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Opportunities and challenges for hepatitis B cure

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ABSTRACT

In spite of the fact that safe and effective vaccines have been available for over 40 years, hepatitis B virus (HBV) remains a major public health problem, as there are 296 million chronically HBV-infected individuals worldwide and 820 000 HBV-related deaths taking place every year. Achieving the goal of HBV cure remains a challenge due to the particularities of the HBV cycle underlying viral persistence. The new understanding of HBV biology and antiviral immune responses has allowed to identify novel drug targets. This has led to a renewed interest in developing new curative strategies and combinations for HBV. In the present review, we aim to summarise the biological and clinical challenges associated with chronic HBV infection. Moreover, we consider the lessons that have been learnt in the past years regarding the preclinical and clinical evaluation of compounds against HBV and how this is driving the field to explore new directions.

INTRODUCTION

The development of antiviral treatments for hepatitis C virus (HCV) infection is one of the most remarkable stories in translational medicine. Since the discovery of HCV in 1989,[1] the scientific community was able to develop the necessary research and diagnostic tools that led to the design of antiviral molecules, nowadays allowing HCV elimination in more than 98% of cases.[2] Although HCV remains a global health burden,[3] the success of these therapies has shown that it is possible to surmount the challenges associated with virally induced liver diseases. In particular, there has been a renewed interest in developing curative therapies for hepatitis B virus (HBV), a pathogen discovered decades before HCV and for which safe and effective vaccines have been available for over 40 years. In spite of this head start, it is estimated that there are 296 million chronically HBV-infected individuals worldwide and 820 000 HBV-related deaths taking place every year.[4] To address the challenges of HBV cure, an important question is to identify the aspects that make HBV elimination such a unique and complex endeavour.

In the present review, we aim to summarise the clinical challenges that stem from the particular biology of HBV. Moreover, we consider the lessons that have been learnt in the past years regarding the preclinical and clinical evaluation of compounds against HBV and how this is driving the field to explore new directions (figure 1).

CHALLENGES FOR HEPATITIS B CURE

The determinants of viral persistence

Particularities of the HBV cycle

One the most relevant characteristics of the HBV viral cycle in regard to its ability to favour chronic infection is the formation of covalently closed circular DNA (cccDNA), which serves as a viral reservoir and template for viral replication.[5] The HBV cccDNA is associated with cellular histones and non-histone proteins and organised into a chromatin-like structure, which regulates its transcription via epigenetic modifications.[5 6] The intrahepatic pool of this highly stable mini-chromosome is maintained via new rounds of infection and intracellular recycling. The dual source of cccDNA, in combination with its long half-life, results in highly stable concentrations that are minimally affected even after long-term antiviral treatment.[7 8] In addition, HBV DNA can persist in the form of integrated sequences within the host cell genome. Although integrated HBV sequences cannot sustain viral replication, they can generate hepatitis B surface antigen (HBsAg) and the transcriptional regulator HBx.[9 10] These integration events occur early during infection, with their number representing a driver for the development of hepatocellular carcinoma (HCC).[11]

Genetic variability of HBV

HBV can be classified into 10 genotypes (A–J) based on an intergroup divergence of ≥8% in their nucleotide sequence.[12] HBV genotypes present particular geographical distributions and have been described to influence patient outcomes, such as hepatitis B e antigen (HBeAg) seroconversion, mutational patterns and response to therapy.[13] Moreover,

