# World Journal of

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Editorial Board Member of *World Journal of Virology*, Ming-Ke Wang, MD, PhD, Associate Chief Physician, Deputy Director, Department of Disease Control and Prevention, Naval Medical Center of PLA, Naval Medical University, Shanghai 200052, China. wmke021@163.com

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*MINIREVIEWS*

# Chronic hepatitis B: New potential therapeutic drugs target

Wattana Leowattana, Tawithep Leowattana

**Wattana Leowattana,** Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand

**Tawithep Leowattana,** Department of Medicine, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand

**Corresponding author:** Wattana Leowattana, MD, MSc, PhD, Professor, Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, No. 420/6 rajavithi road, rachatawee, Bangkok 10400, Thailand. wattana.leo@mahidol.ac.th

## Abstract

Chronic hepatitis B (CHB) infection remains the most causative agent of liver-related morbidity and mortality worldwide. It impacts nearly 300 million people. The current treatment for chronic infection with the hepatitis B virus (HBV) is complex and lacks a durable treatment response, especially hepatitis B surface antigen (HBsAg) loss, necessitating indefinite treatment in most CHB patients due to the persistence of HBV covalently closed circular DNA (cccDNA). New drugs that target distinct steps of the HBV life cycle have been investigated, which comprise inhibiting the entry of HBV into hepatocytes, disrupting or silencing HBV cccDNA, modulating nucleocapsid assembly, interfering HBV transcription, and inhibiting HBsAg release. The achievement of a functional cure or sustained HBsAg loss in CHB patients represents the following approach towards HBV eradication. This review will explore the up-to-date advances in the development of new direct-acting anti-HBV drugs. Hopefully, with the combination of the current antiviral drugs and the newly developed direct-acting antiviral drugs targeting the different steps of the HBV life cycle, the ultimate eradication of CHB infection will soon be achieved.

**Key Words:** Chronic hepatitis B; Hepatitis B surface antigen; Hepatitis B surface antibody; Covalently closed circular DNA; Direct acting antiviral drugs; Functional cure; Entry block; Nucleocapsid assembly modulator; Interfering hepatitis B virus transcription; Inhibiting hepatitis B surface antigen release

**Core Tip:** Current treatment of chronic hepatitis B infection with nucleos(t)ide analogs causes long-term suppression of hepatitis B virus (HBV) DNA levels, significantly

improving hepatocellular injury and extrahepatic complications. However, the risk of hepatocellular carcinoma remains increased. New direct antiviral drugs that target the HBV life cycle, including entry blockers, assembly modulators, covalently closed circular DNA (cccDNA) disruptors, and hepatitis B surface antigen release inhibitors, would lead to hepatitis B surface antigen loss and a functional cure. Moreover, a combination of antiviral drugs with an immune-modulator could enhance the elimination of cccDNA and provide a definitive cure.

# INTRODUCTION

Chronic hepatitis B (CHB) virus infection is a significant public health problem and causes substantial morbidity and mortality. It affects more than 257 million people worldwide, and the first-ever global hepatitis report published in 2017 indicated that in 2015, 887000 persons died from cirrhosis and hepatocellular carcinoma (HCC)[1,2]. The cumulative incidence of CHB infection in children less than 5-years-old fell from 4.7% in the pre-vaccine era to 1.3% in 2015. This reduction in incidence is attributable to progress in immunization coverage. Although we have effective vaccines and potential antiviral drugs to treat CHB patients, the mortality rate of CHB infection still increased over the last 10 years.

Antiviral drugs, such as pegylated-interferon (Peg-INF)-α-2a, Peg-INF-α-2b, Peg-INF-α-1b, and nucleoside or nucleotide analogs (NAs), have been used to treat CHB patients. They strongly suppress HBV replication and slow progression to cirrhosis and HCC. A limitation of the current treatments is the low rate of serological responses because covalently closed circular DNA (cccDNA) persists in the hepatocyte nucleus[3,4]. Hepatitis B surface antigen (HBsAg) loss is uncommon with current therapies, causing the majority of CHB patients to need indefinite therapy. The IFN treatment produces a higher rate of HBsAg loss, but most patients cannot tolerate the adverse events caused by it.

The combination of Peg-IFN and NAs may synergize the treatment effect to enable more CHB patients to achieve HBsAg loss[5,6]. However, a recent randomized controlled, open-label trial did not support the advantage of a combination of Peg-IFN and NAs in CHB patients[7]. Moreover, the patients also need frequent clinical and laboratory monitoring. Numerous clinical trials of drugs that interrupt the HBV life cycle in hepatocytes have been conducted. The novel agents for HBsAg loss include the direct-acting antiviral drugs targeting the different steps of the HBV life cycle and the indirect antiviral drugs modulating host immune response to eradicate HBV[8,9].

This review will address the newly investigated therapeutic drugs, and the results of clinical trials that aim to cure HBV.

# HBV GENOME AND LIFE CYCLE

HBV is a small virus of the *Hepadnaviridae* family which infects hepatocytes, replicates, and persists in the nucleus. HBV particles include the HBV genome, nucleocapsid, and envelope proteins. The HBV genome is partially double-stranded DNA, with approximately 3200 base pairs that form a relaxed circular DNA (rcDNA) genome. The minus (-) strand is the longer-strand DNA which complements pre-genomic RNA (pgRNA). The plus (+) strand is the shorter-strand DNA. The minus (-) strand has four overlapping open reading frames (ORFs), consisting of PreC/C, P, PreS/S, and X. The PreC/C ORF encodes the hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg). The P ORF encodes the HBV DNA polymerase. The PreS/S ORF encodes the large (L), the middle (M), and the small (S) envelope proteins. The X ORF encodes the X protein (HBx)[10].

