Toward Elimination of Hepatitis B Virus Using Novel Drugs, Approaches, and Combined Modalities

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KEYWORDS

• HBV cure • siRNA • CRISPR/Cas9 • Anti-HBV agents • cccDNA • HBsAg • Antiviral therapy

KEY POINTS

- Despite current treatments available for hepatitis B virus (HBV) infection, the inability of the host immune system to completely clear the virus can lead to incurable chronic infection.
- Identification of new targets and development of novel, curative drugs are necessary.
- New therapeutic strategies are critical components of a path toward a possible cure.
- A combination of antiviral agents targeting HBV replication, and drugs restoring or increasing the host immune response could lead to a functional cure.
- Novel modalities could disrupt HBV covalently closed circular DNA and also target integrated viral DNA.

INTRODUCTION

Over the past decades, research efforts have led to the development of several potent nucleoside analog inhibitors such as lamivudine (Epivir), adefovir dipivoxil (Hepsera), entecavir (Baraclude), telbivudine (Tyzeka), and tenofovir disoproxil fumarate (Viread), allowing a large decrease of HBV viremia in chronically infected persons.[1] Nucleoside analog inhibitors in their 5'-triphosphate form are potent inhibitors of DNA polymerase/reverse transcriptase activities of the viral polymerase enzyme. They compete with natural nucleotides and act on several steps of viral DNA synthesis, including initial polymerization, protein priming, or the subsequent DNA strand elongation. It has been suggested that combination therapy using one of these nucleoside analogs

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and interferon could have better virus elimination efficacy than nucleoside analog inhibitor monotherapy, but such studies are difficult to perform because the current monotherapy is already very effective at controlling HBV viral load. However, current nucleoside analog inhibitor treatments do not lead to HBV cure, as indicated by low levels of hepatitis B surface antigen (HBsAg) seroconversion (**Box 1**).[2,3]

Despite the success of current available therapy, subjects who cleared the virus (hepatitis B e antigen [HBeAg] negative, HBsAg negative) can experience reactivation of HBV on treatment interruption or after the use of antiinflammatory or immunosuppressant medications.[4] This strongly suggests that current anti-HBV therapeutics are unable to eradicate the virus from infected liver cells. These limitations have led researchers to continue their drug development efforts toward finding new viral targets that could potentially lead to the discovery of a functional or absolute cure (see **Box 1**).[5]

Considering that HBV covalently closed circular DNA (cccDNA) serves as the template for pregenomic RNA transcription, it is thought to be responsible for virus persistence. At the same time, integration of HBV DNA is thought to be associated with an increased risk of hepatocellular carcinoma development.[6] Accordingly, new therapeutic approaches that target cccDNA directly or indirectly are in development (**Fig. 1**). After recent successes with drug development for hepatitis C virus, the field of viral hepatitis is turning its focus to another major threat to liver health, namely HBV.[7] These new therapeutic strategies will have to address the problems of cccDNA elimination, intrahepatic innate immune response stimulation, HBV-specific immune response restoration and will probably have to include combination of drugs to target multiple steps of the HBV replication cycle.

Box 1
Definitions of cures

Apparent virologic cure

Sustained off-drug suppression of serum HBsAg, HBeAg, and viral DNA.

cccDNA = undetectable or repressed

Normalization of liver function (normal levels of serum ALT and AST).

Risk of death from liver disease: to be determined once long-term survival data have been obtained.

Functional cure

Sustained off-drug suppression of serum HBsAg, HBeAg, viral DNA, and cccDNA.

Normalization of liver function (normal levels of serum ALT and AST).

Comparable with individuals with naturally resolved infection.

Absolute cure – virologic cure

Sustained off-drug suppression of serum HBsAg, HBeAg, and viral DNA.

Normalization of liver function (normal levels of serum ALT and AST).

Elimination of cccDNA.

Presence of HBsAb.

