



Review

Synthetic RNAi triggers and their use in chronic hepatitis B therapies with curative intent



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ABSTRACT

Current therapies for chronic hepatitis B virus infection (CHB) – nucleos(t)ide analogue reverse transcriptase inhibitors and interferons – result in low rates of functional cure defined as sustained off-therapy seroclearance of hepatitis B surface antigen (HBsAg). One likely reason is the inability of these therapies to consistently and substantially reduce the levels of viral antigen production. Accumulated evidence suggests that high serum levels of HBsAg result in exhaustion of the host immune system, rendering it unable to mount the effective antiviral response required for HBsAg clearance. New mechanistic approaches are required to produce high rates of HBsAg seroclearance in order to greatly reduce off-treatment disease progression. Already shown to be a clinically viable means of reducing gene expression in a number of other diseases, therapies based on RNA interference (RNAi) can directly target hepatitis B virus transcripts with high specificity, profoundly reducing the production of viral proteins. The fact that the viral RNA transcripts contain overlapping sequences means that a single RNAi trigger can result in the degradation of all viral transcripts, including all messenger RNAs and pregenomic RNA. Advances in the design of RNAi triggers have increased resistance to degradation and reduced nonspecific innate immune stimulation. Additionally, new methods to effectively deliver the trigger to liver hepatocytes, and specifically to the cytoplasmic compartment, have resulted in increased efficacy and tolerability. An RNAi-based drug currently in clinical trials is ARC-520, a dynamic polyconjugate in which the RNAi trigger is conjugated to cholesterol, which is coinjected with a hepatocyte-targeted, membrane-active peptide. Phase 2a clinical trial results indicate that ARC-520 was well tolerated and resulted in significant, dose-dependent reduction in HBsAg for up to 57 days in CHB patients. RNAi-based therapies may play an important role in future therapeutic regimens aimed at improving HBsAg seroclearance and eliminating the need for lifelong therapy. This paper forms part of a symposium in *Antiviral Research* on “An unfinished story: from the discovery of the Australia antigen to the development of new curative therapies for hepatitis B.”

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1. Introduction

Globally, an estimated 240 million people are chronically infected with the hepatitis B virus (HBV) and more than 750,000 deaths are attributed annually to HBV-related complications, including hepatocellular carcinoma (HCC), cirrhosis of the liver, and liver failure (Fattovich et al., 2004; Lozano et al., 2012; Marcellin et al., 2005; Ott et al., 2012). Persons exposed to HBV after 5 years of age through unsterile medical procedures, unsafe injections, sexual transmission, interfamilial transmission via open cuts or scratches, or other means have a high propensity for developing acute hepatitis, but an approximately 93–95% chance of clearing the virus from the circulation (McMahon, 2005; McMahon et al., 1985). The best patient outcome is seen with clearance of hepatitis B surface antigen (HBsAg) from the serum, with or without development of measurable antibodies to HBsAg (Yuen et al., 2004, 2008). For patients with HBsAg seroclearance, the risk of developing HCC is markedly reduced yet still modestly elevated relative to background levels in the general “healthy” population (Liu et al., 2014). For the 5–7% of HBV-infected adults who do not clear HBsAg, there is a substantially increased risk of developing cirrhosis, hepatic decompensation, and HCC (Fattovich et al., 2004; Ganem and Prince, 2004; Liaw et al., 1988; Marcellin et al., 2005).

In those individuals with HBsAg seroclearance, the virus is still present in the liver and occasionally can be measured in the blood (Yuen et al., 2004). Use of potent immune modulators that suppress the immune system can lead to a loss of immunologic control and reactivation of HBV (Seetharam et al., 2014). For this reason, any drug that effectively treats HBV and leads to seroclearance will not be considered a “sterilizing cure” or “virologic cure” but rather a “functional cure” unless it also leads to complete clearance of viral DNA from all hepatocytes. Given that the HBV genome is not only present in the hepatocyte nucleus in the form of cccDNA but also integrates into the host genome, a full, complete cure seems unlikely with current technologies (see reviews in the present symposium by (Gish et al., 2015; Block et al., 2015; Gozuacik et al., 2001; Mason et al., 2010; Murakami et al., 2005; Saigo et al., 2008)).

Ongoing experience with approved HBV therapies, including interferons (IFNs) and nucleos(t)ide analogue reverse transcriptase inhibitors (NUCs), while showing clear antiviral activity, including reduction in circulating viral DNA often to an undetectable level, high levels of clearance of the hepatitis B e antigen (HBeAg) from the circulation, normalization of liver transaminases, and improvement in hepatic necroinflammation on biopsy, has been disappointing to the hepatology community and patients due to low rates of HBsAg clearance and a high rate of viral rebound after NUC discontinuation (Frenette and Gish, 2009; Fung et al., 2009; Jafri and Lok, 2010; Lok and McMahon, 2009; Seto et al., 2015). Of greater concern, it has been learned that even with effective NUC therapy and good response on the above discussed parameters, these patients remain at increased risk of developing HCC (Hadziyannis et al., 2006).

2. Fundamentals of RNAi

RNA interference (RNAi) is an evolutionarily conserved set of mechanisms that utilize small, non-coding RNAs to regulate the expression of genetic information (Carthew and Sontheimer, 2009). Depending on the precise structure of the non-coding

RNAs and their biological context, different modes of regulation by RNAi are induced. This review focuses on the mode of RNAi that results in the degradation of the mRNA target as this is the mode most commonly employed in RNAi-based drugs, including those designed to target chronic HBV infection (CHB).

The agents used to induce RNAi are short double-stranded oligonucleotides composed of a guide strand that is base-paired to a passenger strand. The guide strand has perfect, or near perfect, complementarity to a sequence in the targeted mRNA. The first demonstration that such a molecule could induce RNAi in mammalian cells involved introduction of synthetic 21mer, double-stranded RNA (dsRNA), named small interfering RNA (siRNA), into cultured cells (Elbashir et al., 2001a). This resulted in sequence-specific reduction of expression of the targeted gene. Since then, the molecular and biochemical mechanisms by which siRNAs trigger RNAi have become better understood (Meister and Tuschl, 2004; Wilson and Doudna, 2013). Briefly, introduction of siRNA into the cell cytoplasm results in its association with protein components of the RNA-induced silencing complex (RISC) (Fig. 1). Once RISC is loaded with the siRNA, the passenger strand is nicked by the RISC component Argonaute2 (AGO2), and further degraded by component 3 promoter of RISC (C3PO) (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005; Ye et al., 2011). This results in the exposure of nucleotide bases of the guide strand, enabling them to base pair with the complementary target mRNA sequence. If the guide strand is sufficiently base-paired with the target mRNA, the phosphodiester linkage in the target mRNA is cleaved by the “slicer” activity of AGO2 between the nucleotides base-paired with nucleotides at positions 10 and 11 relative to the 5′ end of the guide strand (Liu et al., 2004; Meister et al., 2004). The cleaved mRNA is further degraded by general 5′ and 3′-exonucleases. Active RISC is a multiple turnover enzyme (Haley and Zamore, 2004; Liu et al., 2011). Thus, incorporation of a single siRNA into RISC can result in the degradation of multiple mRNA molecules.

