Journal of Clinical and Translational Hepatology **2023**
DOI: 10.14218/JCTH.2023.00067

Review Article

Novel Approaches to Inhibition of HBsAg Expression from cccDNA and Chromosomal Integrants: A Review

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Received: 15 February 2023 | **Revised:** 19 July 2023 | **Accepted:** 16 August 2023 | **Published online:** 19 September 2023

Abstract

Hepatitis B virus (HBV) is a widely prevalent liver infection that can cause acute or chronic hepatitis. Although current treatment modalities are highly effective in the suppression of viral levels, they cannot eliminate the virus or achieve definitive cure. This is a consequence of the complex nature of HBV-host interactions. Major challenges to achieving sustained viral suppression include the presence of a high viral burden from the HBV DNA and hepatitis B surface antigen (HBsAg), the presence of reservoirs for HBV replication and antigen production, and the HBV-impaired innate and adaptive immune response of the host. Those therapeutic methods include cell entry inhibitors, HBsAg inhibitors, gene editing approaches, immune-targeting therapies and direct inhibitors of covalently closed circular DNA (cccDNA). Novel approaches that target these key mechanisms are now being studied in preclinical and clinical phases. In this review article, we provide a comprehensive review on mechanisms by which HBV escapes elimination from current treatments, and highlight new agents to achieve a definitive HBV cure.

Citation of this article: Abdelwahed AH, Heineman BD, Wu GY. Novel Approaches to Inhibition of HBsAg Expression from cccDNA and Chromosomal Integrants: A Review. J Clin Transl Hepatol 2023. doi: 10.14218/JCTH.2023.00067.

Keywords: Hepatitis B; Viruses; Liver disease; Hepatocytes; Gene editing.

Abbreviations: AFT, artificial transcription factor; APOBEC, apolipoprotein B mRNA editing catalytic polypeptide-like; Cas, CRISPR-associated; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CRISPR, clustered regularly interspaced short palindromic repeats; C-TALEN, C/pol TALEN; DAA, direct-acting nucleotide analog; DBD, DNA-binding domain; DDB1, damage-specific DNA-binding protein 1; DHBV, duck hepatitis B virus; DSB, double-stranded break; dslDNA, double-stranded linear DNA; HAT1, histone acetyltransferase 1; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, HBV regulatory X; HDV, hepatitis D virus; IFN-1, interferon type 1; LTβR, lymphotoxin β receptor; NAP, Nucleic acid polymer; NHEJ, nonhomologous end joining; NIRF, Np95/ICBP90-like RING finger protein; NK, natural killer; NTCP, sodium taurocholate co-transporting polypeptide; NTZ, nitazoxanide; PD-1, programmed cell death protein; Peg, pegylated; pgRNA, pregenomic RNA; rcDNA, relaxed circular DNA; sgRNA, single guide RNA; shRNA, short hairpin RNA; SIRT3, silent mating type information regulation 2 homolog 3; SMC-5/6, structural maintenance of chromosomes 5/6; S-TALEN, S/pol TALEN; STOPS, S-antigen traffic inhibiting oligonucleotides polymers; TALEN, transcription activator-like effector nuclease; TLR, Toll-like receptor; ZFs, zinc fingers; ZFNs, zinc finger nucleases.

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Introduction

Hepatitis B virus (HBV) is a common cause of acute disease, and chronic hepatitis that can progress to cirrhosis and hepatocellular carcinoma.[1] The World Health Organization (WHO) estimated that in 2019, 296 million people were hepatitis B surface antigen (HBsAg)-positive. Nearly one million people die from HBV annually.[2] HBV is transmitted through blood and bodily fluids in perinatal and sexual exposure.[3,4] In adults, the vast majority, 80–85%, of acute infections resolve spontaneously. In contrast, in neonates and infants, 80–85% of acute infections result in chronic hepatitis. Treatment with direct-acting nucleotide analogs (DAAs) are generally highly effective in suppression of viral levels, but withdrawal of treatment in the vast majority of cases results in a return to pretreatment levels. Despite suppression of viral replication to low and even undetectable levels, the virus is able to restore levels of replication in the absence of antiviral agents. The aims of this report are to review the mechanisms by which HBV escapes DAA-mediated elimination, and to highlight strategies by which these mechanisms can be exploited in the design of novel agents against HBV and result in sustained virological response.

General principles of HBV replication

The major HBV infectious genome consists of a partially double-stranded relaxed circular DNA form enclosed in a capsid within a viral envelope. Infection involves primarily hepatocytes and is mediated by the binding of the hepatitis B surface antigen (HBsAg) to heparin sulfate proteoglycans on the surface of hepatocytes (Fig. 1).[5] The virus then interacts with sodium taurocholate co-transporting polypeptide (NTCP), a functional receptor for HBV, allowing for viral internalization.[6] Upon entry into hepatocytes, the relaxed circular DNA (rcDNA) in the nucleocapsid travels to the nucleus where its replication is completed by the conversion to covalently closed circular DNA (cccDNA). The latter forms a template for the transcription of viral RNAs.[7] HBV pregenomic RNA (pgRNA) is transcribed from the cccDNA and is encapsidated by the hepatitis B core protein (HBc) in the cytoplasm.[8] Reverse transcription of the RNA pregenome occurs in the cytoplasmic nucleocapsid beginning with binding of DNA polymerase (reverse transcriptase) to the pgRNA stem loop forming nucleocapsids. Within nucleocapsids, about 90% of the pgRNA is reverse transcribed to partially double stranded rcDNA. In the remaining 10%, pgRNA is reverse transcribed to double-stranded linear DNA (dslDNA). The dslDNA nucleocapsids can