The large envelope protein contains the receptor-binding domain and is involved in viral entry into the cytoplasm by receptor-mediated endocytosis. This process involves the sodium taurocholate co-transporting polypeptide (NTCP) receptor in the hepatocyte membrane. After attachment, two pathways for cell entry include endocytosis and fusion of the HBV envelope with the plasma membrane[11]. Then, individual rcDNAs are modified into cccDNAs, packaged into chromatin by histone and non-histone proteins[12]. The cccDNAs are responsible for viral persistence in the nuclei of infected cells. These cccDNAs also use pre-C mRNA and all other sub-genomic mRNAs that code for the main viral proteins.

An HBe protein is translated from the pre-C mRNA transcripts, which have a longer reading frame than HBc protein, and is finally secreted into the bloodstream as HBeAg, the immunoactive biomarker for HBV infection[13]. This replication cycle is concomitant with the release of incomplete sub-viral particles and infectious viral particles. The most abundant of these exported sub-viral particles are particulate forms of viral envelopes formed with such HBs proteins as HBs antigen (HBsAg), a primary immunoactive biomarker for HBV infection in conjunction with HBeAg.

The encapsulated mRNAs are known as HBcAg, which can be detected in serum, even when HBV DNA cannot.

## CURRENT ANTIVIRAL DRUGS AGAINST HBV

Currently, two different therapeutic strategies have been approved to treat CHB patients. These included IFN-α or Peg-IFN-α and direct-acting antivirals comprised of NAs that include nucleoside analogs, lamivudine (LAM), telbivudine (LdT) and entecavir (ETV), or NAs adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF)[14,15].

### IFNs

IFN-α suppresses viral DNA synthesis by stimulating antiviral enzyme production, which results in the clearance of infected hepatocytes, enabling a proportion of CHB patients to achieve a sustained virologic response (SVR). Several studies have demonstrated that IFN-α exhibits an SVR of up to 37%, with a mean loss rate of 33% in HBeAg and 8% in HBsAg. However, other factors influencing SVRs following IFN-α treatment comprised low serum levels of HBV DNA, early infection, treatment-naïve status, HBV genotypes, pre-core HBV mutation detection, chronicity, and co-infection with human immunodeficiency virus (commonly known as HIV)[16]. Due to its limited efficacy, low SVRs, and frequent injections, IFN-α has been replaced with the long-acting Peg-IFN-α.

Peg-IFN-α could prolong the effective half-life of IFN-α, reduce functional dose levels, increase efficacy, and lower side effects[17]. However, randomized clinical trials suggest that Peg-IFN-α effects are better in CHB patients who are HBeAg-positive than in those who are HBeAg-negative. Long-term treatment with Peg-IFN-α in CHB patients with HBeAg-positive status led to viral suppression in 10%–40%, HBeAg loss in 30%–35%, and normalization of alanine aminotransferase (ALT) levels in 35%–50%. Moreover, an HBsAg loss was established in 5% of patients 6 mo after stopping treatment and 10% of patients 3 years post-treatment[18]. Unfortunately, the benefits of Peg-IFN-α treatment vary with patient geographical distributions and HBV genotype, resulting in it not being effective in all CHB patients[19,20-22].

### Nucleosides or NAs

The NAs are the small molecule drugs that directly inhibit the HBV DNA polymerase reverse transcriptase activity, resulting in reduced virion production[23]. Moreover, they also compete with natural nucleotide substrates for the elongating DNA chain, interrupting HBV DNA synthesis[24]. There are six NAs approved for CHB treatment: LAM, ADV, ETV, LdT, TDF, and tenofovir alafenamide (TAF). Long-term treatment with NAs can reduce the cccDNA pool in hepatocytes infected with HBV by inhibiting nucleocapsid recycling. However, they cannot prevent the initial cccDNA formation in newly infected hepatocytes[25].

The first generations of NAs are LAM, ADV, and LdT. The NA approved by the United States Food and Drug Administration in 1998 for the treatment of CHB is LAM, which can compete for cytosine in the synthesis of viral DNA. The CHB patients who were treated with 100 mg LAM for 104 wk achieved 52% virological response. However, after 5 years of treatment, approximately 70% of the patients developed LAM resistance[26,27]. ADV, a phosphonate acyclic NA of adenosine monophosphate,

was approved in 2002. In 2003, Marcellin *et al*[28] reported that after 48 wk of 10 mg ADV treatment in HBeAg-positive CHB patients, 53% had histologic improvement, 21% had undetectable serum levels HBV DNA, and 12% had HBeAg seroconversion. Furthermore, Hadziyannis *et al*[29] demonstrated that after 48 wk of 10 mg ADV treatment in HBeAg-negative CHB patients, 64% had histologic improvement and 51% had undetectable serum levels of HBV DNA. However, long-term treatment with ADV also results in a high drug resistance rate of nearly 30% after 5 years of treatment [30]. LdT, the unmodified β-l enantiomer of thymidine, was approved for CHB treatment in 2007[31]. In 2009, Liaw *et al*[32] reported that LdT was superior to LAM in patients with CHB. They found that the rates of therapeutic response in HBeAg-positive and HBeAg-negative patients treated with 104 wk of LdT compared with LAM were 63% *vs* 48% and 78% *vs* 66%. However, long-term treatment with LdT led to nearly 35% drug resistance after 3 years of therapy[33].

