World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i8.1030

World J Hepatol 2015 May 18; 7(8): 1030-1040
ISSN 1948-5182 (online)

REVIEW

Current and future antiviral drug therapies of hepatitis B chronic infection

Lemonica Koumbi

Lemonica Koumbi, Hepatology and Gastroenterology Section, Department of Medicine, Imperial College London, London W2 1PG, United Kingdom
Author contributions: Koumbi L solely contributed to this work.
Conflict-of-interest: No conflict of interest.

Correspondence to: Lemonica Koumbi, Research Fellow, Hepatology and Gastroenterology Section, Department of Medicine, Imperial College London, St. Mary's Campus, Norfolk Place, London W2 1PG,
United Kingdom. lemonica.koumbi@gmail.com
Telephone: +44-207-5949022
Fax: +44-207-7069161
Received: August 28, 2014
Peer-review started: August 31, 2014
First decision: Novemeber 27, 2014
Revised: January 12, 2015
Accepted: February 4, 2015
Article in press: February 9, 2015
Published online: May 18, 2015

Abstract

Despite significant improvement in the management of chronic hepatitis B virus (HBV) it remains a public health problem, affecting more than 350 million people worldwide. The natural course of the infection is dynamic and involves a complex interplay between the virus and the host's immune system. Currently the approved therapeutic regimens include pegylated-interferon (IFN)-α and monotherapy with five nucleos(t)ide analogues (NAs). Both antiviral treatments are not capable to eliminate the virus and do not establish long-term control of infection after treatment withdrawal. IFN therapy is of finite duration and associates with low response rates, liver decompensating and numerous side effects. NAs are well-tolerated therapies but have a high risk of drug resistance development that limits their prolonged use. The imperative for the development of new approaches for the treatment of chronic HBV infection is a challenging issue that cannot be over-sided. Research efforts are focusing on the identification and evaluation of various viral replication inhibitors that target viral replication and a number of immunomodulators that aim to restore the HBV specific immune hyporesponsiveness without inducing liver damage. This review brings together our current knowledge on the available treatment and discusses potential therapeutic approaches in the battle against chronic HBV infection.

Key words: Nucleos(t)ide analogues; Interferon-α; Drug resistance; Immunotherapy; Hepatitis B therapy

Core tip: Despite significant improvement in the management of chronic hepatitis B virus (HBV) it remains a public health problem. Current therapeutic regimens include pegylated-interferon (IFN)-α and nucleos(t)ide analogues (NAs). Both treatments do not eradicate the virus and have numerous limitations. IFN therapy is of finite duration and has low response rates while long-term NA therapies have a high risk of drug resistance. The development of new therapeutic approaches is imperative. This review brings together current treatments and the ongoing research efforts on evaluating potential therapeutic strategies that target the suppression of HBV replication the restoration of the weak immune responses against HBV.

Koumbi L. Current and future antiviral drug therapies of hepatitis B chronic infection. *World J Hepatol* 2015; 7(8): 1030-1040

Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i8/1030.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i8.1030

INTRODUCTION

Hepatitis B virus (HBV) is a highly transmissible pathogen infecting humans for more than 1500 years[1]. Despite the availability of a prophylactic vaccine today HBV continues to pose one of the most serious and prevalent health problems, accounting for over 1 million deaths annually[2]. HBV is a non-cytopathic virus that can cause a wide spectrum of disease manifestations, ranging from asymptomatic infection to acute self-limiting or fulminant hepatitis, or chronic infection with variable disease activity. Chronic HBV infection (CHB) results in persistent hepatic inflammation and progressive fibrosis that may ultimately lead to hepatic decompensation, cirrhosis, hepatocellular carcinoma (HCC) and liver-related death.