Results of a structure–activity study of the original (canonical) siRNAs found them to have an optimal length of 21 nucleotides with two nucleotide 3′ overhangs when tested in *Drosophila* embryo lysates (Elbashir et al., 2001b). Subsequently, it was reported that double-stranded RNAs (dsRNAs) without 3′ overhangs were also highly effective in mammalian cells (Czauderna et al., 2003). A large degree of flexibility in the length of the dsRNAs has also been demonstrated. Indeed, duplexes 27 base pairs in length were found to be even more effective at gene silencing than the canonical siRNAs when transfected into mammalian cells in culture (Kim et al., 2005). This was true whether these 27mers were blunt-ended or contained 5′ or 3′ two-nucleotide overhangs. These 27mers could act as substrates for the cellular enzyme Dicer, which generated a variety of 21mers. Interestingly, none of the possible 21mers were as potent as the 27mer, leading to speculation that more efficient loading of RISC via Dicer was responsible for their increased potency. However, Dicer-independent gene silencing activity of 25 and 26mers has also been demonstrated (Salomon et al., 2010). In this study, the full-length guide strand was shown to be bound to RISC, indicating that RISC can accommodate longer guide strands than that in the canonical 21mer. This is consistent with structural studies of bacterial AGO bound to a 21 nucleotide guide strand DNA which show that the nucleotides from positions 12 to 17 are disordered and do not make strong contact with the protein (Wang et al., 2009). It is

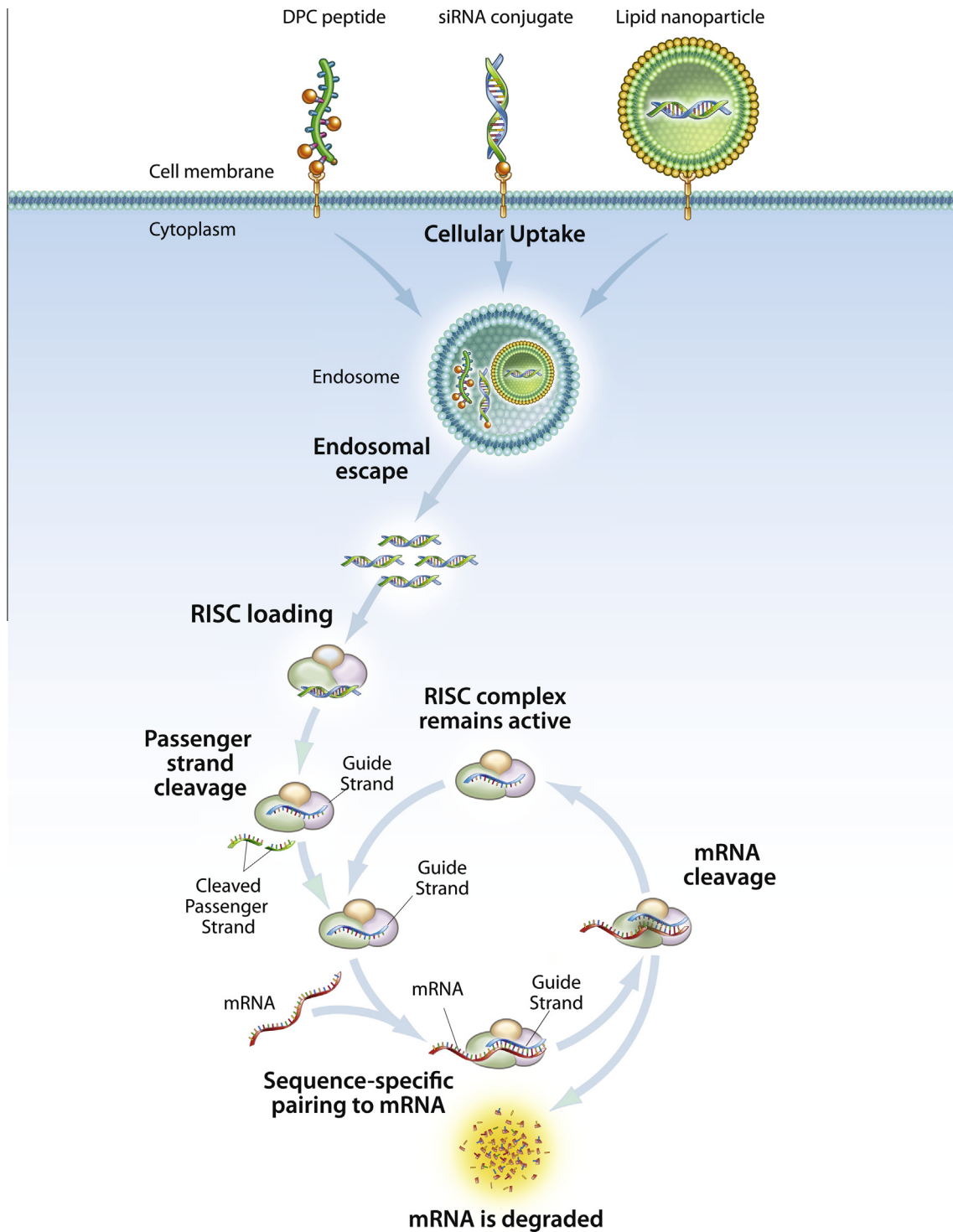


Fig. 1. Schematic of the RNAi mechanism and three clinically relevant delivery technologies used to deliver RNAi triggers to liver hepatocytes. The DPC delivery system utilizes a chemically masked amphipathic peptide (top left) containing *N*-acetylgalactosamine as a hepatocyte targeting ligand (orange balls). This is coinjected with an siRNA or RNAi trigger conjugated to cholesterol (middle). Unmasking of the peptide in the acidic environment of the endosome allows it to interact with the endosomal membrane to facilitate release of the RNAi trigger. The lipid nanoparticle system (left) encapsulates the RNAi trigger in a lipid composition that allows hepatocyte uptake and endosomal escape. The siRNA conjugate system (middle) utilizes a highly nuclease resistant siRNA conjugated to *N*-acetylgalactosamine (orange ball) and does not utilize an active endosomal escape component. All three delivery technologies result in delivery of the siRNA or RNAi trigger to the cytoplasm. Once in the cytoplasm, the siRNA is loaded into RISC and the passenger strand is cleaved and removed. RISC containing the remaining guide strand scans mRNAs until a thermodynamically favorable base pairing occurs. A perfect or near perfect base pair match of the guide strand and mRNA results in mRNA cleavage and subsequent further degradation by cellular nucleases. RISC is then free to engage additional target mRNA molecules.

possible that RISC accommodates longer guide strands by allowing a larger loop in this region, with the 3' end of the guide strand still contacting the pocket of the AGO2 PAZ domain (named after the

proteins Piwi/Argonaute/Zwille). Other structural variants of canonical siRNAs have also been described including shorter duplexes and highly asymmetric designs with short passenger

strands that possess 3' blunt ends, or both 5' and 3' overhangs (Chang et al., 2009; Chu and Rana, 2008; Sun et al., 2008). It is clear that many types of dsRNA molecules besides canonical siRNAs are effective at eliciting RNAi. These are herein referred to as RNAi triggers in order to capture this structural diversity.

