Virology: viral replication, current therapy and progress to a cure

KEY POINTS

- Hepatitis B virus (HBV) can cause persistent infection by the establishment of a viral covalently closed circular DNA (cccDNA), which forms a stable minichromosome in the hepatocyte nucleus.
- In chronic hepatitis B, the cccDNA is largely refractory to elimination by the immune response or by nucleos(t)ide analogue (NA) therapy. Upon immunosuppression or cessation of therapy, the cccDNA can re-initiate virus replication, leading to reactivation of disease.
- HBV has an unusual replication strategy; it employs an error-prone reverse transcription step that allows mutations to be generated. Recent research efforts have identified several new targets for therapeutic intervention which may lead to a functional cure.
- HBV genotypes A-D are found worldwide; genotypes B and C are the major forms in Oceania and Asia. Different genotypes are associated with different rates of disease progression and risk of HCC (1).

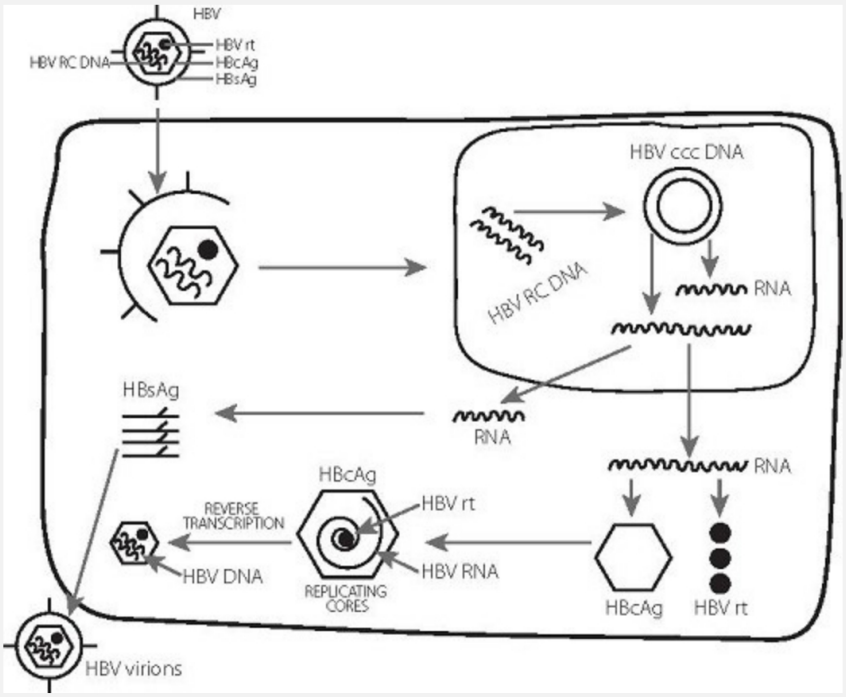
Introduction and pathogenesis

Under normal circumstances, hepatitis B virus (HBV) infection does not directly damage liver cells (hepatocytes). The liver damage associated with acute hepatitis B infection or with chronic hepatitis B (CHB) usually occurs because of the attempts by the host's immune response to clear the virus from infected hepatocytes (2). It is the balance between the host immune response and viral replication that determines the stage of disease during the natural history of hepatitis B infection.

HBV is a member of the family *Hepadnaviridae*. It is an enveloped virus containing a partially double stranded relaxed circular (RC) DNA genome. Novel features of hepadnavirus replication are a reverse transcription step, usually only seen with retroviruses, and production of excess non-DNA containing viral envelope material – the hepatitis B surface antigen (HBsAg), that circulates in the blood in titres that can exceed 10^{12} HBsAg particles/mL. The viral reverse transcriptase (rt) lacks a proofreading capacity; hence, when the virus replicates, it produces a population of closely related variants, known as a quasispecies. This diversity ensures the survival of HBV because, when the virus is under pressure from the immune response or the introduction of an antiviral agent, a resistant viral sub-population will already be present in the pool of newly replicating virus of the person with the infection and such HBV variants can be selected to become the dominant population.

