

# Biology of the hepatitis B virus (HBV) core and capsid assembly modulators (CAMs) for chronic hepatitis B (CHB) cure

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**Abstract:** Hepatitis B virus (HBV) is a hepadnavirus, a small DNA virus that infects liver tissue, with some unusual replication steps that share similarities to retroviruses. HBV infection can lead to chronic hepatitis B (CHB), a life-long infection associated with significant risks of liver disease, especially if untreated. HBV is a significant global health problem, with hundreds of millions currently living with CHB. Currently approved strategies to prevent or inhibit HBV are highly effective, however, a cure for CHB has remained elusive. To achieve a cure, elimination of the functionally integrated HBV covalently closed chromosomal DNA (cccDNA) genome is required. The capsid core is an essential component of HBV replication, serving roles when establishing infection and in creating new virions. Over the last two and a half decades, significant efforts have been made to find and characterize antivirals that target the capsid, specifically the HBV core protein (Cp). The antivirals that interfere with the kinetics and morphology of the capsid, termed capsid assembly modulators (CAMs), are extremely potent, and clinical investigations indicate they are well tolerated and highly effective. Several CAMs offer the potential to cure CHB by decreasing the cccDNA pools. Here, we review the biology of the HBV capsid, focused on Cp, and the development of inhibitors that target it.

**Keywords:** HBV, capsid assembly modulators (CAMs), hepatitis, hepadnaviridae, capsid core

## Introduction

The hepatitis B virus (HBV) was first discovered as an infectious agent in the 1960s by Dr. Baruch Blumberg, who described the presence of what would later be named the HBV surface antigen (HBsAg) in the blood of patients with hepatitis (1). Blumberg's discovery subsequently led to the development of diagnostic screening tests and effective vaccines against HBV by targeting HBsAg, work for which he would later be awarded the 1976 Nobel Prize in Physiology or Medicine (2). As of 2019, the World Health Organization (WHO) estimates that approximately 1.5 million new HBV infections occur each year, with the highest burden of disease currently in the WHO Western Pacific Region and the WHO African Region (3). A fraction of adults (< 10%) with acute HBV infection will develop chronic hepatitis B (CHB), while acutely infected infants are at a much higher risk (~90%); a staggering 296 million individuals are estimated to be living with CHB (3,4). Despite the goals set by the WHO to decrease HBV-related mortality by 65% from 2015 to 2030, global HBV-related mortality is projected to increase by 39% during this time (5,6). Many factors contribute to this increased mortality (recently reviewed (6)) including,

for example, economic disparities in HBV distribution between and within countries, comorbidities and viral coinfections like hepatitis D virus and human immunodeficiency virus (HIV), and societal stigmas that add barriers to receiving HBV healthcare (6,7).

Currently approved strategies to prevent or inhibit HBV are highly effective, including the HBV vaccine (90–95% effective), as well as repurposed nucleos(t)ide analogs (NUCs) from HIV reverse transcriptase (RT) inhibitors like tenofovir and lamivudine (3TC) (4,7,8). Although HBV is effectively inhibited by NUCs, caseation of antiviral therapy leads to unpredictable outcomes and often a rebound of viral load (9). Thus, the current standard of care for CHB is life-long treatment with NUCs. There is another approved class with curative potential, interferon- $\alpha$  or pegylated interferon- $\alpha$  (peg-IFN $\alpha$ ), but it is not well tolerated and suppresses chronic infection in only 30% of patients (10). Currently, CHB has no reliable cure. Therefore, direct-acting agents that target outside the polymerase, alone or in combination, are likely required to achieve HBV eradication (11).

## The HBV replication cycle

The mature, infectious HBV virion is a 42 nm particle

comprised of a viral envelope with embedded HBsAg and contains the relaxed-circular DNA genome (rcDNA) that is covalently attached to the polymerase (P) and encapsulated by the assembled nucleocapsid core (12,13).

