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REVIEW ARTICLE

Hepatitis B virus: new therapeutic perspectives

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Abstract

Current antiviral therapies have dramatically improved the long-term outcomes of patients with chronic hepatitis B virus (HBV) infection. Both interferon (IFN) and nucleos(t)ide analogue (NA) treatments have been shown to reduce the progression of liver disease in chronic hepatitis B (CHB) patients. However, persistent covalently closed circular DNA (cccDNA) can result in a viral relapse after discontinuation of antiviral treatment. On the basis of extensive research on the HBV lifecycle and virus–host interactions, several new agents focusing on viral and host targets are under development to cure HBV. New polymerase inhibitors, tenofovir alafenamide and besifovir provide effective and safer treatment for CHB patients. Agents targeting cccDNA, such as engineered site-specific nucleases and RNA interference therapeutics could eliminate cccDNA or silence cccDNA transcription. Inhibitors of HBV nucleocapsid assembly suppress capsid formation and prevent synthesis of HBV DNA. The HBV entry inhibitor, Myrcludex-B, has been shown to effectively inhibit amplification of cccDNA as well as the spread of intrahepatic infection. Agents targeting host factors that enhance innate and adaptive immune responses, including the lymphotoxin-β receptor agonist, toll-like receptor agonist, immune checkpoint inhibitors and adenovirus-based therapeutic vaccine, could play a critical role in the elimination of HBV-infected cells. With all of these promising approaches, we hope to reach the ultimate goal of a cure to HBV in the near future.

Keywords
chronic hepatitis B – covalently closed circular DNA – hepatitis B virus

Hepatitis B virus (HBV) is an important public health problem and the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) worldwide. The estimated annual incidence of cirrhosis is 2–6% in hepatitis B e antigen (HBeAg)-positive and 8–10% in HBeAg-negative patients. The estimated lifetime risk of developing cirrhosis, liver failure or HCC in HBV patients is as high as 15–40% (1–3). Therefore, effective antiviral agents are urgently needed to delay or even halt the progression from chronic hepatitis to cirrhosis and

Abbreviations
ALT, alanine aminotransferase; CHB, chronic hepatitis B; CI, confidence interval; CTLA-4, cytotoxic T-lymphocyte antigen 4; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; HR, hazard ratio; IFN, interferon; NA, nucleos(t)ide analogue; PEG-IFN-α, pegylated interferon-α; SVR, sustained virological response; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate; TLR, toll-like receptor.

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Key points

- Several meta-analyses have shown that existing chronic hepatitis B treatments can reduce progression of liver disease.
- Persistent covalently closed circular DNA (cccDNA) is the main source of failure to eliminate hepatitis B virus (HBV). Engineered site-specific nucleases and RNA interference therapeutics could clear or silence cccDNA.
- Myrcludex-B targets HBV entry receptor and inhibits amplification of intrahepatic cccDNA as well as the spread of intrahepatic infection.
- The lymphotoxin-β receptor agonist, toll-like receptor agonist, immune checkpoint inhibitor and adenovirus-based therapeutic vaccine could enhance innate and adaptive immune responses to induce non-cytolytic destruction of cccDNA or attack HBV-infected hepatocytes.

HCC. Research and therapeutic developments in the molecular virology of HBV have led to significant advances in the treatment of chronic hepatitis B (CHB). At present antivirals approved for CHB include standard or pegylated interferon-α (PEG-IFN-α) and nucleos(t)ide analogues (NA) (4–6). Although the efficacy of antivirals has markedly improved the long-term outcomes of CHB patients, clearance of hepatitis B surface antigen (HBsAg) is only achieved in a small portion of HBV patients. Therefore, a cure of HBV infection is still a daunting challenge, especially in Asian patients who acquire the virus early in life (7). Several potential strategies focusing on viral and host targets responsible for persistent HBV are under clinical development to cure HBV (8). This article reviews and discusses new therapeutic perspectives of CHB.

