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Review

Nucleic acid polymers: Broad spectrum antiviral activity, antiviral mechanisms and optimization for the treatment of hepatitis B and hepatitis D infection

Andrew Vaillant

Replicor Inc., 6100 Royalmount Avenue, Montreal, Quebec, H4P 2R2, Canada

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A B S T R A C T

Antiviral polymers are a well-studied class of broad spectrum viral attachment/entry inhibitors whose activity increases with polymer length and with increased amphipathic (hydrophobic) character. The newest members of this class of compounds are nucleic acid polymers whose activity is derived from the sequence independent properties of phosphorothioated oligonucleotides as amphipathic polymers. Although the antiviral mechanisms and broad spectrum antiviral activity of nucleic acid polymers mirror the functionality of other members of this class, they exert in addition a unique post entry activity in hepatitis B infection which inhibits the release of HBsAg from infected hepatocytes. This review provides a general overview of the antiviral polymer class with a focus on nucleic acid polymers and their development as therapeutic agents for the treatment of hepatitis B/hepatitis D. This article 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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Contents

1. Introduction 32
2. Antiviral polymers – a history 33
3. Phosphorothioate oligonucleotides as a drug class: conserved polymeric features and pharmacokinetic behaviours 33
4. Nucleic acid polymers: PS-ONs with sequence independent and size dependent broad spectrum activity against viral and other infectious diseases 33
5. NAP effects in viral hepatitis – a novel targets and a novel post entry antiviral activity in HBV infection 35
6. Optimization of NAPs for clinical use in HBV infection 35
7. NAPs: therapeutic effects *in vivo* and current mechanistic hypothesis in HBV 36
8. NAPs: application in the treatment of chronic hepatitis B and D infection 37
9. Future perspectives 38
Acknowledgements 38
References 38

1. Introduction

Hepatitis B virus (HBV) has affected more than 2 billion people worldwide, with recent estimates indicating that 248 million of these individuals still have chronic HBV infection (Schweitzer et al., 2015). The life cycle and molecular biology of HBV infection have been previously described by Gish et al., 2015 in this review series. Complications arising from chronic hepatitis B include the development of liver fibrosis and hepatocelulluar carcinoma (Gish et al., 2015) necessitating therapy to control infection. The disappearance of the hepatitis B surface antigen protein (HBsAg) from the blood (HBsAg loss) is considered the best prognostic indicator for the establishment of control over HBV infection which can endure in

E-mail address: availlant@replicor.com.

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the absence of therapy (Frenette and Gish, 2009; Moucari et al., 2009). While treatment with HBV polymerase inhibitors like entecavir and tenofovir disoproxil fumarate suppress HBV DNA and control the onset of liver disease, they rarely lead to HBsAg loss and require continual therapy (Chang et al., 2006; Lai et al., 2006; Marcellin et al., 2008). Treatment with immunotherapies like pegylated interferon can achieve HBsAg loss but only in a small fraction of patients and combination treatment with immunotherapy and HBV polymerase inhibitors offers only small improvements in the rates of HBsAg loss on therapy (Lau et al., 2005; Marcellin et al., 2008, 2016). As such, there is a need for more effective treatments for CHB infection, particularly those therapies which can increase the incidence of HBsAg loss during therapy.

Nucleic acid polymers (NAPs) are the most recently characterized member of the antiviral polymer family of compounds which share the broad spectrum antiviral activity of this class of antiviral compounds. Importantly, NAPs appear to have a unique ability to block the secretion of HBsAg from HBV-infected hepatocytes not observed with other antiviral polymers. NAPs also belong to another class of compounds, phosphorothioate oligonucleotides (PS-ONs), which can be safely given to human subjects where they naturally accumulate in the liver and enter into hepatocytes. This article provides an overview of the mechanistic properties of antiviral polymers, the general pharmacokinetic properties of PS-ONs and a review of the discovery, characterization and optimization of NAPs for clinical use and their antiviral effects against HBV and HDV infection *in vivo* and in human subjects.