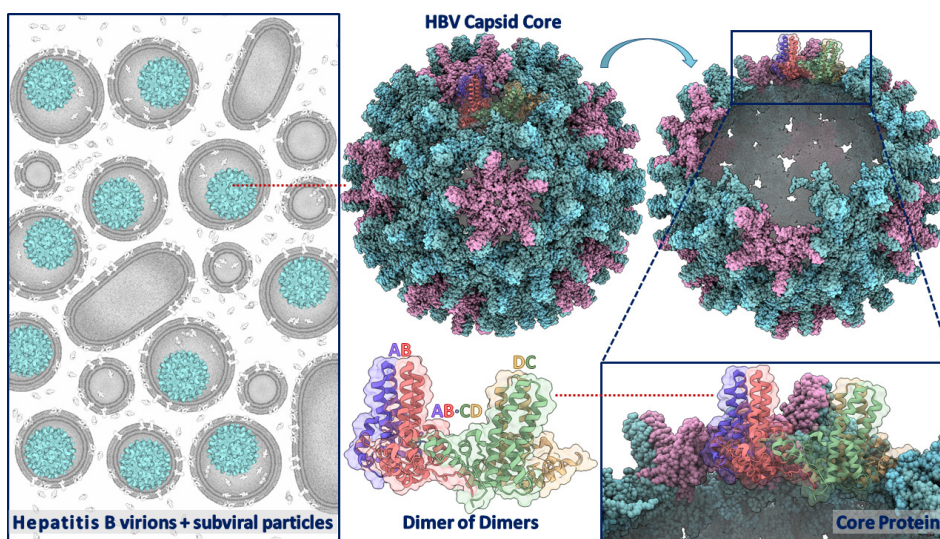
HBV belongs to the hepadnaviridae family of viruses, and as such HBV infects liver cells following HBsAg interactions with the sodium taurocholate cotransporting polypeptide (NTCP), a transmembrane protein specifically expressed in hepatocytes (14). The virus is internalized in an endosome, and the nucleocapsid is released into the cytoplasm by currently unresolved mechanisms (15). In infectious particles, the nucleocapsid is a T = 4 icosahedral core that is an assembly of the multimeric core protein (Cp), or HBV core antigen (HBcAg) (Figure 1). Once in the cytoplasm, the core is trafficked toward the nucleus to proceed through the nuclear pore complex (NPC) in an importin-dependent fashion (12,16). Following nuclear entry, the partially double-stranded and partially single-stranded rcDNA with the covalently attached P protein triggers host cell DNA repair mechanisms. This inevitably leads to the formation of the covalently closed chromosomal DNA (cccDNA), an episome indistinguishable to the host from its own genome, functionally integrating itself into the cell (17,18).

The cccDNA genome is the template for multiple mRNA transcripts of varying size, one of which encodes the full pre-genomic RNA (pgRNA) that can be translated into HBcAg or HBeAg depending on the ORF used, and P (19-21). The HBV P protein is a large, multifunctional protein with Ribonuclease H (RNaseH), RT protein-priming, RNA-dependent DNA-polymerase

(RdDP) and DNA-dependent DNA-polymerase (DdDP) activity (8,21). The interaction of P with pgRNA, specifically with the epsilon RNA stem loop, is required for encapsidation into assembling cores, which has fine-tuned assembly kinetics to make infectious particles (22). The pgRNA serves as the template for RT when creating the rcDNA after encapsidation (21,23). In addition to the infectious virions, referred to as "Dane particles", infected cells excrete non-infectious soluble core antigens (HBeAg) and excrete HBsAg-coated subviral particles (SVP). These SVPs can contain viral mRNA, erroneous cellular mRNA or be entirely devoid of nucleic acid (24). The mRNA translated from cccDNA can be alternatively spliced, leading to production of small/medium and large HBsAg mRNA. The HBsAg precursor proteins are translated at the endoplasmic reticulum (ER) membrane, at different initiation codons, and adopt various conformations to enable the HBV envelope to form and bud (12,19).

### The core protein (Cp) of HBV

The largest transcript from the cccDNA encodes the full HBV genome, the pgRNA. In addition to being packaged into assembling virions, as discussed above, the pgRNA can be used as the mRNA for multiple HBV proteins. pgRNA encodes for P, as well as two in-frame initiation codons that translate precore (p25) or Cp (p21) (12,19,21). When translated starting at the second in-frame initiation codon, Cp is cytosolically translated prior to its assembly into the HBV capsid core. Cp is a 21 kDa protein with 183–185 amino acids, depending on



**Figure 1. Structure of the hepatitis B Virus (HBV) capsid core.** [Left] Rendition of released particles from HBV infection, including mature virions with assembled capsids (PDB: 6HTX) (96) and core protein (Cp) dimers (PDB: 6ECS) (97) as well as both non-infectious subviral particles and excreted HBV E Antigen (HBeAg, PDB: 3V6Z) (51). No full structure of HBV Surface Antigen (HBsAg) is deposited in the PDB; partial HBsAg (PDB: 7TUK) (98) and CD9 (PDB: 6K4J) (99) protein embedded in the default membrane structure from cellPAINT to represent the viral envelope (in grey). The mature capsid shown in blue and all other proteins in white. The P•pgRNA complex or P•rcDNA would be located inside the core. Made with cellPAINT (v2.0) (100). [Right] Two orientations of the HBV Capsid core (PDB: 6HTX) (96) with hexamers in cyan and pentamers in pink. One dimer of Cp dimers has the secondary structure shown with distinct colors in each Cp monomer to display protein-protein interactions. Made with ChimeraX (v1.5) (101).