HBV is the prototype of the *Hepadnaviridae* family and has evolved a distinctive and successful strategy for replication, which allows its indefinite persistence in the liver of the infected host. Upon infection of the hepatocyte, the HBV virion is uncoated in the cytosol and the genome translocates to the nucleus. There, its relaxed circular, partially double stranded DNA is converted into a covalently closed circular DNA (cccDNA) molecule, following completion of the shorter positive-strand and repair of the nick in the negative strand. The cccDNA exists as a stable non-integrated minichromosome and forms the template for the synthesis of four co-terminal mRNA transcripts by the action of host RNA polymerase Ⅱ[3,4]. One of the transcripts, termed pre-genomic RNA (pgRNA), is the template for genome replication and encodes for the core and polymerase proteins. Translation of the transcripts occurs in the cytoplasm and the encapsidation pgRNA into core particles follows[5]. The slightly longer precore mRNA is translated to produce a precore protein that is further proteolytically processed into HBV e antigen (HBeAg). Inside the core particle, the viral polymerase directs the synthesis of the minus DNA strand of the genome by reverse transcription of the pgRNA template, which then serves as the template for plus DNA strand synthesis. Mature core particles containing DNA genomes are then enveloped and released or cycled back to the nucleus to replenish the cccDNA pool to perpetuate chronic infection[3].

The main goal of therapeutic intervention is to achieve a sustained suppression of HBV replication and to improve the quality of life and survival of chronic carriers by preventing progression to cirrhosis, HCC and death. So far, eradication of the virus is impossible and current antiviral treatment aims to reduce liver failure and HCC and to increase survival. The success of antiviral therapy is determined by the HBV surface antigen (HBsAg) and HBeAg serological status, as well as the levels of HBsAg and HBV DNA during the course of therapy. HBsAg seroconversion associates with a remission activity and improved long-term outcome[2]. However, HBsAg clearance is achieved in only 10% of the patients and even in these cases both antiviral options are unable to prevent the replenishment of the cccDNA pool from genomic HBV DNA recycled from the cytoplasm, or to reach efficient clearance of cccDNA-containing hepatocytes[6,7]. This explains the rapid rebound in serum HBV DNA after cessation of antiviral treatment.

Currently there are two therapeutic strategies approved for CHB treatment: five nucleos(t)ide analogues (NAs), which inhibit HBV replication, and the immune-based therapy that includes standard and pegylated interferon-α (IFN-α). Both antiviral treatments are not capable to eliminate the virus and to efficiently control the infection. IFN therapy is of finite duration and associates with low response rates, liver decompensation and numerous side effects, while NAs are long-term, well tolerated therapies but have a high risk of drug resistance development that limits their prolonged use.

This review focuses on current therapies for CHB infection and discusses the development of therapeutic agents that may ultimately lead to the definite eradication of the HBV and cccDNA pool as well as potential immunomodulators that can enhance the host immune responses against HBV that can efficiently control the infection without inducing liver damage.

NATURAL HISTORY OF CHB

The natural history of CHB infection consists five distinct phases of varying duration that are not necessarily sequential and are defined as: immune-tolerant, immune reactive HBeAg-positive, inactive HBV carrier, HBeAg-negative CHB and HBsAg inactive phase[8,9]. The course of the infection is dynamic and is a result of the complex interactions between the virus, hepatocytes and host immune responses. The periodic activation of the host immune system against the infected hepatocytes is an unsuccessful attempt to eradicate the virus that only leads to disease exacerbations and the development of fibrosis, cirrhosis and HCC[10]. The progression of HBV-induced liver diseases depends on the geographical area, the presence of HBsAg and HBeAg mutations and viremia levels[11]. Generally, patients with CHB have a 15%-40% risk to develop cirrhosis and 15% risk to develop compensated cirrhosis, while 60% of the compensated cirrhosis patients risk death[12].

Control of HBV infection involves the elimination of the infected hepatocytes by cytolytic and non-cytolytic mechanisms. The immune system of the host is capable to eliminate the infection as evidenced by the fact that more than 95% of adults spontaneously resolve the infection and that bone marrow transplantation recipients can resolve CHB infection[13,14]. In acute infection viral clearance is succeeded by the development of a robust,

polyclonal and multi-specific, HBV-specific cytotoxic T lymphocytes (CTLs) response to multiple epitopes of the viral nucleocapsid, envelope and polymerase. Furthermore, recovery from acute infection occurs by the non-cytolytic viral eradication mediated by HBV-specific CTLs since in the cases of spontaneous viral clearance only a part of the hepatocytes is being destroyed[15]. Elimination of HBV has been long considered to be T-cell dependent, however, natural killer (NK) cells are now known to be involved early in infection and B cells in the presentation to $CD4^+$ T cells and the production of neutralizing antibodies[16,17].

The complexity of the processes involved in self-limiting infection and natural history of the infection implies the requirement for a combination of therapeutic options. A synergistic approach of boosting the immune response of the host along with and effective viral load suppression is needed to succeed sustained viral clearance and complete eradication of the cccDNA pool in chronic infection.