3. Overcoming the in vivo delivery challenge

The demonstration that RNAi could be used to potently silence the expression of a mammalian gene set off a flurry of activity to harness this cellular pathway for therapeutic uses. However, delivery of siRNAs or other types of RNAi triggers to the appropriate cell or tissue type in a clinically relevant manner proved to be challenging (Akhtar and Benter, 2007; Wang et al., 2010). This is largely due to the fact that RNAi triggers are essentially small, double-stranded oligonucleotides with a highly negatively charged, hydrophilic phosphate backbone. This makes them unable to interact with and transverse cell membranes, and subjects them to rapid filtration from the bloodstream by the kidneys. Those RNAi triggers that do make contact with the cell type of interest are taken up via endocytosis and remain trapped in the endosome where they are subject to degradation by nucleolytic enzymes. This represents a final barrier to effective delivery. Strategies to facilitate escape of intact RNAi triggers from the endosome to the cytoplasm where RISC resides are needed. For a therapy designed to inhibit production of HBV, delivery specifically to hepatocytes would be preferred.

Today, three technologies that allow productive delivery of parenterally administered RNAi triggers to liver hepatocytes in humans with resultant silencing of the target gene expression are in development (Haussecker and Kay, 2015; Lorenzer et al., 2015). These are liposomes or lipid nucleic acid particles (LNPs), in which the RNAi trigger payload is encapsulated or associated with a mixture of cationic and neutral lipids; RNAi trigger conjugates which utilize attachment of a hepatocyte targeting moiety to the RNAi trigger; and dynamic polyconjugates (DPCs) in which the RNAi trigger is conjugated to cholesterol and is coinjected with a hepatocyte-targeted, membrane-active peptide (Fig. 1) (Coelho et al., 2013; Nair et al., 2014; Wooddell et al., 2013). Although very different in their physical constituency and mechanism of delivery, all three technologies have in common the ability to alter the biodistribution properties of the RNAi trigger and enhance its uptake by hepatocytes.

All of these technologies result in hepatocyte uptake of the RNAi trigger via endocytosis (Fig. 1). Localization in the endosome subjects the RNAi trigger to high levels of nuclease activity which is able to quickly degrade dsRNA. In LNPs, the RNAi trigger is encapsulated and thus protected against digestion (Li and Szoka, 2007). For conjugate and DPC systems, the RNAi trigger is not in a complex and therefore must be chemically modified to increase nuclease resistance. The most commonly used modifications involve substitution of the 2'OH group on the ribose ring with 2'OMe and/or 2'F, and limited phosphorothioate substitutions in the sugar phosphate backbone (Allerson et al., 2005; Choung et al., 2006; Czauderna et al., 2003; Volkov et al., 2009). The use of chemical modifications has the added benefit of reducing the likelihood of innate immune system stimulation and other off-target effects that have been described for unmodified RNAi triggers (Jackson et al., 2006; Judge et al., 2006). Protection from nuclease digestion enables the RNAi trigger to remain intact prior to its release from the endosome which is thought to be the rate-limiting step for successful RNAi delivery.

The endosomal release step has been the subject of intensive technological development and evaluation (Dominska and Dykxhoorn, 2010). For LNPs, it is thought that the cationic lipid component interacts with anionic phospholipids in the endosomal membrane to form nonbilayer ion pairs, thus disrupting the

structure and integrity of the endosomal membrane (Hafez et al., 2001; Semple et al., 2010; Torchilin, 2006). The DPC system utilizes the amphipathic peptide melittin-like peptide (MLP) which has demonstrated membrane interactive ability (Wooddell et al., 2013). This activity is inhibited by conjugating it with acid labile chemical masking groups (Rozema et al., 2003). The masking agent is bifunctional and is also used as a linker to attach the hepatocyte targeting ligand *N*-acetylgalactosamine (GalNAc or NAG) to the peptide. Once inside the endosome, the low pH environment triggers unmasking, allowing the peptide to interact with and disrupt the endosomal membrane which allows release of the co-injected RNAi trigger (Wong et al., 2012). The mechanism of endosomal release of RNAi trigger conjugates, when conjugated to GalNAc for hepatocyte targeting, is not known. However, these are typically heavily chemically modified and extremely nuclease resistant. Thus, it is possible that they may take advantage of, or enhance, a tendency for endosomes to release their contents at low frequency (Ohya et al., 2009; Starai et al., 2007). Once the RNAi trigger is released from the endosome to the cytoplasm, it is able to engage with RISC and elicit RNAi.

There is potential for short dsRNAs such as RNAi triggers to induce the innate immune response given the similarity of their molecular signatures to viral RNA (Hornung et al., 2005; Judge et al., 2005). Because of this, clinical trials conducted with RNAi triggers formulated in LNP have incorporated pre-treatment with corticosteroids such as dexamethasone in order to reduce the risk of inflammatory reactions (Coelho et al., 2013; Fitzgerald et al., 2014). However, this is likely more of a concern with LNP formulations than delivery approaches using RNAi-trigger conjugates or the DPC approaches described above due to the relatively large size of LNPs, the potential for cross-linking of immune receptors when multiple siRNAs are presented in a complex, and the propensity of immune cells to take up LNPs (Judge and MacLachlan, 2008). Substitution of the 2'OH group with 2'OMe on the sugar of the RNAi triggers also aids in minimizing the potential for eliciting an innate immune response (Judge et al., 2006). This type of modification is currently being employed in the latest generation of RNAi triggers designed for therapeutic intent.

