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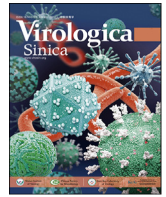

Review

Classifying hepatitis B therapies with insights from covalently closed circular DNA dynamics

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A B S T R A C T

The achievement of a functional cure for chronic hepatitis B (CHB) remains limited to a minority of patients treated with currently approved drugs. The primary objective in developing new anti-HBV drugs is to enhance the functional cure rates for CHB. A critical prerequisite for the functional cure of CHB is a substantial reduction, or even eradication of covalently closed circular DNA (cccDNA). Within this context, the changes in cccDNA levels during treatment become as a pivotal concern. We have previously analyzed the factors influencing cccDNA dynamics and introduced a preliminary classification of hepatitis B treatment strategies based on these dynamics. In this review, we employ a systems thinking perspective to elucidate the fundamental aspects of the HBV replication cycle and to rationalize the classification of treatment strategies according to their impact on the dynamic equilibrium of cccDNA. Building upon this foundation, we categorize current anti-HBV strategies into two distinct groups and advocate for their combined use to significantly reduce cccDNA levels within a well-defined timeframe.

1. Introduction

Chronic hepatitis B virus (HBV) infection constitutes a significant global public health challenge, afflicting nearly 300 million individuals worldwide (Polaris Observatory, 2018; Yuen et al., 2018). Current treatments for chronic hepatitis B, encompassing nucleoside analogs (NAs) and interferon α, can effectively manage viral replication; however, the functional cure rates within a limited treatment duration remain relatively low (Lok et al., 2016b). Accordingly, significant emphasis has been placed in recent years on developing diverse treatment strategies. Indeed, properly classifying these intricate strategies is essential for comprehending and holistically managing them.

The primary goal in developing novel anti-HBV drugs is to elevate the functional cure rate for chronic hepatitis B. A fundamental criterion for achieving a functional cure is the clearance of hepatitis B surface antigen (HBsAg) (Lok et al., 2017; Ning et al., 2019). It is now understood that HBsAg production originates from two primary sources: covalently closed circular DNA (cccDNA) and integrated HBV DNA (Pollicino and Caminiti, 2021; Grudda et al., 2022; Moini and Fung, 2022). Hence, a critical prerequisite for achieving HBsAg clearance is the significant reduction, or even eradication, of cccDNA (Block et al., 2018; Martinez et al., 2021; Li et al., 2022). In light of this perspective, variations in cccDNA levels during treatment have paramount importance, falling under the domain of cccDNA dynamics. In a prior review, we comprehensively analyzed the factors influencing cccDNA dynamics and introduced a preliminary classification of hepatitis B treatment strategies based on these dynamics (Hu and Huang, 2023).

Over the years, numerous reviews have summarized and categorized treatment strategies for chronic hepatitis B (Fanning et al., 2019; Lee et al., 2020a; Ligat et al., 2021; Naggie and Lok, 2021; Roca Suarez et al., 2021; Tsounis et al., 2021; Mak et al., 2022; Dusheiko et al., 2023; Lim et al., 2023; Pan et al., 2023). However, some aspects of these classifications are do not align with cccDNA dynamics. These classifications generally follow this process: first, they categorize strategies according to their fundamental mechanisms, whether they directly target the virus, impact host factors, or modulate the immune system, forming an initial level classification. Subsequently, direct antiviral drugs are further categorized according to the specific stages of the HBV life cycle they target, such as preventing virus entry, inhibiting cccDNA transcription, or interfering with nucleocapsid formation, among other actions. Alternatively, they can be classified according to their effects on cccDNA, including direct inhibition of cccDNA formation, promotion of cccDNA degradation, or modulation of cccDNA transcriptional activity. While objective and easy to grasp, this classification approach does not fully

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align with cccDNA dynamics. In this context, we explore the essence of the HBV replication cycle through a systems thinking perspective and assert the following: 1. HBV with a dynamic replication pattern; 2. This pattern encompasses a continuous closed-loop process without a distinct starting or ending point; 3. All processes and nodes within this closed loop exhibit bidirectional relationships, with each node or process both upstream and downstream of another node or process; 4. Integral molecules carrying the complete genetic information of HBV occupy critical positions within this closed loop; 5. These genetic information carriers exist in dynamic equilibria; 6. Their half-lives vary; 7. cccDNA serves as the rate-limiting node in the clearance of HBV genetic information *in vivo*; 8. Classifying treatment strategies based on their influence on the dynamic equilibrium of cccDNA is a sound approach. Building on this foundation, we divide the primary anti-HBV strategies into two distinct categories: the first aims to reduce cccDNA synthesis, and the second seeks to influence cccDNA decay. We argue that relying solely on the first category of strategies or combining various drugs from this category is insufficient for eliminating cccDNA within a reasonable timeframe. A more effective approach to achieving a functional cure necessitates integrating the first category with the second category of strategies.

2. HBV replication: a dynamic replication pattern without a clear starting or ending point

Current evidence suggests that viruses do not constitute self-sustaining life forms but replicate by exploiting the host's cellular machinery. The instructions guiding virus replication are typically encoded within its genome sequence. Albeit viruses provide the code, it must be deciphered by the host-provided molecular machinery. The host cell's processes, such as transcription, translation, material transport, secretion, and endocytosis, are harnessed to replicate viral genetic information (Fig. 1A). While genetic information is physically encoded as a sequence of nucleic acids, it ultimately gives rise to complex behavior patterns. Consequently, the replication of genetic information entails the reproduction of a specific set of behavioral patterns. Using the example of HBV, its complete genetic information is housed within the cccDNA. It can be posited that HBV replication involves the duplication of cccDNA or the reproduction of HBV viral particles (Fig. 1B). Both of these statements are accurate, although they may not provide a comprehensive description of the process. Both virus particles and cccDNA are nodes within the broader HBV replication cycle. The virus does not merely replicate a single node; instead, it replicates the entire replication cycle. This replication cycle embodies a dynamic closed-loop process, interconnected with no distinct start or end (Fig. 1B). It constitutes a set of behavioral patterns, and the genetic information within the nucleic acid governs the specific content of these behavioral patterns.

The dynamic closed-loop system replicated by the virus centers around replicating its genetic information. Thus, this system's various nodes and links can be differentiated based on the state and location of the viral genetic material. These nodes and links can be visualized in a diagram of the virus's life cycle. In the case of HBV, key discernible links in its life cycle diagram encompass the process of infection and entry into cells, the process of relaxed circular DNA (rcDNA) transportation and release into the nucleus, the process of rcDNA transformation into cccDNA and the formation of minichromosomes, the process of viral RNA transcription using cccDNA as a template, the process of translating viral RNA into various viral proteins, the process of pregenomic RNA (pgRNA) packaging by polymerase and core protein, the process of synthesizing HBV DNA after packaging, and the process of packaging nucleocapsids with the envelop and their subsequent release from cells (Grimm et al., 2011; Karayiannis, 2017; Tsukuda and Watashi, 2020) (Fig. 1A). In these processes, genetic information undergoes conversion and transmission in a closed-loop manner: rcDNA-cccDNA-pgRNA-rcDNA. Although it may seem like the sequence starts with rcDNA, this closed-loop process lacks any distinct starting or ending point. The closed loop concept conveys a vital fact: any link (or node) in the HBV replication is both upstream and downstream of another link or node. For instance, transcribing pgRNA from cccDNA makes pgRNA appear downstream of cccDNA. However, given that pgRNA ultimately serves as a template for synthesizing rcDNA, the rcDNA present in secreted virions may, in turn, infect hepatocytes and form cccDNA. Thus, from the standpoint of the viral population and genetic information replication, pgRNA is an upstream molecule relative to cccDNA.

3. Every node in the closed loop of HBV replication is in a dynamic equilibrium

Emphasizing the HBV replication cycle as a closed-loop process is a systems thinking approach (Kesic, 2019; Momsen et al., 2022), underscoring analyzing various nodes and links in this process from a dynamic perspective. Dynamic analysis primarily considers three parameters: stock, inflow, and outflow. The relationships between these parameters can be visually represented using a stock-flow diagram (Fig. 2A). For instance, we can focus on the HBV virus particles present in the serum and construct a dynamic analytical diagram. At a particular time point, the quantified count of circulating HBV particles is established at, for instance, 10^{10}, with its subsequent alterations contingent upon the influx and efflux rates. Inflow represents the number of newly released viruses into the bloodstream during this period, while outflow is the number of viruses that wane. If inflow equals outflow, the stock remains constant; if outflow exceeds inflow, the virus stock diminishes. Stocks are readily measurable – for instance, measuring serum HBV DNA titers directly reflects the stock. However, flow is more challenging to detect and is often overlooked, explaining why dynamic perspectives on these issues are often elusive.