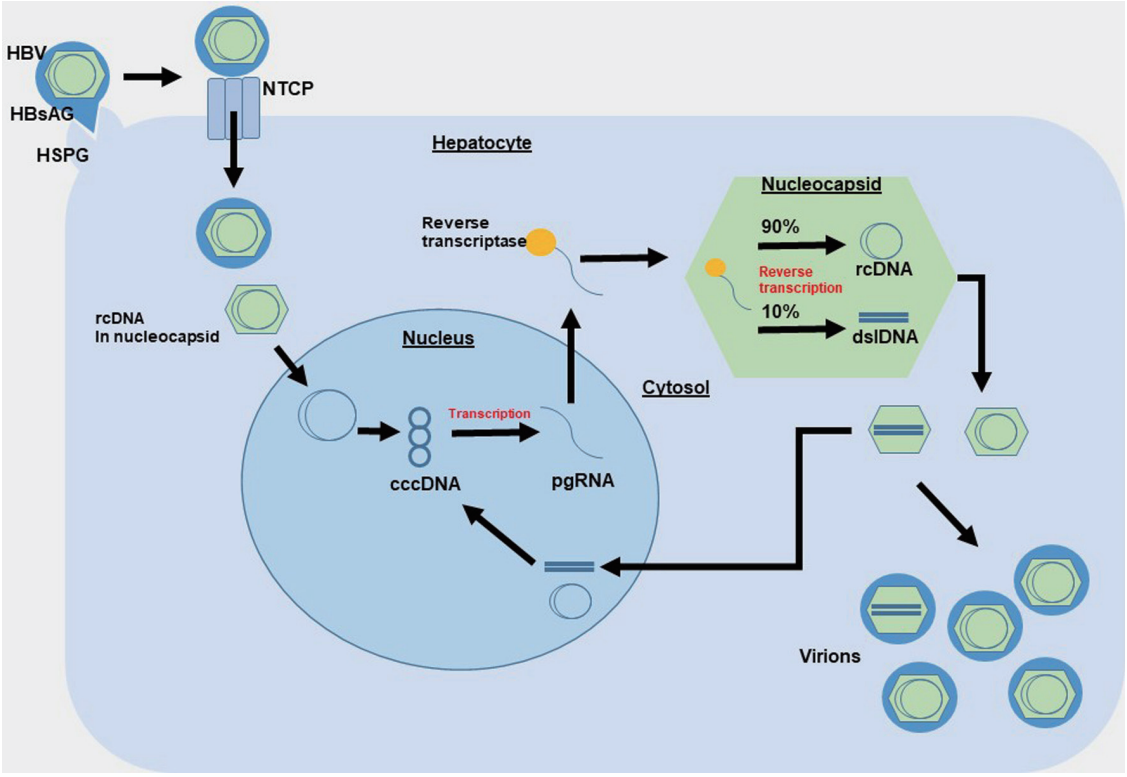

Fig. 1. Mechanism of hepatitis B virus entry and replication. Hepatitis B virus (HBV) enters the hepatocyte following hepatitis B surface antigen (HBsAg) binding to heparin sulfate proteoglycans (HSPGs) and interaction with the sodium taurocholate co-transporting polypeptide (NTCP). Within hepatocytes, relaxed circular DNA (rcDNA) enters the nucleus where it is converted to covalently closed circular DNA (cccDNA), the transcription template for pregenomic RNA (pgRNA). Following transcription, pgRNA is encapsulated by the hepatitis B core protein within the hepatocyte cytoplasm. Reverse transcription of pgRNA occurs in the nucleocapsid, forming rcDNA or double-stranded linear DNA (dslDNA). These reverse transcription products are either enveloped and secreted as new virions, or re-enter the nucleus.

either be enveloped and secreted as new virions, re-enter the nucleus to add to the cccDNA pool, or integrate into the host cell genome. The rcDNA nucleocapsids can be either enveloped along with polymerase and secreted as new virions ready to begin a new infection or re-enter the nucleus and add to the cccDNA pool.[9,10] Because of its overlapping reading frames, linearization of HBV circular DNA results in disruption of genes with loss of replicative capability. However, genes not affected by the linearization can continue to be expressed as integrants. Because cccDNA and integrated HBV DNA forms are stable and protected within the nucleus,[11] they represent key targets for novel therapy for chronic HBV infections. Although linearization terminates HBV replication by integrants, multiple integrants in the same cell could provide complementary gene products in *trans* for complete viral replication. This can complicate attempts to inactivate HBV replication.[12]

Shortcomings of current HBV treatment

The complex nature of HBV-host interaction is the main challenge for new treatment modalities. The major barriers include the presence of a high levels of HBV DNA and HBsAg, the presence of reservoirs for HBV replication and antigen production (cccDNA and integrated chromosomal DNA), and the HBV-impaired innate and adaptive immune response of the host.[13]

HBV inhibits the host immune response through several complex mechanisms including HBV regulatory X (HBx)-dependent downregulation of innate immunity signaling proteins, inhibition of interferon (IFN) type 1 (IFN-1) response, induction of immunosuppressive cytokines, or interference with toll-like receptor (TLR) activity. These mechanisms of immune tolerance have been reported to play a role in HBV chronicity.[14] However, the role of innate immunity in HBV natural immune clearance is controversial. It has been suggested that natural killer (NK) cells may play a role in early infection clearance through the activation of cytokines like IFN-gamma. Other innate immune cells like monocytes could play both pro- and anti-inflammatory roles.[15] Martinet *et al*.[16] revealed that HBsAg is a potential key factor in the dysfunction of the plasmacytoid dendritic cells by altering their interaction with NK cells disrupting the cytolytic activity of NK cells and enhancing immune tolerance.

On the other hand, the adaptive immunity represented in HBV-specific antibody producing B lymphocytes and functional T cells (T-cytotoxic and T-helper) are most important in determining the HBV course of infection. HBV neutralizing antibodies have a role in prevention and modulation of chronic HBV while HBV-specific polyclonal CD8 T cells can lyse infected hepatocytes. They also secrete cytokines that induce the noncytolytic HBV clearance and recruit the inflammatory immune cells. HBV-specific CD4 T cells regulate these processes. The increased antigen burden in chronic HBV infection can functionally exhaust the T cells, causing loss of cytotoxicity, tumor necrosis factor-alpha and IFN-gamma production, and ultimately T cell deletion. Furthermore, co-inhibitory molecules involved in programmed cell death protein 1 (PD-1) are highly expressed by exhausted intrahepatic HBV-specific T cells which further decrease the host immune

reaction against HBV infection.[17]

Objectives of novel HBV therapeutic strategies

The ultimate aim for HBV treatment is to induce HBsAg loss, prevent new hepatocyte infection, regain host immune function, and entirely eliminate HBV DNA. Table 1 highlights various strategies that aim to reduce HBsAg expression.[18–65] As the elimination of cccDNA and integrated HBV DNA remain challenging, the ideal goal of chronic HBV treatment is to achieve functional cure with sustained undetectable levels of HBsAg and HBV DNA after a finite duration of treatment. A more practical goal would be partial cure with detectable HBsAg but with minimal HBV DNA. Patients who achieve a partial cure have better outcomes than patients with untreated viremia but with inferior prognosis to those with functional cure.[66]

Although highly effective, nucleos(t)ide analogs only block reverse transcription and do not directly act on the cccDNA.[67] Additionally, the drugs do not prevent the formation of new cccDNA from incoming virions.[67–70]A phase III clinical trial comparing entecavir and lamivudine confirmed that short-term nucleos(t)ide analog therapy cannot eliminate hepatic cccDNA.[71] Most individuals on nucleos(t)ide analogs must continue treatment indefinitely as the drugs only rarely result in long term HBsAg seroconversion.[72]

Novel cccDNA targets for sustained virological response against HBV

Studies are currently in progress to directly or indirectly inhibit cccDNA transcription/replication which will decrease the antigen burden of HBV. These strategies include inhibiting structures or targets involved in the formation of cccDNA, epigenetic modification including methylation and histone deacetylation that silence cccDNA transcription and HBV replication, improving host immune defense, and employing gene editing techniques to target and cleave cccDNA.

Direct cccDNA inhibitors

Some studies have identified potential targets that inhibit cccDNA directly. For example, Cai *et al*.[18] utilized a cell-based screening strategy to measure cccDNA levels through expression of hepatitis B e antigen (HBeAg). Two disubstituted sulfonamide compounds called CCC-0975 and CCC-0346 reduced levels of cccDNA and rcDNA without directly affecting viral DNA replication. The results suggested that the disubstituted sulfonamide may interfere with the conversion of rcDNA to cccDNA through the inhibition of rcDNA deproteinization, a potentially intermediate step in cccDNA production, though the exact mechanism is unclear. If this result is confirmed, it may offer a new strategy for development of anti-cccDNA agents.

A screen of Chinese herbal remedies for HBV cccDNA inhibitors reported that hydrolyzable tannins, punicalagin, punicalin, and geraniin significantly reduced the production of HBeAg and cccDNA in a dose-dependent manner. It was proposed that the hydrolyzable tannins reduce cccDNA by blocking its formation and promoting its decay.[19] However, the compounds failed to alter viral DNA replication. The evidence suggests that the observed effects of the herbal remedies are directed toward stability or degradation rather than production of cccDNA. These agents may have complementary effects when used in combination with inhibitors of cccDNA replication.

Recently, Amblard *et al*.[20] identified GLP-26, a novel glyoxamide derivative, as a potential HBV inhibitor both *in vitro* and in humanized mice. GLP-26 is an HBV capsid assembly modulator that disrupts the HBV nucleocapsid and likely impacts the stability of cccDNA. GLP-26 inhibited HBeAg secretion and cccDNA amplification effectively. In the humanized mouse model of infection, it reduced HBsAg and HBeAg during and after treatment synergistically with entecavir. A strength of the study was inhibition of the viral load sustained for more than 12 weeks. GLP-26 had good oral bioavailability and did not show signs of mitochondrial toxicity as well when used at concentration less than 50 μm. A weakness of the study was that mice were not reconstituted with a humanized immune system in the model. As a special mouse model was employed, the results in a may not translate to similar results in humans.

