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The current status and future directions of hepatitis B antiviral drug discovery

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Abstract

Introduction—The current standard care of chronic hepatitis B fails to induce a durable off-drug control of HBV replication in the vast majority of treated patients. The primary reasons are its inability to eliminate the covalently closed circular (ccc) DNA, the nuclear form of HBV genome, and restoration of the dysfunctional host antiviral immune response against the virus.

Area covered—Accordingly, discovery and development of therapeutics to completely stop HBV replication, eliminate or functionally inactivate cccDNA as well as activate a functional antiviral immune response against HBV are currently the primary efforts toward finding a cure for chronic hepatitis B.

Expert opinion—We believe that through a consecutive or overlapping three-stage antiviral and immunotherapy program to (i) completely stop HBV replication and cccDNA amplification; (ii) reduce viral antigen load and induce HBV surface antigen (HBsAg) seroclearance through eradication or inactivation of cccDNA and RNA interference-mediated degradation of viral mRNA and (iii) activate a functional antiviral immune response against HBV through therapeutic immunization or immunotherapy, a functional cure of chronic HBV infection can be achieved in the majority of chronic HBV carriers.

1. Introduction

Chronic hepatitis B virus (HBV) infection affects 240 million people worldwide ¹. Approximately one-third of these individuals will develop severe liver diseases, including cirrhosis, hepatocellular carcinoma (HCC) and liver failure. The currently approved antiviral therapeutics are two formulations of alpha-interferon (IFN- α) and five nucleos(t)ide analogues (NUCs). While the NUCs inhibit HBV DNA polymerase activity with varying potencies and barriers to resistance, the antiviral activity of IFN- α against HBV may result from the combinatory effects of activation of host antiviral immune response as well as suppression of HBV covalently closed circular (ccc) DNA transcription and promotion of HBV nucleocapsid decay in hepatocytes ^{2–5}. Clinically, sustained suppression of viral replication with a long-term NUC therapy or through a 48-week of pegylated IFN- α therapy

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has been associated with improvement of liver diseases, prevention of liver decompensation and reduction of hepatocellular carcinoma morbidity and mortality in a portion of treated patients ^{6, 7}. However, HBV surface antigen (HBsAg) seroconversion, the hallmark of a successful immune response to HBV with complete and durable control of infection, or a “functional cure”, is rarely achieved with the current therapies ^{8, 9}.

The reasons for the failure of the current antiviral regimens to achieve the functional cure of chronic HBV infection in the vast majority of treated patients are two folds. First, despite of significant suppression of viral DNA replication and progeny virion production, viral DNA polymerase inhibitors do not efficiently reduce the amounts of cccDNA, the nuclear form of HBV genome that is synthesized from the cytoplasmic relaxed circular (rc) DNA and exists as a minichromosome to transcribe HBV mRNAs ^{10–12}. This notion is supported by the observation that cccDNA becomes the predominant form of HBV DNA replication intermediates in the livers of patients or animals after a long-term NUC treatment ^{13, 14}. Second, despite potent suppression of HBV replication in the livers of treated patients, the dysfunction of HBV-specific antiviral immunity persists. Inability of the immune responses in chronic HBV carriers to recognize cells harboring HBV infection and cure or eliminate the cells actively producing virus is the fundamental reason for the chronicity of HBV infection and the big challenge for development of therapeutics to cure chronic hepatitis B ¹⁵. Therefore, the goals of current drug discovery and development efforts toward a functional cure of HBV infection are (i) complete suppression of HBV replication to prevent the spread of HBV infection to susceptible host cells and the amplification of cccDNA pool in infected cells; (ii) eradication or transcriptional inactivation of cccDNA from infected cells and (iii) restoration of host innate and adaptive immune responses against HBV to achieve a durable control of HBV replication. Because immunotherapeutic approaches to activate host innate and adaptive immune response against HBV have been extensively reviewed recently ^{16, 17}, this article will focus on the development of antiviral agents targeting viral components and their interactions with host cellular proteins to achieve the therapeutic goals.

