EDITORIAL

New horizon for radical cure of chronic hepatitis B virus infection

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Abstract

About 250 to 350 million people worldwide are chronically infected with hepatitis B virus (HBV), and about 700000 patients per year die of HBV-related cirrhosis or hepatocellular carcinoma (HCC). Several anti-viral agents, such as interferon and nucleos(t)ide analogues (NAs), have been used to treat this disease. NAs especially have been shown to strongly suppress HBV replication, slowing the progression to cirrhosis and the development of HCC. However, reactivation of HBV replication often occurs after cessation of treatment, because NAs alone cannot completely remove covalently-closed circular DNA (cccDNA), the template of HBV replication, from the nuclei of hepatocytes. Anti-HBV immune responses, in conjunction with interferon-γ and tumor necrosis factor-α, were found to eliminate cccDNA, but complete eradication of cccDNA by immune response alone is difficult, as shown in patients who recover from acute HBV infection but often show long-term persistence of small amounts of HBV-DNA in the blood. Several new drugs interfering with the life cycle of HBV in hepatocytes have been developed, with drugs targeting cccDNA theoretically the most effective for radical cure of chronic HBV infection. However, the safety of these drugs should be extensively examined before application to patients, and combinations of several approaches may be necessary for radical cure of chronic HBV infection.

Key words: Covalently-closed circular DNA; Genome editing technology; Immune response; Immunotherapy; Program death-1; Interferon-γ; Tumor necrosis factor-α

Core tip: Among the agents used to treat chronic hepatitis B virus (HBV) infection are nucleos(t)ide analogues, which have been shown to strongly suppress HBV replication. HBV replication, however, may be reactivated after cessation of treatment, because complete removal of covalently-closed circular DNA (cccDNA) from hepatocyte nuclei is extremely difficult. Immune responses have been shown to destroy cccDNA, but immune response alone is insufficient for complete eradication of template DNA. Several drugs were

recently developed to block the HBV life cycle in hepatocytes, with drugs targeting cccDNA being, at least theoretically, the most effective for radical cure of chronic HBV infection. The safety of these agents should be extensively examined before their use in patients. Combinations of two or more classes of agent may be necessary for radical cure of chronic HBV infection.

INTRODUCTION

About 250 to 350 million people worldwide are chronically infected with hepatitis B virus (HBV)[1,2], with about 700000 patients per year dying from HBV-related cirrhosis or hepatocellular carcinoma (HCC)[3]. Several anti-viral agents, including interferons and nucleos(t)ide analogues (NAs), have been shown effective, with NA-based treatment strongly suppressing the replication of HBV-DNA and normalizing serum alanine aminotransferase activity, resulting in little or no progression of liver disease[4-6]. NAs target the viral reverse transcriptase, effectively reducing serum HBV-DNA concentrations. However, intrahepatic HBV-DNA, such as converted covalently closed circular DNA (cccDNA), is not a direct target of NAs. cccDNA is a template for all viral RNAs and HBV-DNA replication can be induced to start from residual cccDNA after cessation of treatment with NAs[7]. Small amounts of HBV-DNA can be found in serum long after patients recover from acute HBV infection, suggesting that cccDNA may persist for decades[8]. Thus, cccDNA is difficult to eradicate once infection is established, and should be the main target for the complete eradication of HBV infection. However, measuring intrahepatic cccDNA concentrations is difficult in a clinical setting[9]. The cccDNA levels in HBV-infected human hepatocytes are low, ranging from 1 to 50 copies per hepatocyte[10]. Real-time polymerase chain reaction (PCR) amplification with specific primers for cccDNA or Southern blotting can be used for the detection. However, PCR amplification may be hampered by other co-extracted viral DNA and Southern blotting needs much time and effort. Moreover, the form of cccDNA may be changed during the DNA extraction procedure. Therefore, further investigation should be required to establish the precise evaluation of intrahepatic cccDNA. As an alternative, the reduction in HBV surface antigen (HBsAg) concentration has been reported to partly reflect the decrease in intrahepatic cccDNA, with the goal of treatment for chronic HBV infection being the complete disappearance of HBsAg[11]. Fewer than 10% of patients receiving interferon-based therapy[4-6], and few patients treated with NAs[12,13], achieve complete loss of HBsAg. Various trials have tested agents targeting the life cycle of HBV in hepatocytes, including the elimination of cccDNA.