In theory, detecting inflow necessitates blocking outflow completely and observing the increase in stock over time. Conversely, it is imperative to obstruct inflow and monitor stock depletion over time to detect outflow. Detecting inflow for serum viruses is intricate, given our lack of knowledge about how these circulating viruses degrade and the lack of means to inhibit virus decay. Nevertheless, detecting outflow is feasible due to our capacity to impede new virus production with direct antiviral agents (DAAs). The advent of DAAs has enabled us to unveil the dynamic fluctuations of viruses within the body. For instance, David Ho et al. used protease inhibitors to reveal that HIV circulating in the bloodstream is highly dynamic, with infected individuals having to produce 10.3×10^9 new virus particles every day to counter daily virus decay (with a virus half-life of just 6 h) (Ho et al., 1995; Perelson et al., 1996), suggesting that, in the absence of new virus generation, the virus load would halve every 6 h. Similarly, in 1996, Nowak et al. employed lamivudine to uncover the dynamic changes of circulating HBV in HBV-infected individuals. Some patients were found to produce 10^{11} new virus particles daily, and the half-life of circulating HBV was approximately one day (Nowak et al., 1996).

Virus particles containing rcDNA in circulation constitute a node in the closed-loop replication of HBV genetic information. Meanwhile, pgRNA in hepatocytes can be regarded as another crucial node (Fig. 2B). PgRNA guides the translation of polymerase and core protein and, together with specific host factors, facilitates the conversion and transmission of genetic information by synthesizing new rcDNA. The production of pgRNA stems from cccDNA transcription, representing the inflow, while the outflow pathways entail degradation or packaging and release as virus particles. By impeding pgRNA production, we can determine the half-life of pgRNA. However, since there are currently no specific drugs that can effectively block pgRNA transcription and detecting alterations in pgRNA within the liver is challenging, determining the half-life of pgRNA in human livers remains elusive. Nevertheless, based on data from *in vitro* cell models, it is possible to estimate the half-life of pgRNA, which ranges from 4.5 to 7.7 h (Heise et al., 2006; Imam et al., 2018; Liu et al., 2021). Assuming a similar situation *in vivo*, it suggests that blocking pgRNA production would result in a halving of intracellular pgRNA every 8 h, and the decay rate is faster than that of HBV virus particles in circulation.

4. cccDNA is the rate-limiting node for HBV clearance

In addition to rcDNA and pgRNA, another pivotal node in the replication of HBV genetic information is cccDNA (Fig. 2B). cccDNA serves as the transcription template for all viral RNA and preserves the complete genetic information. Similarly, detecting the outflow of cccDNA necessitates blocking cccDNA synthesis and observing the change in cccDNA quantity over time. cccDNA emerges from the conversion of rcDNA, with various intermediate molecules like DP-rcDNA (or PF-rcDNA) (Gao and Hu, 2007; Guo et al., 2007; Xia and Guo, 2020), situated between rcDNA and cccDNA. Blocking the formation of these intermediate molecules or their transformation into cccDNA can certainly hinder cccDNA synthesis. Moreover, obstructing the synthesis of more upstream rcDNA can prevent cccDNA formation, which is achievable with nucleoside analogs (NAs). As we mentioned in a previous review, there are multiple approaches for estimating the half-life of cccDNA, but the conclusions vary considerably (Hu and Huang, 2023). These disparities are potentially linked to the diversity in hepatocyte lifespan. For analytical convenience, we assume that the half-life of cccDNA is 60 days in the absence of an immune response against HBV. Clearly, in comparison to viral particles and intracellular pgRNA, cccDNA decays at a much slower rate. In other words, when the synthesis of the three components is blocked, cccDNA diminishes at the slowest pace. For example, completely blocking pgRNA synthesis results in a halving of intracellular pgRNA every 8 h and

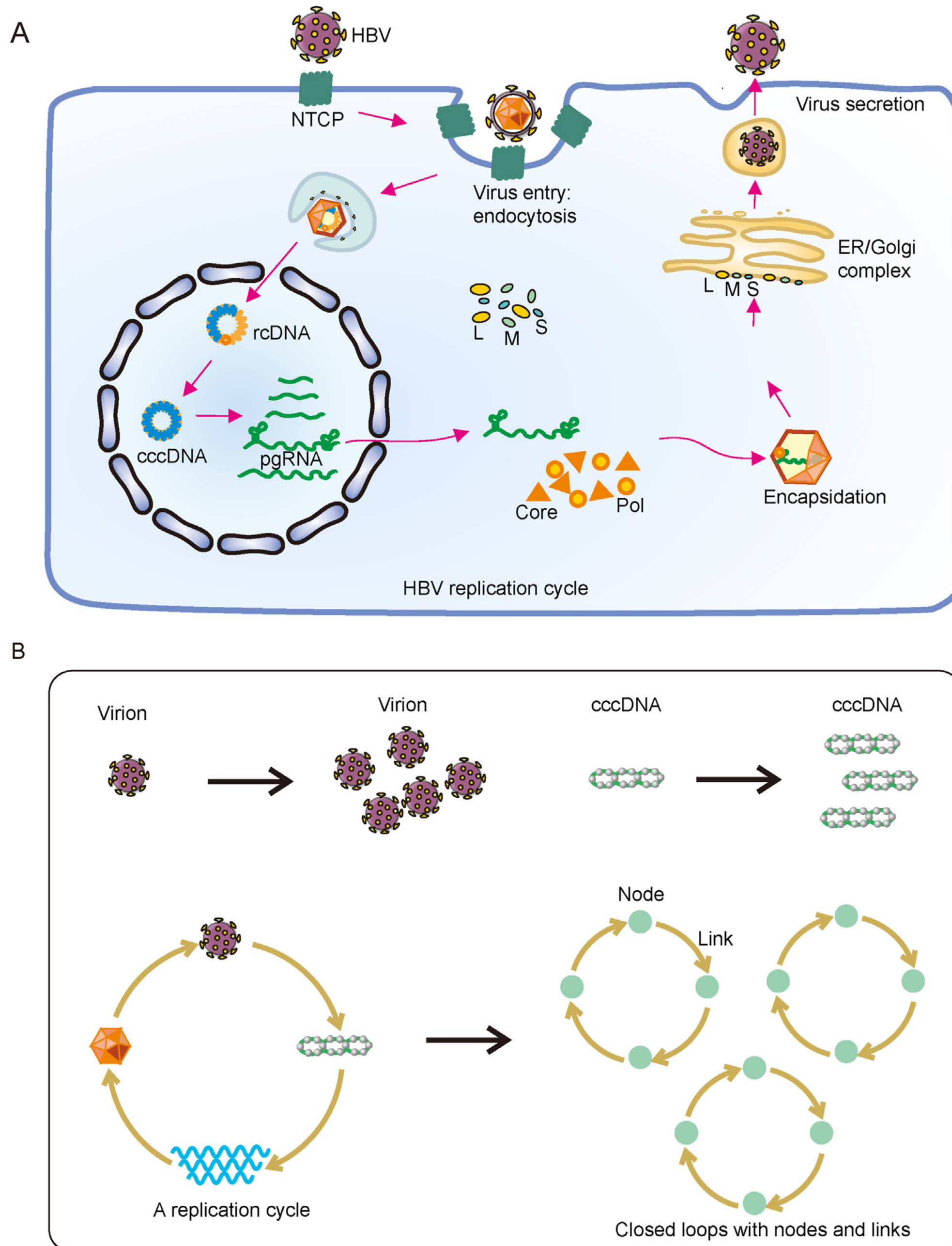

Fig. 1. HBV Replication and its Pattern. **A** Replication cycle of HBV. **B** Representation of HBV replication from a population perspective. HBV follows a genetic information-directed replication pattern, represented as a closed loop without a distinct beginning or end, composed of interconnected nodes and links. Both virions and cccDNA are nodes within this loop. The virus's replication encompasses the entire cycle, highlighting the importance of considering the complete HBV replication process.