Comparable with uninfected individuals.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; cccDNA, covalently closed circular DNA; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen.

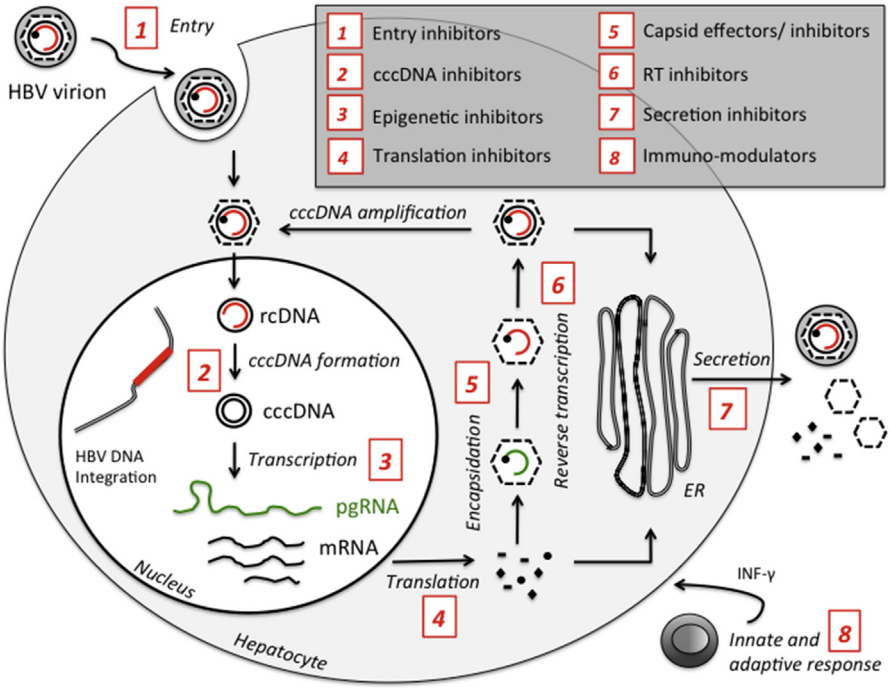

Fig. 1. Schematic representation of the inhibitors of Hepatitis B virus replication cycle. cccDNA, covalently closed circular DNA; ER, endoplasmic reticulum; HBV, hepatitis B virus; INF, interferon; mRNA, messenger RNA; pgRNA, pregenomic RNA; rcDNA, relaxed circular DNA; RT, reverse transcriptase.

New antiviral agents currently in the pipeline include entry inhibitors, relaxed circular (rc)DNA–cccDNA conversion inhibitors and capsid assembly effectors (see **Fig. 1**). Besides these direct acting agents, there has also been a significant development in the area of host-targeting agents. Examples include small interfering RNA (siRNA)–based strategies, RNA interference silencers, CRISPR/Cas9 approaches, HBsAg inhibitors, immunomodulators, therapeutic vaccines and Toll-like receptor (TLR) agonists.[8,9] Using these new investigational approaches, it is hoped that a functional cure for chronic HBV is achieved within the next decade. This review highlights recent progress in developing novel anti-HBV drugs and their mechanisms of action.

CURRENT TREATMENTS AND LIMITATIONS FOR A CURE

Nucleoside analogs are relatively potent inhibitors used for the treatment of chronic hepatitis B. These HBV reverse transcriptase inhibitors are usually well-tolerated and have excellent bioavailability. They are also cost effective in comparison to interferon treatments such as pegylated interferon alfa-2a (Pegasys) and interferon alfa-2b (Intron A). Nevertheless, these drugs have some limitations in terms of HBsAg clearance and cccDNA suppression. Although drug resistance can occur clinically with some of the earlier oral treatment such as lamivudine, drug-resistant viruses are rarely selected with the more recent drugs such as entecavir and tenofovir disoproxil fumarate. It has been thought that combination of nucleoside analog inhibitors could have an additive and synergistic antiviral effect and could reduce the rate of drug resistance. However, combination studies involving 2 nucleoside analogs did not increase

virologic response, because the drugs are already very potent on their own.[10] As a result, because pegylated interferon has a different mechanism of action than a nucleoside analog inhibitor, their combination (tenofovir disoproxil fumarate + pegylated interferon for 48 weeks) showed a greater viral suppression and higher rates of HBsAg loss.[11,12]