2. Antiviral polymers – a history

A variety of polymeric compounds with antiviral activity have been described and although this class of compounds comprises polymers of differing chemical structures (see Fig. 1), they are known to exert their antiviral effects through similar mechanisms governed by polymer length and hydrophobicity. Sulfated polysaccharides such as heparin sulfate or dextran sulfate are perhaps the most widely studied members of this class and have been shown to have a broad spectrum antiviral activity against a wide range of viruses including human immunodeficiency virus, herpes viruses, respiratory syncytial virus, parainfluenza virus, influenza, dengue virus and other encephalitic flaviviruses, hepatitis C, hepatitis B and hepatitis D (Baba et al., 1988a; Hosoya et al., 1991; Lüsher-Mattli, 2000; Lee et al., 2006; Basu et al., 2007; Leistner et al., 2008; Ghosh et al., 2009; Longarela et al., 2013). The antiviral activity of sulfated polysaccharides is not dependent on sugar composition in the polymer chain but is length dependent, with longer polymers having more potent antiviral activity and dependent on sulfate content, with more sulfated glycan polymers having greater antiviral activity (Baba et al., 1988b; Hosoya et al., 1991; Leistner et al., 2008). Sulfation is known to increase the hydrophobicity of polysaccharides (Robinson et al., 1984), suggesting that the conserved requirement of sulfation for antiviral activity defines a large hydrophobic interaction domain somehow important in viral entry. Several studies have demonstrated that sulfated polysaccharides interfere with the initial, non-specific viral adsorption at the cell surface, likely by interfering with the interaction between heparin sulfate glycosaminoglycans and viral fusion glycoproteins or by preventing membrane fusion between the virus and the host cell in viruses with class 1 fusion glycoproteins (Hosoya et al., 1991; Feyzi et al., 1997; Cheshenko et al., 2004; Schulze et al., 2007).

Similar length and hydrophobic dependent broad spectrum antiviral activity has been described for a wide variety of polymers chemically related to sulfated glycans including polystyrene sulfonate, polyvinylalcohol sulfate, polymethylenehydroquinone sulfonate, napthalene sulfonate and carrageenans (Baba et al., 1990; Ikeda et al., 1994; Neurath et al., 2002; Bourne et al., 1999; Rusconi et al., 1996) which are likely a result of analogous chemical features shared by this class of compounds (Fig. 1).

3. Phosphorothioate oligonucleotides as a drug class: conserved polymeric features and pharmacokinetic behaviours

Oligonucleotides are most widely known for their application in hybridization based technologies such as antisense or siRNA which target specific, complementary regions of nucleic acids within the cell (such as mRNA) to affect their degradation via recruitment of nucleases (Bennett and Swazye, 2010; Wilson and Doudna, 2013; Sharma et al., 2014). However, in addition to this sequence specific functionality, single stranded oligonucleotides are also polymers with polyanionic characteristics which are largely conserved regardless of their nucleotide sequence. Similar to sulfation in other antiviral polymers, the hydrophobic (and amphipathic) characteristic of oligonucleotides is enhanced by phosphorothioation of the phosphodiester linkages (Agrawal et al., 1990), rendering phosphorothioated oligonucleotides heparin sulfate-like in their chemical properties (Guvakova et al., 1995; Fennewald and Rando, 1995) similar to other antiviral polymers (Fig 1).