2. Development of novel HBV replication inhibitors

2.1 Inhibition of HBV entry

HBV infection begins by attachment to the surface of hepatocyte *via* the interaction of its large envelope protein (HBL) and cellular heparan sulfate proteoglycan (HSPG) ¹⁸, which is followed by the specific binding of the pre-S1 region of HBL to sodium taurocholate cotransporting polypeptide (NTCP), a liver-specific bile salt transporter and the recently identified cellular receptor of HBV ^{19, 20} (Figure 1). HBV binding to its receptor triggers a caveolin-1-mediated endocytosis to internalize the virus into hepatocytes ²¹. Recently, using a high-throughput infectious cell culture model enabling RNA interference-mediated loss-of-function screening of hepatitis delta virus (HDV) entry and infection, Verrier and colleagues identified glypican 5 as an additional host cell entry factor for HBV and HDV ²². Apparently, specific disruption of the interactions between HBL and host cellular receptor and entry factors will selectively inhibit HBV infection. For instance, myrcludex B, a synthetic HBV preS1 domain-derived lipopeptide, binds to NTCP and efficiently inhibits

HBV and HDV infection of hepatocytes in culture and *in vivo* in humanized chimeric uPA mouse model^{23, 24}. In a phase I clinical trial, myrcludex B showed excellent tolerability and dose-dependent pharmacokinetics²⁵. In a phase Ib/IIa clinical trial in chronically HDV infected patients, myrcludex B monotherapy for 24 weeks significantly reduced HDV RNA serum levels and induced ALT normalization. Furthermore, combination therapy with pegylated IFN- α demonstrated synergistic effects in reducing HDV RNA and HBV DNA serum levels²⁶. Hence, as a first-in-class HBV and HDV entry inhibitor, myrcludex B is very promising for further clinical development.

In addition to myrcludex B, several classes of structurally distinct small molecules, including bile salt analogues and cyclosporin A derivatives, have been shown to bind NTCP and inhibit HBV and HDV infection of hepatocytes^{27, 28}. However, their antiviral efficacy has not been demonstrated in animal models *in vivo*. A major concern regarding the use of NTCP substrate analogues to inhibit HBV/HDV infection is the potential interference with the physiological function of NTCP in bile acid enterohepatic circulation. Interestingly, since the essential amino acid residues in NTCP for HBV/HDV infection and bile acid transportation are overlapped, but not identical, it is thus possible to develop NTCP-targeting drugs that specifically inhibit HBV and HDV infection without compromising the function of NTCP to transport bile acid^{29, 30}.

2.2. Targeting the multi-functional HBV polymerase protein to inhibit HBV DNA replication

Unlike classical retroviral reverse transcriptases that consist of a reverse transcriptase (RT) domain and a ribonuclease H (RNase H) domain, HBV polymerase has two additional domains, a terminal protein domain (TP) and a spacer domain, at its N-terminal region. The unique structure features additional function of HBV polymerase in viral DNA replication³¹. Specifically, HBV polymerase binds a stem-loop structure epsilon (ϵ) at the 5' region of the viral pregenomic (pg) RNA to facilitate the pgRNA and polymerase itself being packaged into nucleocapsid. Within nucleocapsid, polymerase primes the synthesis of the minus strand of viral DNA by catalyzing the formation of a phosphodiester linkage between a hydroxyl residue of a tyrosine located in its TP domain and dGMP with the sequence of 5'-UUC-3' located at the bulge of ϵ as a template. The priming reaction terminates following the synthesis of only 3 nucleotides, which are subsequently transferred to the 3' end of pgRNA at DR1 sequence to continue the elongation of the minus strand DNA toward the 5' terminus of pgRNA. The pgRNA is simultaneously degraded by the RNase H domain of the polymerase, except for its capped 5' terminal region including 5' DR1, which is translocated to the DR2 region of the minus strand DNA to prime the synthesis of plus strand DNA. Apparently, HBV DNA polymerase plays essential roles in packaging pgRNA, priming and elongating viral DNA synthesis as well as degrading viral pgRNA. Upon activation by cellular nucleoside kinases, the NUCs can be incorporated into DNA primer and elongating DNA chains by competition with the natural nucleotide substrates and terminate viral DNA synthesis. However, the NUCs do not inhibit polymerase binding of pgRNA and RNase H activity.