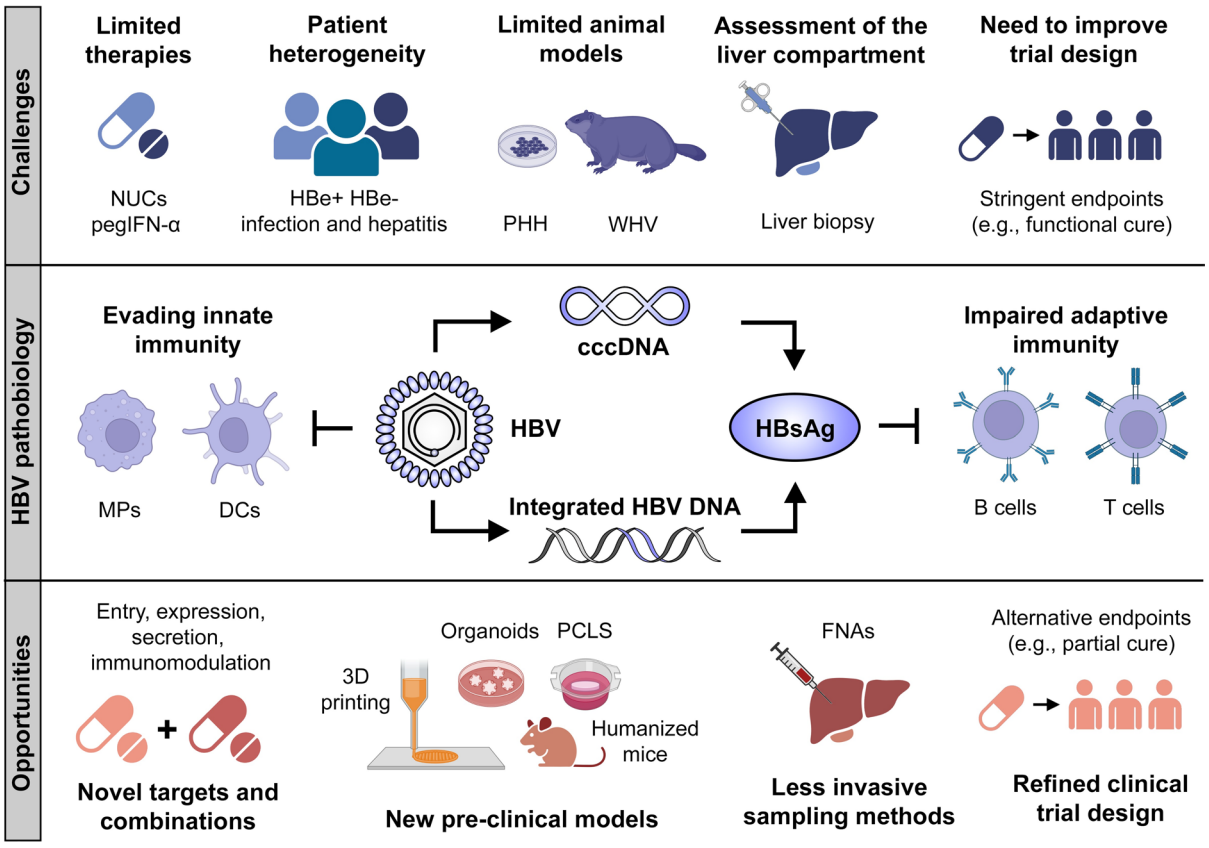

Figure 1 Opportunities and challenges for hepatitis B cure. Particularities of the HBV cycle that favour the development of chronic infection (centre), the clinical challenges stemming from these biological characteristics (top) and the opportunities currently under development to achieve the goal of HBV cure (bottom). cccDNA, covalently closed circular DNA; 3D, three-dimensional; DCs, dendritic cells; FNAs, fine-needle aspirates; HBe, hepatitis B e; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MPs, macrophages; NUCs, nucleos(t)ide analogues; PCLS, precision-cut liver slices; pegIFN-α, pegylated interferon alpha; PHH, primary human hepatocytes; WHV, woodchuck hepatitis virus.

the lack of polymerase activity during reverse transcription of the HBV pregenomic RNA (pgRNA) leads to genetic mutations that ultimately result in the rise of viral quasi-species in infected patients. These viral populations evolve according to their interplay with the host immune responses or antiviral treatment, potentially driving the emergence of HBV mutants able to escape them.[14 15]