Numerous clinical trials have been conducted with single stranded phosphorothioated oligonucleotides (PS-ONs, used as antisense oligonucleotides) over the past two decades from which conserved class behaviours have been established: after administration by intravenous infusion or subcutaneous injection, PS-ONs are rapidly cleared from the blood (1/2 life < 1 h) concomitant with accumulation in peripheral organs, the most significant of which are the liver and kidney (Bennett and Swazye, 2010), ultimately leading to the upake of PS-ONs into hepatocytes by mechanism not yet fully elucidated (Geary et al., 2015). PS-ONs are stable to nuclease –mediated degradation, but degrade slowly overtime, with the primary route of elimination via the kidney (Geary et al., 2015). Importantly, pharmacologically active levels of PS-ON can be maintained in these organs with once weekly dosing which is generally well tolerated with chronic exposure (Bennett and Swazye, 2010). As NAPs are PS-ONs, the application of the lessons learned from effective dosing regimens of other PS-ONs, in the clinic have been successfully applied in the clinical use of NAPs as discussed below.

4. Nucleic acid polymers: PS-ONs with sequence independent and size dependent broad spectrum activity against viral and other infectious diseases

Nucleic acid polymers (NAPs) are single stranded PS-ONs that function independently from any antisense or immunostimulatory effects based on their properties as amphipathic polymers. The complete structure function relationship for the antiviral activity of NAPs as well as their molecular mechanism of action was first completely elucidated in a study describing the specific antiviral effects of NAPs during the entry of HIV-1 (Vaillant et al., 2006). In this study, the entry inhibition effect of NAPs was shown to be sequence independent and size dependent, requiring NAPs longer than 20 nucleotides for significant antiviral effect, with optimal antiviral activity observed for NAPs 40 nucleotides or longer in length. This antiviral activity was also specifically dependent on the amphipathicity conferred by phosphorothioation in a manner independent of the increased nuclease stability present with this modification. The structure function relationship required for inhibition of HIV-1 entry indicated that a large amphipathic protein domain in a protein involved in the entry of HIV-1 into the host cell

PVAS PAVAS SPS (dextran sulfate) PSS PNS NAP (REP 2055) NAP (REP 2139)

Fig. 1. Representative chemical structures of antiviral polymers are shown for polyvinyl alcohol sulfate (PVAS), sulfated poly acrylic poly vinyl co-polymer (PAVAS), sulfated polysaccharides (SPS), polystyrene sulfate (PSS), polynapthelene sulfate (PNS) and nucleic acid polymers (NAP). Composition of phosphate and carboxylic acid groups in PVAS and PAVAS are variable. Sulfated polysaccharides with antiviral activity can vary in sugar content and sulfate content >2 per sugar residue. REP 2055 and REP 2139 are oligonucleotide copolymers of alternating adenosine and cytidine optimized for tolerability and activity in HBV/HDV infection (Table 1). Additional modifications present in REP 2139 (5′methylcytosine and 2′ O-methylribose) to improve NAP administration tolerability in human subjects are shown in bold.

was the target for NAP interaction. This domain was confirmed to be the amphipathic alpha helix triplets found in the fusion core in the gp41 fusion glycoproteins. The NAP-gp41 interaction requires the amphipathic alpha-helices of gp41 to be uncomplexed (with their hydrophobic surfaces exposed), a conformation found only in the pre-fusion conformation of gp41. NAP interaction with this target domain on gp41 exhibited the same sequence independent and phosphorothioation and length dependent features as required for NAP inhibition of viral entry. These interactions were driven by numerous cooperative hydrophobic interactions between the hydrophobic surface of the amphipathic alpha helices in gp41 and the hydrophobic surface of NAPs as depicted in Fig. 2. This study showed that NAPs could function as entry inhibitors by blocking the transition of gp41 into its membrane fusion-competent conformation.

Uncomplexed amphipathic alpha helix triplex structures analogous to those present in the pre-fusion conformation of HIV-1 gp41 are conserved in class 1 fusion glycoproteins from many other viruses susceptible to antiviral polymers (Eckert and Kim, 2001; Lamb and Jardetzky, 2007), consistent with the proposed antiviral mechanism of other antiviral polymers. Following on from the initial study in HIV-1, NAPs were subsequently shown to have the same sequence independent and phosphorothioation (amphipathic) and length dependent antiviral effects in other viruses with class I fusion glycoproteins including herpesviruses, cytomegalovirus, and lymphocytic choriomeningitis virus. (Guzman et al., 2007; Lee et al., 2007; Bernstein et al., 2008; Cardin et al., 2009).