4. Molecular biology of HBV and its susceptibility to RNAi

In order to understand how a therapeutic agent based in RNAi might be suited to the treatment of CHB, it is important to first consider the genetic makeup and organization of HBV. There are several excellent and extensive reviews on the molecular biology and life cycle of HBV; thus we focus here on those most relevant to development of an RNAi-based drug (Seeger and Mason, 2015; Seeger et al., 2007). The HBV genome is remarkable in its compactness and complexity, consisting of a circular, partially double-stranded DNA just 3.2 kilobase pairs (kb) in length (Fig. 2). After viral entry into the cell and uncoating and deposition of the genome into the nucleus, the host DNA repair system converts the partially double-stranded viral DNA into covalently closed circular DNA (cccDNA). The cccDNA is used as a template by the cellular mRNA transcriptional machinery to generate the four families of viral transcripts. All HBV transcripts are overlapping, differing in their 5' initiation sites but sharing a common 3' region and utilizing the same polyadenylation signal. The longest transcripts are approximately 3.5 kb in length and include the pre-C mRNA used in production of the pre-core protein, also known as HBeAg, and a slightly shorter version (pgRNA) that is initiated ~30 base pairs downstream. The pgRNA is used in the production of the core protein and the polymerase-reverse transcriptase which is translated from a different reading frame than core. The pgRNA also represents the pre-genomic RNA and is used as the template by the polymerase-reverse transcriptase during viral replication. This ultimately results in the production of infectious

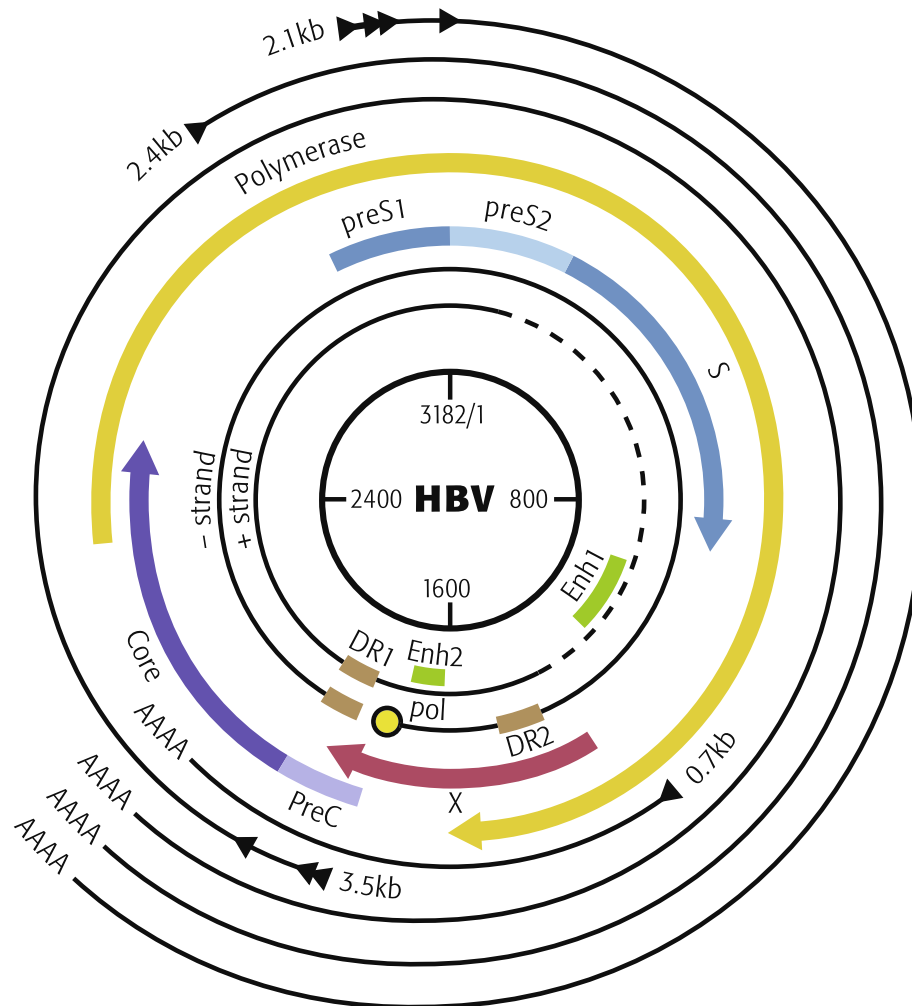


Fig. 2. Organization of the HBV transcriptome. The map coordinates shown are based on the unique EcoRI site at position 3182/1 (inner dial). The major HBV transcripts are shown (outer black lines) along with their 5' initiation sites (black arrowheads) and 3' poly A tails (AAAA). All HBV transcripts utilize the same polyadenylation signal located in the core coding region and thus have overlapping sequences. Coding regions for the HBV proteins are indicated with colored bars. The relaxed circular DNA genome of HBV is also shown with the partially completed + strand (inner black circles) as well as the positions of enhancer 1 (Enh1), enhancer 2 (Enh2), and the direct repeat 1 (DR1) and direct repeat 2 (DR2). The polymerase-reverse transcriptase covalently linked to the 5' end of the - strand DNA is also shown (yellow ball).

viral particles as well as additional copies of cccDNA that are transported back to the nucleus. An alternative priming event can also result in the generation of double-stranded linear (DSL) forms of the viral genome (Staprans et al., 1991). These linear forms are produced at a frequency of about 5–20% and often contain deletions of 5' and 3' genomic sequences that make them defective for replication. Being linear, the DSL form has a higher propensity to integrate into the host genome; integrated HBV DNA is often found in cells of patients with HCC (Fallot et al., 2012; Riviere et al., 2014; Yang and Summers, 1995). It is very likely that HBV integrants are transcribed in a way that can support production of viral antigens (Koshy and Caselmann, 1998). An HBV-specific 2.3-kilobase RNA was detected in human hepatoma cell line PLC/PRF/5 which produced HBsAg (Pourcel et al., 1982). In addition, certain HCC cell lines grown in culture that contain integrated HBV DNA can support the production of HBsAg-containing hepatitis D virus (HDV) virions, suggesting that these cells can produce at least low levels of functional HBsAg (Freitas et al., 2014). HBV integrants have also been detected in normal-appearing hepatocytes in both chimpanzees and humans with CHB (Mason et al., 2009, 2010; Tu et al., 2015). Regardless of their origin, transcripts containing HBV sequences could still be targeted using RNAi-based approaches.

Further downstream of the initiation sites for the 3.5 kb family of transcripts are the initiation sites for the mRNAs used to produce the three forms of the surface protein, L, M and S, which differ in their length at the N-termini. The 2.4 kb transcript (pre-S1 mRNA) is used in the translation of the large surface antigen (L). The 2.1 kb transcripts (pre-S2 mRNA) are initiated downstream of the pre-S1 mRNA and are used to produce the M and S surface antigens. The pre-S2 mRNAs have functional heterogeneity in their 5' ends flanking the initiation codon used to produce M; those initiated upstream are used to produce M and those downstream are used to produce S. A 0.7 kb transcript is initiated the most downstream and is used in the production of the X protein.