CURRENT ANTIVIRAL THERAPY

In view of the natural history of CHB infection it is clear that chronic patients constitute a highly heterogeneous population and therefore require different management strategies. To optimise therapy for individual patient, several factors need to be considered related both to the patient, including age, sex, genetic polymorphisms, lifestyle factors, stage of liver disease and co-infections and to viral characteristics such as viremia, HBeAg-positivity, HBV genotype and viral genome heterogeneity. Furthermore the dosage duration, timing, efficacy, side effects, drug resistance and combination of antiviral agents need to be individually optimised. Unfortunately, current available treatment options require long term use and such attempts are expensive and carry a high risk for the development of breakthrough drug resistance.

NAS

Antiviral therapies for CHB using NAs have become standard treatment modalities. Current NA agents approved for treatment of CHB infection, include lamivudine, adefovir, entecavir, telbivudine, and tenofovir. Administration with NAs leads to a strong and long-term control of virus amplification by interfering with the viral replication cycle. Viral suppression can be reached in up to 95% of the patients[18]. The critical weak point of NA therapy is that it requires life-long administration, has modest effects on HBsAg levels and carries the risk of the development of drug resistance[2]. In addition, in HBeAg-positive patients the rate of seroconversion is as low as 20%-25% following one year of treatment[7]. The major adverse effects of long-term administration include nephrotoxicity and myopathy[19].

NAs are chemically synthesised drugs that competitively inhibit the DNA dependent and reverse transcriptase activity of viral polymerase and therefore inhibiting the reverse transcription of the pgRNA to the first strand of viral DNA. They are mimicking natural nucleotides and during viral replication they are being incorporated into newly synthesised HBV DNA causing chain termination. Moreover, NAs inhibit the synthesis of the HBV negative-DNA strand by reverse transcription and the synthesis of the positive-strand. They reduce significantly the cccDNA pool of infected hepatocytes by inhibiting the recycling of the nucleocapsids that contain viral genomes back to the nucleus but they cannot prevent the initial cccDNA formation in newly infected cells[20]. NAs are, therefore, efficient in blocking the synthesis of new virions and in reducing HBV DNA serum concentrations to undetectable levels but after cessation of treatment viral reactivation does occur due to the persistence of cccDNA. Experiments in woodchuck animals suggest that the effectiveness of NAs in reducing the cccDNA pool may depend on the cell cycle phase of the hepatocytes[21].

Development of antiviral resistance

During long-term therapy with NAs, HBV develops resistance to the drug administered. The resistance rates are higher with earlier generation NAs such as lamivudine, telbivudine, and adefovir. Although entecavir and tenofovir are associated with low risk of resistance for treatment to naive patients, it is still challenging to manage pre-existing antiviral resistance because of the risk of cross-resistance[22]. Emergence of drug resistant variants is commonly accompanied by acute exacerbation of liver disease and in some cases by hepatic decompensation and hence sequential monotherapy with low barrier drugs poses a serious problem[23,24].

The development of antiviral resistance depends on the interaction of viral, drug and patient factors. HBV replicates through the reverse transcription of an RNA intermediate. This step in the replication cycle is particularly prone to errors as the host RNA polymerase Ⅱ has an inherent low copying fidelity, and the viral polymerase/reverse transcriptase lacks proof-reading activity[25]. Considering that HBV is 3.2 kb in size and viral production rate in CHB infection can reach rates as high as 10^{11} virions per day, it has been estimated that 10^7 base pairing errors are produced daily in a chronic patient[26]. Although many of these mutations would be deleterious to the virus, some are advanteous, either by offering a replication advantage, or by facilitating immune escape and therefore predispose to the rise of antiviral resistant mutations[27]. Under the selection pressure exerted by antiviral drugs or immunological responses, the viral mutants that show maximum resistance to the treatment and high replication capacity are selected as primary drug resistance mutants over the wild type quasispecies[28]. The hepatocyte turn over rate is greatly increased in the inflammatory liver and, therefore, the drug resistance variants rapidly spread in

uninfected hepatocytes, occupying the new replication space and becoming the dominant viral quasispecies[26].