There are problems in the evaluation of efficacy of anti-cccDNA agents. This is due in large part to a lack of standardized PCR-based methods that allow accurate cccDNA quantification in HBV-infected samples. For example, Southern blot hybridization is too insensitive, and real-time PCR for this purpose has not been standardized. Another problem is the presence of coexisting replicative intermediates which are identical in sequence to progenitor cccDNA. Furthermore, because cccDNA is located within hepatocytes, accurate quantification currently requires study of liver tissue which is an obvious clinical disadvantage.[73] More research is needed to accurately validate and assess the efficacy of reliable and standardized quantification methods.

Targeting HBV regulatory X protein

Another potential target is the HBx protein which promotes the transcription of the viral genome.[74] HBx assembles a damage-specific DNA-binding protein 1 (DDB1)-containing E3 ubiquitin ligase complex that targets the structural maintenance of chromosomes 5/6 (SMC-5/6), a complex that blocks viral transcription.[74–77] HBx also up-regulates the degradation of apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC)3B which increases cccDNA.[78] Shen *et al*.[80] found that cccDNA specifically associated with an enhancer region on chromosome 19p13.11 that promotes activation of cccDNA transcription mediated by both HBx and a DNA-binding factor called Yin-Yang 1. It has been previously shown that mutations that prevent this interaction inhibit hepadnavirus infection.[21,77,80–72]

HBx-based therapeutics trials are only in preclinical phases. Sekiba *et al*.[22] found that nitazoxanide (NTZ), an antiprotozoan agent, suppressed HBV transcription. Specifically, it decreased cccDNA levels and subsequent viral products through inhibition of the interaction between HBx and DDB1. The initial data suggested that NTZ was a promising HBV therapeutic agent and a potential tool to achieve genomic cure. The effects of NTZ against HBV in this study, while statistically significant, were modest. As NTZ is already used in clinical practice, approval for an HBV indication would likely be less costly and time consuming compared a totally new agent if further studies showed significant decrease in cccDNA.

Cheng *et al*.[23] found that dicoumarol, an inhibitor for NADP(H): quinone oxidoreductase 1, destabilized HBx and blocked cccDNA transcription. Additionally, it decreased HBsAg, HBc protein, and HBV DNA levels in a humanized liver mouse model. The study demonstrated a prolonged sustained inhibitory effect on HBV cccDNA *in vitro* and *in vivo.* Although the study revealed that dicoumarol silenced cccDNA transcription, other mechanisms cannot be excluded as it independently acts as microtubule stabilizing agent and induces reactive oxygen species. However, it also carries a risk of uncontrolled bleeding. Treating chronic hepatitis B (CHB) patients who are already at high risk for bleeding complica-

Table 1. Novel approaches to inhibit hepatitis B surface antigen expression

Category (Refs)	Agent/Mechanism	Design	Advantages	Disadvantages
Screening of novel inhibitors[18,19]	Various	Various	Ability to identify potential targets of cccDNA suppression	Exact mechanisms are unclear; require future study
Direct cccDNA inhibitor[20]	GLP-26, HBV capsid assembly modulator	*In vitro* and humanized mice	Sustained inhibition of viral load	Mice were not reconstituted with a humanized immune system in the model
HBV regulatory X protein[21–24]	Nitazoxanide;[21] Dicoumarol;[22] Protein-carrier HBx vaccine;[23] HBx monoclonal antibody[24]	Various	Promising elimination of HBsAg and or cccDNA	Studies are in preclinical phases
DNA methylation[26–29]	Transcriptional suppression of HBV cccDNA	cccDNA extracted from liver biopsies;[27] Lentiviral vector inducing methylation *in vitro*[29]	Sustained inhibition of viral load	Unclear whether shRNA methylation of HBV cccDNA would occur in non-neoplastic human liver model[29]
Histone acetylation[25,30–33]	Curcumin;[25] Silent mating type information regulation 2 homolog 3 (SIRT3) ;[31] Histone acetyltransferase 1 (HAT1);[32] Np95/ICBP90-like RING finger protein (NIRF)[33]	HepG2 cells;[25] HepG2 cells;[31] Human liver-chimeric mouse model;[32] HepG2 cells and mice[33]	Ability to modulate the HBV cccDNA minichromosome through various mechanisms	Mechanisms of modulation require further study
Apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) protein family regulators[34–40]	APOBEC cytidine deaminases	*In vitro* and *in vivo* studies	Multiple mechanisms by which inhibition can occur	Mechanisms require further study
Nivolumab in patients with chronic hepatitis B[41]	Nivolumab programmed death receptor (PD-1) inhibitor	Clinical study	HBsAg decline in most patients and sustained HBsAg loss in one patient; well tolerated	Small pilot sample warranting further investigation
Toll-like receptor agonists[42,43]	Vesatolimoid;[42] Selgantolimod[43]	Clinical study	Clinical studies showing good safety and tolerance of agents	No significant decline in HBsAg levels
Gene editing	Zinc finger agents[44,45]	*In vitro*	Demonstrated accurate localization of DNA target sites[45]	Not all sequences are available for binding; Potential for off-target cutting
Gene editing	Transcription activator-like effectors nucleases (TALENs)[46,47]	*In vitro*	High precision and can specifically target any DNA sequence	Potential difficulty *in vivo* since a large number of amino acids are required to bind to a single nucleotide
Gene editing	Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)[48–60]	*In vitro*	Does not require engineering of a site-specific nuclease	HBV genomes are highly heterogeneous, though this mechanism has been used to excise full-length HBV genomes from a stable HBV cell line
HBsAg inhibitors[61–63]	Nucleic acid polymers (NAPs);[61] S-antigen traffic inhibiting oligonucleotide polymers (STOPS)[62,63]	Randomized controlled trials	Results demonstrate HBsAg seroconversion and functional cure among patients[61]	Concern for potential toxicity with STOPS[62]
Small molecule cccDNA inhibitor[64]	Myrcludex B, inhibitor of HBV and HDV receptor sodium taurocholate co-transporting polypeptide	Phase Ib/IIa clinical trial	Clinical trial, treatment was well tolerated	HBsAg levels remained unchanged after 24 weeks of treatment
Small molecule cccDNA inhibitor[65]	Bepirovirsen, antisense oligonucleotide that targets HBV mRNAs	Phase IIb clinical trial	Clinical trial, treatment could show promise when incorporated with other combination therapies	HBV DNA loss only occurred in 9–10% of patients who received treatment for 24 weeks

APOBEC, apolipoprotein B mRNA editing catalytic polypeptide-like; Cas, CRISPR-associated; cccDNA, covalently closed circular DNA; CRISPR, clustered regularly interspaced short palindromic repeats; HAT1, histone acetyltransferase 1; HDV, hepatitis D virus; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, HBV regulatory X; NAP, Nucleic acid polymer; NIRF, Np95/ICBP90-like RING finger protein; PD-1, programmed cell death protein; shRNA, short hairpin RNA; SIRT3, silent mating type information regulation 2 homolog 3; STOPS, S-antigen traffic inhibiting oligonucleotides polymers; TALEN, transcription activator-like effector nuclease.

tions with dicoumarol would be challenging. If research on dicumarol analogs reveals agents that retain cccDNA inhibitory effects without affecting coagulation, this class of agents might find better anti-HBV utility.

A protein-carrier HBx vaccine showed a significant elimination of HBsAg and HBV DNA by inducing a systemic CD4+ and CD8+ T cell response in HBV carrier mice.[24] The study demonstrated that HBx-induced adaptive immunity eliminated HBV expressing cells. However, no signs of protective antiHBs were observed indicating that treatment with the HBx vaccine alone is not sufficient to restore the humoral immunity. Another problem is that human immune response to HBx vaccine differs from that of rodents, which are not natural hosts for HBV.