ETV, TDF, and TAF are second-generation NAs with a high genetic barrier to HBV resistance. They are used as the first-line drugs for CHB treatment. In 2005, ETV, a guanosine NA with selective activity against HBV, was launched. The effective concentration ($EC_{50}$) of ETV is 4 nM. This $EC_{50}$ is 100-fold more potent than ADV or LAM in HBV suppression[34]. In 2016, Ahn *et al*[35] reported that ETV had shown durable and increasing viral suppression in 84.6% of HBeAg-positive patients and 96.2% of HBeAg-negative patients over 5 years of treatment. However, the cumulative probability of HBsAg loss at year 5 was 5.2% in HBeAg-positive patients and 4.6% in HBeAg-negative patients. TDF, an acyclic NA with activity against retroviruses, was approved for CHB treatment in 2008. Buti and colleagues[36] reported that 437 patients remained on the study at year 7; among them, 54.5% and 11.8% achieved HBeAg and HBsAg loss in HBeAg-positive patients but only 0.3% of the HBeAg-negative patients achieved HBsAg loss. Although TDF resistance is relatively low, it has been associated with dose-dependent renal toxicity and induced Fanconi syndrome[37,38]. Recently, TAF was approved to be an alternative to TDF because it caused fewer side effects and was suitable for the treatment of CHB patients at risk of renal dysfunction[39]. Moreover, TAF has been demonstrated to be more effective than TDF with continued improved renal and bone safety[40].

### Combination of NA plus Peg-IFN-a

Although the current monotherapy of anti-HBV drugs can suppress viral replication, prevent the progression of CHB to cirrhosis, and decrease the rates of HBV-related HCC in most CHB patients, long-term anti-HBV monotherapy rarely achieves the higher rate of HBsAg loss. Hence, to accomplish the goal of a functional cure in more CHB patients, the combination of NA with Peg-IFN-α has been evaluated. The reason for this is that the two classes of anti-HBV drugs have different mechanisms of action. Thus, their combination would result in a synergistic anti-HBV effect. Several studies have demonstrated that the combination of NA with Peg-IFN-α can substantially enhance the rates of HBsAg loss, but the benefits are mainly limited to a small proportion of patients and depend on HBV genotype and patient geographical distributions[41-44]. Moreover, NAs and Peg-IFN-α treatment have no direct impact on viral transcription or cccDNA. Thus, there is a very high risk of reactivation of HBV and the emergence of downstream disease symptoms after stopping treatment. Therefore, new therapeutic drugs that target different HBV life cycle steps or modulate the host immune system are needed.

## NEW DRUGS TARGETING HBV LIFE CYCLE

### HBV entry inhibitors

**Bulevirtide (Myrcludex B):** NTCP has been demonstrated as a functional receptor for HBV entry into hepatocytes[11]. Therefore, the new drugs targeting viral entry receptors have been proposed as potential agents for preventing uninfected hepatocytes. Bulevirtide (Myrcludex B) is a synthetic lipopeptide of 47 amino acids obtained from the HBV preS1 domain. When bulevirtide binds to NTCP, it will effectively prevent HBV spread among intrahepatic cells and hinder the amplification of intrahepatic cccDNA pool in infected hepatocytes[45,46].

In 2016, Blank *et al*[47] conducted a prospective, open-label, first-in-human, phase 1 clinical trial in 36 healthy volunteers. They found that bulevirtide was well tolerated, with no serious side effects and no immunogenic effects up to the highest dose of 20 mg intravenously. Moreover, the pharmacokinetic model showed that 10 mg and above of bulevirtide subcutaneous injection could reach a target saturation of over 80%

for at least 15 h. Furthermore, Blank *et al*[48] conducted a study to investigate the effects of bulevirtide on plasma bile acid disposition, TDF pharmacokinetics, and perpetrator characteristics on cytochrome (CYP) P450 3A in 12 healthy volunteers. All of the volunteers received 300 mg TDF orally and 10 mg of subcutaneous bulevirtide. They found that bulevirtide increased total plasma bile acid by 19.2-fold without signs of cholestasis, and co-administration of TDF with bulevirtide revealed no relevant changes in TDF pharmacokinetics.

Recently, Wedemeyer *et al*[49] conducted a phase 2b clinical trial in 60 patients with chronic HBV/hepatitis D virus (HDV) co-infection. They randomized 1:1:1:1 into the following four groups: Peg-IFN-α once-weekly (qw) ($n$ = 15, Arm A); bulevirtide 2 mg once daily (QD) subcutaneous (sc) injection + Peg-IFN-α qw ($n$ = 15, Arm B); bulevirtide 5 mg QD sc + Peg-IFN-α qw ($n$ = 15, Arm C); or bulevirtide 2 mg QD ($n$ = 15, Arm D) for 48 wk. They found that HBsAg levels declined by more than 1 $Log_{10}$ in 6/15 (40%) and 2/15 (13.33%) patients from Arm B and Arm C, respectively. Notably, 4/15 (27%) patients from Arm B had undetectable HBsAg levels, and 3/4 (75%) patients established HBsAg seroconversion. Bulevirtide is moving along to phase 3 studies, whereby monotherapy extended or in combination with Peg-IFN-α will be investigated in CHB patients (Table 1).

### cccDNA disruptors

The cccDNA plays a crucial role in the viral life cycle, where it acts as the template for viral transcription, while pgRNA is the template for viral replication. It interacts with histone and non-histone proteins, resembling cellular chromatin within the nucleus [50]. Disruption of cccDNA is considered an optimal target of HBV treatment because its persistence in the nucleus of infected hepatocytes is the crucial reason why HBsAg loss is currently not possible. The blocking of cccDNA formation, enhancing its destruction, and silencing its transcription, are currently under exploration.