Impairment of immune responses during HBV infection

Considering the particularities of the liver immune microenvironment in which HBV infection is established and spread in the liver is equally relevant to understand its persistence. Indeed, the liver presents a wide variety of regulatory mechanisms that induce a bias towards immune unresponsiveness.[16] As immune cells from the blood slowly transit through the liver, the presence of fenestrations in the hepatic sinusoid allows the interaction between lymphocytes and liver resident cells.[17] It is believed that in context of HBV infection, this contact mediates the intrahepatic priming of T lymphocytes by non-professional antigen-presenting cells, such as hepatocytes.[18] The hepatic priming of HBV-specific $CD8^+$ T cells, for example, results in their proliferation and activation, but these lymphocytes fail to differentiate into effector cells, thus contributing to the establishment of a persistent HBV infection.[19] In addition, the persistent exposure of T cells to HBV antigens leads to the establishment of a functionally exhausted phenotype, which is characterised by high expression levels of inhibitory molecules such as programmed cell death 1 (PD-1) and cytotoxic T lymphocyte-associated protein 4.[20–22] As discussed in later sections, the functional restoration of exhausted T cell populations via targeting of these inhibitory checkpoints is an active research field.

The important role of B cell responses in controlling HBV can be exemplified by the use of B cell-targeting therapies (eg, rituximab), which results in HBV reactivation in patients with chronic hepatitis B (CHB) or in those with resolved infection.[23] In this regard, it has been shown that most patients with CHB do not present detectable levels of anti-HBsAg antibodies, with recent evidence suggesting that this stems from an impaired function of HBsAg-specific B cells and not a decrease in their number.[24 25] Hepatitis B core antigen (HBcAg)-specific B cells are present at much higher frequencies than HBsAg-specific B cells and are associated with elevated liver inflammation and viral replication.[26 27] A possible mechanism explaining these observations could be related to the high circulating levels of HBsAg as compared with

HBcAg, resulting in the sequestration of available antibodies as immune complexes.[19]

Barriers in the preclinical evaluation of novel therapies against HBV

Tumour-derived cell lines and primary human hepatocytes (PHHs) are some of the most common in vitro models to study HBV infection. In this context, cell lines overexpressing the HBV receptor sodium taurocholate cotransporting polypeptide (NTCP) represent a flexible system that is able to support the whole HBV cycle.[28] Nonetheless, these cells (eg, HepG2-NTCP) lack multiple cellular components implicated in the immune response, which hinders the evaluation of compounds targeting these signalling pathways.[29] PHHs present a non-transformed phenotype, in addition to several practical advantages that include a relatively simple isolation procedure and the possibility for cryopreservation.[30] However, PHHs cannot be expanded and progressively undergo dedifferentiation when in culture.[31] Moreover, PHHs as a model lack the polarity, zonation and presence of additional cell types that characterise the hepatic microenvironment. The latter point is of particular relevance for the preclinical evaluation of novel therapies against HBV, as an ideal model should allow characterisation of the HBV cycle and the interplay with immune cells, in order to evaluate direct-acting antivirals (DAAs) and host-targeting agents (HTAs).

Immunomodulatory agents can be evaluated in vivo using the woodchuck model in context of woodchuck hepatitis virus (WHV) infection.[32] However, this model differs from HBV infection in several aspects, including genomic divergence, particularities in the mechanisms regulating viral transcription, the course of liver disease with specific integration events and rapid HCC development, and the particular expression pattern of immune components between both woodchucks and humans.[33] Chimpanzees are the only non-human primate model for HBV infection and their use has been fundamental to study the host response against the virus, as well as the development and testing of prophylactic vaccines.[34] However, the HBV field has moved away from this model due to ethical concerns and its ban in many countries.[35] Therefore, current efforts are focused in the development of small animal models for HBV infection. For instance, human liver chimeric mice are based on the engraftment of human hepatocytes in immunodeficient animals.[36] Although this model is inadequate for the evaluation of HTAs, it recapitulates the HBV cycle in its entirety and is therefore useful for the evaluation of DAAs.

Clinical challenges associated with HBV infection

Current therapeutic agents for CHB

Although current therapies based on pegylated interferon alpha (pegIFN-α) and nucleos(t)ide analogues (NUCs) can suppress HBV replication and decrease the risk of complications such as cirrhosis and HCC, HBV is never fully eliminated. Thus, these regimens require indefinite treatment to prevent the virological relapse that usually occurs after treatment discontinuation.[37] Moreover, it is unrealistic to expect all patients to adhere to lifelong non-curative regimens, with a strong patient preference for finite therapy. This is of particular relevance in the case of IFN treatment, as its use is limited by an unfavourable tolerability profile.[38] Finally, the economic burden of long-term treatment and monitoring of these patients is an important issue to consider in highly endemic areas.