Another study demonstrated HBx monoclonal antibody as a potential potent therapeutic agent for HBV. A cell-penetrating antibody targeting HBx was developed by conjugation of HBx antibody and an HIV *tat* protein. The *tat* monoclonal antibody suppressed viral replication and protein production in cell and mice models that mimicked chronic HBV infection.[83] Although significant decreases in HBsAg and HBV DNA levels were observed in mice after a single infusion, virological rebound in serum occurred in most mice between days 7 and 9. More studies are needed to improve cell delivery, efficacy and sustainability to promote HBx monoclonal antibodies as a therapeutic agent.

Epigenetic modification to reduce cccDNA expression

DNA methylation

Epigenetic modifications through DNA methylation and histone deacetylation affect the function of cccDNA.[25,84,85] CpG methylation regulates the transcription of cccDNA.[86] Increased cccDNA methylation is associated with low serum HBV DNA-titer, suggesting suppressed viral activity.[26] Additionally, the methylation of cccDNA in human tissue of CHB patients has been associated with reduced cccDNA mRNA synthesis and viral expression.[27–29] For example, a study on HBV cccDNA extracted from liver biopsies of HBsAg-positive patients found that HBeAg-negative patients had significantly higher positive ratios of cccDNA methylation than those of HBeAg-positive patients.[28] The study showed that methylation of cccDNA is associated with impaired replication of HBV which could result in silencing and ultimately depletion of HBV cccDNA. The study demonstrated that cccDNA may be relevant to HBeAg seroconversion. However, it failed to identify a mechanism of epigenetic modulation.

Another study by Park *et al*.[30] developed a third generation lentiviral vector through cloning of a short hairpin RNA (referred to as shRNA) sequence against the HBx gene into an HIV-based plasmid. It induced methylation and transcriptional suppression of HBV cccDNA in a hepatoma cell line. The study showed that lentiviral vector-mediated of shRNA may be a potential novel tool for suppression and potentially elimination of cccDNA through epigenetic modulation. However, the study did not demonstrate suppression activity in nonneoplastic liver cells and nor demonstrate prolonged suppression of cccDNA. Safety of lentiviral integration would be further required for clinical studies.

Histone acetylation

Acetylated histones bound to cccDNA have been shown to regulate the propagation of HBV.[84] Hyperacetylation of H3 and H4 histones increased transcription of cccDNA and HBV replication.[25] Since HBx can bind to the cccDNA mini-chromosome to promote acetylation and replication of the virus, researchers have identified various targets that help down-regulate histone acetylation.

In a HepG2.2.15 cell line transfected with HBV, Wei *et al*.[31] determined that curcumin, a hypomethylating agent, caused a dose-dependent reduction in HBsAg and HBeAg expression and significant reduction in HBV DNA replication through decreased cccDNA-bound histone acetylation. However, cytotoxicity occurred with concentrations of more than 20 μmol/L. Ren *et al*.[32] demonstrated the effect of silent mating type information regulation 2 homolog 3 (SIRT3), a NAD-1 dependent histone deacetylase, on HBV replication. Ectopic SIRT3 overexpression inhibited HBV replication and transcription by acting cooperatively with methyl transferase to restrict HBV cccDNA. In contrast, gene silencing of SIRT3 increased HBV activity in primary human hepatocytes and HBV-infected HepG2-NA1/taurocholate co-transporting polypeptide cells. However, the reduction in cccDNA in HBV-infected cells did not reach a statically significant difference. Animal studies are needed to more fully evaluate efficacy and toxicity.

Yang *et al*.[33] demonstrated that histone acetyltransferase 1 (HAT1), an important factor in chromatin assembly, regulated the cccDNA mini-chromosome. The authors first demonstrated that HAT1 expression in a human liver-chimeric mouse model increased in HBV-infected humanized mice. They also developed cell lines that could detect and quantify HBV cccDNA and HBV DNA. They found that depletion of HAT1 significantly decreased HBV DNA, HBeAg, and HBsAg in HBV-infected primary human hepatocytes, dHepaRG, and HepG2-NTCP cells. Additionally, qPCR and Southern blot analysis demonstrated that HAT1 was crucial for cccDNA accumulation. They also found that infected nontumorous liver had a higher expression of HAT1 indicating a relationship between the molecule and HBV cccDNA. A strength of the study was demonstration that HAT1 promoted HBV replication and cccDNA accumulation. The authors also demonstrated that HBV upregulated HAT1 to enhance its replication in a positive feedback manner. Weaknesses of the study included a relatively small sample size, 43 liver tissue samples, of which only 39 were positive for HBV DNA and only 24 positive for cccDNA. While the depletion of HAT1, HBV cccDNA and HBV DNA is of interest, the clinical impact of the finding remains unclear.

Qian *et al*.[34] found that Np95/ICBP90-like RING finger protein (commonly known as NIRF), an E3 ubiquitin ligase, reduced the acetylation of HBV cccDNA-bound H3 histones, and inhibited the replication and secretion of HBV through proteasome degradation of HBc proteins. That led to a decreased burden of HBsAg and HBeAg. A strength of the study was the inhibition of replication and viral antigens *in vivo* and *in vitro.* A lack of sustained significant inhibition of HBeAg, HBsAg, HBV DNA, and HBV cccDNA after 72 h in culture cells injected with NIRF compared to the control cells *in vitro* tempers optimism.

Targeting the immune system

APOBEC

APOBECs make up a family of endogenous cytidine deaminases that initiate the destruction of cccDNA in the nucleus.[35–37] They have been shown to inhibit HBV replication through deaminase-dependent and independent mechanisms.[36,38,39] APOBEC3G can bind to the HBc and gain access to DNA during reverse transcription, editing the core associated DNA but not the pgDNA.[40] Furthermore, Lucifora *et al*.[36] demonstrated that activation of lymphotoxin (LT) β receptor

(LTβR) suppresses HBV replication and leads to nuclear cccDNA degradation through upregulation of APOBEC3A and APOBEC3B cytidine deaminase *in vitro*. The study demonstrated a persistent antiviral effect with no rebound in HBV replication. A problem with constitutive expression of LTβR is its association with hepatocellular carcinoma and liver inflammation which make its clinical use as an antiviral challenging.

Various tumor necrosis factor superfamily members are physiological ligands for this receptor and can activate inflammatory, anti-inflammatory survival pathways or induce apoptosis.[36–41]

Nivolumab

As explained earlier, patients with chronic HBV infection have impaired immune response to HBV. This is partially due to chronic exposure of HBsAg. Several approaches to stimulate or decrease inhibition of HBV-specific immune responses have been studied. Gane *et al*.[42] administrated nivolumab, a programmed death receptor inhibitor specifically targeting PD-1, with or without GS-4774, a yeast-based therapeutic T cell vaccine, to chronic HBeAg-negative HBV patients. The study showed that three of 22 patients who received high dose nivolumab had significant decreases in HBsAg levels. However, only one patient had undetectable levels of HBsAg at week 20 which was sustained for 12 months. A strength of this study was evidence that immune checkpoint inhibition can improve the immune response in chronic HBV infection. However, one patient developed an acute flare of alanine aminotransferase. Another weakness was the exclusion of HBeAg-positive patients, a bias toward inclusion of low levels of HBV infection. More studies are needed to assess the safety and efficacy of immune checkpoint inhibition on a larger scale.