This review summarizes and discusses the radical cure (Table 1) of chronic HBV infection, mainly focusing on the elimination of cccDNA.

HBV REPLICATION CYCLE AND THE PRODUCTION OF HBV-RELATED PROTEINS

HBV replication cycle

HBV is a DNA virus that belongs to the family *Hepadnaviridae*, with a 3.2 kb-long partially double-stranded relaxed circular DNA (rcDNA) genome[14]. The life cycle of HBV is shown in Figure 1. HBV virions are thought to enter hepatocytes through a high-affinity interaction between the myristoylated preS1 region of HBV and the surface structures of hepatocytes, including sodium taurocholate cotransporting polypeptide (NTCP)[15-17]. After entry into hepatocytes, uncoated rcDNA is released into the cytoplasm and then enters the nucleus, where it is converted to cccDNA. The cccDNA remains for a long time in the nucleus, where it serves as a template for the transcription of viral mRNA[17,18]. All viral RNAs, pregenomic RNAs (pgRNA) and RNAs encoding the surface proteins, precore and HBx of HBV, are transcribed from cccDNA, with efficient transcription regulated by liver-specific transcription factors[19] and the HBx protein itself[20]. Epigenetic control of cccDNA transcriptional activity, such as acetylation, methylation or phosphorylation, appears to occur[21]. Cytoplasmic pgRNA and polymerase protein are subsequently packaged into envelope proteins, with rcDNA produced from the reverse transcription of pgRNA. Nucleocapsids packaging rcDNA are encapsulated by HBsAg as the envelope protein and released from hepatocytes as virions. The precise understanding of these processes is important for the development of new strategies for the radical cure of chronic HBV infection.

HBV-related proteins and their roles in hepatocarcinogenesis

The HBV-related proteins translated from cccDNA consist not only of the envelope, core and polymerase proteins of HBV, but may play a role in hepatocarcinogenesis itself.

Studies analyzing the role of HBx proteins in hepatocellular transformation and HCC progression have found that low levels of HBx protein are present in non-tumor tissues of HBV-infected liver, whereas high levels of HBx protein are present in HCCs arising in HBV infected individuals, suggesting that this protein has an oncogenic function[22,23]. Moreover, HBx transgenic mice often develop liver cancer[24,25], and HBx protein has been found to accumulate in hepatocytes, affecting the expression of genes associated with signal transduction, cell cycle control, transcription, and immune response[23,26]. Expression of genes on the X-chromosome is regulated epigenetically, including by DNA and histone methyltransferases[27,28], and by microRNAs[29,30].

Table 1	Cure status of hepatitis B virus infection

	Serum HBV-DNA	Serum HBsAg	Intraheptic cccDNA	HBV-DNA-intergrated hepatocytes
Functional cure (clinical cure)	Low	(-)-(++)	(+)	(-)-(+)
Radical cure (virological cure)	(-)	(-)	(-)	(-)