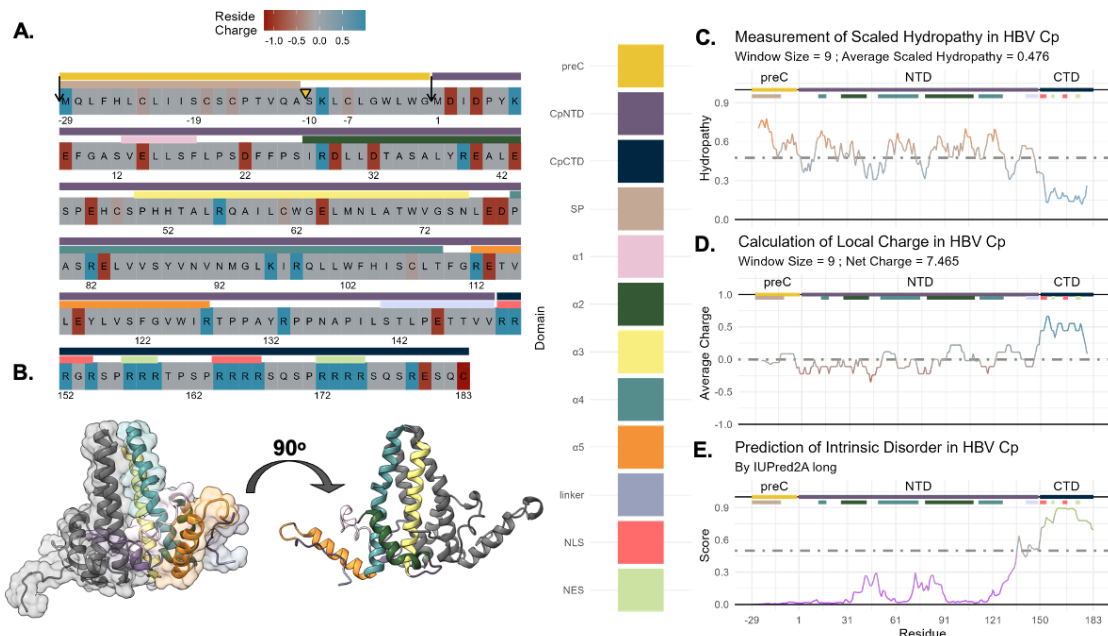
the virus isolate. The N-terminal Domain of Cp ( $Cp_{NTD}$ ) is responsible for capsid assembly and the C-terminal domain of Cp ( $Cp_{CTD}$ ) is responsible for binding nucleic acid and signaling (13,25-29) (Figure 2A).

The  $Cp_{NTD}$  comprises residues 1–149 and, when expressed in *E. coli*, can assemble *in vitro* into particles indistinguishable from native HBV cores; however, the  $Cp_{NTD}$  could not assemble in mammalian cells or *in vitro* with rabbit reticulocyte lysate (29-33). This domain contains five  $\alpha$ -helices ( $\alpha 1$ – $\alpha 5$ ), with  $\alpha 3$  and  $\alpha 4$  acting as the dimerization interface by making a 4-helix bundle (4HB) in  $Cp \cdot Cp$  dimers, and  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 5$  sit perpendicular to the 4HB making the interface for dimer-dimer interactions (Figure 2B). Following translation, Cp monomers will immediately dimerize at the hairpins made by the amphipathic, antiparallel  $\alpha 3$  and  $\alpha 4$ , forming a 4HB, which protrudes out of assembled cores like a spike (Figure 2C) (25,29,30,34-36). The 4HB is stabilized by a disulfide bond between the Cys61 in both monomers, but this linkage is not required for Cp assembly (21,37). Following dimerization, hydrophobic interactions between  $\alpha 5$ 's and multiple contacts throughout Cp create dimer-dimer interactions to form the assembled capsid (36,38,39). The residues 140–149 are frequently referred to as a linker domain connecting  $Cp_{NTD}$  and  $Cp_{CTD}$ , and it has assembly-independent functions (40,41).