TLR agonists

There have been several studies on induction of innate immune response in CHB patients. Vesatolimoid (GS-9620), an oral agonist of TLR7, was studied in 162 HBV patients. Although safe and well tolerated, the study failed to show any significant decline in the levels of HBsAg.[43]

Gane *et al*.[87] evaluated the efficacy of selgantolimod, a TLR8 agonist, in viremic CHB patients. Doses of selgantolimod for two or four weeks did not show a significant decline from baseline in HBsAg or HBV DNA. However, study showed good safety and tolerability profile. More research is required to more fully evaluate efficacy and toxicity of these agents.

Gene editing approaches

It is established that double-stranded breaks (DSBs) stimulate cellular endogenous repair machinery.[88] DNA is typically repaired through two major pathways, nonhomologous end joining (NHEJ) or homology directed breaks. NHEJ results in direct re-ligation of the two ends of the DSB and does not require a DNA template. This may introduce or remove a few nucleotides causing frameshift mutations that could produce truncated proteins or degradation of the mRNA. Homology directed breaks is more complex and functions to repair DSB in a DNA-template-dependent manner. Both pathways are error-prone and can be used to control the DNA repair machinery to engineer a wide variety of genomic alterations.[89,90] Various gene therapeutic approaches have been studied to treat HBV infection through introducing site-specific DSBs in HBV cccDNA. Four major mechanisms have been described: zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems have targeted HBV cccDNA, and meganucleases.[91]

Zinc finger agents

Zinc fingers (ZFs) are multifunctional mammalian proteins that naturally serve as transcription factors. The DNA-binding domain (DBD) of ZF attaches to a certain base triplet. The DBD is connected to the cleavage domain of the FoKL restriction endonuclease. DBD proteins provide the targeting domains while FoKL restriction endonuclease results in cleavage, double-stranded DNA breaks and potential gene knockout through the DNA repair process (Fig. 2).[89,91–93] ZF agent site selection is limited by the fact that not all sequences are available for binding by ZF proteins. Another challenge is off-target cutting by a pair of ZFNs, one that binds the forward strand and another that binds the reverse strand, can double the number of recognized base pairs.[44]

Epigenetic gene silencing is performed by targeting locus-specific, epigenetic changes in effector or repressor domains to inhibit HBV gene expression. cccDNA epigenome editors are currently being developed. In a Hep3B HCC cell line, Singh *et al*.[45] downregulated integrated HBx using an artificial transcription factor (ATF). They created a ZF domain that specifically targeted an 18-bp DNA target in the enhancer region of HBx. In cell lines that expressed HBx, ATF resulted in significant growth arrest. However, the variation of HBV DNA sequence in different patients presents a challenging barrier, as ATF would need to be tailored to individual HBV sequences. Animal studies are needed to more fully evaluate efficacy and toxicity.

Weber *et al*.[94] created three ZFNs to target cccDNA in human embryonic kidney 293 T cells and HepAD238 cells with an encoded adeno-associated viral vector. Through open frame reading, the ZFNs targeted the polymerase/x (1), polymerase/core (2) and polymerase regions (3) to inflict mutations in the DNA. ZFN pair three showed a significant antiviral activity and decreased HBV DNA production.

Without the use of nucleases, Zimmerman *et al*.[95] used ZF proteins to target the cccDNA in duck hepatitis B virus (DHBV). They avoided off-binding by selecting sites based on BLAST searches of the DNA target sites against chicken genome. They screened binding efficiency through a gel shift assay (*i.e*. EMSA) and DHBV enhancers which inhibited the core, small, and large surface protein production were introduced into a DHBV culture system. They concluded that expression of designed ZF proteins in DHBV culture system resulted in decreased viral RNA and protein expression. Strengths of the study included localization of DNA target sites accurately, and demonstration of a lack of off-binding using Western blotting. A disadvantage is that the authors did not demonstrate the effect of ZF proteins *in vivo*. Studies in humanized models may be helpful in more fully evaluating efficacy and toxicity.

TALENs

TALENs are DNA-binding proteins that are produced by xanthomona bacteria.[46] Similar to ZFNs, TALEs can be fused to the catalytic domain of FoKL nuclease to produce TALENs. TALENs can specifically target any DNA sequence and are easy to produce (Fig. 2). However, a large number, 34, of amino acids required to bind to a single nucleotide making them potentially difficult to be delivered *in vivo.*[47]

Bloom *et al*.[96] demonstrated the first targeted nuclease-mediated disruption of HBV cccDNA using TALENs in cell culture and in mice. They generated four TALENS which target-

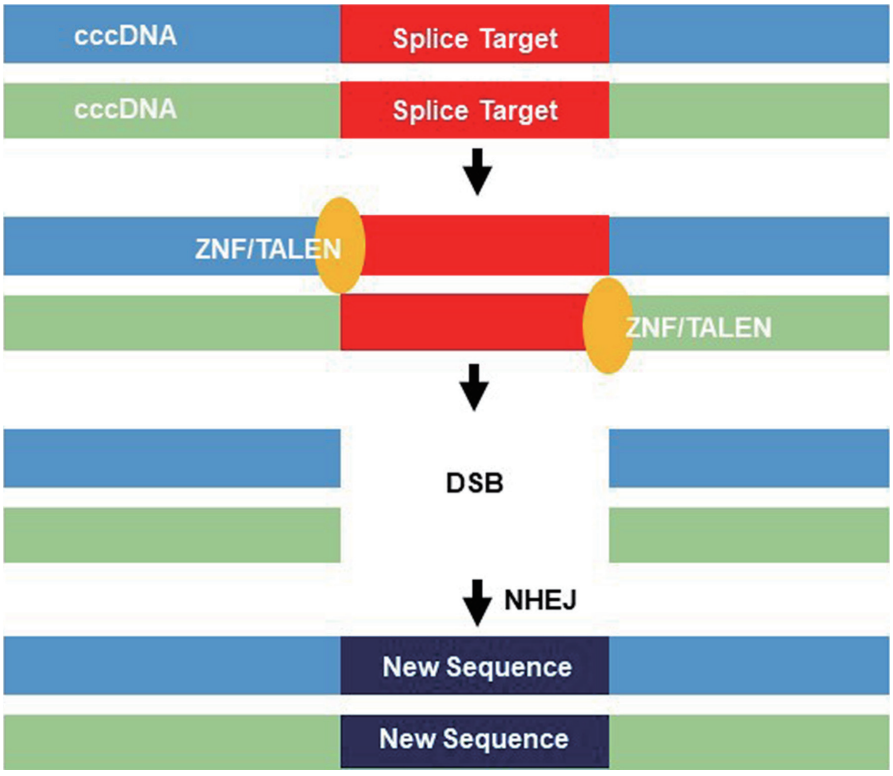

Fig. 2. Gene editing mechanisms of zinc finger nucleases and transcription activator-like effector nucleases. A. Zinc finger nucleases (ZNFs) recognize and bind to target nucleotide triplets. Transcription activator-like effector nucleases (TALENs) operate in a similar mechanism but can directly cleave specific DNA sequences. The nucleases create a double-stranded break (DSB) around the splice target. Nonhomologous end joining (NHEJ) repair causes gene knockout, deletion, correction, or addition. cccDNA, covalently closed circular DNA.

ed specific sites within the S/pol (S-TALEN), C/pol (C-TALEN), and pol ORFs (P-TALEN), of the HBV genome. In Huh-7 cells, there was a significant decrease in the hepatitis B core antigen (HBcAg) in transfected cells. S-TALENs showed increasingly inhibitory effects on HBcAg in a hypothermic environment. The targeted cccDNA was isolated from HepG2.2.15 cells to assess for the TALEN-mediated targeted mutagenesis. PCR-based analysis and primer amplification methods showed no significant contamination with cellular DNA genome or HBV rcDNA. Assays using T7E1 and CEL1 showed that the S-TALEN disrupted 31% of the target molecules that correlated with a decrease in HBcAg secretion in HEPG2.2.15 cells. C-TALEN and P-TALEN showed no significant effect in disruption of HBV DNA.