As new therapeutic strategies are being developed for the treatment of chronic hepatitis B, uncovering new inhibitory mechanisms and potential targets, it is very likely that nucleoside analog inhibitors will have their place in future combinations with future drug candidates.

VIRAL ENTRY INHIBITORS

The HBV viral replication cycle consists of a complex multistep mechanism (see **Fig. 1**), starting with the virus entering the hepatocyte, followed by DNA replication, nucleocapsid formation, and release of virions. HBV entry represents an essential step for spreading and maintaining virus replication. The process involves 2 major interactions between the viral envelope protein pre-S1 and hepatocyte cellular receptors including first, HBV binding to the glycoproteins heparin sulfate proteoglycans followed by its interaction with the sodium taurocholate cotransporting polypeptide. Recently, Hepatera developed a synthetic lipopeptide, called myrcludex-B, which is derived from the HBV L-protein.[13] Studies have shown that the peptide competes with the viral pre-S1 motif for sodium taurocholate cotransporting polypeptide binding, blocking de novo HBV infection. Because of its early effect on the HBV replication cycle, according to the authors, the drug may also efficiently block the amplification of the HBV cccDNA. With this new concept, this inhibitor, which is currently in phase II clinical trials, could have a role in the development of an HBV cure regimen (**Table 1**).[14] Although, there are several other HBV entry inhibitors that can block the in vitro interaction of HBV with sodium taurocholate cotransporting polypeptide such as cyclosporine, ritonavir, ezetimibe, vanitaracin A, and irbesartan, among others; these inhibitors alone cannot lead to a complete inhibition of cccDNA synthesis as observed with Myrcludex-B. However, they might still play an important role by preventing viral entry into cccDNA-free hepatocytes when combined with other antiviral therapies.[15]

THERAPIES TARGETING COVALENTLY CLOSED CIRCULAR DNA

Covalently Closed Circular DNA Formation Inhibitor

HBV has evolved a unique replication cycle that results in the production of large viral loads during active replication without actually killing the infected cell directly. Two of the key events in the viral replication cycle of HBV involve, first, the generation of cccDNA transcriptional template, either from input genomic DNA or newly replicated capsid-associated DNA and, second, reverse transcription of the viral pregenomic RNA to form progeny HBV DNA genomes.[16,17] The HBV cccDNA is associated with viral persistence in HBV-infected hepatocytes.[18,19] Hepatocytes have a long half-life (>6 months or even years); therefore, elimination of cccDNA by hepatocyte turnover is not a major means of clearance. The major limitation of current treatment is the failure to eliminate the preexisting cccDNA pool and/or prevent cccDNA formation from trace levels of wild-type or drug-resistant virus.[20] As a consequence, HBV commonly rebounds after cessation of treatment with nucleoside analog inhibitor, leading different groups to develop assays to screen libraries of compounds to discover new antiviral candidates that can inhibit cccDNA formation.[20] In doing so, disubstituted sulfonamides, such as CCC-0975 and CCC-0346 have been identified as

Table 1
Therapeutics in development for the treatment of hepatitis B chronic infection

Drug	Preclinical	Phase I	Phase II	Phase III	FDA Approved	Target/Type
Nonnucleoside antivirals: interfere with proteins involved in virus replication						
Myrcludex B						Entry inhibitor
ARC-520						RNAi gene silencer
ARB-1467						RNAi gene silencer
ALN-HBV						RNAi gene silencer
Hepbarna						RNAi gene silencer
SB 9200 HBV						RIG 1 and NOD 2 agonist
Rep 2139-Ca						HBsAg release inhibitor
NVR 3–778						Capsid inhibitor
Morphothiadine mesilate (GLS4)						Capsid inhibitor
AIC 649 (Bay 41–4109)						Capsid inhibitor