Lamivudine

Lamivudine is a moderate strength deoxycytidine nucleotide analog but due to its relatively low cost and being the first NA approved, it has a pharmacoeconomic advantage and has been widely used worldwide. Lamivudine inhibits the viral polymerase/reverse transcriptase and is equally effective against the wild-type virus and precore/core mutant variants[29,30]. It is a well-tolerated drug and has been shown to be effective even in patients with severe viral exacerbations and with hepatic failure[31,32]. Long-term lamivudine therapy results in up to 50% HBeAg seroconversions and maintains low levels of HBV DNA and alanine aminotransferases (ALT) in both HBeAg-positive and HBeAg-negative CHB patients[33,34]. However, the development of resistant mutations occurs in 20% after a year and as much as 70% following five years of treatment[35]. The most common mutation that confers resistance to lamivudine is the M204V/I/S mutation and involves a single amino acid substitution within the highly conserved YMDD motif at the catalytic centre of the polymerase[36]. Lamivudine mutations affect the ability of the dNTP-binding pocket to accommodate the drug, which in turn leads to a reduction in the affinity of lamivudine for the reverse transriptase domain[36].

Telbivudine

Telbivudine is a thymidine NA that once administrated is easily phosphorylated to its active triphosphate form[37]. It is structurally similar to lamivudine and has similar resistance profile, is well tolerated and has no dose-limiting side effects[38]. The overall rate of drug resistance development is 22% in HBeAg-positive patients and 9% in HBeAg-negative carriers[39]. Although it is more potent than lamivudine and adenovir, it is cross-resistant with lamivudine and has a considerable risk of drug resistance development[40].

Entecavir

Entecavir is a guanosine NA and inhibits polymerase/reverse transcriptase by competing with the natural substrate deoxyguanosine triphosphate. It inhibits both the wild type and lamivudine-resistant HBV variants, has a high rate of HBV DNA suppression, low drug resistance, low incidence of adverse reactions, and also been shown to improve liver function in patients with decompensation cirrhosis[41]. In clinical trials entecavir was found to be superior to lamivudine in NA-naive and lamivudine refractory HBeAg-positive or HBeAg-negative patients. After five years of therapy in NA-naive patients the risk of entecavir resistance is low but in lamivudine pre-treated patients, entecavir resistance associates with breakthrough in 50% of the patients[42].

Adefovir dipivoxil

Adefovir, an acyclic NA, is a potent inhibitor of viral replication of both the wild type and lamivudine resistance HBV[43]. In addition to acting as a DNA chain terminator it has been reported to induce NK cell activity and to induce endogenous IFN production[44]. The main resistance mutations are located in the palm subdomain of polymerase. Following five year treatment, approximately 30% of the patients develop drug resistance[45]. When adenovir is administered in combination with lamivudine to patients with pre-existing lamivudine resistance, cross-resistance does occur[46].

Tenofovir disoproxil

Tenofovir, another acyclic NA, is a methyl derivative of adenovir and exhibits anti-viral activity in lamivudine resistance HBV. It has been shown to have an additive suppression effect on viral replication when administered in combination with lamivudine, entecavir or telbivudine[47,48].

INTERFERON-BASED THERAPY

Recombinant and lymphoblastoid IFN-α, have been introduced as therapeutic regimens in CHB liver disease since the early 1980s. Conventional IFN-α or Pegylated IFN-α (Peg-IFN-α) induces direct antiviral activity by stimulating the host antiviral immune response and mediating divergent effects on viral replication. Peg-IFN-α has replaced conventional IFN-α treatment as it allows the administration of weekly injections compared to three times schedules of conventional IFN-α, while maintaining similar antiviral efficacy. Peg-IFN-α includes two preparations, Peg-IFN-α and Peg-IFN-α, 2α, that are heterogenous and contain multiple monopegylated isomers.

The response rate of IFN treatment in children is similar to that of adults, being about 30%-40% in those with high ALT levels, but this effectiveness drops to 10% in those with normal levels[49,50]. Nevertheless response rates can change at the end of the therapy because virological relapses commonly occur[51]. Sustained responses have been reported to be about 18%-25% at the end of IFN treatment and in relapsed patients that have been pre-treated with IFN[51,52]. Following IFN treatment factors associated with response to treatment include high ALT levels, low HBV DNA, older age and the absence of previous IFN therapy. Patients with the best outcomes are those with genotype A and high ALT or low HBV DNA, and those with genotypes B or C with both high ALT and HBV DNA levels[53]. Poor responses correlate with the duration of chronicity, the presence of precore mutations, male sex and human immunodeficiency virus (HIV) co-infection. The main advantages of IFN treatment are finite duration, absence of resistance, a higher rate of HBsAg clearance and HBeAg seroconversion (particularly among genotype A and HBeAg-positive patients), improvement of survival rates and a reduction of HCC occurrence[54]. However, the adverse effects of IFN include flu like symptoms, fatigue, bone marrow suppression and exacerbation of autoimmune illnesses and, therefore,