Hepatitis B virus replication: the virus and its life cycle

Figure 2.1 Life cycle of the hepatitis B virus from entering the cell (at the top) to exiting the cell (shown at the bottom)

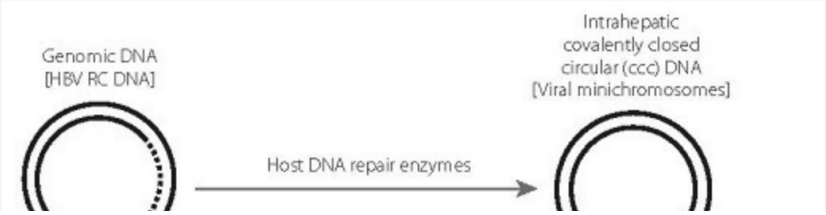


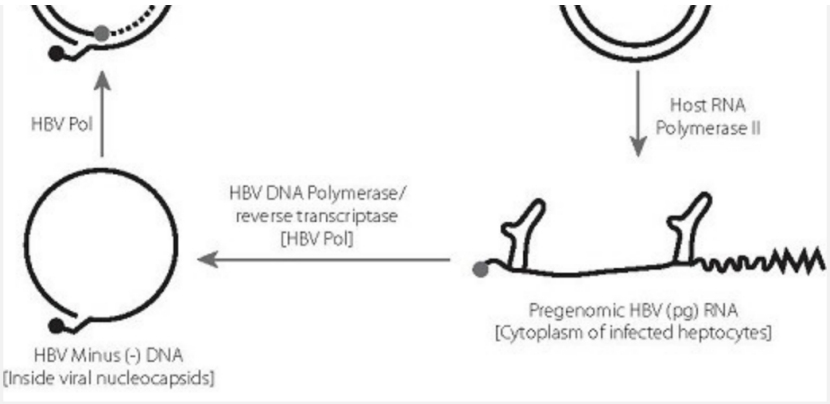
cccDNA: covalently closed circular DNA; HBcAg: hepatitis B core antigen; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; RC: relaxed circular; rt: reverse transcriptase

HBV is a DNA virus. The viral DNA genome is found inside the viral core structure (or hepatitis B core antigen [HBcAg]) along with the viral rt (a reverse transcriptase and DNA polymerase). The core structure is surrounded by the outer viral envelope which is composed of HBsAg. The life cycle of HBV, shown in Figure 2.1, begins when the envelope protein of the virus attaches to the receptor on the surface of the hepatocyte (3) – allowing the virus to enter the cell. Once inside the cell, the viral genomic DNA moves to the nucleus and is converted into a covalently closed circular (ccc) DNA form. The HBV cccDNA, the major transcriptional template of the virus, associates with cellular histone proteins and establishes itself as viral minichromosomes. The half-life of the HBV cccDNA is long which partly explains why HBV can reactivate after years of HBsAg loss or after immune suppression. All viral RNAs are transcribed from the cccDNA template and transported to the cytoplasm of the hepatocyte, where the viral structural proteins and the enzymes involved in viral replication are synthesised. The HBV rt reverse transcribes the HBV pregenomic RNA (pgRNA) into DNA, inside the core particle. The viral envelope proteins then coat the replicating core complexes, creating mature virus that is released from the cell to complete the life cycle. The HBV rt is a unique enzyme and has been identified as a target for nucleos(t)ide analogue (NA) antiviral therapy. In this context, the life cycle of HBV involves two important processes, as shown in Figure 2.2.

- generation of HBV cccDNA from genomic DNA to form a minichromosome, and subsequent processing of the minichromosome by host enzymes to produce viral RNA
- reverse transcription of the pgRNA within the viral core (nucleocapsid) to form HBV RC genomic DNA, thereby completing the cycle (see Figure 2.1).

Figure 2.2 Major processes involved in hepatitis B virus genome replication





Conversion of relaxed circular (RC) DNA into covalently closed circular (ccc) DNA; transcription of cccDNA to produce pregenomic RNA (pgRNA); reverse transcription of pgRNA to make a minus (–) single strand HBV DNA; and HBV DNA polymerase activity to make the RC double stranded DNA, completing the cycle. The cccDNA can only be found in the liver within the nucleus of infected hepatocytes and in the form of a viral minichromosome (4,5)

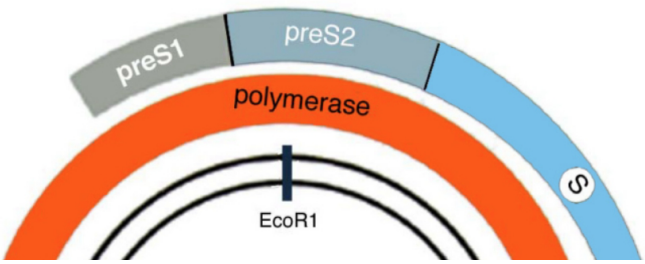
The HBV quasispecies produced by the error-prone rt provides the virus with a strong survival advantage because, on a daily basis, every single nucleotide in the viral genome of 3200 base pairs can be changed. Random mutations from copying errors can lead to phenotypic changes, which in turn may confer a selective advantage. Thus, single and double mutations associated with antiviral drug resistance are often present before antiviral therapy is introduced; for this reason, combination antiviral therapy (cART) is used for many infections such as human immunodeficiency virus (HIV).