NAPs and other PS-ONs with NAP-like functionality also prevent prion protein conversion and block malarial entry into red blood cells with an activity that follows the same structure function relationship as observed for NAP activity against viruses with class I fusion glycoproteins (Clark et al., 1994; Kanagaratnam et al., 1998; Kocisko et al., 2006), effects which are also described for other antiviral polymers (Caughey and Raymond, 1993; Xiao et al., 1996; Vogt et al., 2003). Amphipathic alpha helices analogous to those found in class I fusion glycoproteins are also found in prion proteins where they are involved in conversion of the prion protein to its pathogenic form, and in the Duffy-binding-like domain of erythrocyte binding protein from *Plasmodium sp*. which is involved in malarial entry into erythrocytes (Knaus et al., 2001; Singh et al., 2005) and these domains have been proposed as the target sites for antiviral polymers (Vogt et al., 2003; Kocisko et al., 2006).

These conserved determinants of biological activity of NAPs, not only in viral infection but also in other infectious diseases supports

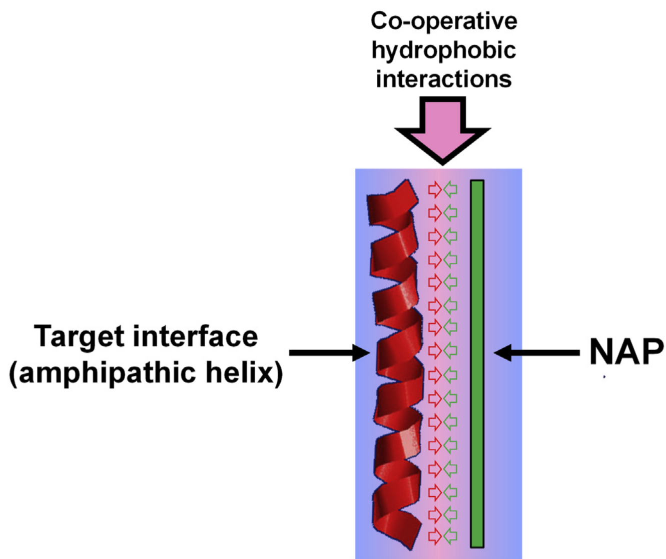

Fig. 2. Current model for universal NAP interaction with uncomplexed amphipathic protein targets. NAP interaction involves a series of cooperative hydrophobic interactions between the hydrophobic surface of an exposed (uncomplexed) amphipathic alpha helix (red) and the hydrophobic surface of NAPs (green). The hydrophobic environment is indicated in pink and the hydrophilic environment is indicated in blue.

a universal model for NAP pharmacology (Fig. 2), with numerous cooperative interactions occurring between the exposed hydrophobic surface of a large, uncomplexed amphipathic protein domain and the hydrophobic side of NAPs (which is enhanced in the presence of phosphorothioation). This interaction prevents conformational changes in the target or its interaction with other amphipathic helices, forming the basis of the biological effect of NAPs.

5. NAP effects in viral hepatitis – a novel targets and a novel post entry antiviral activity in HBV infection

NAPs have also been shown to be entry inhibitors of HCV (Matsumura et al., 2009), having the identical structure function relationship for antiviral effect as observed in viruses with class I fusion glycoproteins. However this study showed that NAPs do not block cell attachment but instead block a downstream, post-binding event required for viral entry. This effect might involve interactions of NAPs with multiple proteins such as the hypervariable region of glycoprotein E2 or apolipoprotein E, which also have NAP-like interactions with heparin sulfate (Libeu et al., 2001; Basu et al., 2004) and are involved in post-binding HCV entry (Basu et al., 2007; Chang et al., 2007).