The  $Cp_{CTD}$  comprises residues 150–183 or 150–185 depending on subtype, and is characterized by intrinsic

disorder, due in part to the presence of 16 positively charged Arg residues and 7 residues (6 Ser and 1 Thr) that can be phosphorylated in this 35–37 amino acid region (26-28,42) (Figure 2, C-E). These sequential Arg residues provide the nucleic acid binding functions of Cp that are needed for encapsulation of pgRNA during core assembly, as well as influencing rcDNA formation and Cp potentially being associated with nuclear cccDNA (17,23,27-29,41,43). The  $Cp_{CTD}$  additionally contains both nuclear localization signals (NLS) and nuclear export signals (NES) that are made of these sequential Arg residues (16). Further, the phosphorylation of  $Cp_{CTD}$  residues encode a complex regulatory system that influences many aspects of HBV replication, including cellular trafficking, disassembly at the nuclear pore complex and RNA encapsidation, which remains the target of ongoing investigations (27,31,32,44,45). For many years, the full-length Cp protein was challenging to recombinantly express and purify; truncated expression constructs made only of the  $Cp_{NTD}$  gave increased stability, and thus, many studies of Cp assembly have relied solely on the  $Cp_{NTD}$ . Now, bacterial codon-optimized expression vectors allow for the efficient production of full-length Cp in *E. coli*, especially helping in the translation of the Arg-rich  $Cp_{CTD}$  (28,45,46). While the  $Cp_{CTD}$  is not required for capsid lattice assembly, it is required for creating infectious HBV virions (47).

Translation of the preCore (preC) begins at the



**Figure 2. Structure and Characteristics of the HBV core protein (Cp).** (A). Sequence and structural motifs in p25; colors to the right (UniProt: P0C6H5). Arrows indicate start codon sites, with the second being used to translate Cp and the first for preCore (preC). The site of the Signal Peptide (SP; brown) cleavage shown with the yellow triangle. Residues are colored by charges at pH 7.0 (42). (B). Two Cp Dimers (PDB: 6HTX) (96), one in gray and the other colored based on the sequence annotation in (A). Made with ChimeraX (101). (C). Shows the local hydropathy of the Cp, with the  $Cp_{NTD}$  hydrophobic helices for assembling the core. (D). Displays the local charge of the Cp, exemplifying the  $Cp_{CTD}$  having a characteristic basic charge for nucleic acid binding. (E). Predictions of intrinsic disorder by IUPred2 ( $> 0.5$  disordered region;  $< 0.5$  ordered region) (102), exemplifying the disordered nature of the  $Cp_{CTD}$ . A, C-E made with idpr v 1.8.0 (42).

initiation codon upstream of the Cp initiation site and generates a precursor protein that contains a 29 residue N-terminal extension of the Cp, which contains a cotranslational ER localization signal (19,20). A segment of the preC N-terminus is processed inside the ER lumen, changing the protein from p25 to p22, and subsequent processing at the C-terminus into p17 prior to ER-Golgi-mediated secretion (Figure 2A; yellow triangle) (48,49). The p22 intermediate and Cp are nearly identical at the sequence level, except for the remaining 10 residue N-terminal extension in p22 (19,48,49). This short leader sequence contains an influential cysteine residue (Cys-7), that forms a disulfide bond with Cys61, creating distinct quaternary structures that result in two HBV-related antigens: HBeAg and HBcAg for p17 and Cp, respectively (13,50,51). Of note, when denaturing Cp, the antigenic region of HBeAg is revealed (49,52). The function of HBeAg is not known and it is dispensable for infection; HBeAg is found in serum of patients shortly after infection and, as such, is used as a marker for active viral replication (53). Additionally, there is emerging interest in tracking the transcriptional activity of cccDNA using the hepatitis B virus core-related antigen (HBcrAg), a mixture of HBcAg, HBeAg, and other preC gene products (19).