A hydrodynamic injection method was employed to assess the effect of S- and C-TALENs on HBV replication measuring HBsAg concentration and viral particles equivalents in mice. More than 90% of HBcAg was knocked down by TALEN S and 70% of viral particles equivalents were decreased by either S-TALEN or C-TALEN The immune-histological assessment and transaminases levels demonstrated minimal toxicity. T7E1 assays demonstrated mutations of 57–87% of amplified HBV DNA in treated mice. Study strengths included demonstrating a significant decrease in HBcAg with a minimal toxicity on liver cells. However, the study failed to demonstrate any significant effect of TALENS on transcription of HBV. mRNA concentrations were similar in both treated mice and controls.[96] Confirmation of these results without using hydrodynamic methods for introduction of the agents may show the potential under conditions closer to clinical applications.

Chen *et al*.[97] designed TALENs to target highly conserved regions among the different genotypes of HBV. L1/R1 TALEN recognized the region around the RNase H (one of the four domains of viral polymerase), while L2/R2 and L3/R3 recognized DNA sequences in the core protein regions. Huh-7 cells were transfected with HBV DNA and plasmid encoding TALENS. Their expression was detected mainly in the nucleus using immunoblotting. CCK8 assays showed no difference in cell viability and cell growth between the control and cells targeted with TALENs. L1/RI and L2/R2 TALENs were able to significantly decrease the HBsAg, HBeAb, and pgRNA production, but only L2/R2 drastically decreased the levels of HBcAg. cccDNA was decreased by 10–20% and 30–40% in L1/R1 and L2/R2, respectively, in TALENs-expressing cells. The authors confirmed their results in a hydrodynamic mouse model with L2/R2 infected cells. Significant decreases in HBsAg, HBeAb, HBV DNA, and pgRNA levels were found. Moreover, they demonstrated a synergistic inhibitory effect by TALENs and INF-alpha on the HBV transcription. A strength of this study was the demonstration of significant decreases in both viral RNA production and transcription across HBV genome with different genotypes (B, C, or D). A weakness of the study is that TALEN L3/R3 failed to show an effect against transcription or replication. The variation in effects between different TALENs suggest that efficacy depended on the target sequence of HBV DNA.

CRISPR/Cas-9

The CRSIPR/Cas-9 system utilizes a short RNA sequence that drives the formation of a DSB at a target site (Fig. 3). It only requires the synthesis of new RNA rather than the engineering of a site-specific nuclease. Various factors influence the

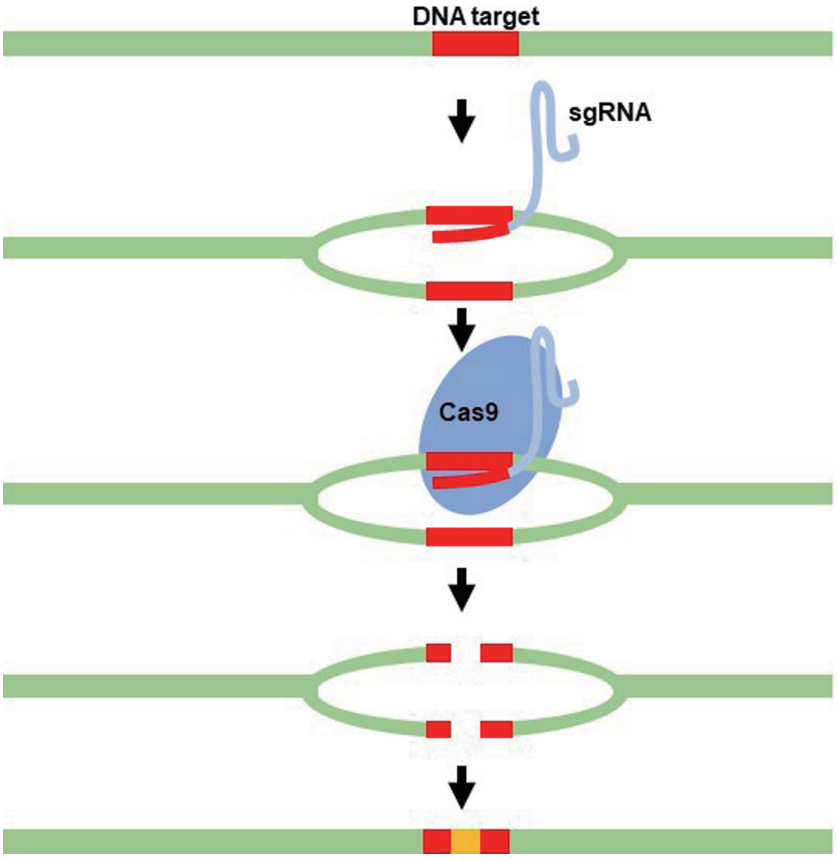

Fig. 3. Clustered regularly interspaced short palindromic repeats/CRISPR-associated gene editing mechanism. Clustered regularly interspaced short palindromic repeats (CRISPR) often utilizes a CRISPR-associated (Cas)-9 nuclease that locates a single guide RNA (sgRNA) and makes a double-strand break, cleaving DNA targets. These breaks trigger DNA repair which can knockout genes, as demonstrated by experiments involving CRISPR/Cas-9 and covalently closed circular DNA (cccDNA).

efficacy of the CRISPR/Cas-9 system including guide RNA design, off-target cutting, Cas-9 activity, and the method of delivery.[98] One challenge of targeting HBV cccDNA using CRISPR/Cas-9 is that HBV genomes are highly heterogeneous.

CRISPR/Cas-9 has been shown to inhibit HBV infections by introducing mutations into the viral cccDNA. Combinations of HBV-targeting nucleases cleaved DNA strands repaired by NHEJ, an error-prone repair mechanism.[48] Seeger and Sohn found that the CRISPR/Cas 9 system was extremely efficient at editing of HBV DNA in HepG2 cells. Using next generation sequencing (NGS), the authors showed a spectrum of mutations in cccDNA following Cas-9 cleavage and repair following NHEJ. Cas-9 cleaved over 90% of HBV DNA, and was 10,000 to 15,000 times more efficient than APOBEC-mediated cytosine deamination following the treatment of infected cells with IFNα. The authors designed single guide RNAs (sgRNAs) called HBx2 and HBx4 that targeted HBx and the overlapping polymerase gene. They also found that approximately 7% of cleaved cccDNA genomes were repaired in a way that might not terminate the function of HBx. The need to use multiple sgRNAs targeting different loci on the HBV genome to inactive cccDNA was recognized.[49]

Kennedy *et al*.[50] used the CRISPR/Cas system to target HBV cccDNA, resulting in decreases in total HBV, cccDNA, and the secretion of HBV antigens including HBsAg. Experimental systems have included *de novo* infection of hepatoma cell lines with HBV produced by HepAD38 cells,[50–58] transfection of plasmids that express HBV into hepatoma cell lines and *in vivo* co-transfection of HBV DNA and CRISPR/Cas expression plasmids in mouse livers.[51,54,56,58,59] Additionally, CRISPR/Cas-9 technology has been used to excise full-length integrated HBV genomes from a stable HBV cell line. Post excision, cccDNA, supernatant HBV DNA, or HBsAg was undetectable for 10 months.[60] However, many of these studies did not consider the high heterogeneity of the HBV genome when identifying and selecting guide RNA (gRNA) targets.[49,50,54,59]