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Table 1
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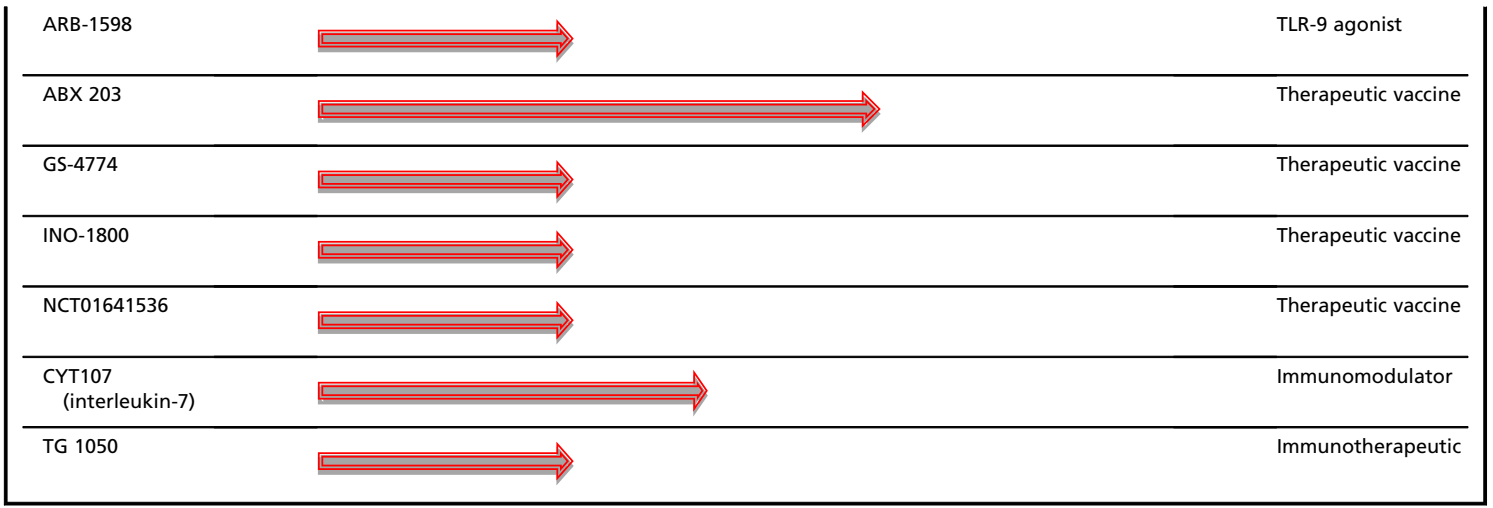
Drug	Preclinical	Phase I	Phase II	Phase III	FDA Approved	Target/Type
CpAMS						HBV core protein
EYP001						FXR agonist
IONIS-HBVRx (ISIS-HBVRx)						Antisense drug
IONIS-HBV-LRx (ISIS-GSK6-LRx)						Antisense drug
CPI-431–32						Cyclophilin inhibitor
ARB-1740						Cyclophilin inhibitor
Noninterferon immune enhancers: boost T-cell infection-fighting immune cells and natural interferon production						
RG7795 (formerly ANA773)						TLR-7 agonist
GS-9620						TLR-7 agonist



Abbreviations: FDA, US Food and Drug Administration; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; RNAi, RNA interference; TLR, Toll-like receptor.
Updated information can be found on the Hepatitis B Foundation website (http://www.hepb.org/professionals/hbf_drug_watch.htm).

inhibitors of cccDNA production.[21] These molecules are believed to interfere with relaxed circular DNA conversion to cccDNA in HepDES19 cells, also inhibiting de novo cccDNA formation. Further development of these disubstituted sulfonamides in combination with other antivirals such as nucleoside analog inhibitors might lead to the elimination of HBV cccDNA.