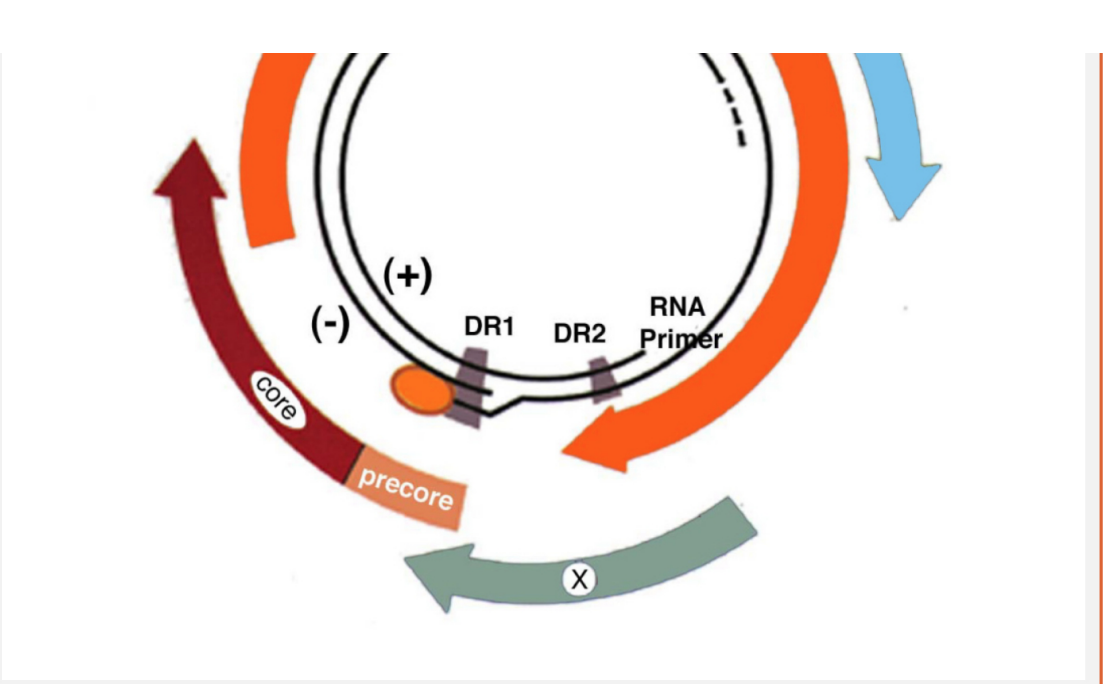
Functional cure of HBV infection refers to *a sustained reduction in virus and other disease markers in the blood even after a drug is stopped*. In addition to suppressing or eliminating viral DNA, functional cure includes loss of HBsAg and appearance of HBV surface antibody (HBsAb). cccDNA persists in hepatocytes in a functional cure but does not initiate viral replication.

Genome organisation

The HBV genome encodes only four genes, but the virus has evolved a remarkably efficient replication strategy. It maximises its information content by using four overlapping reading frames (ORFs), designated P (polymerase), S (surface), C (core) and X (the HBx protein) to encode a total of seven proteins. The longest ORF is that of P which covers approximately 70% of the genome and encodes the viral rtDNA polymerase. The S ORF is located entirely within the P ORF and codes for the three HBV surface (HBs) proteins, the large (LHBs), middle (MHBs) and small (SHBs). The C ORF allows the production of the core protein and with additional precore sequence leads to the synthesis of the hepatitis B e antigen (HBeAg). The X protein is encoded by the smallest ORF. The C and X ORFs also overlap the P ORF (see Figure 2.3).