Initial evaluations of the antiviral effect of NAPs in HBV infection were performed in DHBV-infected primary duck hepatocytes (PDH) (Noordeen et al., 2013a) and confirmed the same sequence independent, size and amphipathic dependent antiviral activity against DHBV infection as found in other viruses. This antiviral activity included the ability of NAPs to block the HBV life cycle both during and after viral entry. This novel post-entry activity was not observed for NAPs in other viral infections (see above) or for other antiviral polymers in HBV infection (Leistner et al., 2008), suggesting a unique potential for NAPs as therapeutic agents in chronic HBV infection. More recent preliminary results from initial experiments in HBV infected HepaRG cells and primary human hepatocytes (Guillot et al., 2015) suggests that phosphorothioated NAPs may also block HBV entry in a sequence independent manner but not in the presence of 2′O methyl ribose modification (i.e. for NAPs like REP 2139, see below) and no post entry antiviral activity has yet been observed in these models. However, efficient uptake and subcellular delivery of PS-ONs is known to be defective in hepatocytes or hepatocyte derived cell lines in tissue culture (Koller et al., 2011) which complicates interpretation of this preliminary data. Verification of these early results and exploration of the post entry effects of NAPs *in vitro* in these models are still ongoing. While the molecular mechanisms of this post entry effect are still under investigation, their effects against DHBV infection *in vivo* and in clinical trials (as discussed below) currently suggest that NAPs block secretion of HBsAg from infected hepatocytes and further may selectively block the secretion of subviral particles.

6. Optimization of NAPs for clinical use in HBV infection

The antiviral activity of NAPs is not only sequence independent but also tolerates modification of all ribose sugars at the 2′ position (Vaillant et al., 2006; Lee et al., 2007; Bernstein et al., 2008; Matsumura et al., 2009; Noordeen et al., 2013b). This flexibility permits the optimization of NAPs for tolerability *in vivo* without impacting antiviral effect by altering sequence composition and or incorporating 2′ ribose modification at any nucleotide position, advantages unique to NAPs. As is the case for all PS-ONs, the tolerability of NAPs can be impacted by pro-inflammatory/immunostimulatory effects mediated by recognition of single stranded oligonucleotides by TLR-7, -8, and -9 or RIG-I (Kawai and Akira, 2009) which recognize single stranded DNA or RNA. Initial animal experiments with the prototypic 40mer degenerate NAP polymer REP 2006 (Table 1) in CMV infected mice and DHBV infected ducks showed that NAPs could prevent CMV infection in the spleen and liver or DHBV infection in the liver but that treatment in either mice or ducks was accompanied by hepatotoxicity and splenomegaly (Cardin et al., 2009; Noordeen et al., 2013b). The tolerability of REP 2006 in DHBV infected ducks was poor enough to require dose reduction shortly after treatment began (Noordeen et al., 2013b). The degenerate nature of REP 2006 (Table 1) results in the presence of CpG motifs capable of stimulating TLR-9 (Krieg, 2002), consistent with its ability to stimulate cytokine induction in PBMCs (Cardin et al., 2009). A second candidate NAP, REP 2031, was designed as a 40mer polycytidine homopolymer (Table 1) with no CpG motifs and was confirmed to have negligible cytokine response in human PBMCs (Cardin et al., 2009), confirming TLR-9 as the primary sensor for NAPs. REP 2031 retained the same antiviral activity as REP 2006 in preventing CMV infection (Cardin et al., 2009) and could also block HCV infection of the liver *in vivo* (Matsumura et al., 2009) and was not accompanied by hepatotoxicity or splenomegaly, illustrating that the antiviral effects of NAPs *in vivo* were not derived from pro-inflammatory/immunostimulatory properties.