Covalently Closed Circular DNA Targeted Endonuclease

New promising systems that specifically use sequence-specific endonucleases to cleave cccDNA and eradicate it from infected hepatocytes have been developed, including the programmable RNA-guided DNA endonucleases (CRISPR/Cas9), transcription activator-like effector nuclease, or zinc-finger nuclease. Promising studies in cell and mouse models with CRIPSR/Cas9 have shown that these systems have the potential to serve as effective tools for the depletion of the cccDNA pool in subjects infected with chronically HBV.[22–24]

CRIPSR/Cas9 specifically reduced total viral DNA levels by up to approximately 1000-fold and HBV cccDNA levels by up to approximately 10-fold, in addition, it also mutationally inactivated the majority of the residual viral DNA in the stably transfected HepAD38 system. Moreover, these Spy Cas9/sgRNA systems showed additive inhibition of HBV DNA accumulation when used in combination with known pharmacologic inhibitors of the HBV reverse transcriptase enzyme in the Hep2.2.15 cells, and in the infected HepaRG cells, reduced both viral production and up to 67% cccDNA formation.[23] In an HBV hydrodynamics-mouse model, the CRISPR/Cas9 system was capable of disrupting the intrahepatic HBV genome (~28%), with significant a reduction but not complete elimination of HBsAg.[24]

SMALL INTERFERING RNA APPROACH

Persistence of chronic HBV infection is markedly demonstrated by an absence of antiviral immune response against the virus. As a result, a continuous production of surface antigen (HBsAg) in the plasma of chronically infected individuals is observed.[25] Three forms of HBsAg are secreted from infected hepatocytes, comprising filaments and spherical particles, with or without virion. The empty, noninfectious particles are the most abundant in the plasma, and may play a role in preventing the immune system from building a specific immune response against HBV. One way to stop secretion of HBsAg from infected hepatocytes is to cease transcription of messenger RNA (mRNA) by using siRNA. These short sequences of nucleotides (siRNA) knock down expression of genes of interest by promoting gene silencing at the posttranscriptional level. Several siRNA-based regimens are currently being developed and evaluated. The promising ARC-520 from Arrowhead Pharmaceuticals is in phase II/III clinical studies (see **Table 1**). This new molecule is composed of 2 distinct siRNA sequences, which was designed to reduce all transcripts of HBV cccDNA and with wide genotype coverage of the HBV genome. To enhance delivery to hepatocytes, ARC-520 was conjugated with cholesterol and then coinjected with a hepatocyte-targeted membrane active peptide. In chimpanzees, ARC-520 treatment resulted in a remarkable 95% decrease in HBV DNA levels and as great as 90% inhibition of secreted HBeAg and HBsAg.[26,27] Similar results were demonstrated in HBeAg-positive patients, however, insignificant suppression of HBsAg was observed in HBeAg-negative chimpanzees or patients, supporting the hypothesis that HBsAg in this case was produced from integrated DNA, which is not targeted by ARC-520.

TKM-HBV/ARB-001467 developed by Arbutus Biopharma is another siRNA regimen in a phase II clinical trial (see **Table 1**), which is currently being evaluated

for its safety and tolerability in HBeAg-negative or –positive subjects receiving nucleoside analog therapy. This molecule targets 3 conserved regions within the HBV genome and seems to clear HBsAg expression from both cccDNA and integrated HBV. Lipid nanoparticles are used to transport it to the hepatocytes, giving it more stability against nucleases.

