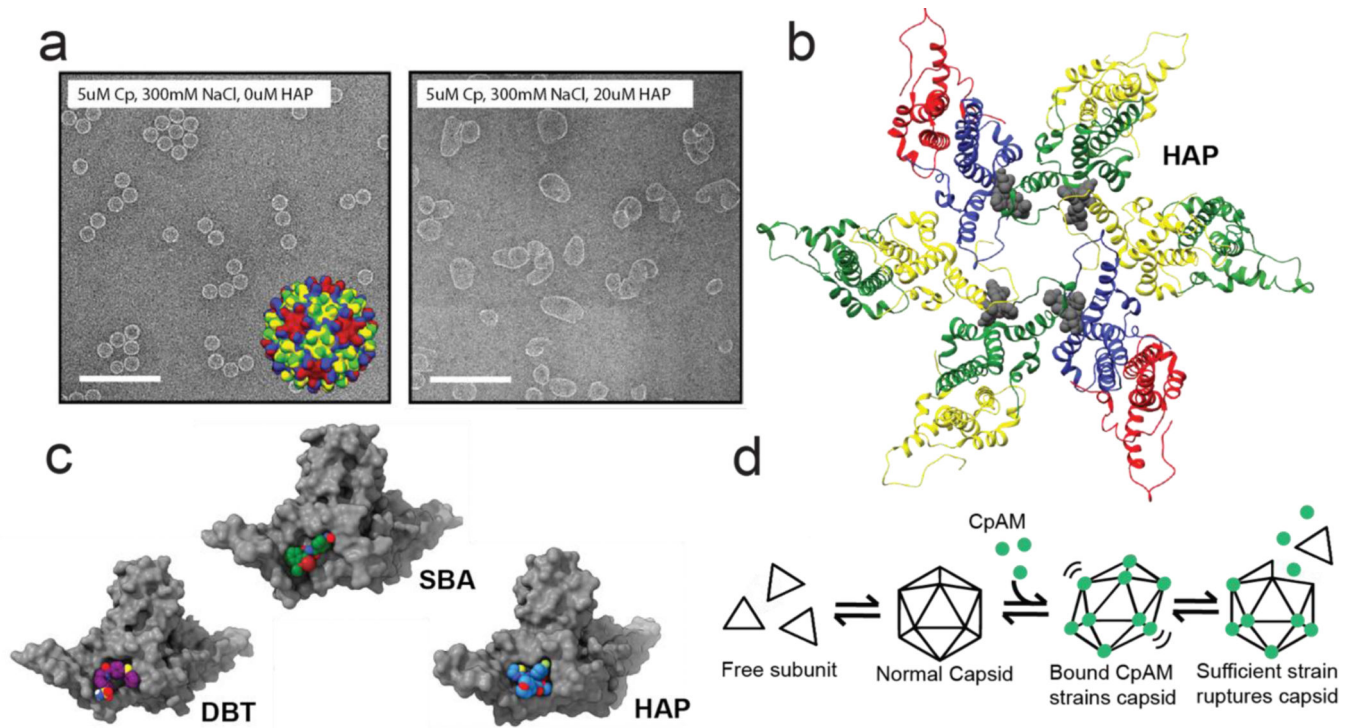
Figure 2. The effects of CpAM binding to HBV core protein.
(**a**) Electron micrographs of assembled HBV Cp dimer. (left panel) Virus-like particles are
~35nm icosahedra. Inset: an model of a T=4 capsid structure with chains colored according
to quasi-equivalent environment. When assembled in the presence of a HAP (right panel),
reaction products include large misassembled structures (40). (**b**) A hexamer fragment of
a T=4 capsid structure in complex with a HAP (44). Coloring is the same as in the inset
in the left panel of **a**, except with the HAP molecules colored gray. The HAP molecules
bind at the interface between two adjacent subunits. (**c**) One site, multiple chemistries:
a selection of CpAMs which target the interface between HBV capsid protein subunits.