The organization of the HBV transcriptome presents an attractive target for the use of RNAi from a molecular standpoint (Fig. 3). The fact that the viral RNA transcripts contain overlapping sequences means that a single RNAi trigger can theoretically result in the degradation of all viral transcripts, thereby directly preventing the production of viral proteins. Such an RNAi trigger would also be expected to directly target pgRNA and impact viral replication levels through an orthogonal mechanism of action to polymerase-reverse transcriptase inhibitors. The cccDNA reservoir in the nucleus may also be decreased due to inhibition of viral

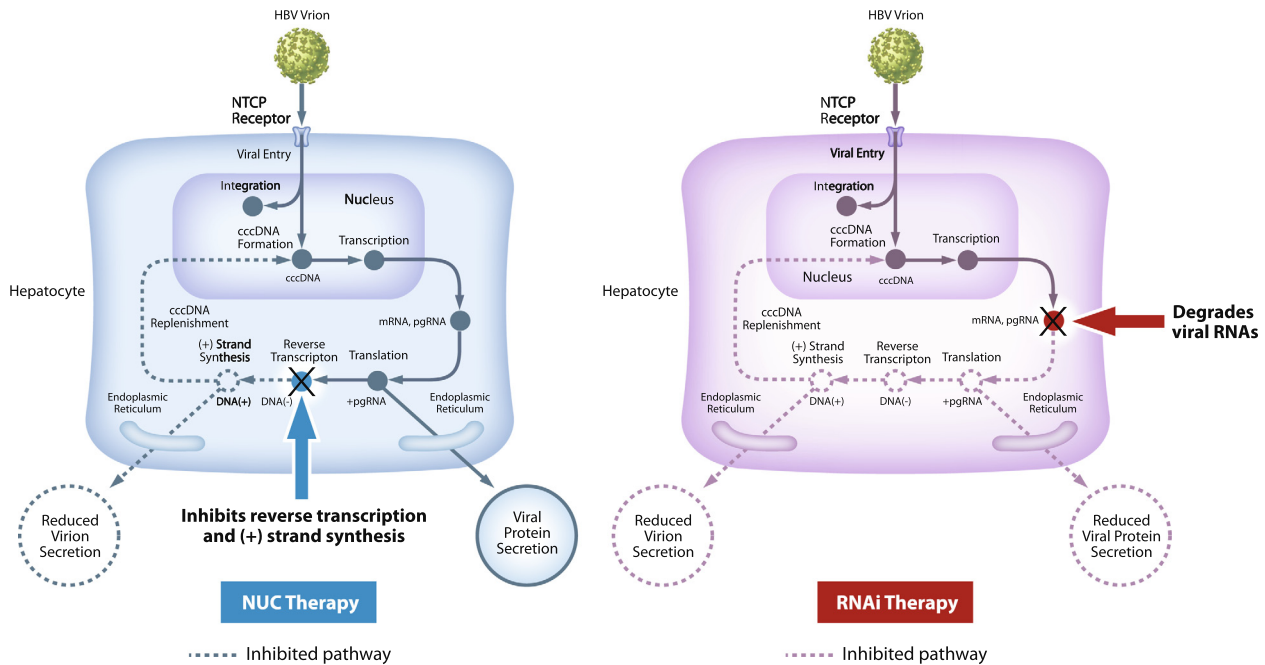


Fig. 3. Target points of NUC and RNAi-based therapies in the HBV lifecycle. NUCs directly inhibit the polymerase-reverse transcriptase (left panel). This results in reduction of viral DNA synthesis, leading to reduced virion production and decreased replenishment of cccDNA in the nucleus. NUCs do not directly affect the production of viral proteins and thus can only gradually reduce HBV protein levels over many years of therapy. In contrast, RNAi-based therapies would directly reduce levels of the targeted viral transcripts, leading to a rapid reduction in viral protein synthesis. The levels of pgRNA can also be affected, resulting in decreased levels of viral DNA synthesis and reduced virion secretion and cccDNA production.

replication, although it would not be directly acted upon by RNAi. Thus, by directly inhibiting viral protein production as well as affecting viral replication, an RNAi-based therapy would affect most aspects of the viral life cycle. This is in contrast to existing direct acting HBV drugs such as NUCs which only directly inhibit HBV replication (Fig. 3).

In addition to these direct antiviral effects, reduction of viral antigens by RNAi may aid in restoration of the immune system, thus enabling it to mount an effective attack on HBV. One hallmark of CHB is the poor immune response to viral antigens (Ferrari, 2015; Ferrari et al., 1990; Jung et al., 1991). A major contributor to this is thought to be the persistent exposure of T cells to high levels of soluble viral antigens, resulting in a stepwise functional impairment and a phenomenon known as T-cell exhaustion (Bertoletti et al., 2009; Chisari and Ferrari, 1995; Kondo et al., 2013). Reducing the level of viral proteins may be one therapeutic strategy to overcome this. One recent study showed that a reduction of viral antigens while under NUC therapy was associated with recovery of T-cell function (Boni et al., 2012). However, while NUCs are highly effective at decreasing viral titer, they are much less effective at lowering key viral antigens in most patients (Chang et al., 2010; Seto et al., 2013). This is because NUCs block only the polymerase-reverse transcriptase, which inhibits viral DNA synthesis, but do not directly affect cccDNA levels or transcription (Takkenberg et al., 2011; Werle-Lapostolle et al., 2004). In contrast, RNAi-based approaches would directly target the mRNAs used in the production of viral proteins, theoretically resulting in faster and more robust lowering of viral antigens compared to NUC therapy (Fig. 3). A relatively rapid decline in HBSAg levels can be a predictor of sustained virological response (SVR) in CHB patients receiving IFN (Moucari et al., 2009; Sonneveld et al., 2013). Unfortunately, there is a dearth of animal models that could be used to test whether antigen reduction using RNAi aids in the restoration of a specific T-cell response. Clinical data will likely be required.

5. Preclinical development of ARC-520

One RNAi-based drug currently in Phase 2 clinical trials for patients with CHB is ARC-520 (Arrowhead Research Corporation, Pasadena, California, USA). This drug is based on the DPC technology described above and contains a hepatocyte targeted, reversibly masked membrane active peptide (NAG-MLP) to facilitate endosomal release of two synthetic RNAi triggers directed towards HBV transcripts. The sequence diversity of HBV genomes is substantial and comprises nine major genotypes (A–I) with greater than 7.5% intergroup nucleotide divergence (Kramvis, 2014). This makes designing an RNAi trigger with broad genotype coverage somewhat of a challenge as perfect or near perfect match to the target sequence in the mRNA is required for maximal activity (Elbashir et al., 2001b; Huang et al., 2009). The substitution rate varies in different regions of the HBV genome. Regions that contain overlapping reading frames or secondary RNA structures required for processes such as HBV replication in non-overlapping regions tend to be more highly conserved (Mizokami et al., 1997; Torres et al., 2013). In the development of ARC-520, by creating an alignment of 2754 full-length HBV genomes present in GenBank, conserved sequences were identified that could be used to generate RNAi triggers cross-reactive with >90% of known HBV genomes (Wooddell et al., 2013). These were then subjected to a specificity filter to eliminate those that could potentially cross-react with human mRNAs in order to avoid sequence-dependent off-target effects. This process resulted in identification of approximately 140 sequences. These sequences were used to generate an initial screening set of RNAi triggers that were tested in cells in culture for potency against a reporter gene harboring HBV sequences. The four most potent sequences were selected for further testing in vivo.