Due to the great success of the approved NUCs in treatment of chronic hepatitis B, development of novel NUCs against HBV is not a high demand. Currently, two tenofovir

prodrugs with reduced renal toxicity than that of tenofovir, GS-7340 (tenofovirafenamide fumarate) and CMX157, sponsored by Gilead Science and Contravir, are at phase III or Phase II clinical trials, respectively ³². Despite the great antiviral potency, accumulating evidence suggests that NUC treatment does not completely inhibit HBV DNA synthesis in hepatocytes ^{13, 33}. Hence, development of non-NUC allosteric HBV DNA polymerase inhibitors as well as drugs that disrupt polymerase binding of pgRNA, priming of minus-strand DNA synthesis or inhibit RNase H activity to completely suppress HBV genome replication, either as monotherapeutic agents or in combination with NUCs, is highly desired ³⁴. Particularly, Tavis and colleagues recently developed biochemical and cell-based assays for discovery of HBV RNase H inhibitors and found that some of the human immunodeficiency virus (HIV) RNase H and integrase inhibitors, including naphthyridinone derivative, β -thujaplicinol ^{35, 36}, 2-hydroxyisoquinoline-1,3(2H,4H)-dione (HID) derivatives ³⁷ and α -hydroxytropolones (α HT) ³⁸, were active against HBV RNase H and inhibited HBV DNA replication by chelating the divalent cations in the RNaseH active site ³⁹.

2.3. Inhibition of nucleocapsid assembly

As mentioned above, HBV DNA replication occurs inside of nucleocapsids. Therefore, inhibition of nucleocapsid assembly will preclude viral DNA replication. Interestingly, assembly of a replication competent nucleocapsid relies on cellular HSP90 chaperone complex-assisted HBV DNA polymerase folding and binding to pgRNA, which triggers the recruitment of 120 copies of core protein dimers to package the pgRNA-polymerase complex into an icosahedral nucleocapsid. Accordingly, inhibition of either HSP90 function or disrupting the interactions between viral polymerase, pgRNA and core proteins should inhibit nucleocapsid assembly. Indeed, it has been demonstrated that inhibition of HSP90 ATPase activity can efficiently inhibit pgRNA encapsidation into capsids ⁴⁰. More importantly, several classes of structurally distinct small molecules have been shown to accelerate capsid assembly kinetics *via* modulation of capsid protein interaction, which results in altered nucleocapsid assembly pathway and formation of either empty capsids or non-capsid polymers.

As illustrated in Figure 2, five chemotypes of nucleocapsid assembly inhibitors have been reported thus far. While heteroaryldihydropyrimidines (HAPs), such as Bay 41-4109 and GSL-4, misdirect capsid assembly to form non-capsid polymers of core proteins ^{41–44}, all other nucleocapsid inhibitors induce the formation of morphologically “normal” capsids devoid of viral pgRNA and DNA polymerase ^{45–48}. Interestingly, crystal structure analyses of HAPs or phenylpropenamides (PPAs) in complex with core protein and capsids revealed that both compounds bind a hydrophobic pocket, designated as HAP pocket, at the dimer-dimer interface near the C-terminal of core protein subunits, with contribution from two neighboring core protein dimers. Binding of these molecules in the HAP pocket induces large scale allosteric conformational changes in core protein subunits and results in quaternary and/or tertiary structure changes of capsids ^{49, 50}. Moreover, a V124W mutation fills the HAP pocket and renders resistance to the inhibition of nucleocapsid assembly by HAPs and PPAs ⁵¹. Intriguingly, it was demonstrated recently that NZ-4, a derivative of bis-heterocycle tandem pairs, induces the formation of morphologically normal, but genome-

free capsids in a core protein C-terminal arginine-rich domain-dependent manner⁴⁷. Pharmacologically, as anticipated, by specifically targeting core protein interaction, all the reported nucleocapsid inhibitors showed excellent antiviral activity against the NUC-resistant strains of HBV^{45, 48}. Thus far, several nucleocapsid assembly inhibitors, such as, GLS4, NVR 3-778 and AB-423, have been shown to inhibit HBV replication in mice models and are currently under preclinical or clinical development^{44, 52}.