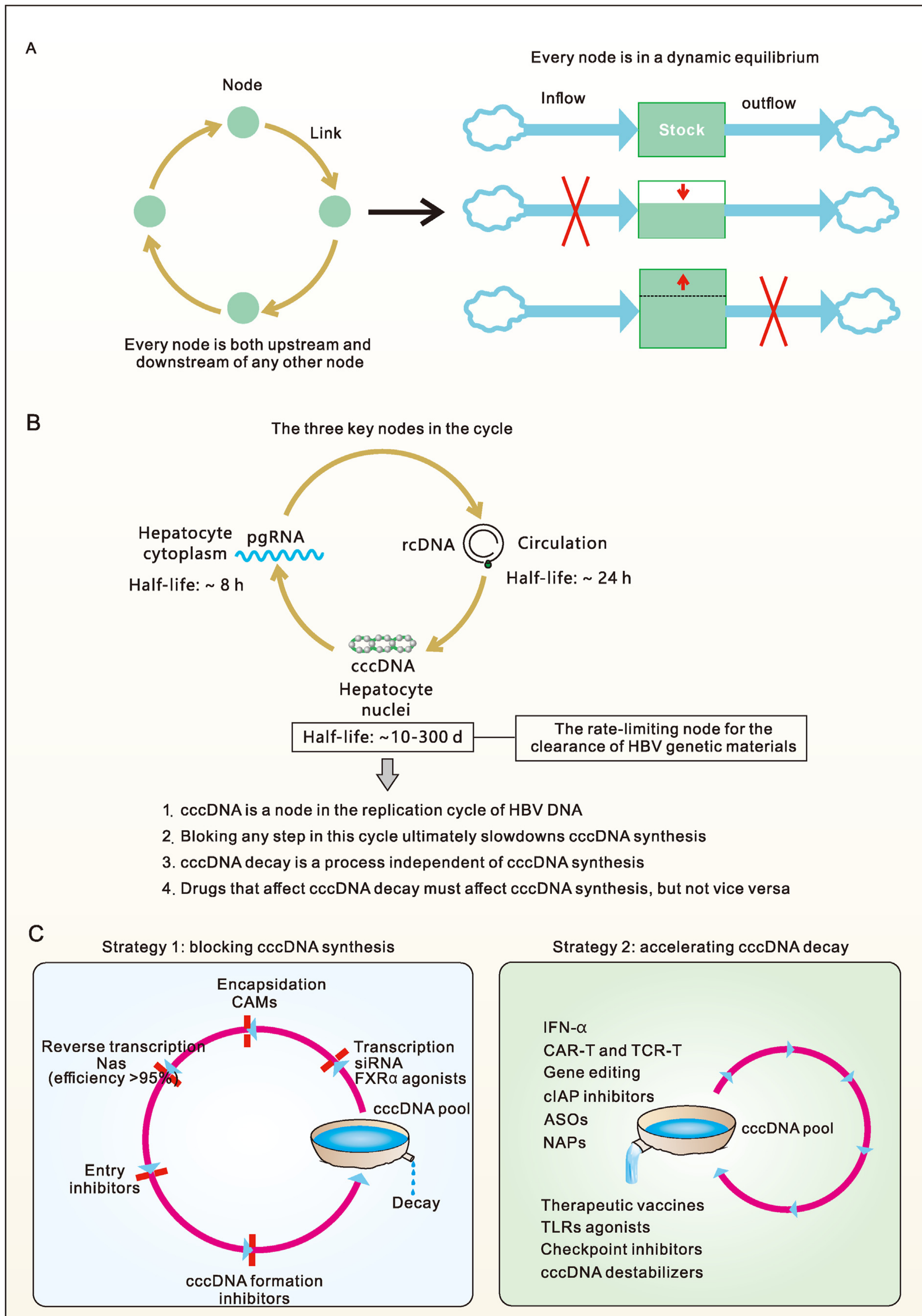

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Fig. 2. Classification of anti-HBV drugs into two categories based on their impact on cccDNA dynamics. **A** Dynamic equilibria of nodes in the HBV replication cycle. Every node within the HBV replication cycle is in a state of dynamic equilibrium. Each node is both upstream and downstream of other nodes, with materials in each node subject to dynamic balance. This equilibrium is described by three parameters: stock, inflow, and outflow. Stock can be directly measured, but assessing outflow or inflow requires observing stock changes over time after blocking inflow or outflow. **B** Three critical nodes in the HBV replication cycle. The replication cycle of HBV involves three pivotal nodes: rcDNA in virions, pgRNA in hepatocyte cytoplasm, and cccDNA in hepatocyte nuclei, each containing complete genetic information of HBV. These nodes exhibit distinct dynamic equilibrium characteristics with varying half-lives. cccDNA is the rate-limiting node for clearing HBV genetic materials from the body. While blocking any step in the replication cycle can reduce cccDNA synthesis, it may not affect cccDNA decay significantly, as cccDNA decay is a process partly independent of cccDNA synthesis. **C** Classification of anti-HBV strategies according to their effects on cccDNA dynamics. Combining various approaches within Strategy 1 may not significantly enhance therapy efficacy, as first-line NAs are already highly efficient at suppressing the cycle. Strategy 1-based approaches, unless accompanied by unexpected effects, are unlikely to impact the cccDNA decay rate or the existing cccDNA pool. To achieve improved outcomes, it is essential to complement Strategy 1 with Strategy 2, which aims to expedite the cccDNA decay rate.

circular rcDNA every 24 h. Halting rcDNA synthesis leads to a halving of circulating rcDNA every 24 h, and without rcDNA replenishment, cccDNA will halve every 60 days (though the presence of rcDNA may cause a slight delay in the halving time). In this context, the production of pgRNA depends on the cccDNA stock, and its decline rate is akin to that of cccDNA. In essence, cccDNA emerges as the rate-limiting node for virus clearance as long as any link in the HBV replication cycle is blocked.

When comparing hepatitis C virus (HCV) and HBV, it becomes evident that HCV's entire replication cycle lacks a genetic information carrier akin to cccDNA. HCV belongs to the positive-strand RNA virus family, and its replication occurs in the cytoplasm (Kim and Chang, 2013; Alazard-Dany et al., 2019). The molecules carrying complete genetic information are all RNA, including positive-strand RNA and negative-strand RNA. Research has shown that in cell models, the half-life of intracellular HCV RNA is merely 2–3 h (Sedano and Sarnow, 2014; Mata et al., 2019), shorter than the half-life of HBV pgRNA (8 h). When replication of HCV RNA is obstructed (such as by NS5B or NS5A inhibitors), intracellular HCV RNA swiftly decays. The half-life of HCV virus particles in circulation is as brief as 45 min (Chatterjee et al., 2013; Nguyen et al., 2017). In essence, the most significant rate-limiting node for eliminating HCV genetic information is intracellular RNA. In contrast to HBV cccDNA, this rate-limiting node presents little impediment. These distinctions create favorable conditions for the cure of HCV infection. Assuming the total amount of HCV RNA in the liver is 1×10^{14} (calculated based on an average of 500 copies per cell) and RNA synthesis is blocked, the virus's genetic material can be entirely cleared from the body within one week, considering a half-life of 3 h. Considering that drug inhibition efficiency does not reach 100%, clinical practice might require a longer time frame. For instance, in a clinical trial, the combination of sofosbuvir + ledipasvir with non-nucleoside analog inhibitor GS-9669 or a protease inhibitor GS-9451 achieved viral clearance within six weeks (Nguyen et al., 2017). Given the substantial differences in the dynamics of key nodes in the replication cycle of genetic information between HBV and HCV, the challenge of curing HBV infection should not be equated with curing HCV infection.

5. Classifying treatments for chronic HBV infection based on cccDNA dynamics

Given that cccDNA functions as the rate-limiting node for clearing the HBV replication cycle, treatment strategies should focus on cccDNA. When a certain approach can reduce cccDNA, it inevitably diminishes the overall scale of the HBV replication cycle. By concentrating on cccDNA, we can comprehensively analyze cccDNA stock and flow. cccDNA stock signifies the total amount of cccDNA in the liver, approximately 10^{10}, estimated at 0.1 copies per cell before treatment. Inflow pertains to the newly synthesized cccDNA over a specific time frame. Based on the previous analysis, factors influencing cccDNA synthesis encompass all links in the HBV replication cycle except for the cccDNA node itself, including RNA transcription, rcDNA synthesis, virus entry into cells, and more, as these links are upstream events of cccDNA synthesis (although it remains uncertain whether changes in these various links have an equal impact on the synthesis rate of cccDNA). Outflow corresponds to the amount of cccDNA lost over a certain period. In the absence of an immune response, cccDNA's lifespan likely parallels that of the hepatocytes in which it is located, with hepatocyte division and death being the primary drivers of its natural decline. The ultimate treatment objective is to reduce the cccDNA stock until it vanishes, which can be achieved by either decreasing cccDNA synthesis or accelerating cccDNA decay, or a combination of both. This categorizes HBV treatment strategies into three primary categories: Strategy 1 is centered on reducing cccDNA synthesis, Strategy 2 aims to expedite cccDNA decay, and the third category encompasses those with dual effects. For the sake of simplification, we will not treat the third category as a distinct entity but rather include it in the second category (Fig. 2C). It should be noted that expediting cccDNA decay naturally results in a decrease in cccDNA synthesis, achieved by limiting pgRNA production. However, suppressing cccDNA synthesis does not necessarily affect cccDNA decay, as cccDNA decay processes operate to some extent independently of the cccDNA synthesis cycle.

Among the approved drugs, NAs inhibit rcDNA synthesis by targeting DNA polymerase and fall under Strategy 1 (Fig. 2C). IFNα can impact cccDNA degradation and transcription and corresponds to Strategy 2. Among the investigated drugs, capsid assembly modulators (CAMs) obstruct rcDNA synthesis by interfering with pgRNA packaging or reduce rcDNA releasing and subsequent cccDNA synthesis by inhibiting capsid disassembly, falling under Strategy 1. Drugs that directly target cccDNA or impact infected hepatocyte, such as CRISPR/Cas9, cccDNA destabilizers (ccc_R08), CAR-T, TCR-T, and others, promote the decay of stock cccDNA and thus align with Strategy 2 (Fig. 2C). Some drugs, including nucleic acid polymers (NAPs) and antisense oligonucleotides (ASOs), might induce hepatocyte death, likely by stimulating immune responses, so they can be classified under Strategy 2. Various immune-based strategies, like therapeutic vaccines and immune modulators, aiming to restore HBV-specific immune responses, fall within Strategy 2.

6. Strategy 1 alone cannot clear cccDNA within a reasonable period

For drugs in Strategy 1, whether used in isolation or combination, it is not feasible to eliminate cccDNA within a reasonable timeframe, assuming that this reasonable timeframe is less than three years, since according to our classification criteria, the first-class drugs can exclusively prevent cccDNA synthesis without affecting its decay, resulting in a very slow reduction in the cccDNA stock.