Prior to in vivo testing, these RNAi triggers were chemically modified using sugar and backbone analogues designed to increase nuclease resistance and minimize the potential for induction of an

innate immune response (Allerson et al., 2005; Choung et al., 2006; Czauderna et al., 2003; Judge et al., 2006; Volkov et al., 2009). These include substitution of the 2'OH of the sugar with 2'OMe or 2'F groups and a phosphorothioate linkage between the terminal 3' nucleotides of the guide strand. A cholesterol moiety was also conjugated to the RNAi triggers to enhance liver uptake (Soutschek et al., 2004; Wong et al., 2012). Two mouse models of CHB were used in these studies. The first was a transiently transgenic pHBV model generated by hydrodynamic tail vein injection of a plasmid harboring the terminally redundant full-length HBV genome (HBV 1.3) (Yang et al., 2002). Typically 5–20% of hepatocytes are transfected using this technique (Zhang et al., 1999). This model expresses all viral RNAs and is competent for HBV replication and production of virions and allows the effects of the RNAi triggers on HBsAg, HBeAg, and viral DNA levels in the serum and viral RNA levels in the liver to be monitored. The second was a transgenic mouse harboring a single chromosomally integrated copy of the HBV 1.3 genome resulting in HBV gene expression and production of virions in the vast majority of hepatocytes (Guidotti et al., 1995). The levels of the 3.5 kb pgRNAs, the 2.4/2.1 kb preS1/S mRNAs, and the HBeAg levels as well as the production of HBV DNA replicative intermediates were monitored using this model. The presence and distribution of hepatitis B core antigen (HBcAg) was also evaluated using immunohistochemistry of liver sections.

All four RNAi trigger candidates were highly active in the pHBV mouse model when co-injected with NAG-MLP, with decreases in HBsAg expression of between 2 and 3 log₁₀ after a single intravenous injection. HBeAg and serum HBV DNA levels, as well as liver levels of HBV RNA, were also dramatically decreased (Wooddell et al., 2013). Two of the RNAi triggers, chol-siHBV74 and chol-siHBV77, elicited the greatest level of knockdown. Further testing of these two RNAi triggers in the pHBV mouse model showed dose-dependent knockdown of HBsAg after coinjection with an equal dose of NAG-MLP on a mg/kg basis (Fig. 4) (Wooddell et al., 2013). Both gave similar levels of knockdown at the two doses evaluated, with the nadir occurring on Day 8 after injection. The duration of effect for formulations containing chol-siHBV74 was slightly longer than with the formulations containing chol-siHBV77, with >1 log₁₀ knockdown of HBsAg lasting approximately 1 month in mice receiving chol-siHBV74. Injection of an equimolar mixture of chol-siHBV74 and chol-siHBV77 with NAG-MLP resulted in a similar level of knockdown at the nadir with a similar duration of effect. No knockdown was observed when the RNAi triggers were injected without NAG-MLP. This indicated that NAG-MLP was required for effective delivery, presumably by enabling endosomal escape of the RNAi triggers. In

addition, no knockdown was observed when NAG-MLP was injected without the HBV RNAi triggers or with a control RNAi trigger, demonstrating that knockdown was also HBV RNAi trigger-dependent, as expected.

The chol-siHBV74 and chol-siHBV77 triggers were further tested in the transgenic HBV mouse model (Wooddell et al., 2013). Coinjection of these RNAi triggers, either alone or in combination, with NAG-MLP resulted in steep reductions in the levels of the 2.4/2.1 kb preS1/S transcripts (encoding HBsAg) in the liver 1 week after dosing as determined by Northern blot (Fig. 5a). The levels of the 3.5 kb transcripts (encoding pre-core, core, polymerase-reverse transcriptase and pgRNA) were also strongly reduced, although to a slightly lesser extent than the 2.4/2.1 kb transcripts. Knockdown of transcripts encoding core, the polymerase, and the pgRNA would be expected to adversely affect HBV replication. Indeed, analysis of HBV replication intermediates by Southern blotting revealed a dramatic reduction in treated mice to below the limit of detection (Fig. 5b). Steep reductions of viral RNA and DNA replication intermediates in whole liver lysates suggested that coinjection of the chol-siHBV74 and chol-siHBV77 RNAi triggers with NAG-MLP was active in at least a majority of hepatocytes. Immunohistochemical staining of liver sections for the presence of core protein confirmed this, as uniform reduction of the cytoplasmic core antigen was observed (Fig. 5c).

The demonstrated efficacy of coinjection of chol-siHBV74 and chol-siHBV77 with NAG-MLP for knockdown of HBV transcripts in the mouse models of HBV infection prompted us to consider using both RNAi triggers in a therapeutic designed to treat CHB. This would enable more extensive genotype coverage than obtained using a single RNAi trigger. Chol-siHBV74 is an identical match to its target sequence in 96.4% of all surveyed HBV genomes and chol-siHBV77 is an identical match to 92.6%. Together they would provide coverage of 99.64% of all genomes (Wooddell et al., 2013). Another possible benefit of using two RNAi triggers would be a theoretical reduction in the frequency of escape mutants that might arise while under therapy as such mutants would need to accumulate at least two resistance mutations, one in each target sequence (Wu et al., 2005). Thus, ARC-520, a therapeutic for treatment of patients with CHB, contains both chol-siHBV74 and siHBV77 in addition to NAG-MLP.

The ability of ARC-520 to reduce viremia and the production of viral antigens in a chimpanzee chronically infected with human HBV has also been examined. This animal had been infected since 1979, was HBeAg(+), and had a very high viral titer of HBV genotype B (10 log₁₀ genome equivalents/ml serum). A single intravenous injection of 2 mg/kg of ARC-520 was well-tolerated and resulted in decreases in serum levels of HBsAg, HBeAg and HBV

