Hepatitis B virus (HBV) infection is a globally distributed health problem. The number of carriers of HBV is estimated to exceed 240 million people worldwide. Compared with uninfected individuals, HBV carriers have an increased risk of developing hepatocellular carcinoma. The prevention of HBV infection is therefore strongly recommended, and a HBV vaccine is available. For carriers, treatment with nucleoside/nucleotide reverse transcriptase inhibitors is available, but infection frequently requires chronic treatment via a lifetime of medication. Thus, curing HBV infection remains a major challenge. However, despite the development of an infectious HBV cell culture system and recent intense research, many aspects of the HBV life cycle remain poorly characterized. In this review, we focus on the current understanding of the HBV life cycle and involved host factors, as well as potential targets for therapeutic intervention against HBV. We also consider possible immunotherapeutic strategies for eliminating HBV, including the removal of cccDNA from infected hepatocytes.

Key words: cccDNA, druggable target, hepatitis B virus, host factor, viral life cycle

INTRODUCTION

Among the confirmed hepatitis viruses, hepatitis B virus (HBV) is the sole DNA virus. HBV (genus Orthohepadnaviridae) belongs to the Hepadnaviridae family, a family that also includes woodchuck hepatitis virus, ground squirrel hepatitis virus, woolly monkey hepatitis virus1 and bat hepatitis virus.2 The HBV virion,3,4 also called the Dane particle,5 consists of a lipid bilayer membrane that incorporates a complex outer envelope that includes a mixture of small, middle and large surface proteins (SHB, MHB and LHB, respectively). The membrane/envelope encloses an inner icosahedral nucleocapsid, also known as the core particle, which is composed of 120 dimers of core protein.6–8 The nucleocapsid contains one copy of a partially double-stranded DNA genome that is covalently cross-linked to the viral polymerase protein. The HBV genome is approximately 3200 bp; although this length is relatively short, the genome encodes viral protein efficiently.

Hepatitis B virus infection is a globally distributed health problem. HBV carriers number in excess of 240 million people worldwide.9 HBV establishes a persistent infection by evading the host immune system, and is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Recently, de novo HBV reactivation during chemotherapy, immunosuppressive therapy and carcinogenesis has been reported; therefore, anti-HBV treatment has the long-term goal of clearance of hepatitis B surface antigen (HBsAg). This HBsAg seroclearance is widely recognized as a functional cure even in case of cccDNA persistence. In the future, such treatment also will need to eliminate cccDNA. However, existing treatments like pegylated interferon (PEG IFN) and nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) have the least effect on complete cure of HBV including the elimination of cccDNA as the ultimate goal of HBV therapy. Therefore, new antivirals with different modes of action for treating HBV are under development. In this review, we focus on current understanding of the HBV life cycle, involved host factors and druggable targets (Fig. 1). We also consider immunotherapeutic strategies for eliminating HBV, including cccDNA, from infected hepatocytes.
ATTACHMENT AND ENTRY

At initial attachment, the preS1 region of the LHB protein binds (at low affinity) to heparan sulfate proteoglycan (HSPG) on the surface of hepatocytes, leading to localized concentration of HBV virions. As a second step, the concentrated HBV virions transfer from HSPG to sodium taurocholate co-transporting polypeptide (NTCP, a liver-specific bile acid and bile salt transporter), which has been identified as a HBV entry receptor. The interaction between the N-terminal myristoylated amino acids in the preS1 region of LHB and NTCP, likely in association with as-yet-unrecognized host factors, provides a spark for incorporation into the hepatocyte via clathrin-mediated endocytosis. Both Rab5 (an early endosome factor) and Rab7 (a late endosome factor) have been reported to regulate HBV infection. However, it remains unclear whether acidification through endocytosis is required, and the process of fusion between the viral envelope and the endosomal membrane is not fully understood.

The identification of NTCP as a HBV entry receptor was a key milestone, enabling the development of a novel cell infection system based on HepG2-NTCP cell lines combined with HBV virions produced in cell culture. Recently, myrcludex B (a HBV preS1-derived lipopeptide) and cyclosporin A (CsA) and its
derivatives (immunosuppressive agents)\textsuperscript{22,23} were shown to directly target NTCP and to exhibit inhibitory effects against HBV entry. Moreover several US Food and Drug Administration-approved drugs (irbesartan, ezetimibe and ritonavir) also have inhibitory activity against NTCP.\textsuperscript{24} Interestingly, (−)-epigallocatechin-3-gallate reduces HBV entry by accelerating the degradation of NTCP.\textsuperscript{25} However, to date, only hepatitis B immunoglobulin (HBIG) has been used regularly in a clinical setting to target HBV infection at the entry stage. Specifically, HBIG has been used to prevent reinfection after liver transplantation, to prevent vertical transmission from HBV-infected mother to child, and for postexposure prophylaxis. Targeting NTCP and other entry factors may provide another strategy against HBV infection.