Oligonucleotides with polypyrimidine tracts (i.e. REP 2031) also form obligate homotetramers (i-motif DNA) at acidic pH (Leroy et al., 1993; Kanehara et al., 1997). This activity neutralizes the amphipathic feature of REP 2031 at acidic pH and also renders it stable to acid hydrolysis at pH 1 (Bernstein et al., 2008). The conservation of antiviral effect of REP 2031 against CMV and HCV infection *in vivo* not only excluded pro-inflammatory/immunostimulatory effects as part of the antiviral mechanism but also demonstrated that the entry-inhibitory effects of NAPs for CMV and HCV involved activity at a neutral pH. Additionally, the pH stability of REP 2031 made possible its delivery through the gastrointestinal tract and when combined with intestinal permeation enhancers, oral administration of naked REP 2031 was shown to have antiviral effect against CMV infection in the liver (Cardin et al., 2009).

However, while REP 2031 was able to block entry of DHBV, it had

Table 1
Optimization of NAPs for clinical use in HBV/HDV infection.

NAP	Sequence[a]	Activity		Tolerability *in vivo*
		In vivo (DHBV)	Clinically	
REP 2006	$(dN)_{40}$	YES	NA	Poor[f]
REP 2031	$(dC)_{40}$ (loss of activity at acidic pH)	NO[c]	NA	Good
REP 2055	$(dAdC)_{20}$[b]	YES[d]	YES	Good[g]
REP 2139	(2′OMeA, 2′OMe-5-MeC)$_{20}$[b]	YES[e]	YES	Good[h]

d = deoxyribonucleic acid, N = degenerate sequence (random A, G, T or C at every position), NA = not assessed.
[a] All linkages are phosphorothioated.
[b] See Fig. 1 for structure.
[c] Blocks viral entry of CMV and HCV *in vivo*.
[d] Also blocks viral entry of HCV *in vivo*.
[e] Unpublished data (Brikh et al., 2015).
[f] Due to CpG content.
[g] Significant administration related side effects in humans.
[h] Administration well tolerated in humans (REP 2139-Ca).

no post-entry antiviral activity in DHBV infected PDH (Noordeen et al., 2013a), strongly suggesting that the unique post entry effect of NAPs in DHBV infection occurs intracellularly in an acidified compartment. *In vivo*, treatment of ducks with REP 2031 was well tolerated, but failed to prevent DHBV-infection in ducks (Noordeen et al., 2013b), indicating that the post entry effect of NAPs is essential for the prophylactic activity in DHBV infection *in vivo*.

An additional optimization of the REP 2031 sequence recovered well tolerated post-entry NAP activity in DHBV infection. This involved "doping" the polypyrimidine sequence of REP 2031 with a purine nucleotide (adenosine) at every other position, yielding REP 2055 (Table 1), a 40mer NAP still devoid of CpG activity (Noordeen et al., 2015) but incapable of tetramerization (and loss of amphipathicity) at acidic pH (Geinguenaud et al., 2000). This NAP was able to prevent DHBV infection in ducks and block liver infection by HCV in Scid/Hu mice and was well tolerated in both models (Matsumura et al., 2009; Noordeen et al., 2013b). Finally, to address administration tolerability issues observed with REP 2055 in human subjects (Al-Mahtab et al., 2016), additional oligonucleotide modifications were introduced to further suppress any residual pro-inflammatory activity which consisted of 5-methylation of each cytosine base and 2′O-methyl modification of each ribose sugar (see Fig. 1 and Table 1). These modifications occur naturally in mammalian DNA (Ehrlich et al., 1982; Kiss, 2001) and are known to block TLR recognition of oligonucleotides (Kariko et al., 2005; Judge et al., 2006; Robbins et al., 2007). This new NAP, REP 2139, was additionally formulated as a calcium chelate complex (REP 2139-Ca) to block chelation effects during administration common to PS-ONs (Mata et al., 2000).