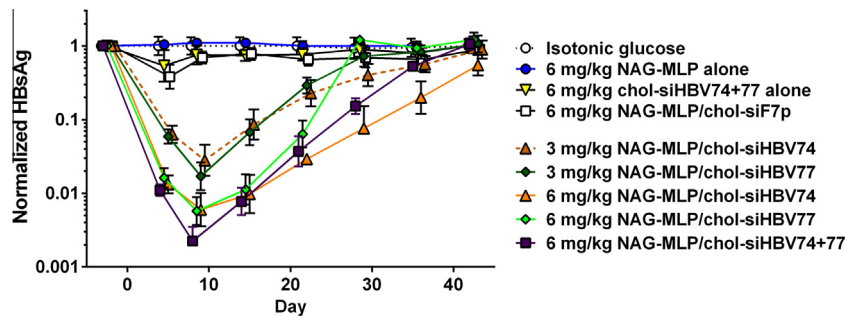


Fig. 4. Dose response comparison of chol-siHBV74 and chol-siHBV77 in the pHBV mouse model. Nonobese diabetic/severe combined immunodeficient (NOD SCID) mice were given a hydrodynamic tail vein injection with 10 μ g minicircle HBV1.3 (MC-HBV1.3). Three weeks later, mice were given one 200 μ l IV injection of isotonic glucose alone ($n = 8$) or 6 mg/kg NAG-MLP alone ($n = 7$); an injection of 6 mg/kg chol-siHBV74 plus chol-siHBV77 (3 mg/kg each) without NAG-MLP ($n = 4$); a coinjection of 6 mg/kg NAG-MLP with 6 mg/kg chol-siF7p (primate F7) ($n = 4$); or a coinjection of the indicated doses of NAG-MLP with equal doses of chol-siHBV74, chol-siHBV77, or chol-siHBV74 plus chol-siHBV77 ($n = 4-11$). HBsAg was measured in serum at the indicated time points before and after injection, mean \pm SD. Figure reproduced with permission from Wooddell et al. (2013).

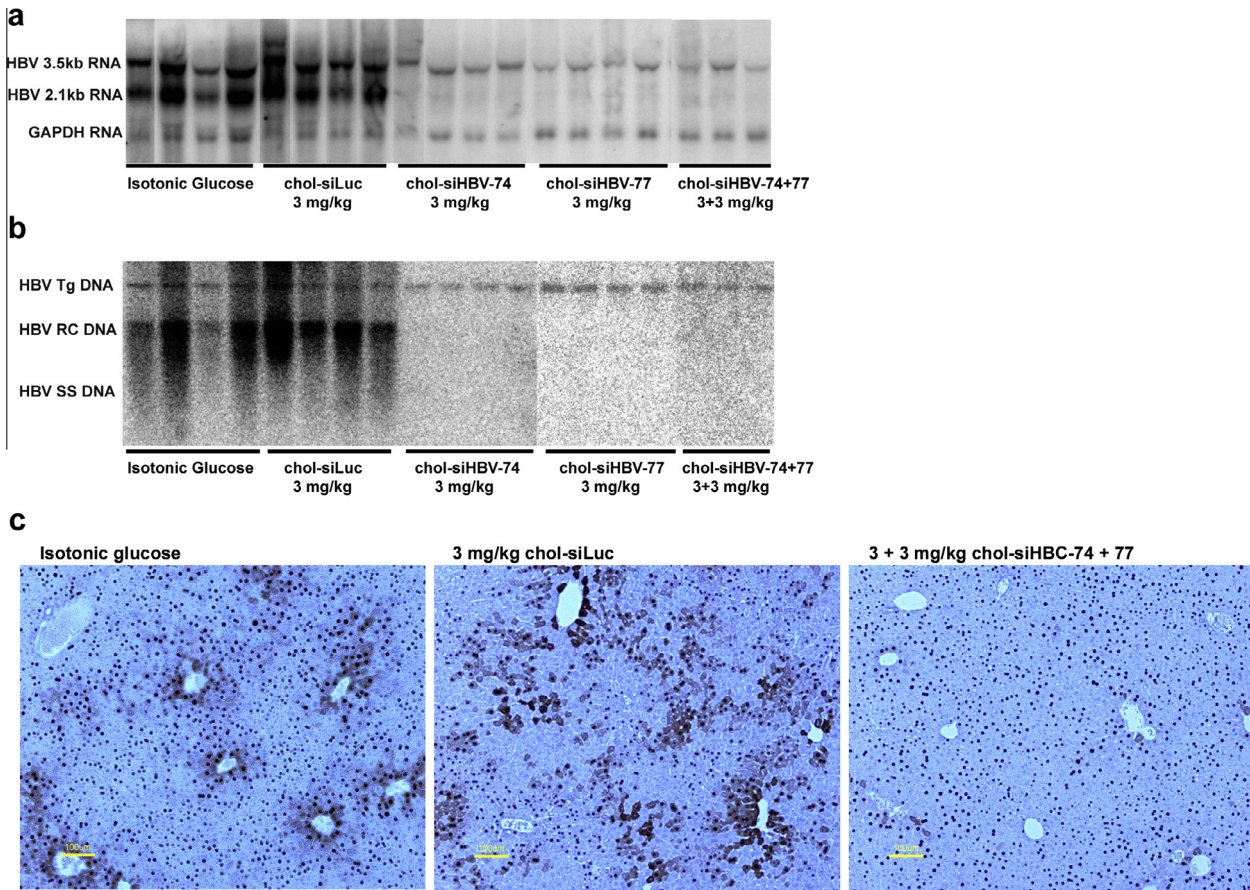


Fig. 5. Efficacy of chol-siHBV74 and chol-HBV77 in transgenic HBV mice. HBV1.3.32 transgenic mice were injected twice, 1 week apart, (c–f, females) with 6 mg/kg NAG-MLP and 3 mg/kg of the indicated chol-siRNA or a combination of 3 mg/kg chol-siHBV-74 plus 3 mg/kg chol-siHBV-77 ($n = 2–4$). The mice were euthanized 7 days after the second injection for evaluation of HBV RNA (a) and HBV DNA (b) in liver. Tg, transgene; HBV RC DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single-stranded DNA. (f) Immunohistochemical detection of HBcAg in hematoxylin-stained sections of liver. Scale bar = 100 μ m. Figure reproduced with permission from Wooddell et al. (2013).

DNA. A subsequent injection of 3 mg/kg 2 weeks after the first injection correlated with increased pharmacological effect, giving 81–96% reductions in these HBV parameters at nadir (Day 29). These reductions were similar in magnitude and duration of effect to those observed in the mouse HBV models receiving similar doses as described above.

6. Clinical experience using ARC-520

ARC-520 has been evaluated in a Phase 1 clinical trial (Clinicaltrials.gov ID NCT01872065) in healthy volunteers and in a single dose Phase 2 trial in CHB patients (Clinicaltrials.gov ID NCT02065336). Interim results from the Phase 1 study showed that single doses of ARC-520 were well tolerated up to 2 mg/kg when administered intravenously (Schluep et al., 2013). Adverse event frequency and severity did not differ between placebo and ARC-520; all adverse events were reported to be mild or moderate. Preliminary results from a Phase 2a, randomized, double-blind, placebo-controlled study to assess depth and duration of HBsAg reduction and safety after a single, intravenous dose of ARC-520 are also available (Yuen et al., 2014). This study was conducted in HBeAg-negative adult CHB patients receiving long-term entecavir. Good tolerability at single doses up to 3 mg/kg and a dose-dependent reduction in HBsAg were observed. When compared to placebo, serum HBsAg was reduced by up to 50% after a single dose of 2 mg/kg and statistically significant reductions were observed for up to 43–57 days. This was the first time that a reduction in HBsAg mediated through RNA interference has been shown

in CHB patients. Neither trial has reported any changes in clinical laboratory measurements indicative of end organ toxicity, nor were drug-related adverse effects reported for vital signs, electrocardiograms or physical examinations.