**UNCOATING AND NUCLEAR IMPORT**

Following entry into hepatocytes, the HBV nucleocapsid (containing the viral genome) is released into the cytoplasm. The nucleocapsid consists of rcDNA covalently cross-linked to the terminal protein domain of viral polymerase. The C-terminal arginine-rich domain of core protein is believed to facilitate the transport of the nucleocapsid from the cytoplasm to a nuclear pore. At the pore, the nucleocapsid is degraded as part of a process called uncoating, such that only the rcDNA enters the nucleus.\textsuperscript{26} Uncoating and nuclear import appear to require microtubules and nuclear importin protein,\textsuperscript{26} but a detailed molecular mechanism for these processes has yet to be determined.

**CCCDNA FORMATION**

The conversion from intranuclear rcDNA to cccDNA progresses through multiple stages, including: (i) release of polymerase from the minus strand of rcDNA; (ii) removal of RNA primer from the plus strand of rcDNA; (iii) synthesis of one DNA strand by trimming and filling in; and (iv) ligation. cccDNA synthesis has been presumed to involve host cellular factors, giving the minimal coding capacity of the HBV genome. Indeed, tyrosyl-DNA phosphodiesterase 2 recently was identified as a host DNA repair factor that has the ability to cleave polymerase from rcDNA, as required for HBV cccDNA biogenesis.\textsuperscript{27} However, other host factors associated with cccDNA formation, including DNA repair, remain unknown. cccDNA forms a minichromosome that associates with 15–16 nucleosomes, each consisting of core histones H2A, H2B, H3 and H4 along with the linker histone H1 and non-histone proteins.\textsuperscript{28} A number of modification factors, including histones, chromatin-modifying enzymes and viral proteins, as well as core proteins and hepatitis B x protein (HBx), have been identified as transactivators and/or epigenetic regulators of cccDNA formation.\textsuperscript{29–31} However, we still lack a detailed molecular mechanism for the formation of cccDNA; targeting of this process remains an unexploited route for inhibition of the HBV life cycle.

ccDNA is the key molecule in the persistence of HBV; elimination of cccDNA remains the ultimate goal for the elimination of HBV infection. Disubstituted sulfonamide compounds were identified as specific inhibitors of cccDNA formation via screening of a small compound molecule library; these compounds were shown to interfere with the conversion of rcDNA to cccDNA.\textsuperscript{32} Recently, it was reported that activation of the lymphotixin-β receptor affects the specific degradation of the nuclear cccDNA by a cytidine-deamination mechanism via APOBEC3B upregulation\textsuperscript{33} resembling the activation of APOBEC3A by IFN-α. DNA cleavage enzyme-based strategies that specifically target cccDNA are being evaluated, and include the use of zinc-finger nucleases, transcription activator-like effector nucleases and CRISPER/Cas9 technology.\textsuperscript{34–37} However, wide clinical application of such treatments will require processes for efficient and accurate delivery of these reagents to HBV-infected cells.

**TRANSCRIPTION AND TRANSLATION**

CCCDNA serves as a template for host RNA polymerase II, which transcribes cccDNA to yield viral RNA of four different lengths: a 3.5-kb (genome-length) RNA, including pregenomic and precore RNA coding for polymerase protein and the precore protein precursor of the secretory hepatitis B e-antigen; 2.4- and 2.1-kb RNA coding for the HBx protein. The transcription of viral RNA is regulated by the PreC/C, PreS, S and X promoters as well as by Enhancer 1 and 2 via interaction with liver-specific host transcription factors.\textsuperscript{38} Interestingly, the HBx protein has a transactivation activity and is involved in transcriptional regulation of viral RNA.\textsuperscript{30} All of the HBV RNA have 5′-cap structures and 3′-poly-A tails. All four viral RNA are exported from the nucleus to the cytoplasm without splicing; the RNA then are translated into different viral proteins.

Viral messenger RNA could be directly targeted by using siRNA,\textsuperscript{39–43} antisense oligonucleotides, and ribozymes. The most promising results have come from a recent phase 2 clinical trial\textsuperscript{43} employing siRNA technology to target HBV-specific transcripts. In this study, an siRNA-based drug, named ARC-520, was well tolerated and resulted in
dose-dependent decreases of HBsAg in chronic hepatitis B patients.

**CAPSID FORMATION, REVERSE TRANSCRIPTION AND DNA SYNTHESIS**

Viral pregenomic RNA (pgRNA) has an ε-stem loop proximal to the 5′-end. Polymerase binding to the ε-stem loop triggers capsid formation. pgRNA is selectively encapsidated within core particles in the cytoplasm together with the polymerase. Within each core particle, viral DNA synthesis is initiated by reverse transcription polymerase activity; following negative strand synthesis and concomitant degradation of the RNA template by RNase H, positive strand DNA synthesis is initiated using short RNA from non-degraded pgRNA as a primer.\(^{44,45}\) Nucleocapsids containing rcDNA then progress to the next step (envelopment) or are recycled by uncoating and importation into the nucleus of the new rcDNA.