Table 1 Potential antiviral drugs for the future treatment of chronic hepatitis B virus

Potential antiviral agents	Mechanisms of action
NAs: MIV-210, elvucitabine, valtorcitabine and clevudine	Inhibition of HBV replication
Lipopeptides: Myrcludex-B	Prevention of viral entry
Disubstituted-sulfonamides: CCC-0975 and CCC-0346	Blockage of the *de novo* cccDNA synthesis
LTR	Destabilization cccDNA minichromosome
Zinc finger nucleases	Disruption of sequences within viral proteins
Epigenetic regulators	Repression of cccDNA transcriptional activity
Small interfering RNA	Silencing of HBV protein gene expression
Phenylpropenamides: AT-61 and AT-130	Prevention of RNA encapsidation
Heteroaryldihydropyrimidines: BAY41-4109	Nucleocapsid destabilization
Synthetic TLR-7 agonists	Inhibition of HBV replication *via* pDC activation
IL8 inhibitors	Increase the potency of IFN-α treatment
REP 9AC amphipathic polymers	Inhibition of subviral particles
Inhibitors of PD-1 and TIM3 receptors	Restoration of T cell function
Immunization with DC pulsed with HBV antigens	Induction of viral specific CTLs
Therapeutic vaccines containing viral peptides	Induction HBV-specific responses
Cytokines: IL12, IL2, IFNγ and TNF-α	Restoration of HBV specific T cell activity
Thymosin alpha polypeptide	Induction of T cell function and NK cytotoxicity

cccDNA: Covalently closed circular DNA; CTLs: Cytotoxic T lymphocytes; DC: Dendritic cells; HBV: Hepatitis B virus; MIV: Lagociclovir valactate; NK: Natural killer; pDC: Plasmacytoid DCs; PD-1: Programmed cell death 1; TLR: Toll like receptor; TIM3: T-cell immunoglobulin domain mucin domain-containing molecule-3; IFN: Interferon; TNF: Tumor necrosis factor; NAs: Nucleos(t)ide analogues; LTR: Lymphotoxin receptor; IL: Interleukin.

patients should be closely monitored[55]. Treatment with IFN-α has been shown to modulate the epigenetic repression of cccDNA activity and its potential role in antiviral treatment is discussed later.

COMBINATION THERAPEUTIC STRATEGIES

Current antiviral monotherapies are not able to eradicate the HBV from the liver, have restricted efficacy, high cost and lead to drug resistance. So far, combination therapy with a number of NAs or with IFN, were not superior in comparison to monotherapy[56-58]. However, a synergistic antiviral effect may confer an additional benefit[59,60]. Combining low barrier resistance drugs, such as lamivudine and adenofir, with or without IFN can increase barrier resistance but does not improve viral suppression and HBsAg clearance as compared to monotherapy with new-generation NAs, like entecavir or tenofovir[61,62]. However, in the absence of alternative antiviral agents, a combination of NAs has been shown to be efficient in patients with partial responses or viral resistance patterns[63].

Considering the shortcomings of antiviral therapies it is imperative to identify novel drug targets to develop new combination therapies that can achieve the clearance of HBV DNA and cccDNA as well as the restoration of immune defence mechanisms. Research on HBV led to the discovery of number of compounds that could potentially complement NAs or IFN therapies (Table 1) and are being further discussed.

HBV LIFE CYCLE INHIBITORS

HBV DNA polymerase

In addition to the approved NAs, there are several novel drugs developed to inhibit reverse transcription. Among them, lagociclovir valactate (MIV-210) is a prodrug with high oral bioavailability in humans and is a potent inhibitor of the replication of the wild type, lamivudine-resistant, adenovir-resistant, and lamivudine-adenovir cross resistant mutant HBV genomes[64]. Other new NAs that show potent inhibition of HBV replication *in vitro*, include elvucitabine, valtorcitabine and clevudine.