3. Eradication or functional inactivation of cccDNA is essential for a functional cure

cccDNA exists as a minichromosome in the nucleus of hepatocyte to serve as a transcription template for viral mRNAs, but cannot replicate itself *via* semi-conservative replication. Instead, cccDNA is synthesized from viral relaxed circular (rc) DNA from infecting virions or progeny cytoplasmic nucleocapsids (Figure 1). The cytoplasmic nucleocapsid rcDNA is delivered into the nucleus *via* importin- β -mediated nuclear transportation pathway and converted into cccDNA, presumably by cellular DNA repair enzymes and other host factors⁵³. The exact copy number of cccDNA may vary among infected cells and in response to different pathophysiological conditions. The average copy number of cccDNA in a HBV-infected hepatocyte is approximately 10. Due to the lack of metabolic labeling studies, the stability or rate of turn-over of cccDNA in infected hepatocytes is unknown. However, using NUCs to inhibit new cccDNA formation in cultured cells and infected animals *in vivo*, the amounts of cccDNA in non-dividing primary hepatocytes is steady for a six-week culture period, but declined with apparent half lives of 9–14, 33–50 and 35–57 days in HBV-infected chimpanzees. WHV-infected woodchuck and DHBV-infected ducks, respectively¹⁵. However, clinical studies demonstrated that although NUC monotherapy for 48 to 52 weeks reduced circulating viremia by 4.7 to 5.5 log₁₀ and cytoplasmic HBV DNA levels in the hepatocytes by approximately 2 log₁₀, reduction of cccDNA was much less pronounced, only by 0.11 to 1.0 log₁₀^{54–56}. Although the mechanisms underlying the apparent stability of cccDNA *in vivo* remains elusive, it is clearly evident from the decade-long clinical experience that prolonged NUC monotherapy is unlikely to eradicate cccDNA in the vast majority of chronic HBV carriers and therapeutic regimens that directly attack cccDNA are in pressing need to achieve a functional cure.

3.1. Targeting cccDNA by sequence-specific DNA-editing technologies

In order to specifically eradicate or functionally inactivate cccDNA minichromosomes, the therapeutic agents must be able to recognize and target the structural features that are unique to cccDNA, but not host cellular chromatin. Obviously, the most unique structure feature of cccDNA minichromosome is its DNA sequence. Therefore, several sequence-specific DNA editing technologies, such as zinc finger nucleases, meganucleases, transcription activator-like effector nuclease (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CAS9^{57–62}, have been explored as potential therapeutic approaches to functionally inactivate or eliminate cccDNA. Recently, using next generation sequencing (NGS) technology, Seeger and Sohn determined the complete spectrum of mutations in cccDNA following Cas9 cleavage and repair by nonhomologous end joining (NHEJ) DNA repair pathway⁶³. The authors elegantly demonstrated that over 90% of HBV cccDNA was

edited by Cas9. Among the mutated cccDNA molecules, approximately 67% had a single nucleotide deletion at position -4 with respect to the protospacer adjacent motif (PAM) sequence. About 10% had a single nucleotide insertion at the cleavage site and 16% exhibited deletions ranging from 2 to 6 nucleotides in length. Moreover, approximately 6% of the cleavage cccDNA had deletions of 3 and 6 nucleotides which maintain the open reading frame of HBx protein. If the one or two amino acid deletion does not compromise HBx function, the results suggest that in order to completely inactivate cccDNA function, more than one sgRNAs targeting cccDNA sequences encoding critical residues of HBV proteins are desired⁶³. Moreover, double cleavage of cccDNA by Cas9 may not only more efficiently inactivate cccDNA function, but also make cccDNA repair more difficult and thus favor its elimination⁶⁴. However, despite all these exciting progresses, several key technical issues, particularly the potential off-target cleavage of host cellular DNA and efficient delivery to all the HBV infected cells, need to be resolved. Considering the fast development in gene editing technologies, clinical applicable gene editing therapeutics for the cure of chronic hepatitis B may be eventually achievable.

