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Commentary

Are novel combination therapies needed for chronic hepatitis B?

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ABSTRACT

The treatment of chronic hepatitis B remains limited to monotherapy with pegInterferon-alpha or one of 5 different nucleoside analogues (NUC). While viral suppression can be achieved in approximately 95% of patients with new-generation NUCs, the rate of HBeAg seroconversion ranges from only 20% with NUCs to 30% with pegInterferon-alpha. HBsAg loss is achieved in only 10% of patients with both classes of drugs after a follow-up of 5 years. Attempts to improve the response by administering two different NUCs or a combination of NUC and pegInterferon-alpha have been unsuccessful. This situation has led researchers to investigate a number of steps in the HBV replication cycle as potential targets for new antiviral drugs. Novel targets and compounds could readily be evaluated in *in vitro* and *in vivo* models of HBV infection. The addition of one or more new drugs to the current regimen should offer the prospect of markedly improving the response to therapy, reducing the future burden of drug resistance, cirrhosis and hepatocellular carcinoma.

Currently available treatments for chronic hepatitis B rely on monotherapy with interferon alpha (IFN) or with nucleoside analogues (NUC), i.e. lamivudine, adefovir dipivoxil, telbivudine, entecavir and tenofovir disoproxil fumarate (Dienstag, 2008). Viral suppression (undetectability of serum HBV DNA) is achieved in up to 95% of patients treated with NUCs (Dienstag, 2008). However, in HBeAg-positive patients, despite the use of more potent NUCs, the rate of HBe seroconversion remains around 20-25% after one year of therapy. HBsAg loss, which is a major treatment endpoint that allows treatment cessation, is achieved in only 10% of patients after 5 years of NUC administration or after 5 years follow-up of IFN treatment (Lampertico and Liaw, 2012). The impact of viral or host genome variability, i.e. viral genotypes or IL28-B polymorphism, on treatment response does not seem to be as convincing as for chronic hepatitis C, although it might be clinically relevant for IFN-based therapy (Lampertico and Liaw, 2012).

One of the main clinical challenges for the management of hepatitis B is to favor access to therapy, especially in resource-poor countries which are also high-endemicity areas, where the consequences of HBV infection are significant (Dienstag, 2008). Despite the fact that the administration of new-generation NUCs can achieve viral suppression in the majority of patients, current drugs cannot clear the viral genome and cccDNA from infected hepatocytes, and long-term administration may select for drug-resistant mutants (Zoulim and Locarnini, 2009). This is in sharp contrast to the situation for hepatitis C, since the latter virus can be completely cleared by antiviral treatment, because its genome is not archived within infected cells (Chung, 2012). The selection of resistant mutants is one of the major issues in countries where HBV is endemic and treatment relies mainly on low barrier to resistance drugs because of cost constraints (Gish et al., 2012). In these countries, the use of sequential monotherapy with drugs having a low barrier to resistance can expose patients to a high risk of selecting multidrug-resistant mutants (Liu et al., 2010; Villet et al., 2009).

The current research trend is to develop antiviral strategies that would lead to finite duration treatment, via the clearance of viral cccDNA and/or HBsAg and the restoration of immune control. It is expected that such strategies would also have a beneficial impact on the prevention of drug resistance (Zoulim and Locarnini, 2009). This may also prove useful for the prevention or delay of HCC development, especially if treatment is initiated at an early stage of liver disease, i.e. before the establishment of a pre-neoplastic stage resulting from molecular and genetic events induced by HBV infection (Mason et al., 2010; Zoulim and Mason, 2012).

Up to now, attempts at therapy with combinations of NUCs targeting different steps in viral DNA synthesis (i.e. priming of reverse transcription, viral minus strand DNA synthesis, plus strand DNA synthesis), or their combination with IFN, have failed to increase the success rate in comparison to monotherapy (Janssen et al., 2005; Lok et al., 2012; Marcellin et al., 2004; Scaglione and Lok, 2012). The use of combinations of NUCs with a low barrier to

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resistance (lamivudine, adefovir) has allowed an increase in the barrier to resistance, but with lower efficacy compared to first-line monotherapy with new-generation NUCs (entecavir, tenofovir) which exhibit a high barrier to resistance. However, the combination of potent NUCs with a high barrier to resistance has not resulted in an increase in the rate of viral suppression and HBsAg loss, even when combined with IFN, which has a complementary mode of action on immune responses. On the other hand, in patients who failed multiple lines of therapy, a combination of NUCs with complementary cross-resistance profiles has been used with success to achieve viral suppression as a rescue strategy (Petersen et al., 2012; Si-Ahmed et al., 2011; Zoulim and Locarnini, 2009).