7. RNAi-based therapeutics as part of the armamentarium for treating CHB

An attractive feature of an RNAi-based therapeutic for the treatment of CHB is the ability of RNAi to directly target HBV transcripts and thus profoundly reduce the production of proteins. Viral antigens are not only required for competent viral reproduction but are also thought to be responsible for the impaired adaptive immune response observed in CHB. Numerous studies have shown that persistent exposure of T cells to viral antigens is a major determinant of functional T-cell impairment in CHB (Bertoletti and Gehring, 2006; Boni et al., 2007; Op den Brouwer et al., 2009; Wherry and Ahmed, 2004; Ye et al., 2015). HBsAg in particular may directly suppress monocytes, dendritic cells, and natural killer cells and lead to exhaustion of CD8⁺ and CD4⁺ T cells (Kondo et al., 2013). Evidence has also been gathered demonstrating the roles of HBeAg and, to a lesser extent, HBcAg in immune suppression (Milich et al., 1998; Riordan et al., 2006; Shimizu, 2012). These data suggest that antigen reduction will be essential to allow functional reconstitution of antiviral T-cell responses. The observation that the RNAi triggers contained in ARC-520 can inhibit the production of circulating HBsAg, HBeAg and intrahepatic core protein in animal models, and HBsAg in HBeAg-negative CHB patients, makes

RNAi an attractive approach for achieving this. Whether or not reduction of viral antigens alone will be sufficient to allow the immune system to recover and clear HBV in CHB patients remains to be seen. Given the experience with HIV and HCV, it would be unsurprising if achieving HBsAg seroclearance in HBV were to require combination therapy, perhaps including an agent targeting direct action on the immune system.

The ultimate goal in treating CHB is loss of HBsAg with or without anti-HBs seroconversion indicating that the immune system has gained control over the virus (Honer Zu Siederdisen and Cornberg, 2014). Indeed, sustained HBsAg seroclearance is the preferred endpoint for CHB therapy since loss of HBsAg is associated with a significant reduction in progression to cirrhosis or HCC (Chen et al., 2002; Lok and McMahon, 2009). Unfortunately, this endpoint is rarely achieved with NUC and IFN therapies, the only therapies currently approved for treating CHB (Wong and Chan, 2013). NUC therapies, while highly effective for reducing viral load, result in only modest reduction of viral antigens. This is likely due to their limited mechanism of action (Takkenberg et al., 2011; Werle-Lapostolle et al., 2004). Nonetheless, patients on long-term NUC therapy often achieve undetectable viral DNA and a normalization of ALT levels. A subset of these seroconvert to anti-HBeAg which is associated with decreased risk of liver decompensation and improved survival, with the incidence of HCC reduced by as much as 78% (Lok and McMahon, 2009; Rijckborst et al., 2010; Sung et al., 2008). HBeAg(–) patients with suppressed viral DNA on NUC therapy also display increased T-cell function. This becomes more complete as a function of HBsAg decline and is maximal in those few patients achieving persistent HBsAg loss (Boni et al., 2012). Unfortunately, seroclearance of HBsAg rarely occurs and cessation of therapy results in virological and biochemical relapse in the majority of patients (Seto et al., 2015). As a result, lifelong NUC therapy is currently recommended.

Treatment with IFN results in a somewhat greater frequency of HBsAg seroclearance, with HBsAg loss reported in 23% of HBeAg(+) patients over an eight-year period (van Zonneveld et al., 2004). The decline in on-therapy HBsAg levels is more rapid and pronounced in patients treated with IFN as compared to NUCs, and a more rapid decline in HBsAg levels in patients undergoing IFN therapy is associated with a greater likelihood of HBsAg seroclearance (Moucari et al., 2009; Reijnders et al., 2011; Sonneveld et al., 2013). This intriguing finding supports the idea that a rapid and deeper decline in viral antigens results in better likelihood of HBsAg seroclearance.

An intriguing approach to treating CHB patients with curative intent would be to combine therapies that target different aspects of the HBV lifecycle and/or act on host factors that contribute to chronicity. Evidence that such an approach may be somewhat more effective than monotherapies is emerging from clinical trials in which IFN therapy is added on to patients receiving NUC therapy, or when patients on NUC therapy are switched to IFN after an 8 weeks overlap period (Kittner et al., 2012; Ning et al., 2014; Ouzan et al., 2013). Patients who responded to these combinations showed higher rates of HBeAg and HBsAg seroclearance than those continuing on NUC therapy alone. Although the reasons why these combinations may be more effective are as yet unclear, they likely involve modulations of both host and viral factors, given the mechanism of action of these drugs.

As discussed above, there is evidence that persistent exposure of viral antigens is a major contributor to immune cell exhaustion, as well as empirical support for the notion that a rapid and deeper decline in viral antigens results in better likelihood of HBsAg seroclearance. The RNAi mechanism is well-suited to reduce viral antigens, as has now been shown in several animal models, including a chronically infected chimpanzee, as well as in humans in a Phase IIa clinical trial designed to evaluate ARC-520. Patients in this

Phase IIa trial were on NUC therapy prior to enrollment with continued usage during treatment with ARC-520. This design allows the investigation of ARC-520 in patients currently receiving the most often used standard of care for CHB. Future trials could be envisioned that would examine the combination of ARC-520 with IFN. This combination is attractive as it would combine the antigen reduction activity of ARC-520 through RNAi, thus enhancing the potential for restoration of T-cell mediated function, with the pleiotropic effects of IFN on viral and host factors. A drawback to the use of IFN is its known adverse effects including flu-like syndrome, headache, myalgia, fatigue, depression and local injection site reactions (Marcellin et al., 2004). However, these could potentially be minimized if the combination therapy proved to have enhanced efficacy, allowing lower doses or shorter treatment durations.

As discussed in other papers in this special issue, other novel approaches to CHB treatment are also being investigated (Kapoor and Kottilil, 2014). Therapies aimed at activating the innate immune system and restoring adaptive immune responses, including TLR agonists, PD-1 inhibitors, and therapeutic vaccines, would be particularly well suited to act synergistically with therapies such as RNAi aimed at reduction of viral antigens. CHB is a complex disease to treat due to its dynamic life cycle and diverse patient profiles, and it will likely require a multi-pronged approach in order to identify therapies that are curative across the patient population. RNAi-based therapies may play an important role in future therapeutic regimes aimed at improving HBsAg seroclearance and eliminating the need for lifelong therapy.

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