Figure 2.3 The hepatitis B virus genome





Shows the circular arrangement of the four overlapping but frame-shifted open reading frames

Genotypes

HBV can be classified into nine genotypes, A to I. There is an additional J genotype of which there is a single isolate. Genotypes A-D are found worldwide; genotypes B and C are common in Oceania and Asia. Genotype E is largely restricted to Central and West Africa, and genotype F to Alaska, Central and South America and Polynesia. Genotype G is limited to a small number of people in Europe and the USA with HBV genotype H is largely confined to Central and South America. Laos and Vietnam appear to be the geographical home of HBV genotype I but it has also been found in East India. Based on a nucleotide divergence many of the genotypes can be further subdivided into subtypes (6).

The different HBV genotypes are associated with different rates of disease progression and risk of hepatocellular cancer (HCC) (1) but current clinical guidelines do not recommend testing for genotype as a part of routine clinical care and it is not reimbursed under Medicare. The geographical distribution of the genotypes and associated risk of HCC does however inform the recommendations for HCC surveillance (see: Hepatitis B related hepatocellular carcinoma). Genotyping is sometimes performed in tertiary settings when drug resistance is suspected.

Common mutants. Mutations affecting hepatitis B e antigen

In addition to coding for HBcAg and HBsAg, the HBV genome encodes the hepatitis B e antigen (HBeAg). The HBeAg protein is thought to act as a tolerogen (7), which means it can down-regulate the host immune response, especially during pregnancy or acute infection, to help the virus avoid elimination. Without HBeAg, it is unlikely that HBV could establish a chronic infection.

During the immunological pressure of HBeAg seroconversion, which is part of the natural history of CHB, the virus has a number of ways of escaping. Two types of mutations have been identified that result in reduced or truncated HBeAg expression.

1. Basal core promoter (BCP) mutants

These mutations are typically found in the precore gene at nucleotide (nt) position 1762 and nt1764, resulting in a reduction in transcription of the precore mRNA (8). Mutations in the BCP, such as the change of an A to a T at nt1762 (designated A1762T), and of a G to an A at nt1764 (G1764A), may be found in isolation or in conjunction with other precore mutations (see below). The double mutation of A1762T plus G1764A results in a significant decrease in HBeAg levels, but not its absolute absence, and these mutations have been associated with an increased viral load in patients. Importantly, these BCP mutations do not affect the transcription of HBV pgRNA or the translation of the core or polymerase protein. Instead, by removing the inhibitory effect of the precore protein on HBV replication, the BCP mutations appear to enhance viral replication by suppressing precore or core mRNA relative to pgRNA (8).

2. Precore mutants

These mutations introduce a translational stop codon at nt1896 (codon 28: TGG; tryptophan) of the HBV precore gene (9). The single base substitution (G to A) at nt1896 (G1986A) converts the TGG codon into TAG (TAG = stop codon) in the second last codon (codon 28) of the precore gene that is located within the epsilon (ε) structure of pgRNA. The ntG1896A forms a base pair with ntT1858 at the base of the stem loop (9). Patients with precore mutant (G1896A) HBV are typically HBeAg negative. Other mutations in the precore gene can block HBeAg production; these include a mutation that abolishes the methionine initiation codon (10). The seroconversion of HBeAg-positive to anti-HBe is normally a marker of disease resolution but for patients with precore mutants, virus replication can be ongoing.

Envelope gene mutations

The current hepatitis B vaccine contains recombinant HBsAg. The subsequent immune response to the major hydrophilic region (MHR) of HBsAg, located from amino acid residues 99 to 170, induces protective immunity in the form of anti-HBs. Mutations within the MHR have been selected during vaccination (11), and following treatment of liver transplant recipients with hepatitis B immune globulin (HBIg) prophylaxis (12). Most vaccine-HBIg escape isolates have an amino acid change from glycine to arginine at residue 145 of HBsAg (sG145R), or aspartate to glutamic acid at residue 144 (sD144E). The sG145R mutation has been associated with vaccine failure (11). It has also been shown to be transmitted, to establish persistent infection and cause disease.

Polymerase mutations: antiviral drug resistance

The treatment of CHB has advanced significantly during the past 20 years with the development of safe and efficacious orally available antiviral NAs. Two synthetic NAs with an unnatural L-conformation – lamivudine and telbivudine – are older agents that are no longer recommended for use in Australia. Adefovir dipivoxil is a prodrug for the acyclic dAMP analogue, adefovir. Adefovir gained approval in 2002, but has largely been replaced by its congener, tenofovir, which is now commonly used as a first-line agent. Tenofovir lacks the potential nephrotoxicity of adefovir; consequently, a higher dose can be used (300 mg/day vs 10 mg/day for adefovir), which may explain its greater efficacy in vivo. The most potent anti-HBV drug discovered to date is the deoxyguanosine analogue, entecavir (13), which is now widely used as a first-line agent for treating CHB.