Second-generation NRTI (e.g., entecavir and tenofovir) target the viral polymerase. NRTI have been shown to reduce HBV viremia by 5–6 logs within 1 year, are well tolerated and select drug-resistant variants at very low rates.\(^{46,47}\) However, NRTI treatment only rarely provides clearance of HBsAg, meaning that many patients will need to take NRTI on a chronic (lifelong) basis.

Phenylpropenamide derivatives were shown to interfere with pgRNA packaging, resulting in the release of immature empty capsids from infected cells. In vitro studies have shown that phenylpropamide derivatives demonstrate synergistic antiviral activity in combination with NRTI.\(^{51}\)

In other work, heteroaryldihydropyrimidines (HAP) have been reported to inhibit both capsid formation and capsid stability.\(^{52–55}\) Interestingly, both phenylpropamide derivatives and HAP have been found to be useful for the treatment of NRTI-resistant viral variants.\(^{48,49}\)

**ASSEMBLY AND SECRETION**

All three of the HBV envelope proteins (SHB, MHB and LHB) have been detected in folded form on the endoplasmic reticulum (ER) membrane. Envelope proteins have been observed to generate empty particles (subviral particles, SVP) in the ER, which are then released through the Golgi body and/or multivesicular body (MVB). Two types of SVP have been seen: spherical and filamentary. In the presence of nucleocapsid, LHB proteins interact with the coated genome to generate a complete HBV virion (a Dane particle). The content ratio of the three envelope proteins is known to differ between Dane particles and SVP; notably, Dane particles are enriched for LHB protein compared with SVP. The mechanism of HBV virion packaging is proposed to consist of the following steps: (i) nucleocapsid is transported to the surface of the MVB through Nedd4 and γ-2-adaptin;\(^{56}\) and (ii) nucleocapsid buds into MVB on contact with HBV envelope proteins via endosomal sorting complex required for transport proteins (Alix, Vsp4 and CHIMP).\(^{57,58}\) However, the process of migration of HBs proteins from ER to MVB remains unknown. Moreover, the pathway for the secretion of HBV virions also remains unclear. Recently, Rab7 was shown to regulate MVB degradation and also to accelerate the disassembly of HBV virions.\(^{59}\)

Several inhibitors targeting HBV secretion and budding are in development, including both preclinical and clinical trials. Notably, amphiphatic DNA polymers, which have been shown to interfere with HBsAg release, show a wide range of antiviral activity against HBV, hepatitis C virus and HIV. Inhibition of HBV secretion is expected not only to reduce viremia but also to facilitate recovery of immunity. However, clinical application of inhibitors of secretion and/or budding will require addressing other issues, including the possible pathological effects of ongoing accumulation of cccDNA and of HBV proteins.

**IMMUNOTHERAPEUTIC AGENTS**

Including degradation of cccDNA, inhibition of transcription, and prevention of encapsidation, IFN-α shows pleiotropic effects on the HBV life cycle.\(^{64,65}\) IFN-α also has a key role as an immunomodulator used to treat HBV infection. However, the rate of cure of HBV infection using PEG IFN monotherapy is very rare, even after 48–72 weeks of treatment. Accordingly, many trials have been conducted by using combination therapy with NRTI and PEG IFN, but the resulting data do not support the use of these combinations.

Other cytokines with potential application for treatment of HBV include IFN-γ, which is known as a main player in non-cytolytic clearance of HBV during acute infection,\(^{66}\) and tumor necrosis factor (TNF), which also is tracked as a marker in the management of HBV infection. Recently, indoleamine 2,3-dioxygenase has been reported to demonstrate IFN-γ-mediated antiviral activity against HBV.\(^{67,68}\) Additionally, cellular inhibitors of apoptosis proteins (cIAP) have been shown to impede TNF-mediated HBV eradication, an observation that has led to the development of cIAP inhibitors as potential HBV treatments.\(^{69,70}\) Toll-like receptor 7 (TLR7) agonists\(^{71–76}\) and therapeutic vaccines\(^{77–79}\) also have been the subjects of vigorous development projects. The restoration of antiviral
immunity will be imperative for the full cure of HBV-infected individuals.

SUMMARY AND CONCLUSIONS

In this review, we described current understanding of the HBV life cycle and associated host factors, including druggable targets that may be used in treating HBV infection. Many of the corresponding drugs are in evaluation via preclinical and/or clinical trials. The combination of present treatments and antiviral drugs in development promises to reduce the severity of HBV infection on a long-term basis. However, we are still a long way away from the complete eradication of HBV, notably including treatments that will permit elimination of cccDNA in carriers. Extended efforts will be needed to obtain a regimen that permits a complete cure of HBV infection.

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REFERENCES


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45 Chotiyaputta W, Peterson C, Ditah D, Lok AS. Persistence and adherence to nucleos(t)ide analogue treatment for chronic hepatitis B. J Hepatol 2011; 54: 12–8.


52 Stray SJ, Bourne CR, Punna S, Lewis WG, Finn MG, Zlotnick A. A heteroaryldihydropyrimidinide activates and can misdirect

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