HBV: Hepatitis B virus; cccDNA: Covalently-closed circular DNA; HBsAg: Hepatitis B virus surface antigen.
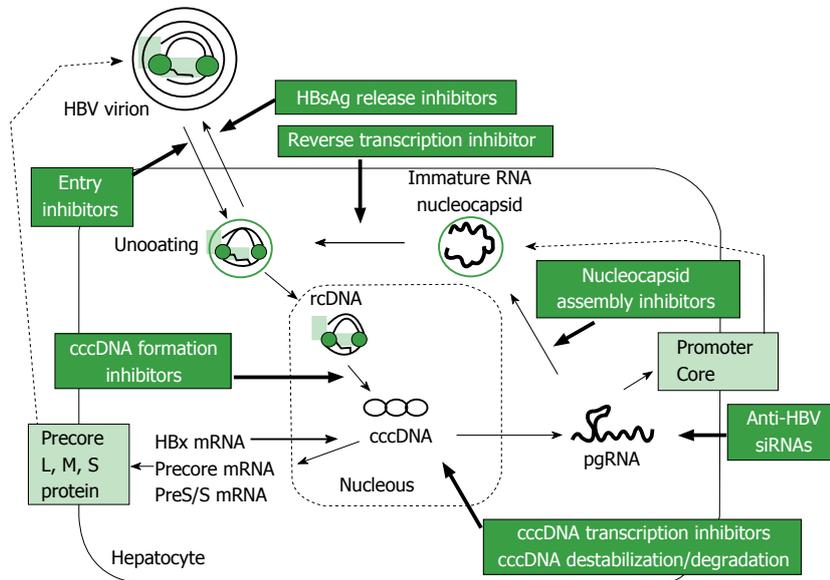

Figure 1 Simplified schema of the hepatitis B virus life cycle and possible targets of therapy. HBV: Hepatitis B virus; cccDNA: Covalently-closed circular DNA; HBsAg: Hepatitis B virus surface antigen; rcDNA: Relaxed circular DNA; siRNAs: Small interfering RNAs; pgRNA: Pregenomic RNAs.

HBx is not only involved in carcinogenesis but in the progression of HCC. HBx has been shown to increase beta-catenin signaling through epigenetic control or micro-RNA[31,32] and to be an independent predictor of survival after HCC resection[33].

HBsAg is also involved in hepatocarcinogenesis. The ground glass appearance of hepatocytes was shown to be a typical histological finding in HBV-infected livers, with this ground glass appearance resulting from the accumulation of HBsAg with preS mutations[34-36]. PreS-mutated HBsAg, especially large HBsAg, was found to accumulate in cytoplasm, leading to the induction of ER stress and oxidative DNA damage[35-37]. Furthermore preS mutations upregulated intracellular signaling *via* hepatocyte proliferation[35,38]. High serum HBsAg levels showed a definite correlation with HCC development in patients with controlled HBV-DNA[39-41]. Like HBV-related proteins, spliced HBV proteins were found to activate intracellular signaling *via* hepatocyte proliferation[42,43]. These findings suggest that not only HBV replication, but the production of HBV-related proteins, should be suppressed to efficiently prevent hepatocarcinogenesis.

IMMUNE RESPONSE AGAINST HBV INFECTION

Immune responses against HBV are involved in both the pathogenesis and control of HBV infection[44-47]. There-fore, understanding the immune response against HBV may result in better control of HBV infection.

Acute infection
Analysis of immune responses that occur during acute HBV infection may provide valuable information on strategies by which immune responses control HBV infection.

A mouse model of acute viral hepatitis B was esta-blished by injecting HBsAg-specific T-cell clones into HBV transgenic mice[48]. Although HBsAg-specific T-cells were found to kill small numbers of HBV-replicating hepatocytes, these T cell clones destroyed intracellular HBV-RNA and HBV-DNA in most infected hepatocytes without killing these cells. This effect was found to be due to interferon (IFN)-γ and tumor necrosis factor (TNF)-α[40,49-51]. Because HBV transgenic mice do not have cccDNA[52], the effects of these cytokines on cccDNA were unclear. In cccDNA-expressing cultured cells, however, IFN-γ and TNF-α inhibited HBV replication and reduced cccDNA in an additive manner[53]. Moreover, the decay of cccDNA was found to require activation of APOBEC3 deaminases[53], which are expressed in liver tissues of individuals with acute, but not chronic, HBV infection. These observations indicate that HBV-specific T-cell activation followed by treatment with anti-viral cytokines, such as IFN-γ and TNF-α, could eradicate HBV without cytolysis.