3.2. Targeting HBV core or X protein to inactivate cccDNA function

As an episomal minichromosome, cccDNA transcription is subjected to epigenetic regulation. It was showed that cccDNA bound H3/H4 hyperacetylation is correlated with high viral replication in HBV infected patients⁶⁵. Furthermore, IFN- α suppresses cccDNA transcription by reducing its H3K9 and H3K27 acetylation, but not increasing H3K9 and H3K27 trimethylation^{2, 66}. Although the HBV genome-wide epigenetic analysis did not reveal any histone modification that is unique to cccDNA minichromosome⁶⁶, many studies did suggest that HBV core and HBx proteins are recruited to cccDNA minichromosome and regulate its transcriptional activity. Therefore, selectively disrupting the interactions between the viral proteins and their host cellular partners for cccDNA regulation may specifically inactivate cccDNA transcription or destabilize cccDNA minichromosomes.

The first direct evidence suggesting that HBV core protein binds to cccDNA is from an electron microscopic and biochemical investigation of the architecture of HBV minichromosome. The studies revealed that core protein is a structural component of viral cccDNA minichromosome and its binding to cccDNA reduces the nucleosomal spacing of the minichromosome and may thus regulate its transcriptional activity⁶⁷. Recent studies further suggested that core protein promotes an epigenetic permissive state of HBV cccDNA by binding on CpG islands of cccDNA⁶⁸. Intriguingly, Lucifora and colleagues found that cytokine-induced DNA cytosine deaminase, APOBEC3A, was recruited to cccDNA minichromosome by interaction with core protein⁶⁹. This remarkable finding implies that core protein can also be hijacked by the host immune system to destabilize and eliminate cccDNA. Hence, pharmacological disruption of core-core and core-host protein interaction may alter cccDNA stability and transcription. It will be interesting to investigate whether the current available nucleocapsid assembly effectors that specifically modulate core protein subunit interaction in capsid assembly would also have effects on the recruitment and function of core protein on cccDNA minichromosomes. It is entirely possible that the cccDNA binding and transcriptional regulation by core protein rely on distinct core-core

and/or core-host protein interaction and thus novel antiviral agents are required to inhibit those functions.

HBx is a 17 kDa viral protein essential for viral DNA replication, which is supported by the facts that HBx-deficient HBV fails to replicate in cultured primary hepatocytes or human hepatocytes in the livers of humanized uPA-chimeric mice *in vivo*^{70, 71}. Although HBx may promote HBV DNA replication in the cytoplasm by modulating cytosolic calcium signaling, cell cycle progression and capsid protein phosphorylation^{72–76}, the recent studies highlight a key role of HBx in activation of cccDNA minichromosome transcription^{70, 77}.

HBx is known to be a trans-activating protein with the ability to stimulate the transcription of episomal DNA, including its own cccDNA minichromosome⁷⁸. CHIP analyses indicated that HBx was recruited to the cccDNA minichromosomes and recruiting epigenetic modifiers to set up a transcription permissive cccDNA epigenetic status altered cccDNA-associated histone modification^{77, 79, 80}. Specifically, HBx had been shown to promote cccDNA-associated histone acetylation as well as recruit histone lysine demethylase-1 (LSD1) to relieve SETDB1-mediated H3K9me3 and HP1 induced condensation of cccDNA minichromosomes⁷⁷. In addition, HBx was also demonstrated to enhance cccDNA transcription by interacting with transcriptional repressor Spindlin1 to prevent its recruitment to cccDNA minichromosome⁸¹ or inhibiting PP1 phosphatase activity to extend the half-life of transcription factor p-CREB⁸². HBx was also reported to interact with a cellular hydroxylase, jumonji C-domain-containing 5 (JMJD5), to promote the transcription of key hepatocyte enriched transcription factors, such as HNF4A, CEBPA, and FOXA3, to enhance cccDNA transcription⁷³. Remarkably, Decorsière and colleagues reported recently that cellular “structural maintenance of chromosomes” (Smc) complex Smc5/6 suppressed the transcription of cccDNA minichromosome and HBx activates the transcription by hijacking DDB1-containing E3 ubiquitin ligase to target the Smc5/6 for degradation⁸³.