Once the critical concentration of Cp has been surpassed, 90 or 120 Cp dimers will spontaneously self-assemble into T = 3 or T = 4 icosahedral capsids, respectively. In the assembled cores, the Cp<sub>NTD</sub> faces outwards with the 4HB protrusions giving the capsid a spike-like appearance, and the Cp<sub>CTD</sub> is generally localized to the interior and is highly flexible. Assembly is characterized by sigmoidal kinetics, similar to a crystal lattice with the rate-limiting step being the formation of a nucleation seed (54). The lag phase is characterized by the formation of a trimer of dimers; individual dimer-dimer interactions *in vitro* are weak (~3.5 kcal/mol), which allows for the thermodynamic editing of improperly associated dimers. Once nuclei form, they rapidly assemble into icosahedral capsids, as free dimers remain. Cp dimers form at least 3 contacts with additive association energies that are enough to form stable capsids even though individual dimer•dimer interactions are weak (54-56). It is important to note that the *in vivo* assembly also can utilize RNA not possible in these Cp<sub>NTD</sub> constructs; in purified capsid-like particles, those with the Cp<sub>CTD</sub> and ssRNA require harsher denaturation conditions (45,47,57). T = 4 capsids are the primary result of assembly products (> 90%) and have a diameter of ~36 nm, while T = 3 capsids have a diameter ~32 nm (58). The ratio of these core sizes, as well as the kinetics of assembly, are impacted by ionic strength and protein concentration with mild conditions favoring T = 4 and few intermediates (28). In addition to the spiked icosahedron, the capsid is characterized by many holes that are permeable to ions, metabolites and inhibitors (25,39,58).

## Capsid assembly modulators (CAMs)

A class of molecules with curative potential are CAMs that bind Cp dimers. Also referred to as core protein allosteric modulators (CpAMs) and Capsid Inhibitors (CIs), these are an exciting development in the field of HBV therapeutics, as NUCs treat but do not cure CHB (59). These Cp-targeting HBV antivirals bind to the same site but are commonly subdivided into two classes: type I CAMs that create aggregated or aberrant capsids that are unable to function (CAM-A), and type II CAMs that enhance the assembly rates of Cp dimer•dimer interactions to make empty cores (CAM-E) (28,60,61). As the capsid has numerous roles throughout the replication cycle, its misassembly can impact multiple steps in acute or CHB infection.

### Class I: CAM-A

The first CAM-A molecule described was BAY 41-4109, belonging to the heteroaryldihydropyrimidines (HAP) family with HBV inhibition at submicromolar concentrations (33,62,63). Oral administration of BAY 41-4109 decreased HBV DNA in liver and plasma of HBV-transgenic mice, in line with 3TC treatment, but also showed a reduction of cytoplasmic HBcAg, a phenotype distinct from the NUC treatment (62). The observed Cp reduction is a result of proteasome activity (63), although an increase in Cp ubiquitylation has not been shown following HAP treatment (33). More recent studies have found nuclear aggregates of Cp, specifically associated with promyelocytic leukemia (PML) nuclear bodies, after multiple days of CAM-A (BAY 41-4109 and BAY 38-7690) but not CAM-E treatment (64,65). Shortly after BAY 41-4109 treatment, Cp aggregates were primarily cytoplasmic, but associated with nuclear envelope (66). Further, it was shown that after infection BAY 41-4109-induced Cp aggregates were perinuclear and targeted for p62-mediated macroautophagy and lysosomal degradation by the host factor STUB1, an E3 ubiquitin ligase (67). It should be noted that STUB1 is a co-chaperone of heat shock protein 70 (HSP70) and HSP90 (68), and both are considered proviral factors for HBV capsid assembly (67,69,70). Though the BAY compounds were not clinically developed, they have served as good probes to characterize the CAM-A chemotype.

Currently, the most promising HAP compounds are RO7049389 and GLS4. RO7049389 is currently in phase 2 trials (NCT04225715), with phase 1 results (NCT02952924) showing infrequent, mild adverse events and a reduction of HBV DNA in treatment-naïve CHB patients after 4 weeks of CAM-A administration comparable to NUC controls (71). Additionally, due to the high prevalence of HBV in Asia (3,6), the safety of RO7049389 was validated in healthy Chinese volunteers in a phase 1 clinical trial (NCT03570658). This showed

the compound was well-tolerated in single (600 mg) and multiple (400 mg twice daily) doses, and the safety profiles were similar between Asian and non-Asian healthy volunteers, though with higher plasma exposure of RO7049389 observed in the Chinese participants (72). The other leading CAM-A molecule currently in phase 2 trials is GLS4 (NCT04147208), a derivative of Bay 39-5493, with nanomolar potency and is fairly well-tolerated and effective when administered at 120 mg daily in combination with 100 mg ritonavir for 28 days (73,74). The safety and efficacy of these CAM-A compounds are exciting, but their long-term application and antiviral resistance remains to be seen.