**Gene editing:** The four ORFs of the HBV genome (surface, core, polymerase, and X protein) are translated into seven essential proteins for viral replication. The blocking of any one of the seven proteins would likely be essential to inhibit viral gene expression. Several small molecules have been developed as sequence-specific RNA-guided (gRNA) nucleases and proteins which can block the formation, enhance the destruction, and silence the transcription of cccDNA, while stimulating cell division [51]. These comprise cleaving sequence-specific DNA targets using the transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats-associated 9 (CRISPR/Cas9) systems that could demonstrate antiviral efficacy[52-54].

In 2014, Lin *et al*[55] demonstrated that the CRISPR/Cas9 system could disrupt the HBV genome both *in vitro* and *in vivo*. They showed that the HBV-specific gRNAs significantly decreased the production of HBV core and HBsAg in Huh-7 cells transfected with an HBV-expression vector. They also reported that the CRISPR/Cas9 system could cleave the intrahepatic HBV genome-containing plasmid and facilitate its clearance *in vivo*, causing a reduction in serum HBsAg levels. In 2015, Kennedy *et al* [56] reported the effective inhibition of HBV DNA production in *in vitro* models of both chronic and *de novo* HBV infection using lentiviral transduction of a bacterial Cas9 gene and single-guide RNAs (sgRNAs) specific for HBV. They showed that Cas9/sgRNA combinations specific for HBV reduced HBV DNA levels by up to 1000-fold and HBV cccDNA levels by up to 10-fold. Moreover, this method could inactivate the mutation of residual viral DNA. They concluded that CRISPR/Cas9 systems could serve as effective tools for disrupting the cccDNA pool in chronically-infected HBV patients.

Furthermore, Liu *et al*[57] showed that HBV-specific gRNA/Cas9 could inhibit the HBV replication of different genotypes *in vitro* and *in vivo* due to error-prone repair of viral DNA templates. Dong *et al*[58] reported that the CRISPR/Cas9 system could be used for disrupting intracellular cccDNA and viral replication in pre-cccDNA-transfected Huh7 cells and a new mouse model carrying HBV cccDNA. Zhen *et al*[59] studied the effects of the CRISPR/Cas9 system targeted to the HBsAg-encoding region of HBV in a cell culture system and *in vivo*. They found that the concentration of HBsAg secreted in the cell culture and mouse serum was decreased by CRISPR/Cas9 treatment. They concluded that a CRISPR/Cas9 system inhibited HBV replication and expression *in vitro* and *in vivo*, and may constitute a new therapeutic strategy for HBV infection. Seeger and Sohn[60] reported that HBV infections could be inhibited up to 8-fold by HBV-specific guide RNAs in NTCP-expressing HepG2 cells. Ramanan *et al*[61] demonstrated that the CRISPR/Cas9 system could specifically target and cleave conserved regions in the HBV genome, causing robust suppression of viral gene

**Table 1 Developing new therapeutic drug targets for chronic hepatitis B**

| Drugs | Mechanism of action | Therapeutic class | Route of administration | Clinical trial | Results |
|---|---|---|---|---|---|
| HBV entry inhibitors | | | | | |
| Bulevirtide (Myrcludex B)[49] | Competition with NTCP | Peptide | Subcutaneous injection | IIb | HBsAg loss in 27% of HBV/HDV co-infected patients after 48 wk of treatment with Bulevirtide + pegIFN-α and 24 wk treatment-free follow-up |
| cccDNA disruptors | | | | | |
| CRISPR/Cas9[67] | Disruption of cccDNA | Gene editing | *In vivo* | Pre-clinical | Significantly improved survival of human hepatocytes in liver-humanized FRG mice and demonstrated a decreasing of total liver HBV-DNA and cccDNA |
| ZFNs[69] | Disruption of cccDNA | Gene editing | *In vitro* | Pre-clinical | Efficiently suppress the cellular template for HBV persistence and inhibit active HBV replication |
| Nucleocapsid assembly modulators | | | | | |
| JNJ-632 and BAY41-4109[73] | Misdirecting the formation of capsid-like structures | Capsid assembly modulators | *In vitro* | Pre-clinical | Induce the formation of morphologically intact viral capsids and prevented formation of cccDNA |
| NVR3-778[78] | Misdirecting the formation of capsid-like structures | Capsid assembly modulator | *In vivo* | I/II | The largest mean reduction in serum HBV DNA levels was achieved from the combination treatment of 600 mg NVR3-778 BD + pegIFN 180 mg subcutaneous weekly (1.97 log10 IU/mL) |
| JNJ-6379[76] | Misdirecting the formation of capsid-like structures | Capsid assembly modulators | Oral | II | No clinically significant changes in levels of HBsAg were observed |
| ABI-H0731[77] | Misdirecting the formation of capsid-like structures | Capsid assembly modulators | Oral | I/II | Dose-dependent reduces in HBV DNA and HBV RNA not HBsAg was seen in both HBeAg-positive and HBeAg-negative patients |
| HBV transcription inhibitors | | | | | |
| ARC-520[84] | Interference viral mRNA | Transcription inhibitor | Intravenous injection | II | CHB patients with high dose significantly reduced HBsAg and persisted for ≥ 85 d after the last dose |
| GSK3389404[85] | Interference viral mRNA | Transcription inhibitor | Subcutaneous injection | I | Dose 120 mg for 4 wk was safe and well tolerate |
| RG7834[87] | Interference viral mRNA | Gene expression inhibitor | *In vivo* | Pre-clinical | Reduced WHsAg by a mean of 2.57 log$_{10}$ and WHV DNA by a mean of 1.71 log$_{10}$ from baseline. However, WHsAg and WHV DNA rebounded to baseline after stopped treatment and WHsAb was not observed. |
| HBsAg release inhibitors | | | | | |
| REP 2055 and REP 2139-Ca[88] | HBsAg release inhibitors | NAPs | Intravenous injection | II | Substantially reduction of HBsAg levels, HBV DNA levels and increasing of serum HBsAb |
| REP 2139-Mg and REP 2165-Mg[90] | HBsAg release inhibitors | NAPs | Intravenous injection | II | Addition of NAPs to TDF + pegINFα-2a significantly increased rates of HBsAg loss during therapy and functional cure after therapy |