Phases of chronic HBV infection and patient heterogeneity

CHB is a highly heterogeneous disease with different clinical phases, stemming from the complex balance between viral replication and immune responses against it. This has led to a classification of chronic HBV infection that takes into account these factors and divides it into four phases: HBeAg-positive chronic HBV infection (HBeAg+, high HBV DNA and normal alanine aminotransferase (ALT)), HBeAg-positive chronic hepatitis B (HBeAg+, high HBV DNA and high ALT), HBeAg-negative chronic HBV infection (HBeAg−, low HBV DNA and normal ALT) and HBeAg-negative CHB (HBeAg− and fluctuating levels of HBV DNA and ALT).[13] These phases do not always progress in a linear manner and patients can go from one phase to another. Classification of patients according to HBV phase is an important predictor of long - term outcome and a valuable mean to define treatment initiation and monitor treatment response.[39]

Monitoring viral and immune parameters in the liver microenvironment

Taking into account the particularities of intrahepatic immune responses and the observation that serum markers do not seem to reflect cccDNA levels at certain disease phases (eg, HBeAg-negative chronic infection),[40] it is considered that intrahepatic cccDNA quantification will be essential in longitudinal studies aiming to evaluate new curative strategies.[41] In this context, core liver biopsy has remained the gold standard for liver histology analysis and CHB staging, as it allows the assessment of host and viral parameters associated with HBV infection. However, as both patients and clinicians favour less-invasive assessments whenever possible, developing relevant less-invasive or non-invasive methods to assess the liver reservoir of HBV is a high priority. Such procedures that could be repeated at short intervals, as in the case of longitudinal studies, would represent an asset to assist the clinical development of novel antiviral strategies.

Design of clinical trials

Considering the above-mentioned biological and clinical characteristics of HBV, the design of clinical trials is crucial in order to evaluate novel therapies for CHB. This is a challenging matter, as there needs to be a balance between selecting the best responders (eg, patients with viral suppression and low HBsAg levels) to provide proof of principle regarding efficacy, against the evaluation of potential therapies in more heterogeneous groups that

represent the diversity of CHB in the real world. Moreover, multiple factors need to be considered, including HBV genotype, ethnic background and stage of liver disease.

As described in the subsequent sections, it is considered that achieving HBV cure with finite treatment regimens will require not only the development of novel agents, but also their use as combination therapies. The concept behind the combination of molecules with different mechanisms of action is to induce: suppression of HBV replication, decrease in viral antigen expression (eg, HBsAg) and activation of the immune response.[41] Defining the need and timing of immunomodulatory therapy is particularly difficult. Indeed, emerging virological and immunological markers to predict patients' response and guide interventions are still at an exploratory stage.

Regarding clinical trial endpoints, the goal of new compounds against HBV would be to eliminate all traces of the virus. However, achieving a *sterilising cure*, with elimination of cccDNA and integrated DNA, seems to be a scenario beyond what can be attained with existing treatments in development.[42] Therefore, *functional cure* was suggested as a new goal and was defined as a sustained (>6 months) HBsAg loss with or without seroconversion to anti-HBsAg antibodies and undetectable HBeAg and HBV DNA after therapy. Unfortunately, very few of the drugs under evaluation have resulted in HBsAg loss at the end of therapy and even fewer have achieved a sustained response. Therefore, there is a need to define alternative clinical trial endpoints that could be useful for the evaluation of novel therapies and combinations against HBV.