7. NAPs: therapeutic effects *in vivo* and current mechanistic hypothesis in HBV

The therapeutic potential of REP 2055 to treat chronic HBV infection was assessed by treating ducks 2 weeks after DHBV infection, which has been previously shown to reliably result in persistent (>280 days) DHBV infection (Foster et al., 2003). A series of experiments evaluating different treatment doses and dosing regimens as long as 28 days were conducted (Noordeen et al., 2015). In these studies, all control animals treated with normal saline maintained a chronic DHBV infection throughout the course of the experiment whereas REP 2055 treatment was accompanied by the reduction and or clearance of duck HBsAg (DHBsAg) in the blood with simultaneous increases in serum titers of anti-DHBsAg antibodies and decreases in serum DHBV DNA. At the end of treatment, DHBsAg was found in the livers of many animals despite clearance from the blood, suggesting that NAPs block the release of DHBsAg from infected hepatocytes. Moreover, although reductions in serum DHBV DNA >3 log were observed in all treated ducks, many maintained persistent serum DHBV DNA titers (10^5-10^6 copies/ml) despite clearance of serum DHBsAg. Similar to HBV infection in humans, DHBV infection results in the production of non-infectious subviral particles (SVPs) that outnumber infectious virus by more than 10,000 to 1 (Ganem and Prince, 2004; Franke et al., 2007). The unusual observation of persistent DHBV DNA titres, despite clearance of serum DHBsAg, is consistent with a selective effect of NAPs on secretion of SVPs (see Fig. 3).

Of particular interest in these *in vivo* studies was the absence of viremia that persisted after REP 2055 therapy was discontinued in these studies: 55% of treated ducks maintained suppression of serum DHBsAg and DHBV DNA which was accompanied by the clearance of DHBsAg, DHBV core antigen, DHBV DNA and cccDNA from the liver during the 16 weeks of follow up after treatment, suggesting the establishment of a durable control of infection with relatively short duration REP 2055 treatment. The durable control of infection observed in DHBV-infected ducks with REP 2055 cannot be achieved in the duck model with other antiviral agents such as pencyclovir, entecavir and adefovir (Lin et al., 1998; Nicoll et al., 1988; Le Guerhier et al., 2003; Foster et al., 2003), which effectively clears circulating DHBV DNA but does not affect circulating DHBsAg levels. This suggests that the unique effect of NAPs of clearing DHBsAg from the blood may play a role in the establishment of durable control of infection observed with REP 2055.

Based on the structure activity relationship of NAPs in DHBV and the antiviral effects observed in the duck model, the current mechanistic hypothesis (Fig. 3) is that *in vivo*, NAPs enter infected hepatocytes and interfere with some aspect of SVP assembly or trafficking through an acidified compartment in the secretory pathway which prevents the release of SVPs into the circulation. This is consistent with the lack of post-entry/*in vivo* activity with REP 2031 and the observation of persistent DHBV DNA (virons) in the circulation in the absence of detectable DHBsAg as discussed above.

SVPs share many biochemical similarities to human serum HDL (Galvanes et al., 1982), suggesting that some aspect of apolipoprotein metabolism may be involved in their assembly and or secretion. Moreover, apolipoprotein H, a component of HDL, is a known ligand of HBsAg (Medhi et al., 1994, 2008). The heparin-like features of PS-ONs (as described above) may drive apolipoprotein interactions that interfere with SVP assembly and or transit but this remains to be proven. The identification of target(s) for NAP interaction which are involved in their post-entry activity in HBV are currently the focus of ongoing investigation.