Viral entry

Myristoylated preS-peptide (Myrcludex-B) is a lipopeptide derived from the pre-S1 domain of the HBV envelope. It can prevent viral spread from infected hepatocytes *in vivo* and reduces the amplification of cccDNA in newly infected hepatocytes[65]. Petersen *et al*[66] demonstrated that it is capable to prevent HBV infection in hepatic cell culture and humanized mice as well as the establishment of hepatitis D virus infection.

Synthesis of cccDNA

Elimination of cccDNA is a prerequisite for a successful therapy and represents a challenging and important antiviral target. Two small molecules that have been reported to specifically target cccDNA synthesis are structurally related disubstituted-sulfonamides and can potentially be used as drugs to block the de novo synthesis of cccDNA[67]. Considering the long nuclear half-life of cccDNA and its dependence on host factors for its activity, eliminating established cccDNA appears to be bigger challenge but evidence suggests that it is not invulnerable to therapy. HBV cccDNA has been shown to be destabilized *in vitro* with inflammatory cytokines and IFN-α by non cytolytic mechanisms while is also eradicated when the infected hepatocytes are being eliminated by host immune mechanisms[68]. Interestingly, a recent study has shown that high doses

of IFN-α and lymphotoxin receptor (LTR) induced the expression of APOBEC3A or 3B resulting in the non-cytopathic reduction of cccDNA in HepaRG cell and primary human hepatocytes[69]. Another target of cccDNA is to identify compounds able to interfere with the regulation of its transcriptional activity. A new approach is the generation of zinc finger nucleases (ZFNs) that target sequences within viral proteins such as polymerase, core and *X* genes[70]. Delivery of HBV-specific ZFNs in cell culture systems was shown to be achieved successfully by vectors and resulted in the efficient disruption of the target genes by the generation of site-specific mutations. However, the delivery of such targeted proteins in chronic patients remains a therapeutically challenge.

Epigenetic control of cccDNA

Epigenetic mechanisms refer to heritable changes in chromatin organization and gene expression independent of the underlying DNA sequence and have been shown to play a key role in HBV replication. Interfering with the epigenetic regulation of cccDNA minichromosome is another promising therapeutic approach. Viral replication and cccDNA transcriptional activity have been shown to be regulated by the acetylation status of cccDNA-bound H3/H4 histones as well as by the recruitment of cellular acetyltransferases and histone deacetylases onto cccDNA in cell culture and primary human hepatocytes[71,72]. Experiments in humanized mice and cell culture demonstrated that treatment with IFN-α induces cccDNA-bound histone hypoacetylation and the active recruitment of transcriptional corepressors onto cccDNA[73]. IFN-α administration was also shown to reduce binding of STAT1 and STAT2 transcription factors to active cccDNA. Identifying, the molecular mechanisms by which IFN-α mediates epigenetic repression of cccDNA transcriptional activity can lead to the development of novel therapeutics. In CHB patient, viral and host DNA methylation density varies significantly has been identified as a host defence mechanism to suppress viral gene expression and replication. Furthermore, an up regulation of DNA methyltransferases has been reported in CHB livers that facilitates the methylation of cccDNA and viral genomes affecting protein production and viral replication[74,75]. It has been reported that host DNA methylation is the main mechanism to inactivate relevant genes in HCC[76]. These findings suggest a potential role of methylation in the future treatment of CHB infection.

Small interfering RNAs

RNA interference (RNAi) is an evolutionary conserved process by which double-stranded RNA induces sequence-specific silencing of homologous genes. RNAi-based therapeutics act in a fundamentally different manner than other therapies. They have the potential to specifically knock down the expression of HBV proteins, including HBsAg and pgRNA, thus reducing viral replication. Experiments in transgenic mice showed that delivery of potent small interfering RNAs (RNAsi) resulted in the long and sustainable repression of viral RNA, proteins and HBV DNA levels[77]. However, the use of RNAsi still remains a therapeutic challenge due to the lack of a safe and effective delivery system to patients.

Nucleocapsid assembly and stability

There are a number of studies aiming at the development of agents that inhibit nucelocapsid assembly or stability. A few non-nucleocapsid molecules have been shown to inhibit the replication of both the wild type virus and of drug resistant variants[78]. These include compounds that belong either to the family of phenylpropenamide (AT-61 and AT-130) and have been reported to prevent RNA encapsidation or to the family of heteroaryldihydropyrimidines (BAY41-4109) that can destabilize nucleocapsids[55,79]. In addition to their impact on replication cycle, these agents can inhibit cccDNA intracellular amplification by inhibiting nucleocapsid recycling to the nucleus in woodchuck animal model[80].