In a chimpanzee model, cccDNA was found to dis-

appear during the course of acute hepatitis B, and HBV-DNA was found to be susceptible to noncytolytic control by cytokines[54]. Moreover, HBV-DNA titers in these livers were reduced before T-cell influx, suggesting that non-T-cells, possibly natural killer cells, may have an important role in the noncytolytic destruction of HBV-DNA in liver during early phases of acute HBV infection[54].

Broad and vigorous CD4^{+} and CD8^{+} T-cell responses have been reported in patients with acute hepatitis B[55]. Moreover, HBV-specific T-cell responses were observed during the incubation period of acute hepatitis, with HBV-DNA reduced before alanine aminotransferase concentration peaked, indicating that noncytolytic eradication of HBV also occurs in acute hepatitis B in humans[56]. However, recovery from acute hepatitis B does not imply complete eradication of HBV, as small amounts of HBV-DNA can be detected in the blood for a long time after resolution of acute hepatitis B[8]. T-cell responses are therefore not sufficient to completely eradicate cccDNA from infected livers, even in acute hepatitis B.

Chronic infection

Immune responses in patients chronically infected with HBV were found to consist of four phases: The immuno-tolerant, immune-active, inactive carrier, and reactivation phases[57]. Although the exact mechanism by which HBV induces immune tolerance is unclear, it may arise from central deletion or peripheral non-recognition of HBV-specific T-cells[58]. Immune tolerance may be broken after several decades by as yet undetermined mechanisms, but these may involve the maturation of dendritic cell (DC) function[59]. Breaking immune tolerance to HBV can lead to the immune-active phase, resulting in some degree of hepatitis. During this phase, suppression of HBV replication is observed in 85% to 90% of patients, leading to an inactive carrier state. Most patients in an inactive carrier state do not need antiviral treatments, but cccDNA may be present in their livers. The cccDNA persisting in inactive carriers may be a template for reactivation of HBV replication. The 10% to 15% of patients who remain in the immune-active phase continue to experience liver inflammation with active replication of HBV, and may be at high risk for progression to liver cirrhosis and the development of HCC. The number of HBV-specific CD8^{+} T-cells was found to be the same in livers with low HBV replication and little hepatitis and in livers with high HBV replication and severe hepatitis[60]. These findings suggest that HBV replication is suppressed by immune surveillance of HBV-specific T-cells in the liver and that these T-cells are important in controlling HBV replication in a noncytolytic manner in inactive carriers. In contrast, HBV-specific immune responses are thought to be dysregulated in livers with active hepatitis, and several possible mechanisms have been proposed.

Impairment of innate immune response

Innate immune system such as pattern recognition receptors, macrophages, DCs, natural killer cells or natural killer T cells are involved in the pathogenesis of HBV infection especially at an early stage of infection[61,62]. HBV has been shown to alter the function of macrophages by modulating the secretion of cytokines[63,64] or type-1 IFN gene expression[64]. Hepatitis B e antigen was shown to directly suppress toll-like receptor (TLR) signaling *via* interaction with Toll/IL-1 receptor-containing proteins such as TRAM and Mal[65]. HBV has been shown to downregulate TLR-2 expression in patients with chronic HBV infection[66]. Thus, innate immunity alteration plays a role, at least in part, in the pathogenesis of chronic HBV infection and TLR-7 agonists have been applied as immune-modulatory components[67,68]. On the other hand, the effect of IFN-α on intrahepatic cccDNA has been recently explored[69], and IFN-α in addition to lymphotoxin-β receptor (LTβR) activation has been shown to induce cccDNA degradation through upregulation of nuclear APOBEC3 deaminases[70]. APOBEC3 can deaminate double-stranded DNA cytidines to uridines[71] and induce cccDNA degradation. IFN-γ and TNF-α produced form T-cells can induce deamination of cccDNA without cytolysis, supporting the essential role of APOBEC3 in reduction of cccDNA[53]. Collectively, type-1 IFN-mediated effects, especially APOBEC3 upregulation, will be a key subject for development of new therapeutics.