Current antiviral agents decrease disease progression and the incidence of hepatocellular carcinoma

At present, seven agents are extensively used for the treatment of CHB: standard interferon-α (IFN-α) or pegylated interferon-α (PEG-IFN-α) and five nucleos(t)ide analogues (NA), including lamivudine (LAM), telbivudine (LdT), entecavir (ETV), adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF). The pharmacological properties of these agents differ. For example, IFN mainly has immune modulatory effects and weak direct antiviral effects while NA have direct antiviral effects only. Several international HBV management guidelines recommend PEG-IFN-α, ETV and TDF as the first-line treatments (4–6).

In hepatitis B e antigen (HBeAg)-positive patients, 1 year of PEG-IFN monotherapy led to HBeAg seroconversion in 29–32% of patients at 6 months off-therapy. HBsAg seroconversion was achieved in 3–5% of patients at 6 months off-therapy (9, 10). Fifteen percent of HBeAg-negative patients treated with 1 year PEG-IFN had a combined response with serum alanine aminotransferase (ALT) normalization and HBV DNA <400 copies/ml. HBsAg loss was reported in 4% at 6 months off-therapy (11). After 4 years of follow-up, HBsAg seroclearance progressively increased to 11% (12).

Among the direct antiviral agents, both ETV and TDF are highly potent nucleos(t)ide inhibitors of the HBV polymerase with a high genetic barrier to drug resistance. Cumulative rates of HBV undetectability in treatment-naïve patients were more than 90% after long-term treatment with ETV (13, 14) and TDF (15), irrespective of HBeAg status. HBeAg seroconversion occurred in 21% of patients after 1-year of ETV and TDF therapy, respectively (16, 17). Of note, HBsAg loss occurred in 11.8% of HBeAg-positive patients after 7 years of TDF treatment (15), mostly in non-Asian patients with HBV genotype A or D infection. In addition to the highly potent viral suppression with ETV and TDF, the high genetic barrier to drug resistance also results in a sustained virological response (SVR). The 5-year cumulative probability of genotype resistance of ETV was 1.2% (18). Similarly, no resistance to TDF was detected after 7 years of treatment (15).

Histological evaluation after long-term treatment with ETV and TDF showed improvement in necroinflammatory and fibrosis scores in most patients (13, 19). Recent studies have shown that the risk of development of HCC was reduced in CHB patients receiving ETV treatment compared to untreated historical controls (20, 21). The effect of long-term ETV therapy on the reduction of the risk of HCC in patients with hepatitis B-related cirrhosis was also investigated in the Cirrhosis Taiwanese Entecavir Multicenter (C-TEAM) study from Taiwan. In this large hospital-based cohort study, 503 patients were enrolled in the control group and 1123 in the ETV group from 24 academic centres. ETV treatment was associated with a 60% reduction in the risk of HCC (adjusted hazard ratio [HR]: 0.40, 95% confidence interval [CI]: 0.27–0.60) in patients with cirrhosis (22). The protective effect of NA in the development of HCC was also confirmed in a population-based cohort study in Taiwan. CHB patients treated with NA had a significantly lower 7-year incidence of HCC (7.32%; 95% CI: 6.77–7.87%) than patients without NA treatment (22.7%; 95% CI: 22.1–23.3%; $P < 0.001$). NA treatment was associated with a reduced risk of HCC, with an adjusted hazard ratio of 0.37 (95% CI, 0.34–0.39; $P < 0.001$) (23). A similar nationwide study in Taiwan also showed that NA-treated HCC patients had a significantly lower rate of recurrent HCC after surgical resection (45.6%; 95% CI: 36.5–54.6% vs. untreated, 54.6%; 95% CI: 52.5–56.6%; $P < 0.001$), and NA treatment was independently associated with a reduced risk

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of HCC recurrence (HR: 0.67; 95% CI: 0.55–0.81; $P < 0.001$) (24). All of these results suggest that NA treatment reduces the risk of HCC in a significant proportion of patients with cirrhosis through long-term viral suppression. However, the viral or host factors associated with the development of HCC in patients with profound viral suppression requires further study.