Li *et al*.[99] pyrosequenced the whole HBV genome in 17 patients with advanced liver disease and 30 chronic carriers and found a variety of viral quasispecies, demonstrating heterogeneity. Liu *et al*.[55] designed eight gRNAs to target 26 conserved regions of different HBV genotypes. The gRNA/Cas-9 systems were co-transfected into HepG2 cells with HBV, and the levels of HBV RNA were detected by northern blotting. Four days after transfection, it was found that all systems significantly suppressed HBV replication compared to controls. CCK8 assays demonstrated no cytotoxicity. Two representative gRNA/Cas-9 systems were amplified and sequenced and found not to have detectable off-target effects. Additionally, the application of multiple gRNA/Cas-9 systems significantly inhibited the replication of HBV all the genotypes used, suggesting that using several gRNAs to simultaneously target conserved regions of HBV may increase the ability to inactivate HBV replication and prevent viral escape from treatment. Moreover, the authors demonstrated inhibition of HBV *in vivo* by injecting mice with the HBV-specific gRNA/Cas-9 systems and HBV. Assays and sequencing

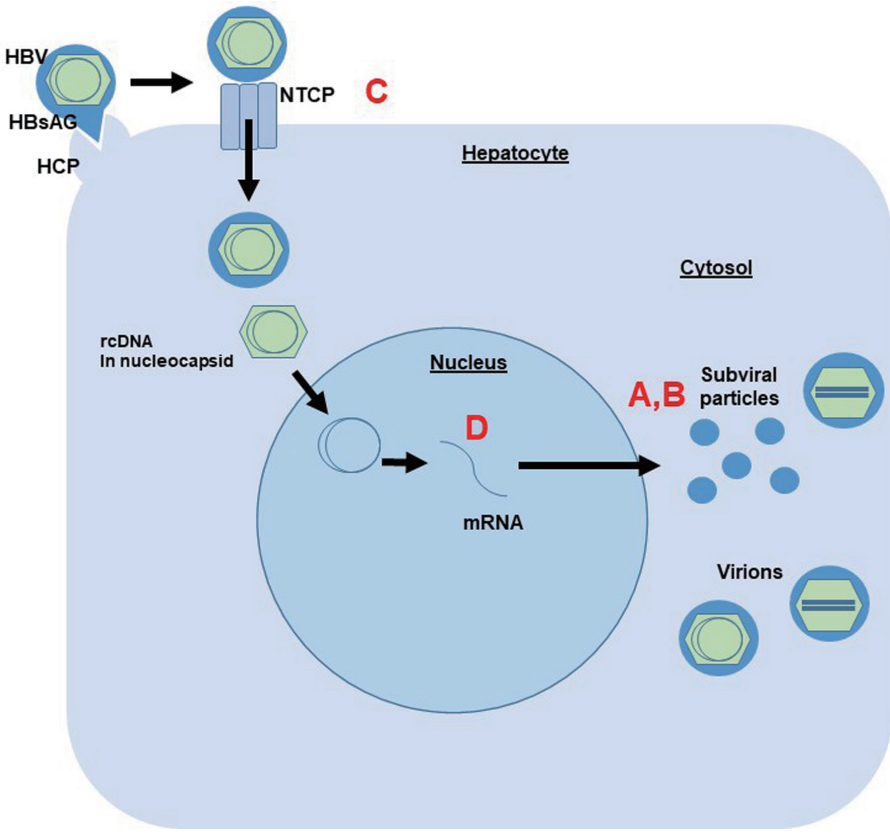

Fig. 4. Mechanisms of novel methods to interfere with hepatitis B surface antigen synthesis (HBsAg). (A) Nucleic acid polymers (NAPs), which interfere with the production of subviral particles that carry HBsAg; (B) S-antigen traffic inhibiting oligonucleotide polymers (STOPS), which inhibit expression of HBsAg; (C) Myrcludex B, a small molecule cccDNA inhibitor that targets the sodium taurocholate co-transporting polypeptide (NTCP); and, (D) Bepirovirsen, an antisense oligonucleotide that targets Hepatitis B virus (HBV) mRNA.

showed that these systems could introduce approximately 11% mutagenesis *in vivo*. A weakness of this study is that only the human codon-optimized SpCas9 plasmid was used which only covered a portion of the HBV genotypes and subgenotypes even though the gRNA/Cas-9 systems targeted conserved regions.

To show direct cccDNA targeting, Martinez *et al*.[61] used HBV-specific gRNAs and CRISPR/CAS-9 to determine the effect on cccDNA after gene editing in HBV-infected HepG2-NTCP cells. The gRNA/CAS-9 was delivered using ribonucleoproteins. In several single and dual combinations, persistent reduction in RNA, HBsAg and HBeAg levels were observed most significantly in dual combination with targeting Sp5 and Sp7. The study demonstrated the formation of new small transcriptionally active HBV variants. The study pointed out the presence of episomal HBV DNA variants after the cleavage by CRISPR/CAS-9.

The use of nuclease-based therapy in HBV therapy faces several obstacles including safety, efficacy, and specificity. There is potential toxicity associated with gene targeting as nucleases may be considered as foreign bodies by immune system.[89] In addition, DSBs could result in aberrant chromosomal rearrangements with unexpected adverse effects. Targeted and efficient delivery remains a major challenge as gene editing components are relatively large in size.[90] Various delivery methods are being explored including the use of viruses such adenoviruses or adeno-associated viruses and nonviral methods such as lipid-based nano-formulations.[91] Another challenge is the high sequence heterogeneity within HBV genotypes which make it difficult to find effective gRNAs to target conserved HBV sequences across different genotypes.[89–91]

Novel inhibitors of HBsAg

Figure 4 shows various novel mechanisms of agents for inhibition of HBsAg synthesis and expression. Targeting integrated HBV DNA could help eliminate an important source of HBsAg expression which is partially responsible for immune tolerance and T cell exhaustion.[17]

Nucleic acid polymers

Nucleic acid polymers (NAPs) are oligonucleotide-based, broad-spectrum antiviral agents. They act on the apolipoprotein interaction involved in the assembly and release of HBV subviral particles which are made of HBsAg. The elimination of HBsAg can improve the efficacy of the immune therapy and potentially achieve functional cure.[62]

Bazinet *et al*.[63] studied two NAPs, REP 2165 and REP 2139 combined with pegylated (Peg) IFN and tenofovir disoproxil fumarate in HBeAg-negative CHB patients. Patients were randomly assigned to combination therapy (with REP 2165 or 2139) or only TNF and Peg-IFN. The study demonstrated a significant decrease in HBsAg levels in the experimental group. Furthermore, 60% of patients who received the combination therapy had HBsAg seroconversion and 39% achieved functional cure. Levels of HBsAg did not differ significantly between patients who received REP 2165 or REP

2139. There was a greater elevation in the levels of transaminases in the experimental group, and was associated with symptoms. The levels correlated with the initial increase in HBsAg, and normalized during therapy. The study did not demonstrate the mechanism of NAPs in suppressing HBsAg. Evidence of hepatotoxicity as reflected by aminotransferase elevations is a concern. It is possible that other congeners that have similar anti-HBV effects without hepatotoxicity may be developed.

S-antigen traffic inhibiting oligonucleotide polymers

S-antigen traffic inhibiting oligonucleotide polymers (STOPS) are a class of oligonucleotides that are similar to NAPs. They can potentially inhibit HBsAg synthesis through the sequestration of cellular proteins that are required for proper expression and folding of HBsAg. A challenge of using STOPS in clinical practice is potential toxicity.[64] Currently, the safety and efficacy of the first STOP, ALG-010133, are being studied in preclinical phases.[65]

Viral entry inhibitors

Myrcludex B

Myrcludex B is a linear, chemically synthesized, myristoylated peptide. It acts as a specific entry inhibitor of the HBV and hepatitis D virus (HDV) receptor NTCP and ultimately blocks entry of HBV and HDV into hepatocytes (Fig. 4). In addition, because bile acids themselves have been shown to promote HBV transcription and gene expression, myrcludex B was shown to inhibit HBV replication on a transcriptional level at a post entry step by interfering with the farnesoid X receptor alpha. In hepatoma cell lines with stable NTCP expression, myrcludex B significantly reduced the levels of HBsAg in cell cultures. The results were consistent in in mouse studies in which HBsAg, HBeAg, and HBV DNA were all significantly decreased by myrcludex B adminstration.[100] A limiting factor was that the effect was only seen after 5 days of treatment which indicates that only sustained treatment may impair replication. The authors did not comment on the effect of the myrcludex on the physiological function as a bile acid transporter and its importance in bile acid homeostasis in liver cells which might be challenge for clinical use of myrcludex B. The study is important because it shows the role of myrcludex B in inhibiting HBV replication at a post entry level.