cccDNA: Covalently closed circular DNA; CHB: Chronic hepatitis B; CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/CRISPR-associated 9; HBsAb: Hepatitis B surface antibody; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HDV: Hepatitis D virus; NAPs: Nucleic acid polymers; NTCP: Sodium taurocholate co-transporting polypeptide; pegIFN-α: Pegylated interferon-alpha; TDF: Tenofovir disoproxil fumarate; WHsAb: Woodchuck hepatitis surface antibody; WHsAg: Woodchuck hepatitis surface antigen; WHV: Woodchuck hepatitis virus.

expression and replication both *in vitro* and *in vivo*, and extended this antiviral activity to a virus isolated from patients. They also reported that upon continuous Cas9/sgRNA, a sharp decline of cccDNA and HBV proteins resulted in a *de novo* infection model.

Wang *et al*[62] evaluated the efficiency of each gRNA and 11 dual-gRNAs on the suppression of HBV (genotypes A-D) replication using the measurement of HBsAg or HBeAg in the culture supernatant. They found that all dual gRNAs could efficiently suppress HBsAg and HBeAg production for HBV of genotypes A-D, and the efficacy of dual gRNAs was significantly increased compared to the single gRNA used alone. Karimova *et al*[63] identified cross-genotype conserved HBV sequences in the S and X region of the HBV genome targeted for specific and effective cleavage by a Cas9 nickase. This technique could disrupt episomal cccDNA, and chromosomally integrated HBV target sites in reporter cell lines and HBV replication in chronically and *de novo* infected hepatoma cell lines.

In 2019, Kostyushev *et al*[64] evaluated CRISPR/Cas9 systems from four different species using co-expressed cell lines with gRNAs targeting conserved regions of the HBV genome. They found that the CRISPR/Cas9 systems from *Streptococcus pyogenes* (Sp) and *Streptococcus thermophilus* (St) targeting conserved regions of the HBV genome could block HBV replication and degrade over 90% HBV cccDNA by 6 d post-transfection. They concluded that the St CRISPR/Cas9 system represented the safest system with high anti-HBV activity.

In 2020, Yang *et al*[65] investigated the utility of CRISPR/Cas-mediated "base editors" (BES) in inactivating HBV gene expression without cleavage of DNA. They found that Cas9-mediated base editing is a potential strategy to cure CHB by permanently inactivating integrated HBV DNA and cccDNA without double-strand breaks of the host genome. Recently, Kayesh *et al*[66] evaluated the effects of adeno-associated virus 2 (AAV2) vector-mediated delivery of 3 selected from 16 gRNAs. These gRNAs/Cas9 significantly suppressed HBV replication in cells, with WJ11/Cas9 demonstrating the highest efficacy. Furthermore, AAV2/WJ11-Cas9 also substantially inhibited HBV replication and significantly reduced cccDNA in the tested cells. It also enhanced ETV actions when used in combination due to different modes of action. They concluded that AAV2/WJ11-Cas9 significantly suppressed HBcAg, HBsAg, and HBV DNA along with cccDNA in the liver tissues without significant cytotoxicity in humanized chimeric mice. A pre-clinical study was reported by Stone *et al*[67], in which HBV-specific AAV-*Staphylococcus aureus* (Sa)-Cas9 therapy significantly improved survival of human hepatocytes in liver-humanized FRG mice and demonstrated a decrease in total liver HBV DNA and cccDNA; in addition, a good tolerance profile was found. The investigators concluded that this approach was safe and feasible for *in vivo* gene editing therapy in CHB infections, and it may be a plausible method to cure CHB patients.

In 2010, Cradick *et al*[68] demonstrated the effective cleavage of viral DNA targets by HBV-specific ZFNs within cultured cells. Moreover, the cleaved fragments were mis-repaired, which could potentially inactivate HBV. The authors suggested that AAVs can transfect 100% of mouse hepatocytes and could be used to deliver ZFNs to the human livers. In 2014, Weber *et al*[69] evaluated three ZFNs that target sequences within the HBV polymerase, core, and X genes. They demonstrated that HBV-targeted ZFNs could efficiently suppress the cellular template for HBV persistence and inhibit active HBV replication, causing them to be potential candidates for cccDNA disruptors (Table 1).

Overall, gene editing techniques have demonstrated the usefulness of destroying HBV cccDNA *in vitro* and *in vivo* and shown the therapeutic potential in acute and chronic HBV infection. Gene editing is at an exciting stage, and the future of curative anti-HBV regimens for chronic HBV infection may well entail the use of it combined with other drugs.

### Nucleocapsid assembly modulators

HBV capsid has numerous functions in the HBV life cycle, including reverse transcription, genome packaging, and intracellular trafficking. It is an excellent target for the development of new antiviral drugs[70]. The capsid assembly modulators (CAMs) can disturb pgRNA encapsidation and HBV DNA replication by misdirecting the formation of capsid-like structures[71]. There are two categories of CAM: type I represented by heteroaryl-dihydro pyrimidine, which misdirects the formation of aberrant structures; and type II represented by phenylpropenamides and sulfamoyl-benzamides, which accelerate the formation of morphologically intact empty capsids [72].