Unmet needs of existing CHB treatment: clearance of cccDNA and better prevention of hepatocellular carcinoma

Although there has been significant progress in the treatment of CHB in the past decade, there are still unsolved problems. First, although a higher rate of sustained off-treatment response is achieved with IFN than with NA, its serological response rate is still far from satisfactory. Most patients do not obtain clearance of HBsAg. In contrast, since NA do not affect HBV covalently closed circular DNA (cccDNA), viral relapse is common after discontinuation of NA, even in patients who have achieved therapeutic endpoints (25–29). Second, NA reduces the risk of development of HCC through long-term viral suppression in a significant proportion of patients with cirrhosis. However, HCC still occurs in patients in persistent virological remission (30). In addition, maintained viral suppression under NA therapy has been associated with a lower risk of HCC in Asians but not in Caucasians (31). These data suggest that NA reduces, but does not eliminate, HCC. Thus, new treatment strategies to clear intrahepatic HBV cccDNA are urgently required for patients who are at a high risk of developing cirrhosis and HCC. Finally, the definition of a cure to HBV must still be clarified. International guidelines state that the ideal therapeutic endpoints for CHB include HBeAg/HBsAg loss or seroconversion in HBeAg-positive patients, and HBsAg loss or seroconversion in HBeAg-negative patients (4–6). To evaluate these treatment goals, the definition of a cure must be confirmed. In principle, a complete or sterilizing cure of HBV requires either clearance of HBV cccDNA or purging HBV-infected hepatocytes (32). At present achieving HBV cccDNA clearance could be difficult and we lack the biomarkers to confirm it. On the basis of stable off-drug suppression of HBV viraemia, cccDNA and HBsAg seroconversion, a functional cure would be the feasible goal of novel treatments (32, 33).

Towards a cure to HBV

A review of the existing data shows that eradication of HBV infection is still a challenge especially in Asian patients who acquire the virus early in life. Fortunately, thanks to extensive research of the hepatitis B viral life cycle and virus–host interactions (Fig. 1), several novel therapeutic approaches to cure HBV that focus on viral and host targets responsible for persistent HBV have been proposed and are being actively investigated (Table 1) (8).

New polymerase inhibitors

Most NA that have been approved to treat the HBV polymerase target DNA elongation. ETV and TDF also impair protein priming (34). However, the major limitation of long-term NA therapy is the development of drug resistance. Novel NA are thus being developed in advanced clinical trials.

Tenofovir alafenamide (GS-7340)

Tenofovir alafenamide (TAF) is a prodrug of tenofovir. In patients with the human immunodeficiency virus (HIV) infection, TAF showed more potent anti-HIV-1 activity and higher intracellular tenofovir levels as well as low-level plasma tenofovir concentrations than TDF (35). In an *in vitro* study, TAF resulted in high levels of the pharmacologically active metabolite tenofovir diphosphate than TDF (36). In a recent study, non-cirrhotic, treatment-naïve CHB patients were randomized to TAF 8, 25, 40, 120 mg and TDF 300 mg for 28 days. Whatever the TAF dose, the mean change in serum HBV DNA in patients receiving TAF treatment were similar to those receiving TDF (37). In addition, TAF has no renal transporter (organic anion transporters, OAT1 and OAT3)-dependent cytotoxicity, which may result in an improved renal safety profile (38).

Besifovir (LB80380)

Besifovir (LB80380) is a novel and potent acyclic nucleotide phosphonate with a similar chemical structure to adefovir and tenofovir. In a multicenter-randomized trial, there were no differences in the proportions of patients achieving undetectable HBV DNA, normalization of ALT or HBeAg seroconversion between besifovir and ETV therapy (39, 40).

Thus, both TAF and besifovir provide effective and safer treatment for CHB patients, but still do not clear HBV cccDNA.