6.1. Theoretical calculation

Given that Strategy 1 does not expedite cccDNA decay, the existing cccDNA pool must decrease due to "natural forces". In patients without an effective immune response against HBV, these "natural forces" are mainly driven by hepatocyte turnover, involving cell death and division processes (Fourel et al., 1994; Zhu et al., 2001; Mason et al., 2009; Allweiss et al., 2018; Tu et al., 2022; Ding et al., 2023; Hu and Huang, 2023). The impact of these "natural forces" on the cccDNA pool in CHB patients can be unveiled by blocking cccDNA replenishment through NAs. The cccDNA pool declines with an observed half-life (as discussed in our previous study (Hu and Huang, 2023)). Assuming an optimistic

scenario where the cccDNA pool has a half-life of 60 days, it would still require over six years to clear 10^{10} cccDNA copies from the liver, even with an agent that completely halts cccDNA synthesis.

6.2. Heterogeneity in cccDNA half-life

As calculated above, if the first class of drugs is expected to clear cccDNA within three years, it requires that the half-life of cccDNA is less than 30 days. Therefore, how long the half-life of cccDNA becomes important. There are two main methods for estimating the half-life of cccDNA. The first method is based on the second phase of serum viral load decline during short-term (one-month) NAs treatment. The other depends on the change in the abundance of intrahepatic cccDNA before and after NAs treatment. As we mentioned in a previous review, different estimation methods lead to different results: as the treatment time is prolonged, the decline in cccDNA becomes slower, or the half-life of cccDNA becomes longer. During short-term treatment (one month), the half-life is 7–18 days (Tsiang et al., 1999; Wolters et al., 2002a, 2002b; Goncalves et al., 2021); during mid-term treatment (one year), it is more than 10 weeks (Werle-Lapostolle et al., 2004; Wong et al., 2006; Takkenberg et al., 2011; Zheng et al., 2014; Bowden et al., 2015; Lai et al., 2017); and when the observation period is longer (such as 10 years), the half-life of cccDNA can be as long as 300 days (Boyd et al., 2016; Lai et al., 2017; Wang et al., 2022). This phenomenon leads us to believe that the half-life of cccDNA is heterogeneous, likely due to the heterogeneity of hepatocyte turnover rate (or lifespan).

Measurements of rat, mouse, and human hepatocyte lifespans have shown that there is indeed significant heterogeneity in hepatocyte lifespan. H3 thymidine labeling and tracking of rat hepatocytes revealed that although 41% of labeled cells disappeared (died or divided) within 60 days, the lifespan of the remaining labeled cells could exceed six months (Macdonald, 1961). Similar experiments in mice showed that the proportion of labeled cells decreased from an initial 33.7%–16.6%, 10.9%, and 7.9% at 100, 200, and 300 days, respectively (Magami et al., 2002). Although about half of the labeled cells had a lifespan of less than 100 days, nearly a quarter had a lifespan of more than 300 days. For ethical reasons, it is not feasible to use similar labeling methods to observe the lifespan of human hepatocytes. However, Heinke et al. recently used changes in the ^{14}C content of hepatic genomic DNA (^{14}C birth dating) to study human hepatocyte lifespan and turnover rate (Heinke et al., 2022). It was found that human hepatocytes could constantly renew, with an average age of less than three years, but the renewal rates of different hepatocytes varied significantly, with diploid hepatocytes renewing at a rate 7 times faster than polyploid hepatocytes. Although 95% of diploid cells are renewed within a year, 12% of polyploid hepatocytes can survive for 10 years. It can be imagined that if HBV infects these cells, cccDNA formed in them may also exist for more than ten years. If HBV infects these long-lived hepatocytes and forms cccDNA, even the strongest first-class drugs will be powerless because they have no direct effect on these existing cccDNA.

6.3. The efficacy of Strategy 1 in clinic trials

6.3.1. Nucleot(s)ide analogs

Among the first class of drugs, NAs have been clinically applied for a long time, with good safety and reliable efficacy (Fung et al., 2011; Lampertico et al., 2016). It has become the main antiviral drug for treating chronic hepatitis B and should also be the mainstay of future functional cure drug combinations for chronic hepatitis B.

During treatment with NA, the serum HBV DNA load follows a significant biphasic decline trend, characterized by a rapid decline in the first phase and a slower decline in the second phase (Tsiang et al., 1999). The duration of the first phase depends on the drug's efficiency in inhibiting HBV DNA production. For instance, with an inhibition efficiency of 90%, the duration of the first phase theoretically lasts approximately 3–4 days (duration referring to the time from the start of drug administration to the point of inflection in the decline rate of HBV DNA). If the inhibition efficiency is 99%, the duration extends to 6–7 days; with an efficiency of 99.9%, it extends to about 10 days. This pattern is determined by the serum HBV DNA dynamics. This biphasic decline has been observed in patients receiving various treatments, including NAs, CAMs, siRNA, and combination therapy. The first phasic decline is attributed to the decay of serum virion and the second phase is mainly driven by the turnover of infected hepatocytes or the decline of cccDNA (Tsiang et al., 1999; Hu and Huang, 2023).

Combination therapy based on NAs must yield additional benefits compared to NAs monotherapy to be considered valuable. Therefore, in clinical trials, comparing the efficacy of NAs monotherapy with NA-based combination treatments is a crucial aspect of assessing the clinical value of new drugs. Drugs with different mechanisms of action can theoretically produce synergistic effects, making them an attractive strategy.

6.3.2. Capsid assembly modulators

Besides NAs, CAMs have received significant attention among the first class of drugs. CAMs can be categorized into two groups (Cole, 2016; Nijampatnam and Liotta, 2019; Kim et al., 2021; Taverniti et al., 2022; Zoulim et al., 2022; Bassit et al., 2023). The first group disrupts the normal assembly of core protein polymers into nucleocapsids by binding to the core protein, including heteroaromatic dihydropyrimidine (HAP) molecules. The second group induces the formation of empty, non-functional nucleocapsids, lacking pgRNA, after binding to the core protein, and this group includes benzoylimide (PP), sulfonylbenzamide (SBA), and 2-oxoacetamide (GLP) molecules.

In a phase 1b clinical trial of GLS4, a HAP-class drug (Zhang et al., 2021), 24 untreated patients with chronic hepatitis B were randomly divided into two groups: a GLS4 (plus ritonavir) combined with entecavir (ETV) treatment group and an ETV monotherapy group. After 28 days of treatment, it was found that the decrease in HBV DNA in the combined treatment group (120 or 240 mg dose) was not as significant as in the ETV monotherapy group (decline of −1.42, −2.13, and −3.5 $\log_{10}$ IU/mL), and no synergistic effect was observed.

JNJ-56136379 belongs to the second group of CAMs. In a phase 2 clinical trial (Janssen et al., 2023), 232 patients with chronic hepatitis B were randomly assigned to a 75 mg or 250 mg monotherapy group, an NA monotherapy group, or a JNJ-56136379 + NA combined treatment group with a treatment period of ≥24 weeks. The results showed that at 24 weeks of treatment in HBeAg(+) patients, the decreases in HBV DNA in the JNJ-56136379 (75 mg) + NA, JNJ-56136379 (250 mg) + NA, and placebo + NA groups were 5.53, 5.88, and 5.21 $\log_{10}$ IU/mL, respectively, indicating a certain synergistic effect. The dynamic change curve of serum HBV DNA titer revealed that the decrease in the combined treatment group was greater than that in the NA monotherapy group at one week after treatment. Assuming that the decay rate of HBV in the blood circulation is the same and ignoring individual differences among patients, this difference can be attributed to variations in inhibitory efficiency. However, more detailed data are required to estimate inhibitory efficiency precisely. On the other hand, during the second phase (2–24 weeks) of HBV DNA decline, there was no significant difference in the decline rate (slopes of the curves) among different groups, indicating that there might not be a substantial difference in the decay rate of cccDNA, consistent with the theoretical analysis results that the decay rate of cccDNA is independent of cccDNA synthesis.

Several other CAMs, including Espiritu et al. (2023), ALG-000184 (Chen et al., 2023), EDP-514 (Feld et al., 2022), ZM-H1505R (Jia et al., 2023), and more, are currently undergoing trials. ALG-000184, a class II CAM, has demonstrated strong efficacy with picomolar potency in inhibiting HBV DNA in cell models. In addition, ALG-000184 has inhibited the production of HBsAg *in vitro* through the regulation of the de-novo establishment and replenishment of cccDNA, suggesting a secondary mechanism of action (Zhang et al., 2020). In a phase 1 clinical trial, ALG-000184 combined with ETV for 48 weeks resulted in a reduction of serum HBV DNA levels by up to 5.2 $\log_{10}$ IU/mL, and the

serum HBsAg levels of five patients in the 300 mg dose group also decreased, with a maximum decrease of 0.78 $\log_{10}$ IU/mL) (Hou et al., 2023). Interestingly, all five patients exhibited elevated ALT, indicating transient hepatocellular injury. Whether this injury reflects a mechanism distinct from other CAMs requires further investigation.

6.3.3. Entry inhibitors

Strategies that interfere with the HBV infection of hepatocytes also fall under type I. These strategies may involve monoclonal antibodies targeting S or preS1, peptides, and small molecules targeting the HBV receptor, sodium taurocholate co-transporting polypeptide (NTCP) (Yan et al., 2012). While these approaches can disrupt the binding of HBV to the receptor and potentially accelerate the decline in circulating virus levels, they primarily affect the synthesis rate of cccDNA and do not impact the decay rate of existing cccDNA. Additionally, though anti-S monoclonal antibodies can rapidly reduce circulating HBsAg levels by accelerating decay, their contribution to rebuilding HBV-specific immunity may be limited (Fumagalli et al., 2020). Furthermore, because monoclonal antibodies do not directly influence the rate at which integrated HBV DNA and existing cccDNA generate HBs, they require continuous administration to suppress circulating HBsAg, raising cost and cost-effectiveness concerns.