Dysfunction of dendritic cells

DCs are the most potent antigen-presenting cells, stimulating both T- and B-cells. In patients with chronic hepatitis, the cytokine-induced maturation of circulating myeloid DCs is impaired, possibly by exposure to high amounts of HBV or HBsAg[72,73]. Dysfunctional DCs may act as tolerogenic antigen-presenting cells, resulting in a failure to induce HBV-specific immune responses.

Alteration of the hierarchy of epitope-specific CD8^{+} T-cell responses

In acute hepatitis B, the CD8^{+} T-cell response to the immunogenic epitope HBc18-27 (HLA-A2 restricted epitope) is dominant. In contrast, HBc18-27-specific CD8^{+} T-cell responses are low and CD8^{+} T-cell responses against less immunogenic envelope (183-191) are dominant in chronic hepatitis B[74]. Although the mechanisms underlying changes in the major epitope to CD8^{+} T-cell response are not yet known, they may account, at least in part, for the different CD8^{+} T-cell responses observed in patients with acute and chronic hepatitis.

Regulatory T-cells

Regulatory T-cells (Tregs) expressing the forkhead family transcription factor, Foxp3, are specialized cells that have a major role in the maintenance of immunological self-tolerance by suppressing self-reactive cells[75]. Tregs express CD25 [interleukin (IL)-2 receptor α-chain] and/or cytotoxic T-lymphocyte antigen-4 (CTLA-4), which are excellent inhibitors of IL-2 production or downregulation of CD80 and CD86 on DCs by a CTLA-4-dependent mechanism[76].

The numbers of CD4^{+}CD25^{+}FoxP3^{+} Tregs were higher in the livers of patients with chronic hepatitis B,

suggesting that these cells suppress intrahepatic HBV-specific T-cell responses, leading to insufficient immune control of HBV replication in the liver[77].

Inhibitory receptors

Program death (PD)-1 is a surface receptor critical for the regulation of T-cell function[78,79]. Binding of the ligand PD-L1 to PD-1 on T-cells results in the antigen-specific inhibition of T-cell proliferation, with a molecule related to T-cell exhaustion found in the livers of patients with chronic hepatitis B. T-cell exhaustion is characterized by poor cytotoxic activity and cytokine production, as well as by the expression of inhibitory receptors, including not only PD-1 but lymphocyte activation gene-3, CTLA-4, T-cell immunoglobulin domain and mucin domain-3, and CD244[66]. These inhibitory receptors are thought to be induced by persistent exposure of intrahepatic T-cells to HBV or HBV-related proteins[80]. Exhaustion of T-cells could also account for impaired T-cell responses in the livers of patients with chronic hepatitis B, and blockade of these receptors could be therapeutic.

Patients with high serum HBV-DNA concentration have been reported likely to progress to cirrhosis and eventually HCC[81]. Transition of immune-active patients to an inactive state with low HBV-DNA replication by the direct stimulation of HBV-specific T-cells or removal of immunosuppressive factors, may be sufficient to inhibit progression to cirrhosis or HCC. Inactive HBV carriers may not require specific treatment, because spontaneous HBsAg develops at a rate of 1% to 1.9%/year in these patients, making the development of HCC rare[82]. Therefore, an inactive HBV carrier may be regarded as in a state of functional cure (Table 1). However, HBV replication may be reactivated, either spontaneously or during treatment with an immunosuppressive or anticancer agent, resulting in a higher risk of hepatocarcinogenesis than in the general population[83]. The rate of HCC development was recently reported to be greater in patients with high than with low serum HBsAg concentrations, even in inactive HBV carriers with low serum HBV-DNA concentrations[36,37].