Strategies targeting cccDNA

Persistent HBV cccDNA is the main cause of failure to achieve a cure to HBV and of viral relapse after NA therapy is stopped (41). Novel drug targets must eliminate cccDNA or silence cccDNA transcription.

Engineered site-specific nucleases that induce DNA double-strand breaks are currently used to inhibit HBV replication. The three most commonly used engineered DNA-binding proteins used to target cccDNA are the zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the RNA-guided

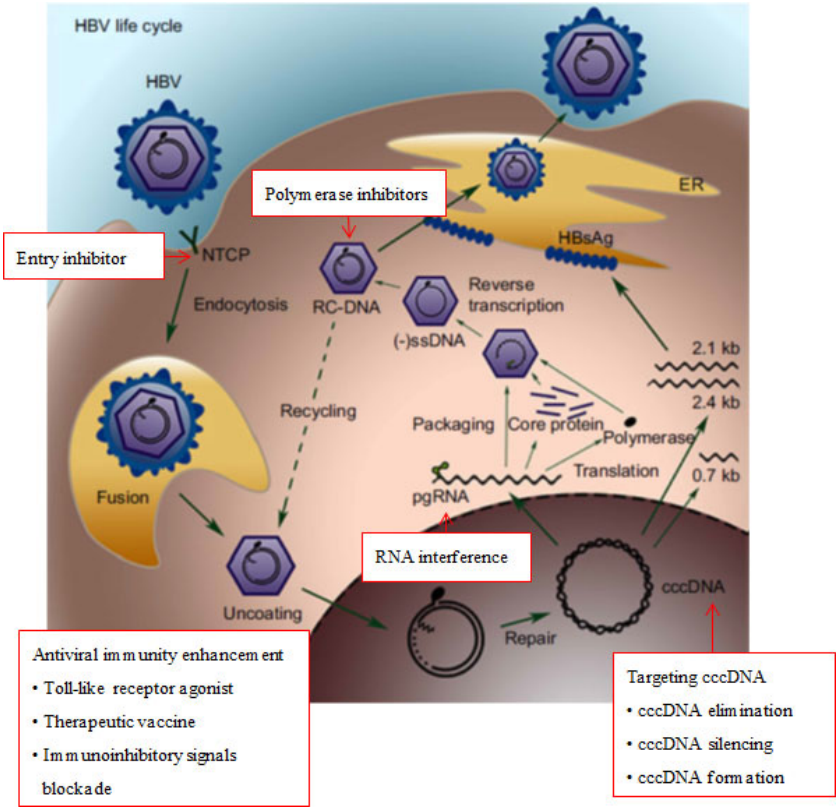

Fig 1. Model of the replication of hepatitis B virus and suggested mechanisms of action for novel antiviral molecules. cccDNA: covalently closed circular DNA, ER: endoplasmic reticulum, HBV: hepatitis B virus, NTCP: sodium taurocholate cotransporting polypeptide, pgRNA: pregenomic RNA; RC-DNA: relaxed-circular DNA; ssDNA: single-stranded DNA. Adapted from Yang *et al.* (41).

clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein endonucleases (42). By blocking the transcription of cccDNA, ZFNs can be used to inhibit viral transcription and replication of duck HBV (43). An *in vitro* study has also shown effective cleavage of viral DNA targets by HBV-specific ZFN within cultured cells (44). TALENs are newly developed sequence-specific nucleases that target HBV-specific sites to disrupt sequences and suppress markers of viral replication. Bloom *et al.* reported that engineered mutagenic TALENs targeting S or C open-reading frames caused mutation in 35% of cccDNA molecules (45). TALENs targeting the conserved regions of HBV DNA significantly reduced the viral production of HBeAg, HBsAg, HBcAg and pregenomic RNA, and resulted in a decreased cccDNA levels and misrepaired cccDNA in Huh7 cells transfected with monomeric linear full-length HBV DNA (46). The CRISPR/Cas system is a novel genome editing tool for site-specific cleavage of DNA targets directed by a synthetic guide RNA base-paired to the target DNA sequence (47, 48). Our recent study showed that the CRISPR/Cas system with eight HBV-specific guide RNAs targeting different regions of the HBV genome significantly reduced the production of HBV core and surface proteins in Huh-7 cells transfected with an HBV-expression vector. The CRISPR/Cas9 system could thus disrupt HBV-expressing templates both *in vitro* and *in vivo* (49).