OPPORTUNITIES FOR HEPATITIS B CURE

Novel therapeutic targets and combinations against HBV

Inhibition of HBV entry

Because de novo infection is a central step in the maintenance of the cccDNA pool and the persistence of HBV infection, targeting viral entry would be a sensible approach to halt progression of the viral cycle. Bulevirtide, a synthetic peptide containing 47 amino acids of the HBsAg pre-S1 domain, was developed to compete with NTCP and prevent virion uptake by hepatocytes.[43] Although the clinical evaluation of bulevirtide monotherapy was unsatisfactory regarding its effect to decrease HBsAg levels,[44] entry inhibition may still represent a useful approach in combination with agents targeting other steps of the HBV cycle. Indeed, blocking new rounds of infection could be an adequate strategy to favour clearance of HBV-infected hepatocytes harbouring cccDNA as a consequence of cell turnover. Bulevirtide is currently developed to treat coinfections with hepatitis D virus (HDV), a situation in which the underlying high turnover of viral infection has supported the clinical development of this antiviral agent.[44 45]

Monoclonal antibodies against HBsAg are a potential strategy to neutralise viral particles and prevent HBV entry. Moreover, this approach could present the advantage to decrease circulating HBsAg levels, resulting in a reinvigoration of immune responses and enhanced viral clearance. VIR-3434 is a neutralising antibody against HBsAg, which has been Fc-engineered in order to extend its serum half-life and increase binding to activating Fc gamma receptors supporting a potential vaccinal effect. VIR-3434 has been reported to neutralise HBV infection in vitro and to decrease circulating HBsAg levels and HBV spread in vivo.[46] Regarding its clinical evaluation, preliminary results have reported that VIR-3434 is well tolerated, with the majority of patients presenting at least a 1 log IU/mL drop in HBsAg in the first week after treatment.[47] Early reports from a subsequent phase II study showed that combination of VIR-3434 with the small interfering RNA (siRNA) VIR-2218 achieved reductions in HBsAg of >2.5 log IU/mL.[48] Triple combination of VIR-3434, VIR-2218 and pegIFN-α is currently under phase II evaluation (NCT04856085).

Capsid assembly modulators

Capsid assembly modulators (CAMs) are a class of molecules that interfere with the HBV cycle by favouring spontaneous capsid nucleation or accelerating their formation, which leads to the production of aberrant or empty capsids devoid of pgRNA.[49] Moreover, it has been proposed that this could have an effect on the formation of nascent cccDNA in de novo infected cells by preventing newly formed capsids from cycling back to the nucleus. While there was initial excitement for the use of CAMs,[50–52] discouraging results from later trials have dampened the enthusiasm for these agents. Preliminary results from an attempt to stop therapy in patients with no detectable serum markers of HBV replication after a year of treatment with an NUC/CAM (ie, vebicorvir) combination showed that this led to an immediate relapse.[53] Consistently, a large phase II trial showed limited effect on HBsAg and HBeAg levels.[54] This suggests that with the use of the currently available CAMs, the reservoir of transcriptionally active cccDNA had not been eliminated from the liver. In addition, liver toxicity with the use of some CAMs and reports of an apparent antagonism with siRNAs have left the future of CAMs uncertain.[55–57]

It would be important to determine if new generation of CAMs can exert a direct effect on the cccDNA pool and if their use would require longer duration of treatment in order to achieve HBsAg loss.

Targeting HBV RNA

The compact nature of the HBV genome and its overlapping open reading frames offers the opportunity to target multiple HBV transcripts with individual siRNAs or antisense oligonucleotides (ASOs). These strategies could interfere not only with HBV replication (eg, targeting pgRNA), but also decrease HBsAg production in order to reinvigorate immune responses against HBV. Some of the compounds under evaluation include the siRNAs JNJ-3989, VIR-2218, AB-729 and RG6346, and the ASO bepirovirsen.

Although siRNA monotherapy or their combination with NUCs is associated with on-treatment HBsAg responses, HBsAg loss is rarely achieved. However, strategies combining pegIFN-α with siRNAs seem to have an additive effect on HBsAg levels. In this context, preliminary results from the combination of VIR-2218, an N-acetylgalactosamine (GalNAc)-conjugated siRNA, with pegIFN-α have shown that this led to HBsAg loss in 30.8% of patients with CHB under NUC therapy. Although the long-term durability of this approach still needs to be confirmed, combinations using these agents could be an important strategy for HBV cure.[58 59]