Some other CAM-A molecules include KL060332 (75), a HAP molecule under a phase 1a trial in China, and preclinical compounds JNJ-890 (76), HAP\_R01 (77), and ZW-1847 (78). A unique, non-HAP molecule with CAM-A phenotype is Ciclopirox, which inhibited HBV at 5 mg/kg in mice (79). Interestingly, ciclopirox olamine®, an antifungal agent, was tested for oral safety and tolerability as an anti-apoptotic gene suppressant for treating hematological malignancies (NCT00990587), indicating its safety for use in future trials investigating HBV inhibition in clinical trials (79,80).

#### *Class II: CAM-E*

Both CAM-A and CAM-E bind to the same hydrophobic pocket at Cp dimer•dimer interfaces, but the cores formed following only CAM-E treatment are noninfectious, vacant icosahedral capsids. CAM-E molecules enhance the rate dimer-dimer interactions, overcoming the rate-limiting step in assembly, and form icosahedral capsids that are devoid of P•pgRNA. Unlike the CAM-A class made primarily of the HAP chemotype that results in aggregates, CAM-E molecules are much more diverse and rapidly lead to native-like assemblies. Some CAM-E scaffolds include phenylpropenamides (PPAs), glyoxamoylpyrroloxamides (GLPs), sulfamoylbenzamides (SBAs), and sulfamoylpyrroloamides (SPAs).

One of first reported CAMs and the best studied CAM-E molecule is AT-130, which belongs to the PPA class (61,81). AT-130 inhibits HBV with submicromolar potency in cell culture but has not been tried in clinical trials. As the prototypical scaffold for the CAM-E chemotype, AT-130 has been shown to increase the kinetics of capsid assembly through interactions near Cp<sub>NTD</sub> linker and  $\alpha$ 5 that alter the interface of dimer•dimer interactions, as well as distorting the 4HB, resulting in intermediates that are primed to assemble (82-84). These result in icosahedral capsids without the P•pgRNA needed for HBV replication, or partially completed assembly intermediates (84,85).

Another preclinical CAM-E class that has great potential is the GLP class, with the first GLP compound patented in 2015 (61,86). GLP compounds are

exceptionally potent, with the compound GLP-26 having nanomolar potency in humanized mice (87). GLP-26 was further shown to be well tolerated and highly effective at inhibiting HBV in both non-human primate studies and primary human cardiomyocytes, indicating safety for further investigations (88).

Both the SBA and SPA classes are some of the most clinically developed CAMs. One of the first successful clinical investigations into CAM safety was the SBA compound NVR 3-778 (NCT02401737), which was tolerated but not as effective as approved NUCs and thus was discontinued (28,89). An SBA under clinical investigation is JNJ-64530440 (phase 1b, NCT03439488) with potent antiviral activity in patients with CHB and was well tolerated (90). Additionally, the SPA JNJ-6379 (JADE NCT03361956), recently reported phase 2 trials results of a reduction in HBV DNA and RNA when given in combination with a NUC. They note that multiple patients had the T33N mutation emerge during monotherapy (91). This underscores the likely importance of combination therapy in future HBV therapeutics.

Some other promising CAM-Es molecules include ALG-000184, a potent inhibitor with successful phase 1 trials (NCT04536337) (92), Canocapavir (ZM-H1505R) that was well tolerated (Phase 1b, NCT05470829) (93), and GST-HG141 (phase 1, NCT04386915 & NCT04868981) (94). Many CAMs have been reported and are under investigation in clinical trials without their exact CAM phenotype described (Recently reviewed (28,55)). Additionally, a novel HBV antiviral class has been reported as a cccDNA inhibitor; the molecule ccc\_R08 is hypothesized to target an unknown host factor and decreased preexisting cccDNA pools, unlike currently reported CAMs (95).

#### **Conclusions**

At the present, approximately 3.5% of the world's population lives with CHB, though most are unaware (3,6). Key to curing CHB is elimination of cccDNA, and the currently approved and widely used NUCs suppress HBV replication but do not achieve a cure (18,55). Due to their ability to clear cccDNA, CAMs that modify Cp dimer•dimer interactions have the potential to redefine the standard of care for treating HBV infection and could subsequently achieve a cure for CHB (11,59). The long-term effects of CAM treatment and likely emergence of CAM-induced antiviral resistance remain to be investigated. However, the progress in CAM development and successful investigations into HBV capsid biology over the last two and a half decades give promise for highly effective and well characterized treatments to potentially cure the hundreds of millions currently living with CHB.

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