Among agents targeting the conversion of relax-circular DNA to cccDNA two structurally related disubstituted sulphonamides, called CCC-0975 and CCC-0346, were recently identified and confirmed as inhibitors of cccDNA production (50).

Strategies targeting inhibition of nucleocapsid assembly

Hepatitis B virus replication can be prevented by effective inhibition of nucleocapsid assembly. The inhibitors of nucleocapsid formation include phenylpropenamide derivatives (for example AT-61, AT130) (51, 52), heteroaryldihydropyrimidines (Bay 41-4109) (53) and sulphamoylbenzamide derivatives (54). These compounds either inhibit capsid formation (heteroaryldihydropyrimidines) or prevent the encapsidation of viral pregenomic RNA into nucleocapsid (phenylpropenamide and sulphamoylbenzamide derivatives). Because inhibition of viral replication occurs at the step prior to viral DNA synthesis, nucleocapsid formation inhibitors are effective against both wild-type and NA-resistant HBV (54).

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Table 1. Emerging treatments for chronic hepatitis B

Target	Mode of action	Compound
Virus		
HBV Polymerase	Polymerase inhibitor	Tenofovir alafenamide (GS-7340) Besifovir (LB80380)
cccDNA	Site-specific cleavage of DNA	ZFNs, TALENs, CRISPR/Cas
	Inhibition of relax-circular DNA to cccDNA conversion	Disubstituted sulphonamides
	Inhibition of nucleocapsid assembly	Phenylpropenamide derivatives Heteroaryldihydropyrimidines Sulphamoylbenzamide derivatives
HBV RNA	Knock down HBV RNA, viral proteins and HBV DNA	RNA interference
Host		
NTCP	Entry inhibitor	Myrcludex-B
Innate immunity	Induce APOBEC3A and APOBEC3B	Lymphotoxin-β receptor agonist
	Exogenous interferon stimulation	Toll-like receptor agonist
Adaptive immunity	CD8 T cells activation	Therapeutic vaccine Programmed death -1 inhibitor

APOBEC, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3A and 3B; cccDNA, covalently closed circular DNA; CRISPR/Cas, clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems; HBV, hepatitis B virus; NTCP, sodium taurocholate cotransporting polypeptide; TALENs, transcription activator-like effector nucleases; ZFNs, zinc-finger nucleases.

Strategies targeting HBV RNA

RNA interference-based therapeutics could specifically knock down the expression of viral proteins, including HBsAg, and viral replication (55). ARC-520 is a combination of hepatocyte-targeted, N-acetylgalactosamine-conjugated melittin-like peptide with liver-tropic cholesterol-conjugated small interfering RNAs directed against conserved HBV RNA sequences that effectively knocks down HBV RNA, proteins and DNA levels (56, 57). By combining HBV gene silencing and induction of IFN in the liver, 5′ triphosphorylated small interfering RNAs induced a strong expression of IFN-β in liver cells and showed transient but strong inhibition of viral replication (58).