Clinical trials of monoclonal antibodies have not shown better-than-expected results. In a clinical trial testing the anti-HBs monoclonal antibody Tuvirumab, 9 out of 10 patients discontinued treatment early due to its poor efficacy, and HBsAg levels exhibited only temporary decreases (van Nunen et al., 2001). Another phase 1 clinical trial evaluated the effect of a mixed formulation of two monoclonal antibodies, HBV-ABXTL (Galun et al., 2002). The results indicated that different dose groups (injected once a week for a total of 4 times) displayed similar response patterns: HBV DNA and HBsAg rapidly decreased after each drug administration, becoming undetectable in some cases, but gradually increased before the next drug administration, sometimes returning to pre-treatment levels. A study by Lee et al. assessing the effect of another recombinant monoclonal antibody, Lenvervimab, showed a response pattern of HBsAg similar to previous studies (Lee et al., 2020b). HBsAg levels rapidly decreased after drug administration but significantly rebounded between administrations. Twenty days after drug withdrawal, HBsAg levels returned to near pre-treatment levels (Lee et al., 2020b).

Myrcludex B (bulevirtide) is the first inhibitor targeting NTCP for HBV entry (Volz et al., 2013; Blank et al., 2016; Cheng et al., 2021). It binds to the HBV receptor NTCP, competitively inhibiting viral infection of cells. However, it primarily reduces the entry of rcDNA into cells and the subsequent synthesis of cccDNA without affecting existing cccDNA. As such, drugs of this type are not expected to yield better anti-HBV effects than NAs. In two phase 2 clinical trials, bulevirtide combined with TDF or IFNα did not significantly reduce HBsAg levels (Bogomolov et al., 2016; Wedemeyer et al., 2023). Bulevirtide has demonstrated efficacy in treating HDV infection and has been approved for this purpose (Wedemeyer et al., 2023). When combined with TDF, it can reduce the serum HDV RNA levels of 77% of HDV-infected individuals by more than 2 $\log_{10}$ IU/mL or below the detection limit. However, this does not necessarily translate to the same effectiveness against HBV infection. The difference is attributed to the fact that HDV, like HCV, is also an RNA virus, and its replication cycle lacks DNA molecules to store genetic information. Due to the short half-life of RNA molecules in liver cells, after blocking the synthesis of new HDV RNA, existing RNA molecules can decay and disappear relatively quickly.

6.3.4. FXRα agonists

The regulation of HBV cccDNA's promoter and enhancer activity involves multiple transcription factors, among which some are nuclear receptors highly concentrated in hepatocytes, such as HNF4α, PPARα/RXRα, and FXRα (Kim et al., 2016; Radreau et al., 2016; Turton et al., 2020). In both cell and mouse models, the FXRα agonist GW4064 has demonstrated its ability to decrease HBV RNA and cccDNA levels (Mouzannar et al., 2019). Another FXRα agonist, Vonafexor, was evaluated in a clinical trial (Erken et al., 2021). The results indicated that at a dose of 400 mg/day, HBsAg levels decreased by 0.1 $\log_{10}$ IU/mL after one month of treatment. However, HBV DNA levels did not show a significant reduction, suggesting a relatively weak therapeutic effect.

6.3.5. siRNA

We classify siRNA into Strategy 1 based on clinical evidence suggesting that it does not affect cccDNA decay. The primary mechanism of action for siRNA is degrading target mRNA and inhibiting its translation. Notably, in 2018, the FDA approved the first siRNA drug for hereditary transthyretin amyloidosis treatment (Aimo et al., 2022), sparking significant interest in the potential of siRNA therapeutics. Typically, siRNA is designed as a 21-base pair double-stranded structure with a 2-base pair overhang at the 3' end. To enter cells, siRNA usually requires modification, such as N-acetylgalactosamine modification, to leverage the asialoglycoprotein receptor on hepatocyte surfaces. Inside the cell, the siRNA's guide strand (antisense strand) forms an RNA-induced silencing complex (RISC) with various host cell factors, enabling RISC to bind the target RNA sequence and exert its inhibitory function (Hui et al., 2022).

ARC-520, the first siRNA drug for chronic hepatitis B treatment (Wooddell et al., 2013), exhibited promising results in a phase 2 clinical trial. A single dose of ARC-520 reduced HBsAg levels by 1.4 $\log_{10}$ IU/mL in HBeAg(+) patients, albeit without significantly affecting HBeAg(−) patients. Further investigations suggested that ARC-520 failed to target HBV RNA transcribed from integrated HBV DNA (Wooddell et al., 2017). In an improved clinical trial of ARC-520, patients on NA treatment were randomly assigned to receive ARC-520 or a placebo. The ARC-520 treatment group received four doses (one per month). The results indicated that ARC-520 reduced HBsAg levels by 0.54 $\log_{10}$ IU/mL in HBeAg (+) patients and by 0.38 $\log_{10}$ IU/mL in HBeAg(−) patients (Yuen et al., 2020). Accordingly, prolonged ARC-520 treatment produced better outcomes (Yuen et al., 2022a). However, its development was discontinued due to concerns regarding excipient toxicity. Other siRNA drugs include RG-6346, AB-729 (Mak et al., 2021), and more. In clinical trials, they generally managed to reduce HBsAg by 1–2 $\log_{10}$ IU/mL but complete HBsAg clearance was rarely achieved (Man-Fung, 2021; Gane E. J. et al., 2023).

The potential of combining siRNA with NAs or CAMs was explored in clinical trials. In a phase 2a clinical trial, 84 chronic hepatitis B patients received three doses of JNJ-3989 at various doses while continuing NAs. The results showed that 97.5% of these patients experienced an HBsAg reduction of >1 $\log_{10}$ IU/mL (Yuen et al., 2022b). A phase 2b clinical trial, REEF-1, examined the effects of siRNA (JNJ-3989), CAM (JNJ-6379), and NA in combination or triple therapy (Yuen et al., 2023b). In this trial, 470 patients were randomly assigned to different treatment groups, each receiving at least one drug, and were followed up for 24 weeks after 48 weeks of treatment. Importantly, 19% (18/94) of patients in the JNJ-3989 (200 mg) + NA group met the criteria for NA withdrawal at 48 weeks. 9% (8/94) of patients in the JNJ-3989 (100 mg) + JNJ-6379 + NA group met the criteria for NA withdrawal, compared to 2% (1/45) in the NA monotherapy group. However, achieving HBsAg negativity was uncommon in all treatment groups.

7. Strategies to accelerate cccDNA decay

Accelerating cccDNA decay can be achieved by either noncytopathic or cytopathic effects. Pegylated interferon α (PEG-IFNα) is the only available treatment that can accelerate cccDNA degradation by non-cytopathic effects (Liu et al., 2013; Lucifora et al., 2014). However, limited efficacy, side effects, and the need for injection make PEG-IFNα an unsuitable option for terminating chronic HBV infection. Although the exploration of mechanisms by which PEG-IFNα degrades cccDNA deepens our understanding of its pharmacological action (Lucifora et al., 2014), it has yet to result in more effective agents targeting cccDNA.

7.1. CAR-T and TCR-T cell therapy

The recovery from acute HBV infection requires an HBV-specific cellular immune response (Shin et al., 2016; Iannacone and Guidotti, 2022). Activated HBV-specific CD8$^+$ effector T cells can control HBV infection by killing hepatocytes expressing HBV antigens (Webster et al., 2000; Thimme et al., 2003; Asabe et al., 2009). After recovery from acute infection, most infected individuals have not completely cleared the virus from their bodies. About 20–30 years after recovery from infection, trace amounts of viral DNA can still be detected in the circulation of some individuals, suggesting that these recovered individuals rely on immune responses to continuously suppress the residual trace amounts of the virus in the body (Michalak et al., 1994; Rehermann et al., 1996). Chronic HBV-infected individuals in the immune tolerance phase lack effective immune responses to HBV. HBeAg/HBeAb seroconversion indicates that the infected person has acquired a cellular immune response against HBe/HBc (Milich and McLachlan, 1986). This immune response has a partial protective effect, reducing the viral load of infected individuals by approximately 3 $\log_{10}$ IU/mL (Volz et al., 2007). However, the immune response against HBs is still lacking, resulting in the maintenance of a considerable level of viral infection.

Using CAR-T or TCR-T technology to modify T cells so that they can recognize and kill HBs-expressing liver cells is theoretically an effective strategy for treating HBV infection (Bertoletti and Tan, 2020; Tan and Schreiber, 2020). The expression of HBs in liver cells is not limited to those infected with HBV but also includes those containing integrated HBV DNA. Killing these cells will help reduce the antigen load in the body. In a humanized liver mouse model, it was found that transfer of TCR-T cells could reduce viral markers by 4–5 $\log_{10}$ IU/mL, even to undetectable levels (Wisskirchen et al., 2019). However, 8–10 weeks after cell transfer, virological markers began to rebound. The combination of Myrcludex B enabled long-term control of HBV infection. There are currently few reports of clinical trials of CAR-T or TCR-T therapy for chronic hepatitis B. In a clinical trial for HBV-related hepatocellular carcinoma, CD8$^+$ T cells were isolated from 8 patients, then transfected and expressed TCR targeting HBs antigen epitopes, and then these cells were autologously infused multiple times. The results showed that the HBsAg levels of most patients decreased, but the magnitude was limited ($<$1.5 $\log_{10}$ IU/mL) (Meng et al., 2021).