In a phase II clinical trial, the ASO bepirovirsen has shown promising results as monotherapy or in combination with NUCs, reporting HBsAg loss in ~10% of patients by the end of 24-week follow-up.[60] It is worth noting that bepirovirsen is not GalNAc-conjugated, thus potentially internalised not only by hepatocytes, but also by non-parenchymal cells in the liver such as macrophages. Indeed, preliminary results suggesting the activation of Toll-like receptor 8 (TLR8) by bepirovirsen have been shown in transgenic mice expressing the human version of this receptor.[61]

Inhibition of antigen secretion

Nucleic acid polymers (NAPs) are a class of compounds that block the release of subviral particles from HBV-infected hepatocytes. As in the case of agents targeting HBV expression, NAPs could present the advantage of decreasing circulating levels of HBsAg and thus potentially favour clearance of the virus by the immune system. Combination of NAPs (ie, REP 2139 or REP 2165) with pegIFN-α and tenofovir has been reported to achieve high rates of HBsAg seroconversion (50%) and HBsAg loss (35%) after 1 year of treatment-free follow-up. Interestingly, a significant number of patients presented ALT flares at the time of HBsAg decline.[62] Clinical evaluation of REP 2139 in context of HBV/HDV coinfection showed that its combination with pegIFN-α was well tolerated and associated with significant HBsAg declines and HDV RNA clearance, which was sustained up to 3 years off-treatment.[63] Although the results look promising, further mechanistic and clinical studies in larger patient cohorts will be required to assess the role of NAPs in future therapeutic combinations.

Innate immunity activators

Considering that natural HBsAg clearance is based on immune mechanisms, there has been a major focus on developing immunomodulatory approaches to achieve a cure of HBV. Besides the long use of IFN-α for the treatment of CHB, the clinical evaluation of agents targeting innate immune responses has included agonists of retinoic acid-inducible gene I (RIG-I), TLR7 and TLR8. The RIG-I agonist inarigivir was reported to inhibit HBV replication via induction of IFN-α in vitro. Despite an initial assessment concluding that inarigivir was well tolerated following 12 weeks of treatment,[64] a second longer clinical trial reported severe toxicity in several patients and the development of multiorgan failure and death in one individual.[65]

Similarly, initial reports of the TLR7 agonist GS-9620 in the WHV and chimpanzee models were promising.[66–68] However, its clinical evaluation was disappointing, as no significant decreases in HBsAg were observed despite target engagement.[69] More recently, clinical evaluation of the TLR8 agonist selgantolimod in NUC-suppressed patients reported HBsAg loss in 5% of participants and HBeAg loss in 16% of them, with a mean HBsAg reduction of <1 log IU/mL.[70]

Checkpoint inhibitors

Checkpoint inhibitors reinvigorate pre-existing antiviral immunity by preventing the action of cell components and pathways that limit immune responses. Considering that these factors are upregulated in HBV-specific T cells, checkpoint inhibitors could represent a sensible strategy to restore T cell responsiveness.[71] Preliminary results from the phase II clinical evaluation of ASC22 (envafolimab), a humanised anti-programmed death-ligand 1 antibody, in virally suppressed patients reported a mean HBsAg decrease of 0.38 log IU/mL. Moreover, 42.9% of patients with baseline HBsAg ≤100 IU/mL obtained a sustained HBsAg loss.[72]

Therapeutic vaccines

Unlike checkpoint inhibitors, therapeutic vaccines prime new antiviral responses. This strategy mainly relies on the induction of effective $CD4^+$ and $CD8^+$ T cells and to a lesser extent on B cells and the production of antibodies. Although the use of therapeutic vaccines has shown disappointing results,[73 74] new approaches in their development and combination have sparked interest in this strategy once again.