The siRNA-based approaches for HBV are especially beneficial because HBV viral RNA transcripts have their sequences overlapped. This facilitates the synthesis of a single siRNA trigger that could degrade all viral transcripts simultaneously and prevent viral proteins secretion. However, there are 3 main drawbacks with regard to siRNA approach for HBV therapeutics. (i) Specific delivery to hepatocytes in vivo: because of their small size and highly negatively charged hydrophilic phosphate backbone, siRNA are rapidly filtrated by the kidney and are cleared from the blood stream before achieving their target. (ii) The siRNA that reach the cell membrane of hepatocytes can be trapped easily in the endosome and undergo degradation by nucleolytic enzymes. (iii) Undesirable off-target effects of siRNA and innate system stimulation are also a concern. Despite these obstacles, novel chemical modifications seem to minimize the chance of cross-reactivity with human mRNAs to occur. These approaches can also enhance efficient delivery of siRNA to the cytoplasm where they can react with RNA-induced silencing complex and prompt specific degradation of the HBV mRNAs.[27]

More recently, Benitec Biopharma developed BB-HB-331 based on a similar approach pertaining to DNA-directed RNA interfering strategy.[28] BB-HB-331 is a recombinant DNA construct, capable of continuously expressing short hairpin RNA that in turn can silence permanently the targeted viral mRNA expression with a single treatment. They revealed the results of an in vivo study conducted in humanized mouse PhoenixBio, showing a 98.5% elimination of circulating HBV (reduced serum HBV DNA by 1.83 logs), a 94.5% reduction of intracellular liver HBV DNA, and almost complete suppression of serum antigens HBeAg and HBsAg (92.6% and 97.6%, respectively), and reduction of HBV viral RNA and cccDNA levels.

CAPSID ASSEMBLY AND CORE PROTEIN EFFECTORS

The HBV nucleocapsid is well-recognized to have an important role in the viral replication cycle. It is believed to play an essential role in HBV genome packaging, reverse transcription, intracellular trafficking, and maintenance of chronic infection.[29] Several small molecules including heteroarylpyrimidines have been shown to target the capsid protein homodimers that rearrange to form the nucleocapsid. They have been identified to disrupt the capsid assembly, thus leading to inhibition of HBV replication both in vitro and in vivo.[30,31] BAY 41-4109 (AiCuris) was the first heteroarylpyrimidine to be developed and reached phase I, but because of toxicity, solubility, and other issues, it seems to have been abandoned.[8] Despite hepatotoxicity in rats at a high dosage,[32] it was shown to inhibit the virus replication in HBV transgenic mouse[33] and, more important, effectiveness against lamivudine– and adefovir dipivoxil–resistant viruses.[33,34] Based on these results, HEC Pharm developed more recently another heteroarylpyrimidine named morphothiadine mesilate GLS4, which entered a phase II clinical trial in China.[35] Early studies have demonstrated that this new heteroarylpyrimidine was more potent and significantly less toxic than analog BAY41-4109.[36] GLS4 was found to misdirect capsid assembly leading to the formation of aberrant capsids without primarily affecting core protein levels.[37] Because these molecules may also have an impact on cccDNA stability, it is suggested that they may contribute to discovery of an HBV cure.[38]

Another class of small molecules known as sulfamoylbenzamides has been identified to interfere with the capsid, and potently inhibit the formation of pregenomic RNA-containing capsids.[39] NVR 3-778 is a sulfamoylbenzamide compound having a pangenotypic antiviral activity, developed by Novira (later acquired by Johnson & Johnson) that recently reached human phase IIa producing significant virus loads reduction (a 1.7-log reduction of serum HBV DNA and 0.86 log for HBV RNA, at 600 mg twice a day for 41 days). NVR 3-778 has shown encouraging pharmacokinetic properties, and was well-tolerated in human volunteers.[40] It has also been shown to inhibit the production of HBV DNA and RNA particles, especially in combination with pegylated interferon. Because their mechanism of action is still not completely clear, this new class of small molecules represent a promising cohort of molecules with curative potential when combined with other small molecule inhibitors.