The identification of other drug targets is therefore needed to develop true combination therapies for HBV infection. Several targets and novel compounds are currently being evaluated in *in vitro* and *in vivo* experimental models, which could potentially complement NUC or IFN-based therapy. As shown in Fig. 1, these targets include viral entry, the formation of viral cccDNA and its epigenetic regulation, the RNAse H activity of the viral polymerase (which remains a poorly studied antiviral target), nucleocapsid assembly, and effectors of both innate and adaptive immunity involved in the control of viral infection. These new developments should pave the way for the clinical evaluation of novel combination strategies in the near future.

With respect to viral entry, it was shown that myristoylated preS-peptide (Myrcludex-B), a lipopeptide derived from the pre-S1 domain of the HBV envelope, can prevent HBV infection in hepatocyte culture as well as *in vivo* in humanized uPA/SCID mice, in which the liver is repopulated by human hepatocytes (Petersen et al., 2008). The same group used this mouse model to show that treatment with this HBV entry inhibitor efficiently hindered the establishment of infection by hepatitis delta virus (HDV), a defective virus which requires the HBV envelope for its infectivity (Lutgehetmann et al., 2012). Since there is currently no specific antiviral for HDV infection except interferon alpha, these data provide the rationale for the clinical evaluation of this compound in patients who are co-infected with HBV and HDV.

The formation of cccDNA represents a very important antiviral target. The cellular and biochemical events required for this process involve the transport of nucleocapsid to the nucleus, and the transformation of the relaxed circular DNA genome into cccDNA via the removal of the viral polymerase covalently linked to viral minus strand DNA, the removal of the short RNA primer for plus strand DNA synthesis, the completion of plus-strand DNA the removal of the viral minus-strand DNA redundancy and the ligation of DNA strand extremities (Seeger and Mason, 2000). These steps seem to involve several nuclear enzymes, for which it may be difficult to target a function specific to the viral life cycle (Sohn et al., 2009). Administration of nucleoside analogues failed to prevent the initial formation of cccDNA after *de novo* infection of hepatocytes, while their long-term administration to already infected cells or individuals seems to decrease the pool of already