The therapeutic vaccine VTP-300 was evaluated in NUC-suppressed patients with or without the PD-1 inhibitor nivolumab. Preliminary results from this evaluation showed that only 16.6% of patients treated with VTP-300 alone achieved HBsAg declines of 0.7–1.4 log IU/mL. Patients who received VTP-300 and low-dose nivolumab achieved HBsAg declines of 1.15 log IU/mL, which persisted 8 months after the final dose.[75]

Encouraging preliminary results have been reported from the clinical evaluation of CHB targeted immunotherapy (CHB-TI), a strategy consisting of the administration of viral vectors in a heterologous prime boost regimen combined with adjuvanted recombinant HBc and HBs proteins. CHB-TI treatment in patients with CHB was associated with an increase of HBV-specific $CD8^+$ T cells.[76] Similarly, an alternating immunisation strategy based on the combination of the arenavirus vectors GS-2829 and GS-6779 has shown promising preclinical results. Indeed, GS-2829/GS-6779 administration in cynomolgus macaques induced a strong polyfunctional CD8 T cell immunity, as well as an anti-HBsAg antibody response.[77]

Finally, an interesting concept that has been explored in preclinical models consists of the combination of agents that reduce HBsAg expression (eg, siRNAs) with therapeutic vaccines.[78] By decreasing antigen levels before vaccination, T cells may be better able to respond to vaccine antigens. It is currently unclear for how long and to which extent HBV antigens would need to be reduced before T cells respond to a therapeutic vaccine. In addition, it remains to be determined if additional strategies will be necessary to revive the activity of exhausted T/B cells. In this context, CHB-TI is currently being evaluated in combination with bepirovirsen for patients with CHB under NUC treatment (NCT05276297), similarly to the combination of VTP-300 with AB-729 (ACTRN12622000317796).

Directly targeting cccDNA

Although currently at the preclinical stages, strategies aimed to deplete the intrahepatic cccDNA pool remain a high priority, as this could drastically change the perspective for HBV cure.[79] Indeed, approaches to directly target cccDNA using clustered regularly interspaced short palindromic repeat/Cas9, base and new base editors that do not induce double-stranded DNA breaks have shown promising results in vitro and in animal models.[80–82] Further characterisation of their effect and safety profiles, as well as improvements in their delivery, could make these strategies a valuable tool for future combination therapies. More recently, it has been reported the discovery of a small molecule (ccc_R08) that is able to decrease the HBV cccDNA reservoir in multiple models. Although the exact mode of action of this compound remains to be determined, the analyses presented in this work suggest it to be most likely mediated by the modulation of host regulatory networks.[83 84]

New preclinical models for the characterisation of therapies against CHB

Aimed to circumvent the limitations of classic in vitro approaches and more closely recapitulate the cellular context observed in vivo, advanced three-dimensional (3D) culture models have emerged as a viable alternative to study the liver. In this regard, 3D microfluidic PHH cultures have been reported to better recapitulate the liver microenvironment, as they present functional bile canaliculi and a complete cell polarisation. Moreover, this type of system allows HBV infection and the co-culture of PHHs with additional cell types, such as Kupffer cells (KCs).[85] In this model, activation of KCs with lipopolysaccharides was able to reduced HBsAg levels, suggesting that 3D microfluidic cultures could be a useful tool to characterise novel compounds targeting innate immune responses against HBV. Other in vitro models, such as liver organoids, have been reported to support the entire HBV cycle, which can be experimentally halted by the use of tenofovir or bulevirtide.[86] Considering that organoids can be expanded and biobanked, this could represent a practical approach for the screening of novel molecules against HBV.[87] Moreover, it has recently been shown that liver organoids can be adapted to liver-on-chip systems, exhibiting enhanced in vivo-like functions and potential utility for drug-induced liver injury (DILI) risk assessment. Using this model, tenofovir/inarigivir-associated hepatotoxicity was observed and correlated with the clinical manifestation of DILI reported in patients.[88] Finally, ex vivo models such as precision-cut liver slices (PCLS) have the advantage of retaining the complex multicellular architecture of the hepatic environment, while offering the practical aspects of an in vitro model. PCLS have been described to allow HBV infection, having the potential to be a valuable tool for the preclinical characterisation of novel HTAs against HBV.[89]

Although there are ethical concerns associated with the use of animal models, the in vivo characterisation of therapeutic compounds against HBV is an approach that presents several advantages. Indeed, these models provide the ability not only to examine the hepatic microenvironment, but also the interorgan relations the liver shares. Moreover, there is the possibility to establish mice that present a humanised immune system and chimeric liver. These mice have been reported to support the HBV cycle and to develop an immune response against it, with NUC therapy decreasing HBV loads and restoring a naïve-immune phenotype.[90 91]