TOLL-LIKE RECEPTOR

TLR agonists have antiviral effects. TLR-7 agonist activates the innate immunity by stimulating plasmacytoid dendritic cells to produce interferon-alpha and other cytokines/chemokines and induce the activation of killer cells as well as cytotoxic lymphocytes. Therefore, this new approach with agonist-induced activation of TLR-7 can trigger both innate and adaptive immune responses and may represent a new strategy to treat chronic viral infections. GS-9620 (Gilead) is a small molecule with agonist activity. It binds to TLR-7, leading to subsequent activation of several transcription factors, including nuclear factor κB and interferon regulatory factors. GS-9620 has recently entered phase II clinical trials in combination with tenofovir versus tenofovir monotherapy.[41,42]

Other Therapeutics with Potential

Caspase activators, RIG 1 activators, cyclophilin inhibitors, RNase H inhibitors, and therapeutic vaccines are also being evaluated (see **Table 1**.). Some of these strategies, such as therapeutic vaccines, seem very promising, but are still in development and will have to overcome any possible toxicity and problems related to immune-enhancing approaches variable in treated subjects.[9] An impressive reduction of HBsAg has been demonstrated with the novel nucleic acid polymer Rep 2139-Ca (Replicor) alone or in combination with pegylated interferon alpha 2a in subjects chronically infected with HBV or coinfected with hepatitis delta virus.[43] This compound is in a phase II clinical trial and has the ability to block the formation of surface antigen protein by inhibiting the interaction of apolipoproteins with these subviral particles.[44] Recently, a new in vitro approach was developed to facilitate the direct interaction of small molecules with the human HBV polymerase. With a large-scale production of this enzyme coupled with its structural and biophysical characterizations,[45] Voros and colleagues validated their new system using a small molecule—metal-dependent and -binding modulator of HBV polymerase, calcomine orange 2R—which inhibits not only the duck HBV polymerase, but also human HBV polymerase. It remains to be determined whether this drug would interact synergistically with nucleoside analog inhibitors that also target the viral polymerase.

Another approach targeting microRNA could also have a role toward an HBV cure. MicroRNA-122 (miR-122) is a noncoding RNA involved in liver development and hepatic function, which has also been found to play a role in the regulation of HBV replication. It has been shown that miR-122 plays a role in viral persistence; a decrease in miR-122 is correlated with enhancement of HBV replication through a cyclin G1-P53–dependent pathway. Based on these observations, Li and colleagues[46] found that all 4

HBV mRNAs were harboring an miR-122 complementary site, revealing a novel mechanism by which viral mRNAs mediate host miRNA activity, contributing to the regulation of liver cancer cell proliferation, invasion, and tumor growth. Moreover, recent studies have shown that transfection of miR-122 expression vector into HepG2.2.15 cells repressed the transcription and expression of the protein N-myc downstream-regulated gene 3 (NDRG3), contributing to HBV-related hepatocarcinogenesis.[47] Thus, given the broad interactions of miR-122 in HBV chronic infection and HBV-related hepatocarcinomas, this miRNA represents a target for the development of new anti-HBV therapies.

SUMMARY

Compared with the currently available therapies that decrease and suppress the HBV viral DNA levels to undetectable levels, the new investigational drugs and approaches described herein have the potential to decrease or eliminate cccDNA and/or HBsAg. It is believed that combinations of antiviral agents targeting HBV replication and drugs restoring or increasing the host immune response could lead to a functional and perhaps an absolute cure within a decade.[9] After the recent success of therapy for hepatitis C virus infection, the viral hepatitis community has turned its focus on the discovery of novel HBV-associated biomarkers and therapeutic targets. It is hoped that the recent surge in anti-HBV drug discovery efforts will lead to the development of novel therapeutic strategies that could represent a path to cure for the more than 300 million individuals who are suffering from chronic hepatitis B infection worldwide.

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