IMMUNOMODULATORS

Besides interfering with the viral life cycle, other therapeutic approaches aim to the restoration and duration of the immune responses against HBV. An increasing number of studies have been reporting a number of potential immunomodulators that can be effective in CHB treatment (Table 1).

Innate responses

The important role of the innate immunity in controlling HBV infection has gained significant ground the last years and several studies have focused on the development of compounds that can manipulate NK cell immunity. In CHB infection, NK exert potent antiviral activities either directly by the lysis of infected hepatocytes or indirectly by modulating viral specific T cells while they also contribute to the pathogenesis of liver injury[81]. Furthermore, it has been proposed that HBV inhibits the innate system *via* the suppression of toll like receptor (TLR) induced antiviral signalling[82]. TLR7 and TLR9 ligands or agonists have been shown to inhibit viral replication by the production of vast amounts of type Ⅰ and Ⅲ IFNs *via* the activation of plasmacytoid dendritic cells (pDCs)[83]. Experiments in chimpanzee and woodchucks have shown that a synthetic TLR-7 agonist reduced serum and liver viremia as well as HBsAg and increased the expression of IFN-α and interferon stimulated genes[55]. This compound has reached Phase Ⅰ clinical trials[84]. Treatment with entecavir has been reported to restore TLR2 expression in infected cells while administration of TLR2 ligand repressed HBV replication[85]. These findings suggest that a combination of TLRs agonists with NAs could provide a promising therapeutic approach. Another compound that is being evaluated for its antiviral capacity is the REP 9AC Replicor, which is a nucleic acid-based amphipathic

polymer. It has been shown to facilitate innate responses *via* the inhibition of subviral particles from infected hepatocytes[86].

Interleukin-8 (IL8) chemokine is an important mediator of innate immunity and T cell function. In patients undergoing HBV reactivation, serum IL8 levels have been shown to parallel viremia levels[16,87]. Specific inhibition of IL8 has been shown to increase the potency of IFN-α treatment in HBV transfected hepatic cell lines and the addition of recombinant IL8 was reported to rescue almost completely viral replication following IFN-α treatment[87]. The development of an IL8 blockage strategy combined with IFN-α treatment can be another encouraging future therapeutic approach.

Viral specific T cell responses

CHB infection is characterized by the hyporesponsiveness of HBV-specific $CD4^+$ T cell and CTL that is considered to be caused from the presence of large quantities of virions and viral particles in the tolerogenic environment of the liver, particularly in childhood. The dysfunction of viral specific T cells has been associated with defects in co-stimulatory pathways. The negative regulation of T cell function associates with defects in co-stimulatory pathways and in particular with the increased expression of inhibitory receptors programmed cell death 1 (PD-1) and its ligand 1, T-cell immunoglobulin domain, mucin domain-containing molecule-3 (TIM3) and CD244 as well as the impairment of DCs and the increased frequencies of T regulatory cells (Tregs)[55,88,89]. Restoration of T cell function could, at least partially, be achieved by the blockage of the negative regulatory pathways including inhibitors of such receptors, *e.g.*, anti-PD-1 mAb, and anti-apoptotic drugs that block TIM3[13]. Another potential therapeutic strategy is to activate DC function, by DC-based immunotherapy. Immunization of DCs pulsed with HBV antigens has been shown to induce viral specific CTLs responses, to overcome tolerance against HBV and to reactivate B cell responses in transgenic mice[90]. Tregs that significantly contribute to T cell tolerance in CHB were reported to reduce the response to treatment in IFN-α non-responders whereas administration of entecavir reduced their frequencies and function[89,91]. Expansion of HBV core antigen (HBcAg)-specific CTLs is shown to be essential in HBV replication control and leads to the activation of endogenous DC and HBsAg-specific CTLs without inducing liver damage[90]. Therefore the suppression of Tregs and HBcAg can also be considered as potential approaches in immunotherapy.