Collectively, these results suggest that induction of immune control against HBV infection may result in functional cure of HBV infection. Functional cure, however, may be an unstable condition, allowing progression to cirrhosis or HCC under various conditions. Although radical cure (Table 1) is desirable, it is problematic because of the difficulty in eliminating HBV cccDNA from the liver.

THERAPEUTIC STRATEGIES FOR HBV INFECTION

Immunotherapy

Radical cure of HBV infection could be achieved by both the elimination of cccDNA in the liver and the destruction of HBV-DNA-integrated hepatocytes. The primary goals of immunotherapy in HBV-infected individuals include the induction or stimulation of HBV-specific immune re-sponses, leading to the killing of infected cells or the degradation of HBV-RNA and HBV-DNA in a noncytolytic manner, inhibiting progression to liver cirrhosis and hepatocarcinogenesis. Although immune responses involving cytokines such as IFN-γ and TNF-α can eliminate cccDNA[50,53], cccDNA is not completely eliminated even after resolution of acute hepatitis B[8], suggesting that immune responses alone may be insufficient to achieve radical cure of HBV infection.

Induction or stimulation of HBV-specific immune responses

Efforts to stimulate HBV-specific T-cells have included immunizations with HBV-peptides, viral proteins, DCs, and DNA, as well as treatment with cytokines[84]. Because HBV-specific T-cells in patients with chronic hepatitis B are exhausted by long-term exposure to high levels of HBV-related antigens, activation of those cells by immunization would be ineffective without functional restoration of the cells by blocking the inhibitory signals responsible for T-cell exhaustion. Blockade of PD-1, CTLA-4 or Tim-3 has been shown to restore exhausted HBV-specific T-cells[80], suggesting that the combination of immunization and blockade of inhibitory signals would be effective in activating HBV-specific T-cells.

Other immunotherapeutic approaches to HBV infection include administration of cytokines, such as IFN-γ, IL-6, IL-1β, LTβR-agonists and/or TLR-7 agonist, as well as IFN-γ and TNF-α which were shown to cause silencing or degradation of cccDNA[67]. This strategy may be more effective in the complete eradication of HBV infection than strategies involving the activation of HBV-specific cells, suggesting that only cytokine administration results in the elimination of cccDNA.

Elimination of HBV-infected hepatocytes by a novel approach

A novel approach to eliminate HBV-core containing hepatocytes[85] was based on findings showing that elimination of HBV is impaired by cellular inhibitor of apoptosis proteins (cIAPs), which inhibit the TNF-α-mediated death of HBV-infected cells[86]. This led to testing the effects of inhibitors of cIAPs, including birinapant and other Smac mimetics, on HBV-infected hepatocytes. These inhibitors of cIAPs resulted in the rapid reduction in serum HBV-DNA and HBsAg concentrations, possibly by eliminating HBV-core containing hepatocytes. However, the effects of those drugs on cccDNA are unclear.

Immunotherapeutic strategies for HBV-DNA-integrated hepatocytes

Three main mechanisms are responsible for hepatocarcinogenesis: (1) the oncogenic potential of the HBV-related proteins, HBsAg and HBx; (2) HBV-DNA integration into the host genome, dysregulating the cell cycle by the introduction of deletions, cis/trans-activations, and/or translocations, and/or inducing generalized genomic instability; and (3) persistent inflammation in the

liver causing rapid turnover of hepatocyte regeneration, enhancing the instability and/or mutagenesis of host genomes.