For CAR-T and TCR-T cell therapy strategies, unfavorable factors include the limited longevity of the transferred cells in the body, the challenge in accessing and eliminating certain hepatocytes, and the potential difficulty in identifying infected hepatocytes with low HBV antigen expression. Moreover, treatment costs and adverse effects are also significant concerns. Conversely, the inherent challenge lies in the potential difficulty of eradicating the virus directly with exogenous effector T cells, given the persistence of the naturally occurring cellular immune response in recovered patients with acute infections. However, it remains plausible that T-cell therapy could contribute to the establishment of a targeted immune response against HBV, although this aspect warrants further exploration.

7.2. Gene editing

The HBV gene sequence differs from that of the host genome, which provides convenience for treating HBV infection using gene editing technology. Based on CRISPR/Cas9 and similar technologies, strategies have shown favorable results in cell and animal models, which have been extensively reviewed (Yang and Yang, 2021; Cai et al., 2023). The CRISPR/Cas9 based on *Streptococcus pyogenes* Cas9 (SpCas9) system has been widely used in previous studies. The binding of sgRNA to the HBV DNA sequence leads to Cas9 cleavage, resulting in site-specific DNA double-strand breaks (DSBs). DSBs induce the NHEJ repair process, resulting in the inactivation of the target gene through the formation of frameshift mutations (Martinez et al., 2022). Due to the integration of HBV DNA into the chromosomes of some hepatocytes, the formation of DSBs also leads to the risk of mutations in the host genome. Using base editing systems can help avoid this problem. For example, the cytosine base editor (CBE) can convert C-G base pairs to T-A base pairs (Kantor et al., 2020; Porto et al., 2020). Using this, it is possible to introduce a stop codon at the appropriate gene position to inactivate gene expression without causing DSBs. To be suitable for AAV vector delivery, researchers have developed smaller Cas9s, such as *Staphylococcus aureus* Cas9 (SaCas9) (Ran et al., 2015) and *Campylobacter jejuni* (CjCas9) (Kim et al., 2017). The use of AAV to deliver the SaCas9 system for treating HBV infection has also been tested in cell and animal models (Stone et al., 2021). Recently, Yi et al. co-deliveryed Cas9 mRNA and guide RNAs by SM-102-based lipid nanoparticles into mouse and tree shrew models and observed efficient editing of hepatitis B virus episomal and integration DNA (Yi et al., 2023). So far, there have been no reports on the clinical research of CRISPR/Cas9 for treating chronic hepatitis B.

Despite the hopes placed on it, some issues still need to be addressed before the clinical application of this strategy can be finalized, including safety issues, hepatocyte delivery efficiency issues, and editing efficiency issues after limited administration. Due to the large number of liver cells, even if only a few are infected, the total number is still considerable. It is extremely difficult to completely edit the cccDNA in these cells. Stone et al. showed in experiments on human hepatocyte chimeric mice that after AAV transduction of anti-HBV SaCas9, less than 1% of the sequence was successfully edited, resulting in no significant viral replication when using anti-HBV SaCas9 alone (Stone et al., 2021). Mohammad et al. screened a sgRNA (WJ11) that had a good inhibitory effect on HBV replication in cultured cells and evaluated the effect of AAV2/WJ11-Cas9 in a human hepatocyte chimeric mouse model. The results showed that compared with the control group, the circulating and intrahepatic HBV DNA levels in the treated group were reduced by less than 50% (Kayesh et al., 2020). These data suggest that there is still a long way to go before CRISPR/Cas9 can be used to treat chronic hepatitis B.

7.3. Orally available cccDNA destabilizer

Recently, a study by Li et al. identified ccc_R08 as the first orally available cccDNA destabilizer from 84600 compounds through a screening campaign based on HBV-infected human primary hepatocytes (PHHs) and using secreted HBsAg as the readout (Wang et al., 2023). In HBV-infected PHHs, treatment with ccc_R08 inhibited the levels of extracellular HBsAg, HBeAg, and HBV DNA, as well as intracellular viral HBV DNA and HBV RNA, with IC50 values ranging from 0.2 to 5 μmol/L. Additionally, ccc_R08 reduced cccDNA in PHHs and another cell line, HepDES19. These findings suggest that the effect of ccc_R08 on cccDNA is not a secondary effect resulting from the inhibition of HBV replication or transcription.

Although ccc_R08 did not affect body weight in the mouse model, it was associated with two safety flags. Hence, a derivative of ccc_R08, named ccc-R09, which did not have these issues, was further tested. ccc-R09 showed less potent activity (IC50 = 19 μmol/L) on secreted HBsAg in PHHs than ccc_R08. Unfortunately, in the uPA-SCID mouse model, ccc-R09 did not show a persistent anti-HBV effect. HBsAg and HBeAg levels in ccc_R09-treated mice decreased by 0.35 $\log_{10}$ and 0.2 $\log_{10}$, respectively, one week post-treatment. However, the on-treatment viral marker rebounded at day 21 and day 28 post-treatment. One explanation for this phenomenon is that replenishment of cccDNA by new virus infections or intracellular recycling of rcDNA compensated for the decay of cccDNA. Accordingly, combining an entry inhibitor (such as myrcludex B) or NAs to block the replenishment of cccDNA might help clarify whether ccc-R09 accelerates the established cccDNA pool in this animal model.

The discovery of ccc_R08 and its derivatives ignite new hope to degrade cccDNA in a noncytopathic manner. Although the clinical usage of such agents might not be seen very soon, the iteration of this approach based on clarified mechanisms and targets will help to achieve a functional cure for CHB.

7.4. Antisense oligonucleotide (ASO)

We classify ASO as the second type, unlike siRNA (which falls into the first category), based on clinical trial evidence suggesting a different mechanism of action. While ASO and siRNA bind to target mRNA and induce its degradation, notable distinctions exist. ASO has a complementary sequence of single-stranded DNA with a length of 15–25 bp, flanked by RNA-like sequences on either side. This design increases ASO's affinity for the target sequence and enhances its resistance to nuclease degradation (Hui et al., 2022). Unlike siRNA, unmodified ASO can enter hepatocytes through receptor-mediated endocytosis and primarily localizes in the cytoplasm (Miller et al., 2018), leading to the need for more frequent dosing. Additionally, ASO can directly bind to mRNA, recruiting RNase-H to degrade the target sequence (Liang et al., 2017), bypassing the requirement for RISC assistance.

GSK3389404 is an N-acetylgalactosamine (GalNAc)-modified ASO that targets the HBX ORF, a common region for all HBV RNAs (Han et al., 2019). In a phase 2 clinical trial, patients receiving NA therapy were given GSK3389404 weekly (120 mg) for 12 weeks, resulting in a 0.75 $\log_{10}$ IU/mL decrease in HBsAg. However, 12 weeks after treatment cessation, it returned to pre-treatment levels (Yuen et al., 2022c). GSK3228836 (Bepirovirsen) (Han et al., 2022) is an unmodified version of GSK3389404 and has shown the best effect so far in clinical trials. In a phase 2 clinical trial, treatment-naive or NA-treated chronic hepatitis B patients were subcutaneously injected with different doses of GSK3228836 on days 1, 4, 8, 11, 15, and 22 after the start of the trial. On day 29, the average HBsAg decrease in 12 treatment-naive patients was 1.56 $\log_{10}$ IU/mL, and in 5 patients receiving concurrent NA therapy, the average HBsAg decrease was 1.99 $\log_{10}$ IU/mL. One patient in each group achieved temporary HBsAg seroconversion (Yuen et al., 2021). In a subsequent phase 2b clinical trial, 457 treatment-naive or NA-treated patients were randomly assigned to different dosing regimens. It was found that among patients receiving continuous NA therapy, those receiving weekly doses of 300 mg for 24 weeks achieved HBsAg negativity in 26% of cases, and 12% were still able to maintain HBsAg negativity 24 weeks after treatment cessation. Treatment-naive patients receiving only GSK3228836 therapy, with weekly doses of 300 mg for 24 weeks, achieved HBsAg seroconversion in 29% of cases, and 14% were still able to maintain HBsAg negativity 24 weeks after treatment cessation (Yuen et al., 2022d).