In summary, accumulating evidence suggests that both core and HBx proteins can be recruited to cccDNA minichromosome and modulate its transcription activity via multiple mechanisms. Interestingly, a screening of FDA approved drug library found that erbinafine, a antifungal medicine, suppressed HBx-mediated HBV transcription *via* unknown mechanism⁸⁴. Further investigation on the mechanism of core and HBx recruitment to and regulation on cccDNA transcription should reveal and validate molecular targets for the discovery of antivirals to selectively inactivate cccDNA function.

3.3. Phenotypic screening of compounds targeting cccDNA

In addition to rationally discovering compounds that selectively target viral proteins and their interacting cellular partners essential for cccDNA function, recent technical development in HBV infection cell culture systems^{19, 20}, establishment of a HepG2-derived stable cell line expressing epitope-tagged HBeAg in a cccDNA-dependent manner⁸⁵ and production of recombinant HBV reporter viruses⁸⁶ will allow for high throughput screening of compound libraries to phenotypically identify compounds that destabilize or functionally inactivate cccDNA.

4. Induction of HBsAg seroclearance and seroconversion

Clinical studies indicate that while resolution of an acute HBV infection is associated with a polyclonal and vigorous HBV-specific T-cell response, chronic HBV infection is always associated with a weaker or barely detectable HBV-specific T-cell response⁸⁷. Failure to induce a functional antiviral immune response in chronic HBV infection is due to the prolonged exposure of T cells to the high quantities of viral antigens in the liver and high levels of HBsAg in circulation, which results in depletion or functional exhaustion of HBV-specific T cells. The exhausted T cells are characterized by the increased expression of negative co-stimulatory molecules and dysregulation of co-stimulatory pathways. Accordingly, lowering intrahepatic viral antigen load and/or reducing HBsAg antigenemia may be essential for the recovery of functional antiviral immune response to durably control HBV infection. However, how much the reduction of viral antigen load or HBsAg antigenemia is required for a spontaneous or treatment-induced recovery of anti-HBV immune response in chronic carriers remains to be determined. Clinical studies of spontaneous and treatment-induced HBsAg seroclearance/anti-HBs seroconversion and a recent experimental study in mice may be helpful to answer this important question, at least in part.

Spontaneous HBsAg seroclearance, defined by the loss of serum HBsAg on two occasions at least six months apart and remaining absent up to the last visit, is rare, but consistently occurs at an annual of spontaneous HBsAg seroclearance varied from 0.12 to 2.38% in Asian countries and 0.54 to 1.98% in Western countries⁸⁸. Prolonged NUC therapy reduces viral load in the vast majority of treated patients by more than 4.5 log, but does not increase the rate of HBsAg seroclearance. However, 48 to 52 weeks of pegylated IFN- α therapy increases the incidence of HBsAg seroclearance by 3 to 6 folds⁸⁹. Clinically, spontaneous and treatment-induced HBsAg loss/seroconversion is associated with the stable control of HBV infection and excellent outcome, if without pre-existing cirrhosis and viral superinfection. However, only 17%, 56% and 76% of the carriers have detectable anti-HBs at 1, 5 and 10 years after HBsAg seroclearance⁹⁰. The observed slow anti-HBs seroconversion after spontaneous and treatment-induced HBsAg seroclearance in chronic HBV carriers suggests that (i) seroclearance, but not lowering the level of HBsAg, may be required to restore the antiviral immune response; (ii) spontaneous restoration of effective antiviral immune response to sustained control HBV infection after HBsAg seroclearance may not immediately occur; and (iii) therapeutic vaccination/immunotherapeutics may be required to accelerate the activation of effective antiviral immune response after HBsAg seroclearance. Moreover, a recent study demonstrated that the immune tolerance status to HBV in AAV-HBV infected mice is attributed to the level and duration of circulating HBsAg⁹¹. Intriguingly, depletion of circulating HBsAg by a monoclonal anti-HBsAg in tolerant mice could gradually reduce tolerance and reestablish B cell and CD4⁺ T cell responses to subsequent Engerix-B vaccination⁹¹. Furthermore, HBsAg-specific CD8⁺ T cells induced by the addition of a TLR agonist resulted in clearance of HBV in both peripheral blood and liver⁹¹. These results re-enforce the notion that circulating HBsAg plays an important role in induction of immune tolerance to HBV and HBsAg seroclearance with following immunization or immune agonist therapy is required to promote the

restoration of humoral and T cell immune response to achieve a durable control of HBV infection, or a functional cure.