Lamivudine, telbivudine and adefovir resistance

Older antiviral agents, lamivudine, telbivudine and adefovir which are no longer recommended for use in Australia, are sometimes used by people arriving from countries overseas. Resistance to these agents develops over several years via different mechanisms.

Lamivudine resistance increases progressively during treatment at rates between 14% and 32% annually. At year four of therapy, rates of lamivudine resistance reach over 70% in HBV mono-infection, and exceed 90% in HBV–HIV co-infection (14,15). Factors that increase the risk of development of resistance include high pretherapy serum HBV DNA and alanine transaminase (ALT) levels, and incomplete suppression of viral replication (14,16). Lamivudine resistance does not usually confer cross-resistance to adefovir or tenofovir unless the change rtA181T is selected. However, the presence of rtM204I/V confers cross-resistance to the other L-nucleoside analogues, including telbivudine and, to a lesser extent, entecavir which affects the choice of antiviral therapy if there is a history of lamivudine use.

High maternal viral loads have been shown to be a risk factor for neonatal immunoprophylaxis failure, and lamivudine was previously used during the third trimester to reduce the risk of perinatal transmission. Recent evidence has shown that viral variants conferring lamivudine resistance are not only present at baseline but importantly are rapidly selected by the time of delivery (17). The presence of such variants can complicate future clinical management and tenofovir rather than lamivudine is now the recommended therapy for reducing the risk of maternal-to-child transmission.

Resistance to lamivudine is conferred by mutations that result in replacement of methionine at amino acid position 204 in the tyrosine-methionine-aspartate (YMDD) catalytic site motif (C-domain) of the rt by valine (rtM204V) or leucine (rtM204I) (18). In a few cases, the B-domain change at rtA181T (alanine to threonine) is also responsible for primary resistance to lamivudine. For other L-nucleosides such as telbivudine, the B-domain (alanine to threonine or valine - rtA181T/V) and C-domain (rtM204I) changes are the main substitutions associated with the development of resistance. The rtM204I change can occur in isolation, but rtM204V is found only in association with other substitutions – predominantly with rtL180M (leucine to methionine), and occasionally with both rtL180M and rtV173L. These additional changes partly compensate for the loss of replication fitness that can be associated with the development of drug resistance (19).

Resistance to adefovir has been associated with changes in domain B (rtA181T/V) and D (N236T – asparagine to threonine) of the rt (20). HBV resistance to adefovir occurs less frequently (about 2% after 2 years, 4% after 3 years and 18% after 4 years) than resistance to lamivudine. The rtN236T change does not significantly affect sensitivity to lamivudine (20), but the rtA181T/V change confers partial cross-resistance to lamivudine.

Entecavir and tenofovir resistance

Resistance to entecavir in patients naïve to therapy is rare, and occurs almost exclusively in patients who had already developed lamivudine-resistant HBV (21). Entecavir resistance appears to require the initial presence of rtM204V/I, followed by mutations that encode at least one additional entecavir signature substitution at rtI169T or rtT184G (threonine to glycine - B-domain), rtS202I (serine to isoleucine - C-domain) or rtM250V (E-domain) of HBV Pol. The rate of entecavir resistance at 1 year in those with prior lamivudine resistance is approximately 10%, therefore in this setting tenofovir is the preferred NA and if entecavir is used, a higher dose than in treatment naïve people (1.0 mg) is recommended.

To date primary resistance to tenofovir has not been clearly documented; however, clinically, it has been shown that the presence of adefovir resistance can impair tenofovir efficacy (22).

Multidrug resistance

Multidrug-resistant (MDR) HBV has been reported in patients who received sequential treatment with different NA monotherapy, and rtA181T should be considered a MDR variant (23). The development of multidrug resistance will influence the efficacy of rescue therapy, as in the case of MDR HIV (24). Successive evolutions of different patterns of resistant mutations have been reported during long-term lamivudine monotherapy (25). The isolates of HBV with these initial mutations appear to be associated with decreased replication fitness compared with wild-type HBV; however, additional mutations that can restore replication fitness are frequently detected as treatment is continued (26).