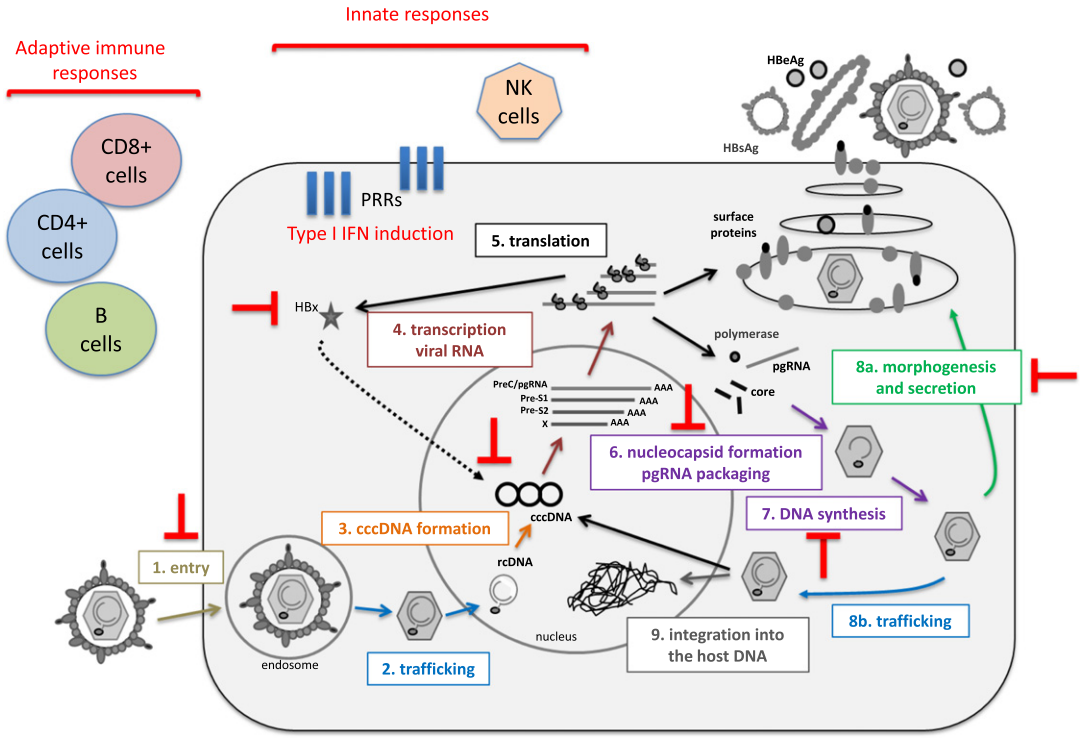

Fig. 1. The HBV replication cycle and targets for combination antiviral therapy. HBV is a DNA virus in the Hepadnaviridae family; its replication cycle has been reviewed in detail elsewhere (Seeger and Mason, 2000). After viral entry, the naked HBV nucleocapsid is directed towards the nucleus, and the HBV genome is translocated into the nucleus. The relaxed circular DNA (rcDNA) genome is then converted into covalently closed-circular DNA (cccDNA), the template for viral transcription. After RNA transcription and subsequent protein synthesis, the pregenomic RNA (pgRNA) is encapsidated and reverse transcribed into new rcDNA. Mature nucleocapsids are then either directed to the secretory pathway for envelopment and the release of new virions, or redirected towards the nucleus to amplify the cccDNA pool. The HBV genome can also be integrated into the cellular host genome; this is not necessary for viral replication, but it is one of the key factors involved in oncogenesis. Viral clearance requires the concerted action of different components of the immune response, including innate and adaptive responses (Bertoletti and Ferrari, 2012), as well as infected cell killing by host immune responses and replacement of infected cells by non-infected cells (Summers et al., 2003). The HBV genome is spontaneously variable, and mutations are generated at each replication cycle. The fittest mutants can be selected during antiviral treatment with NUCs (Zoulim and Locarnini, 2009). The main targets for antiviral therapy are depicted in red, and include: (1) inhibition of the main steps of viral replication: viral entry, cccDNA formation and its epigenetic regulation, the HBx protein functions, nucleocapsid assembly and pgRNA packaging, viral DNA synthesis, and viral morphogenesis; (2) modulation of innate immune responses, which rely on HBV sensing by infected cells via pathogen recognition receptors (PRR) subsequently leading to type I IFN induction, with the role of NK cells being debated; (3) modulation of adaptive immune responses to restore the function and activity of anti-HBV T and B cells.

established cccDNA by the inhibition of the recycling of nucleocapsids containing viral genomes to the nucleus and by the clearance of infected cells from the liver (Delmas et al., 2002; Le Guerhier et al., 2000, 2001; Werle-Lapostolle et al., 2004). Interestingly, it was recently reported that small molecules may specifically target cccDNA formation. Two structurally related disubstituted-sulfonamides compounds were identified and may potentially serve as proof-of-concept drug candidates to eliminate cccDNA from chronic HBV infection (Cai et al., 2012).

Another way to target cccDNA is the modulation of its transcriptional activity. A first approach was to design zinc finger proteins targeting the duck hepatitis B virus enhancer. After cotransfection of vectors encoding these proteins and DHBV in cultured cells, it was shown that zinc finger proteins are able to bind to the DHBV enhancer and interfere with viral transcription, resulting in decreased production of viral products and progeny virus genomes (Zimmerman et al., 2008). The delivery of such targeted proteins remains a therapeutical challenge. Interfering with cccDNA associated chromatin proteins is another exciting approach. Indeed, the acetylation and/or methylation status of the histones bound to cccDNA affect its transcriptional activity. It was shown in cell culture and in humanized mice that IFN administration induces cccDNA-bound histone hypoacetylation, as well as active recruitment to the cccDNA of transcriptional corepressors (Belloni et al., 2012). IFN-α treatment also reduced binding of the STAT1 and STAT2 transcription factors to active cccDNA. This may represent a molecular mechanism whereby IFN-α mediates epigenetic repression of cccDNA transcriptional activity, which may assist in the development of novel therapeutics.

The use of anti-sense or small interfering RNAs mainly represents a proof of concept that targeting the expression of a specific viral or host gene can inhibit viral replication, but still remain a therapeutic challenge, especially in terms of delivery to patients.

Targeting the HBx protein is also a potential antiviral approach. Indeed, it was shown that this protein is required for viral infection *in vivo* (Zoulim et al., 1994). More recently, it was shown in primary hepatocyte cultures and in HepaRG cells that the HBx protein is necessary to initiate and maintain viral replication. Interestingly, although equal amounts of nuclear cccDNA demonstrated comparable uptake and nuclear import, active transcription was only observed with HBx-expressing viral genomes (Lucifora et al., 2011). This may be consistent with the observation that nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function (Belloni et al., 2009). A more detailed knowledge of HBx function should help to target this key regulator of viral replication.