In 2017, Berke *et al*[73] conducted the study to evaluate the CAM JNJ-632 and CAM BAY41-4109, novel and potent inhibitors of HBV replication, *in vitro* across genotypes A to D. They found that it can induce the formation of morphologically intact viral capsids. They prevented the formation of cccDNA in a dose-dependent fashion when added with the viral inoculum. Moreover, it also reduced intracellular HBV RNA, HBeAg, HBcAg, and HBsAg concentrations in the cell culture supernatant. They concluded that CAMs have a dual mechanism of action that inhibits early and late steps of the viral life cycle, whereas NAs did not. In 2018, Lam *et al*[74] conducted a pre-clinical characterization of CAM NVR3-778 in HepG2.2.15 cells, mice, and dogs. They found that CAM NVR3-778 suppressed HBsAg, HBeAg, and intracellular HBV RNA production in primary human hepatocytes. Furthermore, it can block cccDNA formation during *de novo* infection and the subsequent transcription and viral protein translation steps. Furthermore, Klumpp *et al*[75] performed a comparative study of NVR3-778 to determine the *in vivo* antiviral efficacy and effects on innate and endoplasmic reticulum stress responses alone or in combination with Peg-IFN-α and compared with entecavir in 61 uPA/SCID mice with humanized livers. Mice were infected with an HBV genotype C preparation and then waited for 8 wk. They were randomly assigned to six groups (control, NVR3-778, entecavir, Peg-IFN-α, NVR3-778 + entecavir, or NVR3-778 + Peg-IFN-α) for 6 wk. Ultimately, the mice given NVR3-778 or entecavir alone for 6 wk showed reduced serum levels of HBV DNA compared with controls or mice given Peg-IFN-α. Moreover, the most considerable HBV DNA serum level reduction was demonstrated in mice given NVR3-778 + Peg-IFN-α. Serum levels of HBsAg and HBeAg were reduced in the groups that received Peg-IFN-α.

In 2020, Zoulim *et al*[76] performed a double-blind study of 57 treatment-naïve patients with HBeAg-positive or -negative CHB infection without cirrhosis. They were randomly assigned to five groups to receive either 25 mg (100 mg loading dose), 75 mg, 150 mg, or 250 mg JNJ-6379 or placebo daily for 4 wk, with an 8-wk follow-up period. They found that all doses of JNJ-6379 tested were well tolerated, demonstrated dose-dependent pharmacokinetics, and had potent antiviral activity in patients with CHB. However, no clinically significant changes in levels of HBsAg were observed. Recently, Yuen *et al*[77] conducted a phase 1/2, randomized, placebo-controlled study to explore the safety, pharmacokinetics, and pharmacodynamics of ABI-H0731 in healthy subjects and patients with CHB in two parts. In part 1, healthy adults were randomly assigned to receive single oral doses of ABI-H0731 (100, 300, 600, or 1000 mg) or matching placebo, or once-daily or twice-daily doses ABI-H0731 800 mg or matching placebo for 7 d. In part 2, HBeAg-positive or HBeAg-negative CHB adults were randomly assigned to receive ABI-H0731 (100, 200, 300, or 400 mg) or matching placebo once daily for 28 d. Overall, ABI-H0731 was safe and well-tolerated. There were no serious adverse events, nor clinically significant drug-related, dose-dependent, or treatment-emergent laboratory findings. ABI-H0731 showed dose-related activity with once-daily dosing. The mean maximal HBV DNA reductions from baseline of 1.7 $Log_{10}$ IU/mL at 100 mg to 2.8 $Log_{10}$ IU/mL at 300 mg after 28 d for the HBeAg-positive and HBeAg-negative patients. The authors concluded that dose-dependent reduction in HBV DNA and HBV RNA with ABI-H0731 occurred in both HBeAg-positive and HBeAg-negative patients. There were no serious adverse events related to the 1600 mg daily doses in healthy subjects or patients with CHB infection receiving doses up to 300 mg once daily.

Furthermore, Yuen *et al*[78] also performed a phase 1/2 study to examine the safety, pharmacokinetics, and antiviral activity of NVR3-778 in 73 patients with HBeAg-positive CHB infection without cirrhosis. The study had eight cohorts comprised of one placebo cohort and seven treatment cohorts. The four dose-escalation cohorts received NVR3-778 of 100 mg (10 cases), 200 mg (10 cases), or 400 mg once daily (QD) (8 cases), or 600 mg twice daily (BD) (8 cases). The fifth cohort was treated with 600 mg NVR3-778 BD + Peg-IFN 180 mg subcutaneous weekly (10 cases). The sixth cohort was treated with Peg-IFN 180 mg subcutaneous weekly + placebo (10 cases). The seventh cohort was treated with 1000 mg NVR 3-778 BD (7 cases). The eighth cohort was treated with a placebo. The investigators found that mean HBV DNA decline was minimal with low once-daily doses of NVR3-778, but when daily dosing was increased to 1200 mg/d, HBV DNA reductions became substantial. The fourth cohort (600 mg NVR3-778 BD) showed a mean HBV DNA reduction of 1.72 $Log_{10}$ IU/mL. The most significant mean reduction in serum HBV DNA levels was achieved from the combination treatment of 600 mg NVR3-778 BD + Peg-IFN 180 mg subcutaneous weekly (1.97 $Log_{10}$ IU/mL). They concluded that NVR3-778 treatment for 28 d up to a dose of 1000 mg BD was well tolerated. Substantial and correlated decreases in serum HBV DNA and HBV RNA concentrations were demonstrated with the higher-dose cohorts and were notably most excellent for combination treatment with NVR3-778

and Peg-IFN. They do not evaluate serum HBsAg, HBeAg, immunomodulatory effects, and effects on cccDNA persistence. These encouraging data suggested that CAMs can result in a substantial reduction in HBV DNA and HBV RNA levels. Longer-term treatments alone or combined with other antiviral agents will be needed to investigate whether CAMs will result in HBeAg, HBsAg, and cccDNA loss (Table 1).