Another adjuvant of potential benefit is CpG DNA, a synthetic oligonucleotide that preferentially stimulates Th1 responses, with the production of IL12 and IFNγ[92]. Immunization of transgenic animals with HBsAg vaccine supplemented with CpG DNA led to clearance of serum HBsAg and the development of anti-HBs, with concurrent down-regulation of HBV mRNA production in the liver. Adoptive transfer experiments of T cells from such animals showed that they were able to partially control transgene expression in the liver and to clear HBsAg without an antibody requirement[92]. A CpG-containing HBsAg vaccine was shown to overcome hyporesponsiveness normally seen in immunized orangutans[93]. Similarly, it was shown that cytokines from peripheral blood mononuclear cells from HBV-negative individuals stimulated with CpG ODN strongly inhibited HBV viral replication, HBsAg and HBeAg production from infected HepaRG and HepG2 cells[94].

Therapeutic vaccination

Therapeutic vaccination is another approach that can be used in attempts to achieve long-term antiviral treatment. An effective vaccine should both induce a strong antigen-specific immune response and the subsequent deployment of immune response to HBV in the liver. Currently, the vaccines that are being evaluated in CHB patients and experimental animals include recombinant proteins, specific peptides, DNA vaccine or DNA delivered by viral vectors. Clinical trials using vaccines containing HBcAg and HBsAg peptides showed a reduction of HBV replication that that were not accompanied by HBsAg clearance[95,96]. However, a recent vaccine formulation that comprised HBsAg and HBcAg particles and was delivered together with a saponin based ISCOMATRIX adjuvant in transgenic mice induced the activation HBsAg- and HBcAg-specific CTLs and the high production of their antibody[97].

Cytokines and thymosin

Several cytokines are involved in the defective immune responses and can be used as adjuvant compounds to break the immune tolerance in CHB infection. Among them, IL12 has been reported to restore viral specific-T cell hyporesponsiveness and to down-regulate PD-1 inhibitory receptor[98]. Combined therapy of lamivudine and recombinant IL2 was shown to increase HBV-specific T cell activity and to induce HBeAg seroconversion[99,100]. Treatment with lamivudine combined with IFNγ and tumor necrosis factor-α was shown to induce a stronger inhibition of cccDNA and in the efficient suppression of viral replication without the development of cytotoxicity[101]. Thymosin alpha 1 (Ta1) is a synthetic polypeptide that has immunomodulating activity and has been shown to promote T cell activity, IFNγ and IL12 production as well as NK-induced cytotoxicity[102]. Treatment with Ta1 has been demonstrated to reduce significantly viral replication in chronic patients and woodchuck animals[103,104]. Long-term combination therapy of lamivudine and Ta1, but not with peg-IFN-α, was found to be superior to monotherapy and correlated with HBeAg seroconversion[27,105]. The conflicting results on the benefits of Ta1 in combination therapy suggest that more clinical studies are required to further evaluate this compound.

CONCLUSION

Although antiviral therapy of CHB infection has improved dramatically during the last decades an effective treatment is still not available and CHB remains a serious

clinical problem worldwide. Current available antiviral options suppress viral replication and improve patient survival but they do not eradicate the virus and the cccDNA pool resulting in viral reactivation after cessation of treatment and in the development of liver disease progression. The goal of new therapeutic strategies is to eliminate or control HBV and to allow access to therapy in poor high-endemicity areas, where the consequences of HBV infection are more severe. Experience with the treatment of HIV and HCV has proven that combination therapy with compounds targeting multiple steps in the replication cycle would be more efficient than mono-therapy. Research efforts focus on the identification of novel compounds that inhibit viral entry, nucleaocapsid assembly, reverse transcription and cccDNA formation and stability. Besides interfering with the viral life cycle, an increasing number of studies have reported several promising immunomodulators that aim to restore the HBV specific T cell hyporesponsiveness and to boost the innate immune arm of the host, while blocking potential pathways of liver damage. The development of such agents would help to improve existing therapeutic regimens and provide new opportunities for more efficient combination therapies. New strategies should be clinically evaluated by large-scale trials or by the use of relevant experimental models. Because access to chimpanzees is restricted, human HBV replication is being now being studied in humanized mice. Even if these mouse models are useful in validating novel antiviral compounds have the critical weak point of an immune-deficient host that doesn't reflect the situation of human liver environment.

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P- Reviewer: Castiella A, Robert KI, Rodriguez-Castro KI, Sargsyants N, Urganci N, Xu R
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