Several attempts have been made to develop inhibitors of nucleocapsid assembly or stability. A few non-nucleosidic molecules, belonging to the family of phenylpropenamides (AT-61 and AT-130) (Delaney et al., 2002) and heteroaryldihydropyrimidines (BAY41-4109) (Deres et al., 2003), can prevent RNA encapsidation or destabilize nucleocapsids, respectively. These antiviral compounds were shown to inhibit the replication of wild-type HBV as well as HBV mutants resistant to nucleoside analogues (Billioud et al., 2011). Beside their effect on viral DNA synthesis and virion production, these agents may potentially inhibit the intracellular amplification of cccDNA via the inhibition of nucleocapsid recycling to the nucleus, and may have other beneficial effects by modulating interactions between HBV and its hosts, for which the exact mechanisms need to be unraveled. Interfering with other steps of viral morphogenesis and virion infectivity through the modulation of viral envelope glycosylation by alpha-glucosidase inhibitors represent other relevant approaches to be developed (Block et al., 1998; Lazar et al., 2007). Other groups have also tried to use triazolo-pyrimidine derivatives to decrease viral envelope protein secretion in experimental models in the perspective of restoring specific immune responses against viral envelope epitopes (Yu et al., 2011).

Beside the inhibition of viral replication, other antiviral strategies are to enhance specific immune responses against HBV. Based on recent knowledge of the role of innate responses in the control of HBV infection (Durantel and Zoulim, 2009), several approaches have been evaluated to determine, among others, the effect of TLR2 or TLR7 stimulation in the woodchuck and chimpanzee models, respectively. For instance, it was shown that a TLR7 agonist can induce IFN-alpha and interferon stimulated gene (ISG) expression in chimpanzees, which was associated with reduced serum and liver viral load (Lanford et al., 2010). Transient elevations of serum transaminase levels were observed. The data were consistent with immune elimination of infected hepatocytes. Another recent study showed that entecavir administration can restore TLR2 expression in infected cells, and that administration of TLR2 ligands inhibited viral replication (Zhang et al., 2012). It would be interesting to test whether the combination of a NUC with a TLR2 agonist results in an enhanced antiviral effect.

In chronic HBV infection, defective T cell function is probably maintained by the effect of the prolonged exposure of T cells to large quantities of viral antigens and by the tolerogenic features of both liver cells and liver resident cells (Bertoletti and Ferrari, 2012). These two combined mechanisms can result in the deletion of HBV-specific T cells or in their functional inactivation (exhaustion), which is characterized by an increased expression of negative co-stimulatory molecules and dysregulation of co-stimulatory pathways, such as PD-1/PD-L1, which affect antiviral T cell responses. In principle, restoration of immune control could follow different strategies (Bertoletti and Ferrari, 2012). The inhibition of viral replication and decline in HBV antigens could lead to partial restoration of anti-viral HBV-specific T cell functions and inhibition of HBV suppressive effects. Blockade of negative regulatory pathways could be effective, by partially restoring HBV-specific T cell functions. Anti-apoptotic drugs may reduce HBV-specific T cell apoptosis and fight against T cell exhaustion. The *de novo* reconstitution of functionally active HBV-specific T cells or activation of heterologous T cells is also another potential strategy. Besides these targeted immune strategies, attempts to deliver therapeutic vaccines (with recombinant proteins, specific peptides, DNA vaccine or DNA delivered by viral vectors) have been evaluated in chronically infected patients or animals, and may represent an interesting treatment option to be further evaluated in association with NUCs, at least in selected patient populations. These different studies have been reviewed recently (Michel et al., 2011).

These new strategies should be evaluated in the most relevant experimental models, which have been more difficult to handle than those of HCV infection, which have permitted the rapid development of direct-acting anti-HCV drugs from bench to bedside (Manns et al., 2007). Infectious cell-culture models for HBV rely on primary hepatocyte culture and the HepaRG cell line (Gripon et al., 1988, 2002), which are the only robust models used to study the entire HBV life cycle, but are still tedious to work with, compared to the traditional HepG2 or Huh7 hepatoma cell lines, which are used to study the late stages of HBV replication after transfection of replication-competent constructs. These cell culture models were critical for pinpointing important steps in viral replication and virus-host cell interactions, leading to the identification of potentially new antiviral targets. The *in vivo* infectious models are based on the use of ducks or woodchucks naturally or experimentally infected with their respective hepadnavirus. Because access to chimpanzees is restricted, human HBV replication is currently studied in humanized uPA/SCID mice (Petersen et al., 2008). However, these mouse models have the disadvantage of using an immune-deficient host. Nevertheless, these experimental models are useful for validating new targets and elucidating the

mode of action of new antiviral compounds. This knowledge will be essential to design clinical trials for true combination strategies, which currently represent one of the major clinical challenges for the long-term control of chronic hepatitis B.

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