### HBV transcription inhibitors

After HBV enters the infected hepatocytes, partially double-stranded DNA (pdsDNA) moves to the nucleus and is converted to cccDNA. Furthermore, it is wrapped by histones to form a mini-chromosome. RNA interference (RNAi) and antisense oligonucleotides are mechanisms in which a double-stranded RNA (dsRNA) inhibits gene expression by degrading mRNA or blocking a specific gene's translation pathway. RNAi can directly target HBV transcripts and induce their degradation, causing gene silencing. Antisense oligonucleotides are small nucleic acids complementary to the target transcript, that induce degradation after binding. Hence, targeting the viral mRNA using RNAi and antisense oligonucleotides may be an effective method to control HBV infection. Many studies of RNAi and antisense oligonucleotides are in progress[79-81].

In 2017, Schluep *et al*[82] conducted a phase 1 randomized, double-blind, placebo-controlled study to evaluate the safety, tolerability, and pharmacokinetics of ARC-520 injection in 54 healthy volunteers (36 ARC-520 *vs* 18 placeboes). They found that ARC-520 was safe and well-tolerated. In the same year, Wooddell *et al*[83] conducted a phase 2 randomized, double-blind, placebo-controlled study to determine the safety, tolerability, and pharmacological effect of ARC-520 in 40 CHB patients with or without preceding nucleos(t)ide viral replication inhibitors (NUC) treatment. They found that ARC-520 resulted in a rapid and potent decrease in serum HBV DNA. However, the reduction of HBsAg was only demonstrated in HBeAg-positive patients. Follow-up studies in chimpanzees showed that the HBsAg being produced in the HBeAg-negative patients was predominantly derived from an integrated virus, which ARC-520 did not target.

In 2020, Yuen *et al*[84] conducted 2 randomized, multicenter studies to evaluate in-depth HBsAg decline using 1 mg/kg or 2 mg/kg ARC-520 compared with placebo at four monthly doses in 58 HBeAg-negative and 32 HBeAg-positive CHB patients concomitantly with NUC. They found that both HBeAg-negative and HBeAg-positive high-dose groups had significantly reduced HBsAg compared with placebo, with mean reductions of 0.38 and 0.54 Log IU/mL, respectively. Moreover, HBsAg reductions persisted for 85 d in HBeAg-negative patients and > 85 d in HBeAg-positive patients after the last dose of ARC-520. They concluded that ARC-520 was active in both HBeAg-negative and HBeAg-positive CHB patients treated by NUC. However, absolute HBsAg reductions were moderate, which may occur due to HBsAg expression from integrated HBV DNA.

In 2019, GSK3389404, an antisense oligonucleotide, was studied by Han *et al*[85]. The investigators conducted a randomized, double-blind, phase 1 study to assess the safety and pharmacokinetics of GSK3389404 in healthy subjects. Four single ascending-dose cohorts (10 mg, 30 mg, 60 mg, and 120 mg subcutaneously) and three multiple ascending-dose cohorts (30 mg, 60 mg, and 120 mg once weekly for 4 wk) each comprised 6 subjects randomized to GSK3389404 and 2 subjects randomized to placebo. They reported that there were no serious adverse events (SAEs) or withdrawals due to SAEs. GSK3389404 dosing has been tested up to 120 mg for 4 wk with an acceptable safety and pharmacokinetic profile and suitable for further clinical evaluation in CHB patients.

In 2018, Mueller *et al*[86] reported that RG7834, a novel oral HBV gene expression inhibitor, could reduce the levels of viral proteins and lower viremia. RG7834 is a small-molecule compound belonging to the dihydroquinolizinones chemical class similar to RNAi but through a different mechanism. They found that oral treatment of HBV-infected humanized mice with RG7834 Led to a mean HBsAg reduction of 1.09 $Log_{10}$ compared to entecavir, which had no significant reduction on HBsAg levels. In 2020, Menne *et al*[87] conducted a study to evaluate the potency of RG7834 alone and in combination with ETV or woodchuck interferon-α (wIFN-α) in the woodchuck model of chronic HBV infection. RG7834 could reduce woodchuck hepatitis virus (WHV) surface antigen (WHsAg) by a mean of 2.57 $Log_{10}$ from baseline and WHV DNA by a mean of 1.71 $Log_{10}$. ETV + wIFN-α reduced WHsAg and WHV DNA by 2.40 $Log_{10}$ and 6.70 $Log_{10}$, respectively. RG7834 combined with ETV and wIFN-α significantly decreased WHsAg and WHV DNA concentrations by 5.0 $Log_{10}$ and 7.46 $Log_{10}$, respectively. However, WHsAg and WHV DNA rebounded to baseline after stopping treatment, and WHsAb was not observed. Notably, both RNAi and antisense

oligonucleotides do not eliminate cccDNA, and rebound of HBsAg levels to pretreatment points after stopping treatment has been reported. Therefore, it is likely to be used in combination with other drugs (Table 1).