Strategies targeting HBV entry receptor

Recently, the sodium taurocholate cotransporting polypeptide (NTCP) has been confirmed as a specific binding receptor of the pre-S1 domain of the HBV large envelope protein for HBV entry into the host cell (59). Based on these results Myrcludex-B, a synthetic lipopeptide derived from the pre-S1 domain of the HBV envelope protein targeting NTCP, has been developed and shown to effectively inhibit HBV entry *in vitro* and *in vivo* (60, 61). In humanized mice infected with HBV, 6 weeks of Myrcludex-B treatment completely blocked the rise of serum viral load and HBsAg concentrations. The results showed that Myrcludex-B inhibited amplification of intrahepatic cccDNA as well as the spread of intrahepatic infection (60). Myrcludex-B mainly interfered with the viral entry process and virion productivity was not affected during treatment. Myrcludex-B combined with existing antiviral agents could synergistically control HBV, both by reducing viraemia and by blocking *de novo* infection (60, 62).

Strategies targeting host immune responses

Agents targeting host factors to enhance innate and adaptive immune responses could play a critical role in the clearance of HBV-infected cells. Lucifora *et al.* recently reported that IFN-α and lymphotoxin-β receptor agonists up-regulated apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 3A (APOBEC3A) and APOBEC3B cytidine deaminases in HBV-infected cells, resulting in non-cytolytic clearance of cccDNA and reducing cccDNA, HBV DNA and HBsAg levels (63). The therapeutic potential of lymphotoxin-β receptor agonists, in combination with NA or future antivirals, must be evaluated.

The toll-like receptor (TLR) family is important regulator of innate and adaptive immune responses to various pathogens (64). Exogenous IFN stimulation by TLR agonist may reinstate endogenous IFN-α responses and result in innate and adaptive immune reconstitution (65). GS-9620, a selective oral TLR7 agonist, could induce prolonged suppression of HBV DNA in serum and the liver in chronically infected chimpanzees. Furthermore, serum HBsAg and HBeAg levels, and the number of HBV antigen-positive hepatocytes were reduced while hepatocyte apoptosis was increased (66). Similarly, GS-9620 can induce a SVR in woodchucks infected with woodchuck hepatitis virus (67). A recent phase Ib study showed that GS-9620 was safe and associated with induction of peripheral IFN-stimulated gene 15 production in CHB patients (68). The data suggest that the TLR7 agonist is a potential therapeutic approach to control or eliminate HBV infection.

Antiviral immunity plays an important role in the control of HBV infection, and therapeutic vaccines have been considered a promising strategy. Martin *et al.* developed a novel adenovirus-based therapeutic vaccine, TG1050, encoding three HBV antigens or domains, including core, polymerase and envelope proteins. They showed that long-lasting HBV-specific memory CD8(+) T cells could be induced by TG1050 in mouse models

(69). Although reduction in the HBsAg and HBV DNA levels was observed in mouse models, the extent of reduction might not be enough to eradicate persistent HBV or control viral replication in humans (70).

In addition to therapeutic vaccines, several alternative approaches are proposed. In patients with CHB, high viral load leads to exhaustion and dysfunction of T cells by induction of inhibitory costimulatory molecules, such as programmed death-1 (PD1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (71). HBeAg-positive patients with high viral load show increased PD-1 and CTLA-4 expression on HBV-specific CD8(+) T cells (72). PD-1 blockade increased CD8(+) T-cell proliferation and enhanced HBcAg-specific IFN-γ production in intrahepatic lymphocytes (73–75). These results indicate that blockade of immunoinhibitory signals through these immune checkpoint inhibitors might restore immune dysfunction and enhance antiviral T-cell immunity. In the future, novel immune modulatory agents could be combined with long-term NA therapy to achieve protective immunity against HBV with a subsequent cure of HBV (8, 65).

Conclusions and perspectives

Although long-term viral suppression significantly reduces the degree of liver damage and risk of end-stage liver disease, such as cirrhosis and HCC (76–78), current antiviral treatments fail to cure most CHB patients because of persistent HBV cccDNA. cccDNA can be eliminated by non-cytolytic destruction of cccDNA or immune-mediated killing of HBV-infected hepatocytes (41). Several potential strategies to cure HBV focusing on cccDNA and host targets responsible for persistent HBV are under active development. By combining these promising approaches we hope to reach the ultimate goal of curing HBV in the near future.

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