Based on the available data, bepirovirsen has a better effect on reducing HBsAg than siRNA. This difference may be related to the liver cell injury induced by bepirovirsen. The potential relationship between hepatocyte injury and drug efficacy has been reported in a small-sample clinical trial of ARC520 (Yuen et al., 2022a). Three patients with HBeAg (+) showed temporary ALT elevation during the medication period, while their HBsAg levels decreased significantly. However, in larger clinical trials, another siRNA drug, JNJ-3989, did not induce liver cell injury (Yuen et al., 2023b). The ALT levels of all treatment-naïve patients in all groups showed a downward trend. Patients in the JNJ-6379 group, the JNJ-3989 200 mg group, and the triple therapy group who achieved virological suppression before treatment exhibited slightly increased ALT levels, remaining within the normal range. Unlike siRNA, 8 of 18 patients (44.4%) with initial treatment experienced an increase in ALT during treatment with bepirovirsen. Three of five patients receiving continuous NA treatment experienced elevated ALT after receiving 300 mg of bepirovirsen. These ALT elevations were temporary and self-limited. There is a correlation between elevated ALT and decreased HBsAg (Yuen et al., 2021). Generally, a larger increase in ALT corresponds to a larger decrease in HBsAg. Most patients with HBsAg decline of ≥ 3 $\log_{10}$ IU/mL experienced elevated ALT. In further clinical trials for bepirovirsen, 17% (39/225) of NA-treated patients and 41% (93/227) of previously untreated patients had ALT levels that exceeded the upper limit of normal at least 3 times during treatment and follow-up, but the correlation between ALT elevation and HBsAg decline was not analyzed in the report (Yuen et al., 2022d). An interesting comparison for bepirovirsen is GSK3389404. GSK3389404 is a GalNAc-modified bepirovirsen, but its efficacy in clinical trials is unsatisfactory. In a phase 2a clinical trial, patients receiving a 120 mg dose (12 times per week) of GSK3389404 experienced an average HBsAg decrease of 0.75 $\log_{10}$ IU/mL. Three patients showed a decrease in HBsAg by more than 1.5 $\log_{10}$, but none showed an increase in ALT (Yuen et al., 2022c). Although there were two cases of ALT elevation, they were not considered to be related to drug effects.

The effectiveness of bepirovirsen appears to be linked to liver cell damage. We hypothesize that this effect could be due to various mechanisms, including the following: (1) Bepirovirsen, an unmodified ASO, is primarily located in the cytoplasm after entering the cell, while its GalNAc modification GSK3389404 is mostly found in the endosome. This divergence in subcellular localization could potentially facilitate the more convenient recognition of bepirovirsen's single-stranded DNA by cytoplasmic DNA sensors, thus instigating a natural immune or injury response. A phase 1 clinical trial in healthy subjects seems to support this theory, as GSK3389404 resulted in a minor increase in ALT in 4.1% of subjects (Han et al., 2019), whereas bepirovirsen caused a notable elevation in ALT in 23.8% of subjects (Han et al., 2022). (2) Bepirovirsen, in the cytoplasm, can potentially form numerous DNA-RNA hybrids with HBV RNA. These hybrids might function as effective inducers of natural immune responses and apoptosis. Research by Magdalena et al. suggested that RNA-DNA hybrids known as R-loops can be detected by cellular receptors like cGAS and TLR3, leading to the activation of IRF3 and apoptosis (Crossley et al., 2023). Although cells can recognize double-stranded RNA-induced natural immune response through melanoma differentiation-associated protein 5 (MDA5) or retinoic acid-inducible gene I (RIG-I) (Chen and Hur, 2022; de Reuver and Maelfait, 2023). In contrast, RNA-RNA double strands formed between siRNA and HBV RNA appear less effective in eliciting a therapeutic liver cell injury response, as demonstrated in clinical trials.

7.5. Nucleic acid polymers (NAPs)

NAPs are single-stranded thiophosphate oligonucleotides, typically 40 nucleotides in length. Unlike ASOs and siRNAs, they exert non-sequence-specific effects, primarily by forming high-affinity interactions with certain proteins, such as DNAJB12, through amphipathic α-helices. *In vitro* studies have demonstrated that NAPs selectively target the assembly and secretion of subviral particles (SVPs) by degrading unassembled HBsAg without impacting the production of HBV DNA and HBV RNA (Blanchet et al., 2019; Vaillant, 2022). It should be noted that SVPs contain HBsAg in significantly higher quantities than virions and represent the primary source of HBsAg *in vivo* (Chai et al., 2008; Mohebbi et al., 2018). Consequently, focusing on SVPs proves to be an effective strategy for reducing HBsAg levels. SVPs mainly follow the ER-Golgi intermediate compartment (ERGIC) pathway (Chai et al., 2008), for secretion, which is distinct from the MVB pathway used by virions (Selzer and Zlotnick, 2015). Therefore, interventions that target SVPs need to operate within the ERGIC pathway. It is worth mentioning that NAPs exhibit limited inhibitory effects on HBsAg secretion in human hepatoma cell lines (Boulon et al., 2020), likely due to their confinement to endosomes when introduced into cells via methods like electroporation or liposome transfection. However, the effectiveness of NAPs can be revealed by employing drugs that facilitate the release of NAPs from endosomes, such as UNC7938 (Blanchet et al., 2019). Interestingly, it has been reported that NAPs demonstrate no activity in transgenic mice, human hepatocyte chimeric mice, or woodchucks (Schoneweis et al., 2018). However, they exhibit substantial efficacy in reducing HBsAg levels in ducks infected with DHBV (Noordeen et al., 2013). In human trials, NAP monotherapy can reduce HBsAg levels by up to 7 $\log_{10}$ IU/mL in some patients, although achieving a functional cure remains challenging (Al-Mahtab et al., 2016). Combination therapy involving NAP, PEG-IFNα, and TDF administered for 48 weeks has resulted in a high HBsAg seroconversion rate, with 60% of patients (24/40) experiencing

this effect (Bazinet et al., 2020). During the 48-week follow-up after treatment cessation, 39% of these patients (14/40) maintained HBsAg negativity, which is encouraging. Notably, an analysis of HBV DNA change curves revealed that individuals who responded well to NAPs maintained a stable rate of HBV DNA decline 3–10 weeks post-treatment initiation, and this trend persisted. Moreover, the characteristics of HBsAg decline mirrored those of HBV DNA decline (Bazinet et al., 2021a, 2021b), suggesting the involvement of specific factors that expedite the decay of the cccDNA pool. These factors, at least in part, may encompass hepatocyte injury, as individuals who responded well often experienced transient ALT elevations during NAP treatment. These elevations appear to be correlated with the magnitude and timing of HBsAg decline. Nonetheless, whether this hepatocyte injury is immune-related, linked to the restoration of HBV-specific immunity, or if NAPs induce non-specific immune responses remains unclear.

7.6. Inhibitors of cellular inhibitor of apoptosis proteins (cIAP)

cIAPs exert their anti-apoptotic function by inhibiting NF-κB signaling. This inhibition occurs through the promotion of IκB kinase (IKK) degradation. NF-κB plays a pivotal role in activating and regulating the TNF pathway, thereby preventing cell death induced by TNF and promoting cell survival (Gyrd-Hansen and Meier, 2010; Silke and Meier, 2013; Hrdinka and Yabal, 2019). Inhibiting cIAPs, therefore, leads to increased TNF-mediated cell death. Mammals possess three primary IAP types: cIAP1, cIAP2, and XIAP. Studies have shown that mice deficient in cIAP1 and cIAP2 are more likely to clear HBV (Ebert et al., 2015), implying that cIAPs represent potential effective targets for combating HBV infection (Liu et al., 2018). Birinapant, a cIAP1 inhibitor, can reportedly mimic the effects of the endogenous protein second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pI (Smac/Diablo), natural antagonizer of IAPs (Martinez-Ruiz et al., 2008). In a mouse model of HBV infection involving hydrodynamic injection of HBV DNA, Birinapant was able to promote viral clearance. This effect was dependent on TNFα, as its efficacy was abrogated when an antibody neutralized TNFα. The mechanism underlying Birinapant's promotion of viral clearance appears to involve the induction of liver cell injury. A single dose of Birinapant reduced the number of HBcAg-positive hepatocytes, accompanied by an increase in the number of TUNEL-positive cells, and a temporary rise in ALT levels (Ebert et al., 2015). Interestingly, when control mice without HBV replication were treated with birinapant, no TUNEL-positive hepatocytes were observed. This suggests that birinapant's effect is specific to HBV infection. One possible explanation is that HBV infection upregulates the level of TNFR1 in liver cells, making them more sensitive to TNFα. Unfortunately, a phase 1 clinical trial of Birinapant was terminated due to the observation of cranial nerve paralysis. A second-generation highly selective IAP inhibitor, APG-1387, was developed by Ascentage Pharma. In a phase 1 clinical trial, 4 weeks of APG-1387 treatment did not result in serious side effects. By day 28, the average HBsAg level had decreased by 0.04 $\log_{10}$ IU/mL in the 30 mg dose group, suggesting a relatively mild effect (Ascentage Pharma, 2022). However, this result might be influenced by the clinical trial's design. All subjects were previously untreated patients who initially received 4 weeks of APG-1387 treatment before continuing with NA treatment. Assuming that APG-1387's primary mechanism of action is similar to that of Birinapant, inducing apoptosis in infected hepatocytes, the death of some infected hepatocytes would lead to a reduction in the cccDNA pool and a decrease in viral replication. Nonetheless, the newly formed hepatocytes would replace the dead ones. Since the viral load was not significantly decreased (most hepatocytes remain uninfected), these newly generated hepatocytes may become infected, thus compensating for the cccDNA pool loss. Given this analysis, it might be worth considering a clinical trial protocol involving the addition of APG-1387 to patients who have already undergone treatment and achieved a virological response.