### HBsAg release inhibitors

HBsAg release inhibitors function under the same exact mechanism as the RNAi and antisense oligonucleotides that block the release of subviral HBsAg particles. Circulating HBsAg is an immunoinhibitory factor that blocks the innate immune response. Clearance of circulating HBsAg is a crucial step in the functional control of HBV infection and permits anti-HBs seroconversion. In 2016, Al-Mahtab *et al*[88] conducted two studies to evaluate REP 2055 and REP 2139-Ca, nucleic acid polymers (NAPs), in 8 and 12 CHB patients, respectively. The results from both studies showed that NAP monotherapy was accompanied by 2-7 $Log_{10}$ reductions of HBsAg levels, 3-9 $Log_{10}$ reductions in HBV DNA levels, and the appearance of serum hepatitis B surface antibody (HBsAb) (10-1712 mIU/mL). Eight of the nine patients treated with the combination of NAP and immunotherapy experienced HBsAg loss, and all nine patients experienced substantial increases in serum HBsAb antibody titers before treatment was stopped. Moreover, 1 year after the REP 2055 therapy was stopped, a rebound of serum HBV DNA > 1000 copies/mL or HBsAg > 1 IU/mL was not observed in 3/8 CHB patients. Suppression of serum HBV DNA > 1000 copies/mL or HBsAg > 1 IU/mL was further maintained for 290 and 231 wk in 2 of these patients. For REP 2139-Ca treatment, 8 patients achieved HBV DNA < 116 copies/mL after treatment withdrawal. The rebound of serum HBV DNA > 1000 copies/mL or HBsAg > 1 IU/mL occurred over 12 to 123 wk in 7 patients but was still absent in 2 patients at 135 and 137 wk of follow-up. The authors concluded that NAP could elicit significant antiviral responses during treatment which may improve the effect of immuno-therapy. NAPs may be a potentially useful component of future combination therapies for the treatment of CHB.

In 2017, Bazinet *et al*[89] conducted an open-label, non-randomized, phase 2 trial to assess the safety and efficacy of REP 2139 and Peg-INF-α-2a in 12 patients with CHB HDV co-infection. The results showed that 6 patients had HBsAg concentrations < 50 IU/mL by the end of treatment. Five patients maintained the level of suppression at the end of 1-year follow-up. Six patients had HBsAb titers > 10 mIU/mL at the end of treatment (five had maximum HBsAb levels of 7681-86532 mIU/mL during treatment), which were maintained at the end of 1-year follow-up. By the end of 1-year follow-up, normalization of serum aspartate aminotransferase (AST) and ALT occurred in 9 of 12 patients. They concluded that combined REP 2139 and Peg-INF-α-2a therapy is well-tolerated, safe, and establishes functional control of HBV and HDV co-infection and normalization of serum AST and ALT in a high proportion of patients 1 year after therapy. In 2020, Bazinet *et al*[90] performed an open-label, phase 2 study of the safety and efficacy of REP 2139 or REP 2165 combined with TDF and Peg-INF-α-2a in 40 HBeAg-negative CHB patients. Forty patients were randomly assigned to groups that received 48 wk of experimental therapy (TDF + Peg-INF-α-2a + REP 2139-Mg or REP 2165-Mg) or 24 wk of control therapy (TDF + Peg-INF-α-2a) followed by 48 wk of experimental therapy. At 48 wk, when patients completed the TDF + Peg-INF-α-2a + NAPs regimen, HBsAg concentrations were ≤ 0.05 IU/mL in 24 of 40 (60%) patients, while all of the patient's achieved seroconversion with HBsAb up to 233055 mIU/mL. During 48 wk of treatment-free follow-up, virologic control persisted in 13 of 40 (32.5%) patients, whereas functional cure persisted in 14 of 40 (35%) patients with persistent HBsAg loss. They concluded that the addition of NAPs to TDF + Peg-INF-α-2a significantly increased rates of HBsAg loss during therapy and functional cure after therapy. However, these results should be carefully applied for Asian race because Van Hees *et al*[91] found that Caucasian patients had more than 6-fold increased chance of HBsAg loss compared to other ethnicities. Further studies regarding ethnicity and HBsAg loss are needed. Thus, NAPs alone or combined with TDF or Peg-INF-α-2a may allow better functional control of HBV infection (Table 1). A longer duration of NAPs treatment would be needed to identify their sustained virological effects and potential risk for adverse events.

## CONCLUSION

Tremendous progress has been explored in understanding the pathophysiology and treatment of CHB over the past 20 years. The CHB current treatment with a potent and a high genetic barrier NA (ETV, TDF, and TAF) can suppress the viral replication to an

undetectable level in most CHB patients. They also prevent the progression of CHB to cirrhosis and markedly reducing the rates of HBV-related HCC. Regardless of viral suppression by NAs, there are many obstacles to achieve a functional cure or HBsAg loss in CHB patients. HBV could persist in the hepatocyte nucleus by continuously replenishing the cccDNA with a long half-life and the integrated forms of viral DNA. Moreover, the defective immune response and the inefficient innate immune response prevent HBV-infected hepatocytes from being cleared by host immunity.

HBsAg loss with or without HBsAb seroconversion is one of the most desired endpoints of new drug development. Targeting HBsAg by inhibiting the entry of HBV into hepatocytes, disrupting or silencing HBV cccDNA, modulating nucleocapsid assembly, interfering HBV transcription, and inhibiting HBsAg release are the primary targets for functional cure in CHB patients. However, newly developed drugs still have limitations in being used alone without IFN and NAs to induce HBsAg loss. Interestingly, a new strategic therapy in treating chronic HBV infection is to use a combination of multiple drugs, including a backbone of a NA, one or more new direct-acting antiviral drugs, and at least one immunomodulator. With the collaborative efforts of basic research scientists and clinical experts, the ultimate elimination of CHB infection is likely to be achieved soon.

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