Concerning the therapeutic approaches to lower HBV antigen load and particularly, induce HBsAg seroclearance, we consider that the most efficient way is to reduce viral protein expression in infected cells by eradication or inactivation of cccDNA with the technologies discussed in the previous section or RNA interference (RNAi)-mediated degradation of viral mRNA. Due to the improvement in RNA chemistry and hepatocyte-targeted delivery technologies in recent years, RNAi technologies, such as ARB-1467, ARC-520, AVV/HB-BB-331 and ALN-HBV, have now been demonstrated in preclinical animal models and human clinical trials to efficiently suppress HBV protein expression and reduces viral load ⁹². Their effects on immune restoration by decreasing viral antigen load are under investigation. In addition to reducing viral antigen expression, compounds that selectively inhibit HBsAg secretion from hepatoma cells have also been reported ^{93, 94}. However, their mode of action and *in vivo* efficacy in reducing HBsAg levels remain to be determined. Interesting, nucleic acid polymers (NAPs), sequence-independent phosphoro-thioated oligonucleotides, was reported to inhibit HBV entry and HBsAg secretion. REP9-AC (REP 2055), a 40- nucleotide poly-cytidine amphipathic DNA polymer, have been demonstrated to efficiently reduce viral load and surface antigen of duck hepatitis B virus and induce antibodies against surface antigen in duck ⁹⁵. REP 2139 in combination therapy with pegylated IFN alpha-2a is in clinical trial for chronic HBV and HDV co-infection ⁹⁶.

5. Conclusions

To achieve a functional cure of chronic hepatitis B, *i.e.*, HBsAg seroclearance and anti-HBs ceroconversion, in the vast majority of treated patients, therapeutic agents targeting multiple unexplored viral and host cellular functions, particularly nucleocapsid assembly, cccDNA metabolism as well as innate and adaptive immunity against HBV, are currently under preclinical or clinical development. A complete and periodically updated list of HBV antiviral and immunotherapeutic agents under preclinical and clinical development can be found in the “Drug Watch” program of Hepatitis B Foundation ⁹⁷. Currently, the major hurdles for the development of curative therapeutics are the lack of cell culture and animal models supporting robust HBV infection and high throughput assays to efficiently identify and evaluate therapeutic agents in biologically relevant systems. Immune competent animal models supporting persistent HBV infection is particularly important for development of immunotherapeutics to induce a functional antiviral immune response that durably controls HBV infection. Obviously, development of the *in vitro* and *in vivo* HBV infection models will also facilitate the investigation of HBV replication, cccDNA biology and immunopathology, which should provide basis for rational development of therapeutics to reach the clinical endpoints of chronic hepatitis B treatment.

6. Expert opinions

Ideally, cure of HBV infection should be the sterilizing elimination or eradication of the virus from infected individuals and return the patients to their pre-infection risk of death from liver diseases. However, it is well established that even persons with naturally resolved

acute HBV infection have a risk of death from hepatocellular carcinoma (HCC) three times greater than those who have never been infected. Moreover, the facts that persistence of HBV cccDNA can be detected even in the liver of patient resolved HBV infection and HBV replication can be reactivated by immune suppression therapies in individuals with naturally resolved acute hepatitis B decades ago suggest that “cure” of HBV infection by host immune responses is at least not always associated with the clearance of the virus, but most likely through a tight immune surveillance of the virus to minimize its pathological consequences. It is, therefore, unrealistic or even unnecessary to develop therapeutics to completely eradicate HBV (cccDNA). Instead, we believe that the overall goal of antiviral therapy for chronic HBV infection is to induce a durable off-drug control of HBV infection, which has been associated with improvement of liver diseases, prevention of liver decompensation and reduction of hepatocellular carcinoma morbidity and mortality^{6, 7}. The key to achieve a durable off-drug suppression of HBV replication is the therapeutic activation of a functional antiviral immune response, which is practically indicated as HBsAg to anti-HBs seroconversion in treated patients. Hence, we consider that HBsAg seroclearance and anti-HBs seroconversion are the realistic endpoints for novel anti-HBV therapies.