Despite occupying the same site, each molecule has a distinct binding mode, and associated
phenotype (47, 49, ••52) (**d**) A simplified reaction schematic for how CpAMs disrupt pre-
formed capsids. Increasing the per-contact association energy causes favorable, but modified
pairwise orientation that induces global strain. Strained capsids will rupture to relieve strain
(53). Components in this figure are extracted from or adapted from figures in references
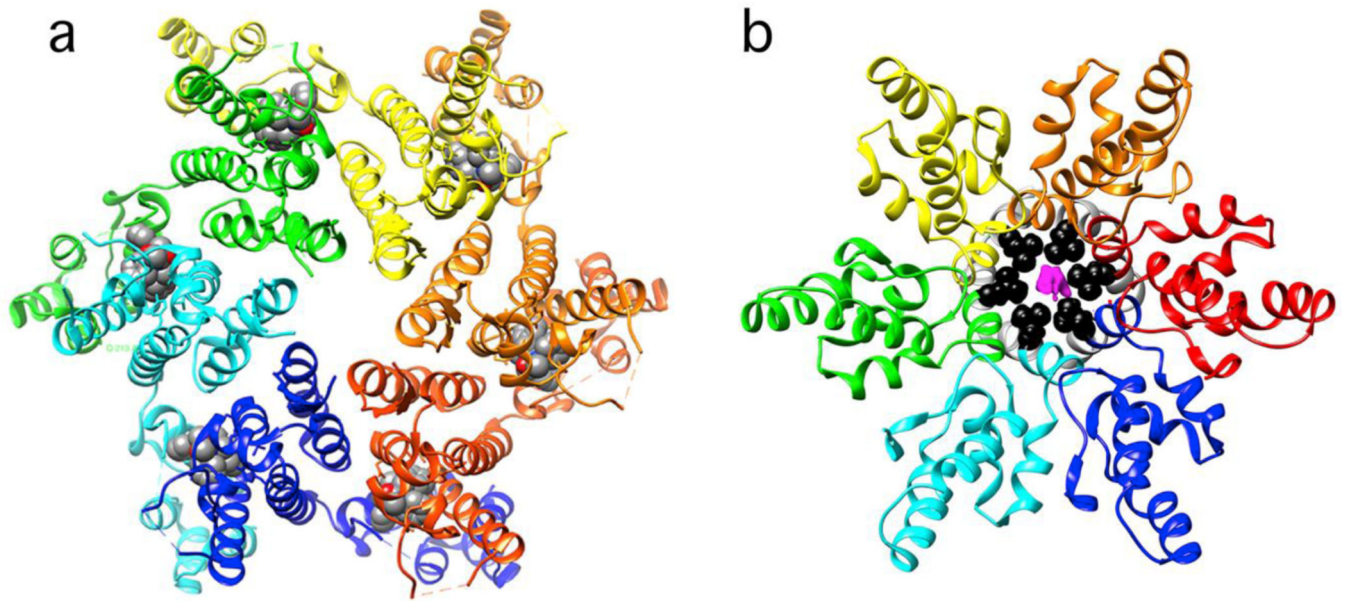(40), (44), and (••52).

Figure 3. The HIV CA domain with hexamer-stabilizing small molecules.
(**a**) A ribbon diagram of a top view of a mature CA hexamer with bound P74 (PDB: 4u0e). The P74 molecule (space filling with carbons in gray) fits into a pocket largely formed by the NTD (top) and completed by the CTD from the neighboring subunit (CTDs are partially obscured by the NTD). (**b**) A CTD-SP1 construct representing a fragment of immature Gag with bound bevirimat (PDB: 6n3u). The CTD is on top. The last residue in the CTD, L363 (black), and SP1 (gray) are partially obscured. Electron density for bevirimat (magenta), contoured at 1.1 σ, fills a cylindrical gap in the ring of helices, presumably stabilizing them and blocking accessibility to protease.