Improvements in the design of clinical trials

Regardless of the approach employed, it is fundamental for future clinical trials to have a better understanding of the viral and immune changes taking place within the intrahepatic compartment.[92] In this context, fine-needle aspirates (FNAs) represent a promising alternative to liver biopsies that could allow sequential liver sampling during the natural history of the disease or antiviral therapy, while minimising risk and discomfort to patients. Indeed, characterisation of liver FNAs by single-cell technologies has already provided a great opportunity to dissect intrahepatic immune responses.[93] Moreover, quantification of cccDNA and 3.5 kb RNA in FNAs by droplet digital PCR has proved as reliable as in liver biopsies.[94] The use of FNAs has started to be applied in clinical trials, as in the B-Fine Study (NCT04544956) or the IP-cure-B Study (NCT05045261), which will include repeated FNA sampling in order to characterise the immune changes associated with bepirovirsen treatment or selgantolimod followed by NUC cessation, respectively. Moreover, efforts have been made to standardise cccDNA quantification in research laboratory assays.[95] These new data will support studies to better stratify patients and guide the use of therapeutic agents until new reliable biomarkers and non-invasive methods are available.[96] Nonetheless, promising candidates have emerged in recent years, including the quantification of hepatitis B core-related antigen and circulating HBV RNA to assess the cccDNA reservoir in a non-invasive manner.[97–100]

Regarding clinical trial endpoints, it is important to consider that the best predictor of HBsAg loss is low

HBsAg levels before treatment. Therefore, future trials should stratify patients according to baseline HBsAg levels. Patients with low HBsAg levels could also take part in clinical trials designed to evaluate monotherapy or short treatment using combination approaches. Finally, the use of stringent definitions of success during clinical evaluation (eg, functional cure) could have a negative impact in the development of new therapies, potentially leading to an early halt of molecules able to improve the clinical management of HBV infection. Therefore, alternative endpoints have been suggested such as *partial HBV cure*, defined as HBsAg positive at low levels, HBeAg negative and undetectable serum HBV DNA after discontinuation of a finite course of treatment.[42] Considering that partial HBV cure is rarely achieved following NUC discontinuation, this alternative endpoint could represent a useful milestone in the path towards functional cure.

PERSPECTIVES

The landscape of therapeutic options against HBV has expanded considerably in the past years as drug discovery efforts are progressing. Now, it will be necessary to refine our understanding of which combinations are more often associated with favourable outcomes, the appropriate timing for their use and the patient populations that could benefit the most from these interventions. In this context, therapeutic strategies targeting HBV transcripts (eg, siRNAs, ASOs) or the secretion of viral antigens (eg, NAPs) have reported the highest rates of HBsAg decline and HBsAg loss. Although the long-term durability of this effect remains to be established, the use of these molecules as a therapeutic backbone could be an important approach for HBV cure. Moreover, their combination with immunomodulatory agents could help to restore HBV-specific immune responses. Therefore, longitudinal studies aimed to characterise viral and immunological responses in the intrahepatic compartment could be useful not only to monitor patients under treatment, but also to gain highly valuable mechanistic insights and the identification of novel CHB biomarkers. Altogether these investigations should provide new insight in the path towards the best combination strategies that could include potent inhibition of viral replication and blockade of cccDNA turnover, decrease of viral antigen expression to modify the T and B cell exhausting environment, immune invigoration through innate immunity boosting or immune checkpoint inhibition, and specific stimulation (or replacement) of adaptive immunity.[9 41] Currently, the most promising approach under investigation is the use of ASO (ie, bepirovirsen) in combination with NUCs which has just entered phase III clinical trials (NCT05630807). For instance, it will be interesting to see in bepirovirsen-treated patients if add-on therapies with other modes of actions or treatment stopping strategies will enhance the rate of HBsAg loss.

Although the path towards HBV cure has not been straightforward, the multiple molecules under clinical evaluation, the development of novel preclinical models and sampling techniques are important milestones that on the long term will contribute to address this unmet medical need.

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