As illustrated in Figure 3, in our opinion, the therapeutic endpoints can be achieved through three consecutive or overlapping stages of therapies. The goal of the first stage is to completely inhibit HBV replication to stop the spread of infection and amplification of cccDNA pool in virally infected cells. This goal can be achieved with combination therapies of NUCs and novel HBV replication inhibitors currently under preclinical and clinical development. For patients with stronger residual immune response to HBV, complete suppression of HBV replication with the combination therapeutic regimens may result in gradual elimination of infected cells by immune response and hepatocyte turnover, which will reduce antigen load and favor the restoration of antiviral immune response and ultimately achieve a functional cure. However, for the majority of the chronic carriers, complete inhibition of HBV replication may not be able to reduce the number of virally infected cells. Therefore, the goal of the second stage therapy is to reduce viral antigen load and induce HBsAg seroclearance. This goal can be achieved by inhibition of viral protein expression, such as elimination or functional inactivation of cccDNA and/or siRNA-mediated degradation of viral mRNA. Due to the important role of secreted HBsAg in the induction of immune tolerance to HBV, therapeutics to induce intracellular degradation, inhibit secretion or accelerate the clearance from circulation of HBsAg will also be helpful. By reducing viral antigen load and particularly clearing HBsAg from circulation, the exhausted HBV-specific immune response may recover spontaneously. Because spontaneous anti-HBs seroconversion may take years after HBsAg seroclearance, the goal of the third stage therapy is to accelerate the recovery of exhausted immune response against HBV by therapeutic immunization and/or immunotherapy. Currently, several therapeutic immunization regimens are in preclinical and clinical development. TLR7 agonists and checkpoint blockade therapy with antibodies against PD1 is in clinical trial⁹⁸. Taking together, therapeutic agents to more potently suppress HBV replication, reduce viral antigen load and restore host HBV-specific antiviral immune response are currently under

development. In our opinion, combination application of the three categories of therapeutic agents should finally provide a functional cure for the vast majority of chronic HBV carriers.

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- Of outstanding interest

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Figure 1. HBV replication cycle in hepatocyte and targets of currently available antiviral therapeutics

Briefly, HBV infects hepatocyte by binding to its cellular receptor NTCP and entering into the cells *via* endocytosis. Upon arriving at the cytoplasm, nucleocapsid delivers viral relaxed circular (rc) DNA into the nucleus to be converted into cccDNA by cellular DNA repair machinery. The cccDNA serves as a transcriptional template for production of all viral RNAs, which are subsequently exported to the cytoplasm to translate viral proteins and serve as a pgRNA to be packaged into nucleocapsid. Within the nucleocapsids, pgRNA is reverse transcribed into single-stranded (ss) DNA and then rcDNA. In addition, capsid protein can also assemble into viral RNA/DNA-free empty capsids. Interestingly, while rcDNA-containing mature nucleocapsids can be enveloped and secreted as virions *via* multi-vesicle bodies (MVB), pgRNA containing nucleocapsids as well as empty capsids can also be enveloped and secreted as virion-like particles. In the blood of HBV infected individuals, the number of empty capsid-containing virion-like particles is approximately 100-fold more than virions. However, the number of pgRNA-containing virions is approximately 10 to 100-fold less than virions. In addition to virion-like particles, HBV-infected hepatocytes also secrete empty envelope particles or filaments in the amounts of 1000 to 10,000-fold more than virions.

Figure 2. Five chemotypes of representative HBV capsid assembly inhibitors

Bay 41-4109, GLS4 and 4-methyl HAP 34a are heteroaryldihydropyrimidine derivatives.

DVR-23 is a sulfamoylbenzamide. AT-61 is a phenylpropenamide.

Compound 3711 is a pyridazinone.

Figure 3. Illustration of the three-stage therapeutic program of chronic HBV infection

The contribution of current drug discovery efforts on the cure of chronic hepatitis B is highlighted in the approach boxes. The thickness of the arrows indicates the likelihood of the outcomes.