7.7. Immune modulators

The natural recovery from adult acute HBV infection hinges on a specific immune response to HBV. However, this response becomes deficient (tolerant) in chronic HBV infection. Reestablishing or reconstructing the body's specific immune response to HBV is a critical approach toward ultimately curing chronic hepatitis B. This endeavor encounters substantial challenges primarily due to a lack of clear understanding regarding the reasons for immune tolerance in chronic HBV infection. Past studies have suggested mechanisms contributing to this immune tolerance involve clonal clearance or anergy of HBV antigen-specific immune cells, inhibitory effects of regulatory T cells (Treg), and the exhaustion of effector T cells (Iannacone and Guidotti, 2022). Designing effective strategies based on these mechanisms might hold therapeutic potential.

7.7.1. TLR agonists

TLR7 is a pathogen recognition receptor mainly expressed in the lysosome/endosome of plasmacytoid dendritic cells (pDC) and B cells. It is widely acknowledged that TLR7 can recognize pathogen-associated molecular patterns in viral single-stranded RNA (Li et al., 2023). Upon activation, TLR7 induces pDCs to produce IFN-α and other cytokines (Akira and Takeda, 2004). It can also activate NK cells and cross-activate cytotoxic T cells. GS9620, a potent TLR7 agonist, displayed encouraging results in a chimpanzee study, reducing circulating HBV DNA levels by 2.2 $\log_{10}$ after 8 weeks of treatment. Notably, HBsAg and HBeAg levels decreased by 61% and 93%, respectively, compared to baseline (Lanford et al., 2013). However, clinical studies of GS9620 did not yield the expected results. Evaluation of different doses of GS9620 in patients already receiving NA treatment (HBV DNA negative, HBsAg+) revealed that while GS9620 treatment increased T cell cytokine production and enhanced NK cell function, it did not significantly reduce HBsAg levels (Boni et al., 2018). Similarly, a phase 1 clinical trial of TLR7 agonist RG7854 demonstrated that treatment with this agonist increased the expression of various genes, including IFN-γ, ISG15, and IP10, in previously untreated and previously treated NA patients. Nevertheless, the reduction in HBsAg was modest, with an average decrease of 0.14 $\log_{10}$ IU/mL (170 mg dose group) and a maximum decrease of 0.4 $\log_{10}$ IU/mL in NA-treated patients (Yuen et al., 2023a).

TLR8 represents another pattern recognition receptor primarily expressed on the endosomes of monocytes/macrophages and myeloid dendritic cells (Li et al., 2023). GS-9688 (selgantolimod) is a TLR8 agonist that induces human PBMCs to produce IL-12, IL-18, TNF-α, and IFN-γ (Mackman et al., 2020; Amin et al., 2021). It also activates NK cells and stimulates the proliferation of $CD8^+$ T cells (Amin et al., 2021). In a model of HBV infection in primary human hepatocytes, GS-9688 treatment led to reductions in HBV DNA, RNA, HBsAg, and HBeAg levels. In a clinical trial involving 48 chronic hepatitis B patients, GS-9688 was administered in different treatment groups for 24 weeks with NA. The results showed that 26% of patients (10/39) treated with GS-9688 experienced a decrease in HBsAg greater than 0.1 $\log_{10}$ IU/mL at 48 weeks, with only one patient achieving a decrease greater than 1 $\log_{10}$ IU/mL (Gane et al., 2023).

7.7.2. Immune checkpoint inhibitors

Multiple studies have revealed that the abundance of HBV-specific $CD8^+$ T cells in chronic HBV-infected individuals is lower than in those with acute HBV infection, with detection predominantly limited to chronic patients with low viral load (Sobao et al., 2002; Boni et al., 2007; Bertoletti and Kennedy, 2019). These cells often exhibit an “exhausted” phenotype, marked by reduced proliferative capacity and lower production of IFN-γ, IL-2, and TNF-α (Das et al., 2008; Park et al., 2016; Rossi et al., 2023). The exhaustion of $CD8^+$ T cells is associated with high expression of co-inhibitory molecules such as PD-1 and CTLA-4 (Schurich et al., 2011; Park et al., 2016). Consequently, inhibiting these inhibitory molecules can restore HBV-specific immunity in

chronic hepatitis B patients. In a phase 1b clinical trial, the administration of a single 0.3 mg/kg dose of the anti-PD1 monoclonal antibody nivolumab led to a mean decrease of 0.48 log_{10} IU/mL in HBsAg at 24 weeks, with one patient achieving HBsAg clearance (Gane et al., 2019). Combining nivolumab with a therapeutic vaccine (GS-4774) did not yield improved efficacy. A phase 2b clinical trial demonstrated that the anti-PD-L1 antibody envafolimab, when combined with NA treatment in HBeAg-negative infected individuals, resulted in a decrease of greater than 0.5 log_{10} IU/mL in HBsAg in 21% of patients (7/33), with three cases achieving HBsAg negativity (Wang et al., 2022).

7.7.3. Therapeutic vaccines

While prophylactic vaccination can effectively protect against HBV infection, achieving therapeutic objectives through vaccination in the context of chronic infection has proven challenging (Cargill and Barnes, 2021). Clinical trials of therapeutic HBV vaccines have so far yielded disappointing results. Despite various attempts involving antigen selection, source, and immune carriers, a promising therapeutic vaccine has yet to emerge. GS-4774 is a recombinant vaccine expressed in yeast comprising HBsAg, HBcAg, and HBx. In healthy individuals, GS-4774 vaccination induces low to moderate immune responses. However, in chronic HBV-infected patients with suppressed HBV DNA and relatively low HBsAg levels, the efficacy of GS-4774 vaccination, when added to NA treatment, is limited. Only 3 out of 178 patients exhibited a decrease in HBsAg greater than 0.5 log_{10} IU/mL (Lok et al., 2016a). Another study combining GS-4774 with TDF did not outperform TDF alone (Boni et al., 2019). TG-1050, a therapeutic vaccine utilizing a replication-defective human adenovirus vector, was designed to express a truncated core protein, a modified polymerase, and a T-cell epitope-rich domain in the surface antigen as immunogens. However, in a phase 1 clinical trial, only a modest decrease in HBsAg, less than 0.2 log_{10} IU/mL, was observed after TG-1050 administration (Zoulim et al., 2020). ε PA-44, a nanoparticle vaccine using liposomes as a carrier, consists of a 44-amino acid synthetic peptide encompassing a CTL epitope core 18–27 derived from HBc, a $CD4^{+}$ T cell epitope derived from tetanus toxoid (830–843), a B cell epitope from PreS2 133–143, and a palmitic acid molecule to promote the CTL response. In a phase 2 clinical trial, HLA-A2-positive and HBeAg-positive patients received six doses of ε PA-44. After 76 weeks, HBeAg seroconversion was achieved in 38.8% of patients in the 900 μg dose group, compared to 20.2% in the placebo group. However, the decrease in HBsAg in various treatment groups was limited (Wei et al., 2022).

Importantly, therapeutic vaccines aim to stimulate the body's specific immune response to HBV antigens, especially HBsAg. Notably, many chronically infected individuals eventually achieve spontaneous HBeAg seroconversion after reaching adulthood (Yuen et al., 2005; Chen et al., 2010; Chu et al., 2022). This indicates that the body has developed a specific immune response to HBeAg. In contrast, the rate of spontaneous HBsAg seroconversion in chronically infected individuals is notably lower, only about 1% per year (Yeo et al., 2019; Zhou et al., 2019), much less than the rate of HBeAg seroconversion. Studying the mechanisms of immune reconstitution against HBeAg could potentially provide insights to help restore the immune response against HBsAg. However, this research field faces significant challenges due to the absence of animal models that can accurately replicate chronic HBV infection in humans (Liu Y et al., 2021; Ploss et al., 2021).

8. Conclusions and future directions

In conclusion, the persistence of cccDNA as a stable genetic reservoir poses a significant challenge to the complete eradication of HBV and achieving a functional cure for chronic hepatitis B. To attain this goal, prevention of cccDNA synthesis alone is insufficient; a combination of strategies that expedite cccDNA decay is imperative. Among the novel strategies, ASOs and NAPs have demonstrated promising effects in accelerating cccDNA decay in specific patient groups. This effect appears to be an unanticipated outcome of their mechanisms and is partly associated with cytotoxic effects, which may be related to bothnon-specific and specific immune responses. This phenomenon offers a new perspective for designing and optimizing chronic hepatitis B cure strategies, suggesting that liver cell damage resulting from treatment may have a beneficial role in the therapeutic process. Additionally, the regulation and training of the host immune system to help chronically infected individuals rebuild specific immunity against HBV represents a curative strategy. However, the path to achieving this remains unclear, as our understanding of immune tolerance mechanisms in chronic HBV infection, particularly the mechanisms that allow the body to partially overcome this tolerance, is limited due to the absence of small animal models accurately replicating HBV chronic infection. This knowledge gap significantly hampers the development of effective immune-based strategies.

In summary, the road to achieving a functional cure for most chronic hepatitis B patients in a limited treatment timeframe may involve a multistage process. Combining NAs or interferon with a drug capable of influencing cccDNA decay has the potential to significantly improve the functional cure rate. This approach represents a promising direction for future research and clinical development in the field of chronic hepatitis B therapy.

Conflict of interest

The authors of this study declared that they do not have any conflict of interest.

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