Therefore, if future advances in therapeutic modalities result in the complete elimination of cccDNA, hepatocarcinogenesis resulting from HBV-DNA integration into the host genome should be addressed. HBV-DNA integration into the hepatocyte genome has been observed in 86.4% of HBV-related HCCs and in 30.7% of adjacent liver tissue[87]. Integration of HBV-DNA into areas of the host genome encoding genes that regulate cellular proliferation, such as telomerase or proliferation signal transduction genes, may lead to cis-/trans-activation, inducing malignant transformation[88]. Furthermore, integration of HBV-DNA may induce genetic instability by altering the expression of oncogenes, tumor suppressor genes and microRNAs[87,89]. In addition, a viral-human chimeric transcript was reported to function as a noncoding RNA and promote hepatocarcinogenesis[90]. Integration of HBV-DNA into the host hepatocyte genome of transiently infected individuals has been reported to be a rare event, occurring in 0.01%-0.1% of hepatocytes[91]. Further investigations are needed to determine the mechanism by which HBV-DNA integration into the host genome induces carcinogenesis. The immune cytolysis of cells expressing HBV-related peptides may be the only strategy that effectively eliminates HBV-DNA-integrated hepatocytes. However, if non-immunogenic regions of HBV-DNA are integrated, elimination of those cells by immune attack would be impossible.

Taken together, these findings indicate that immunotherapy against HBV can control viral replication and reduce cccDNA, but may not be sufficient to completely eradicate HBV-infected or -integrated hepatocytes.

Inhibition of HBV replication

Currently available NAs can efficiently reduce viremia but cannot eliminate intracellular cccDNA. However, complete suppression of HBV polymerase can result in the complete elimination of cccDNA through the death of cccDNA-containing hepatocytes after one natural lifespan of these cells[92]. Among the agents being tested are prodrugs of HBV polymerase inhibitors[93]. These include prodrugs of tenofovir, such as AGX1009 (Agenix) and TAF (GS-7340, Gilead Sciences), which have been evaluated in phase 3 trials[93,94], and CMX157, a lipid conjugate of tenofovir, which has been evaluated in phase 1/2 trials[93,95]. RNase H inhibitors are also being tested, based on the specificity of HBV replication, which depends on the RNase H activity of HBV polymerase to degrade pgRNA[10]. Evaluations of selective inhibitors of HBV polymerase RNase H activity[96] suggest that they might be more effective when combined with NAs[93].

Destruction of cccDNA

Eradication of cccDNA in hepatocytes is essential to achieve radical cure of established HBV infection. Several trials have targeted cccDNA. For example, gene silencing techniques, such as small interfering RNAs (siRNAs) or antisense oligonucleotides (ASOs), have been evaluated for their ability to reduce viremia and cccDNA. Although siRNAs may have promising activity, methods to effectively deliver them to hepatocytes have not been determined[97]. RNAi can inhibit all steps of HBV replication, and ARC-520 has been tested in a phase 2 trial in patients with chronic hepatitis B[95]. In contrast, a single injection of ASO, consisting of liver-targeted peptides, into a mouse model of chronic HBV infection was shown to reduce HBV-RNA, proteins and HBV-DNA for a long time, suggesting that ASO may become a promising treatment in patients with chronic HBV[98]. Furthermore, disubstituted sulfonamide was shown to selectively inhibit the formation of cccDNA[99].