A clinical trial compared daily myrcludex B with entecavir administration in patients with CHB. One cohort received myrcludex B daily for 24 weeks followed by Peg-IFNα-2a for 48 weeks, another cohort received myrcludex B daily in combination with Peg-IFNα-2a weekly for 24 weeks followed by 24 weeks of Peg-IFNα-2a alone, and the last cohort received only Peg-IFNα-2a weekly. The primary endpoint was HBsAg response at week 12 of therapy. Secondary endpoints included the responses of HBsAg at 24 weeks and those of HDV RNA, HBV DNA, and ALT to therapy at 24 and 48 weeks, as well as at the end of a 24-week treatment-free follow up period. The inhibitor caused a significant decrease in HDV in chronic hepatitis D patients after 24 weeks of treatment. However, HBsAg levels remained unchanged. In a cohort receiving daily myrcludex B in combination with weekly Peg-IFNα for 24 weeks, HBD DNA decreased significantly at the end of the study. A strength of this study was that the monotherapy myrcludex B was well tolerated and its combination with Peg-IFNα did not increase frequency or severity of adverse events.[101] A weakness of this clinical trial was the small sample size. Although the study reported that the agents were well tolerated, if IFN is required for optimal effects of myrcludex B, patient acceptance of IFN side effects could be a practical clinical issue.

In mouse reconstituted human hepatocytes that were infected with HBV, Volz *et al*.[102] showed that daily administration of myrcludex B inhibited viral spreading from the initially infected human cells demonstrated by decreased HBcAg levels after 6 weeks of infection. Furthermore, intrahepatic cccDNA loads remained stable compared to the values found in mice sacrificed 3 weeks post-infection. A study strength is the demonstration of an inhibitory effect of myrcludex B on the intrahepatic cccDNA pool. It was suggested that myrcludex B might hinder conversion of rcDNA to cccDNA, the mechanism by which this occurs was not clarified.

Small molecule cccDNA inhibitors

Bepirovirsen

Yeun *et al*.[103] assessed the safety and efficacy of bepirovirsen (GSK3228836), an antisense oligonucleotide. Bepirovirsen targets HBV mRNA and may have an immune stimulatory activity mediated by TLR8. The study included 475 patients, half receiving nucleotide analogue therapy. Sustained HBsAg and HBV DNA loss occurred in 9–10% of patients who received bepirovirsen for 24 weeks. Adverse effects occurred more in the experimental group than the placebo group, and included pyrexia, fever, and elevated liver aminotransferases. The loss of HBsAg that was observed predominantly in HBeAg-negative patients who potentially have integrated HBV sequences in their genome. This suggested but did not prove that bepirovirsen targeted integrated HBV DNA. There was no apparent response in HBsAg-positive patients who were not on nucleotide analoguetreatment. That observation might be related to the increased baseline levels of HBsAg and its role in prediction the response to HBV therapies. The study showed a relative low efficacy of bepirovirsen. However, there is a potential for enhancement with other combination therapies and more selective baseline characteristics of the patients. Currently, the durability of response is under investigation.

Discussion

Complete eradication of cccDNA and ultimately complete cure of HBV infection remains elusive with many challenges ahead. One of the main challenges is the need for efficient targeted delivery that could decrease degradation of the agent by untimely elimination, and avoid side effects and toxicity to nonhepatic cells at the same time. Polymeric nanoparticles and lipid nanoparticles have been shown to improve inhibition of viral replication and clearance of cccDNA. The understanding of the tissue and intracellular environments has enabled the utilization of receptor specific ligands in the design of the nanocarrier systems.[104] Several examples of targeted delivery to hepatocytes have been described[105] Receptor mediated-delivery with high affinity ligands may have advantages of specificity if the receptors are cell-specific. Any nonspecific uptake by scavenger receptors such as those present on Kupffer cells may decrease the efficiency of delievery.[105,106]

The most commonly used viral vectors are retroviruses, adenoviruses, and adeno-associated viruses. Although many studies approached increasing the transfection efficiency of viral vectors, there are many concerns regarding their cytotoxicity to hepatocytes and potential inflammatory response.[105] Hydrodynamic-based delivery has been used in

small animals but the rapid injection large fluid volumes is likely to increase the risk serious adverse effects making that technique unlikely to become a clinical application.[107] Despite numerous developments, novel targeted drugs delivery methods remain in early phases.[108]

To treat HBV-infected patients with agents that affect cccDNA, the efficacy of such novel anti-HBV agents can only be monitored by accurate quantitation of cccDNA levels. However, such measurements currently present a major technical challenge.[73] Obstacles include the current need for liver tissue, the lack of standardized PCR-methods, and the presence of coexisting identical replicative intermediates to cccDNA. More studies are needed to develop more sensitive techniques to overcome the small size liver samples, low levels of viral DNA during therapy and the need to investigate the number of infected hepatocytes harboring cccDNA in the liver.[109,110] Therefore, there is an urgent need for a reliable and convenient standardized cccDNA assay. Tu *et al*.[111] developed a new assay called cccDNA inversion quantitative PCR which showed high precision and sensitivity. However, that study did not demonstrate that cccDNA inversion quantitative PCR could be used to assay clinical isolates from patient samples.

Another challenge of gene editing approaches is the potential for off-target effects resulting in toxicity. There are multiple mechanisms under investigation to reduce off-target effects. For example, for CRISPR/Cas-9, improvement, and engineering Cas-9 variants as suitable sgRNAs, finding new versions of gene editors that do not induce DSBs, and improving methods to deliver Cas-9/sgRNA into target cells have been developed.[112] Similar strategies can be developed for novel HBsAg treatments through gene editing techniques.

Lastly, as new agents are developed, it is necessary to consider the logistical, global health challenge of distributing new medications to areas with a higher viral burden. The prevalence of the virus is highest in the resource-limited regions of China, South America, Southeast Asia, and sub-Saharan Africa. Distribution of new agents can be limited by geography, public health infrastructure and surveillance, and government cooperation.[113] As new novel agents are investigated in clinical trials, it is also important to consider the global representation of HBV. Ideally, new agents should be studied in clinical trials in areas most burdened by chronic HBV, as these populations would benefit most from treatment.

Conclusions

A rapid surge in the development of novel agents has sparked hope that it might be possible to finally cure HBV. However, fulfillment of that hope appears to remain in the distant future as significant challenges remain due to the biological characteristics of cccDNA and integrated HBV DNA. Targeted delivery, sustained action, without off-target effects remain the most challenging objectives. Creative solutions are needed to make PCR-based assay methods affordable, minimally invasive, and readily available for quantification of cccDNA accurately in HBV infections. Novel agents targeting multiple steps of the viral life cycle may be necessary to achieve effective inhibition of HBsAg and cccDNA. While promising, even the most advanced agents will require more investigation to make HBV cure a reality.

Acknowledgments

This work was made possible by the Herman Lopata Chair in Hepatitis Research.

Conflict of interest

GYW has been an editor-in-chief of *Journal of Clinical and Translational Hepatology* since 2013. The other authors have no conflict of interests related to this publication.

Author contributions

Proposed concept for review and revised manuscript with critical revisions (GYW), drafted the manuscript (AHA and BDH).

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