HBV genotype appears to play no role in the development of resistance or response to therapy.

Alternative antiviral therapy – interferon

The development of longer-acting pegylated forms of interferon (IFN) has stimulated renewed interest in treating patients with immunomodulatory agents. However, the drawbacks of pegylated IFN (PEG-IFN) treatment are similar to that of conventional interferon, with low efficacy and high toxicity. One advantage of the pegylated form, however, is that treatment is finite: usually 48 weeks. Furthermore, IFN therapy is effective against NA-resistant HBV, and is not associated with any changes in the HBV polymerase region (27). Recent research has used the quantification of serum HBsAg as a clinical biomarker to identify the patient subgroup most likely to respond to PEG-IFN treatment (28). This may allow the use of response guided therapy for CHB and provide higher rates of HBsAg seroclearance.

HBV genotype does appear to play a role in IFN response, with patients with genotype B having a greater response rate than those with genotype C. Similarly, patients with genotype A have a superior response rate than those with genotype D.

New antiviral drug targets

Recent advances in understanding HBV replication have identified a number of potential antiviral drug targets. These include blocking virus entry using the peptidomimetic agent, Myrcludex B. This drug has been constructed to mimic the first 48 amino acids of HBV Pre-S1 (L) protein, one of the HBV surface proteins, and it has been shown to competitively inhibit viral entry (29). Clinical trials with this agent have revealed the important finding that it is possible by using entry inhibitors to protect newly formed cells from (re-)infection with HBV (Urban S, personal communication in 2018).

Myrcludex B has been granted provisional approval for treating chronic hepatitis D infection in combination with PEG-IFN in Europe. The next major target is to inhibit the generation and processing of the major transcriptional templates (cccDNA). Several molecular-based approvals using CRISPR-Cas9 (30)have shown selective destruction of cccDNA, but it has been challenging to develop an appropriate delivery system for these therapies. The core protein has been shown to be amenable to inhibition with a novel sulphonamide group and the antiviral agents of the heteroary/dihydiopyrimidine (HAP) class are effective inhibitors of nucleocapsid assembly by selectively binding core monomers and preventing core dimerization (31). Several pharmaceutical companies are in phase II development with these inhibitors.

The last group of direct-acting antiviral agents target HBV mRNA using RNA interference (RNAi) technology. Significant progress has been made in platform deliveries for these compounds and companies are moving into phase II studies after promising phase IB data demonstrated proof of concept. These RNAi active compounds are potent inhibitors of HBV transcription-translation and have resulted in substantial inhibition of HBV-associated protein, especially HBsAg subviral particles, the HBx protein and HBeAg.

In parallel with these advances, immune-based approaches using Toll-like receptor (TLR) agonists, RIG-I agonists, anti-PD-1/PD-L1 antibodies to restore immune exhaustion and even re-engineered T-cell receptor adoptive therapies are also being evaluated. In order to achieve effective control of HBV and even to consider discontinuation of therapy, combinations of direct acting antivirals with these immune active agents will most likely be required to achieve the new treatment goal of functional cure. This goal has been defined as loss of HBV DNA, loss of HBeAg, loss of HBsAg and production of anti-HBs, off antiviral therapy.

Potential new classes of HBV therapy

Antiviral agents	Immune-based approaches
Entry inhibitors	Toll-like receptor agonists
Capsid assembly inhibitors	RIG-1 agonists
CRISP-Cas 9 technologies	Anti-PD1/PD-L1 antibodies
RNAi agents	Therapeutic vaccines

Conclusion

Resistance has not been a critical issue in the management of patients with CHB in Australia because long-term therapy with the first-line NAs (tenofovir disoproxil and entecavir) have a high genetic barrier and good safety profile. However, in many countries with a high prevalence of CHB, those NAs most compromised by viral resistance (lamivudine and telbivudine) are cheap and are still commonly used. One of the important lessons learned from the HIV paradigm is that resistance is likely to occur if viral replication is present during treatment, as occurs with some existing monotherapy regimens that use L-nucleosides (32). New initiatives from the pharmaceutical industry have resulted in a plethora of clinical trials with agents targeted to other aspects of the HBV life cycle. Theoretically, combination therapy with these and existing standard-of-care NAs will reduce not only the viral load and quasispecies pool, but also reduce the risk of selecting resistance, provided that the antiviral agents used do not select for mutual cross-resistance. Hopefully, these advances will allow a functional cure of CHB and in some instances, true virus elimination.

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