In addition, several genome editing technologies have been developed to silence sequence-specific cleavage of cccDNA. These include zinc finger nucleases (ZFNs)[100,101], transcription activator-like effector nucleases (TALENs)[102,103], and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated system (Cas). These sequence-specific genome editing technologies could induce double-stranded breaks at certain DNA sites. ZFNs consist of a zinc finger domain, which contains a sequence-specific binding site, and a *Fok I* nuclease domain. ZFNs form heterodimers and induce double-stranded breaks at targeted sites. These breaks are subsequently repaired by homology-directed repair or non-homologous end joining. The specificity of ZFNs may be context-dependent, resulting from interactions between DNA binding domains and neighboring zinc fingers[104]. TALENs have transcription activator-like effector specific DNA binding activity, with DNA-binding sites more specific than those of ZFNs[105]. However, both ZFNs and TALENs require pairs of site-specific nucleases for each target to produce customized proteins[9]. In contrast, CRISPR/Cas technology is a novel genome-editing method, which is more useful than ZFNs or TALENs[106]. CRISPR/Cas loci encode RNA guided endonucleases, which are induced by immune responses against foreign genetic elements such as bacteriophages and plasmids[107]. The type 2 CRISPR/Cas system from *Streptococcus pyogenes* is a chimeric single-guide RNA with Cas9 protein[108]. The CRISPR/Cas9 system was shown to suppress HBV replication in cultured cells and in mouse models[109-117], reducing both HBsAg[109,110,112-116] and cccDNA[110,113-115,117]. These findings suggest that genome editing technology, such as a CRISPR/Cas system, may be a potential therapeutic option for the complete eradication of HBV infection in future. However, cleavage of cccDNA and subsequent DNA repair may introduce mutations into the host genome. These mutations may be harmful to the host, resulting in the possible development of malignancy[9,118,119], suggesting the need for further improvements in efficacy and safety prior to the therapeutic use of these systems.

Future perspectives on radical cure of chronic HBV infection

Various trials have assessed agents that can terminate

Table 2 Tgerapectic agents against hepatitis B virus currently in clinical development

Mode of actions	Target	Stage of development	Ref.
Entry inhibitions	NTCP	Myrcludex in phage 2	[14,123]
cccDNA			
Formation inhibitions	DSS	Preclinical	[99]
Transcription inhibitions	ASO	IONIS-HBVRx in phase 1	[98]
Destabilization/degradation	ZFN	Preclinical	[100,101]
	TALEN	Preclinical	[102,103]
	CRISPR/Cas9	Preclinical	[109-117]
SiRNA	PgRNA	ARC-520 in phase 2	[95]
Nucleocapsid assembly inhibitions	Capsid formation	BAY4109 in phase 1	[93,95]
		NV1221 in phase 1	[93,95]
Reverse transcription inhibitions	Polymerase	TAF in phase 3	[93,94]
		Cmx157 in phase 1/2	[93,95]
HBsAg release inhibitions	HBsAg secretion	Preclinical	[109]
	HBsAg secretion	Rep2139 in phase 1/2	[110]
Immune modulating	TLR-7 agonist	GS-9620 in phase 2	[67,68]
	HBV-specific	Preclinical	[84]
	cIAPS	Preclinical	[86]

cccDNA: Covalently-closed circular DNA; ZFN: Zinc finger nuclease; TALEN: Transcription activator-like effector nuclease; CRISPR: Clustered regularly interspaced short palindromic repeat; Cas: CRISPR associated system; pgRNA: Pregenomic RNAs; HBsAg: Hepatitis B virus surface antigen; TLR: Toll-like receptor; HBV: Hepatitis B virus; cIAPS: Cellular inhibitor of apoptosis proteins; DSS: Disubstituted sulfonamide; ASO: Antisense oligonucleotides; TAF: Tenofovir Alafenamide; NTCP: Na$^+$/taurocholate cotransporting polypeptide.

the HBV life cycle in hepatocytes, including inhibitors of HBV-DNA polymerase, virus entry, core assembly and HBsAg secretion (Table 2)[93,95,120,121]. Especially Myrcludex B, a synthetic lipopeptide that targets NTCP, has been shown to efficiently prevent viral spread and has been applied in clinical trials[15,17,122,123]. These agents, including Myrcludex, are not themselves sufficient to eliminate HBV from chronically infected hepatocytes, as shown by the remaining cccDNA in the nuclei and HBV-DNA-integrated hepatocytes. Immunotherapy may potentially eliminate both cccDNA and HBV-DNA-integrated hepatocytes, but its effects would be limited. Although drugs targeting cccDNA in hepatocytes are theoretically ideal for complete eradication of HBV, no single drug or strategy, whether currently available or under development, has shown the ability to completely eliminate HBV with established safety and efficacy. Future trials, testing combination of different agents or strategies, will be necessary.

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