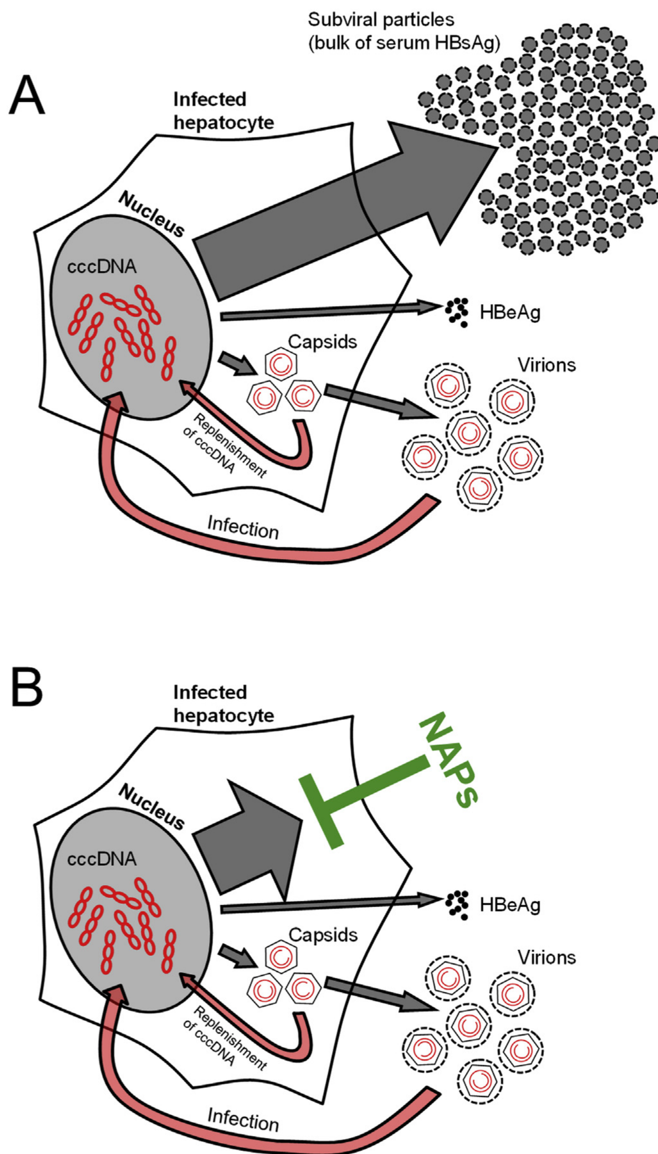

Fig. 3. Particle production in HBV infected hepatocytes (A) and current model for the mechanism of action of NAPs (B). In this model, the secretion of subviral particles is selectively blocked by NAPs.

8. NAPs: application in the treatment of chronic hepatitis B and D infection

The clinical impact of REP 2055 on treatment naïve subjects with HBeAg positive chronic HBV infection was assessed in a small proof of concept clinical trial (REP 101 study, Al-Mahtab et al., 2016) using dosing regimens drawn from previous clinical studies with antisense PS-ONs targeting liver disease (Kastelein et al., 2006). In this trial, patients either had demonstrable maternal transmission of their HBV infection or were HBsAg positive for at least 6 months prior to treatment. The effects of REP 2055 monotherapy in these patients were remarkably similar to those observed in DHBV infected ducks *in vivo*: in 7/8 patients, treatment was accompanied by reduction or clearance of serum HBsAg up to 7 logs from baseline (in some cases resulting in HBsAg loss) which was accompanied by the appearance of anti-HBsAg antibodies, HBeAg seroconversion and reduction of serum HBV DNA. Moreover, during therapy, some patients experienced protracted periods of HBsAg loss where substantial titers of HBV DNA (~10^5 copies/ml) still persisted in the blood suggesting that the post-entry mechanism postulated in DHBV infection (selective blockage of SVP particle secretion) might also be occurring in human HBV infection. Four responder patients also experienced transient elevations in liver transaminases. These transaminase flares were not accompanied by any other evidence of liver dysfunction and were timed with the reduction or clearance of viremia and self-resolved with continuing REP 2055 therapy. Also similar to the previous duck studies, 3/7 responders maintained suppression of serum viremia (serum HBV DNA < 1000 copies/ml and HBsAg < 1 IU/ml) for 1 year after treatment was stopped, with two of these patients currently having no detectable serum HBV DNA and HBsAg ≤ LLOQ (0.05 IU/ml) 4.5–5 years off treatment.

Chronic REP 2055 treatment for more than 1 year was well tolerated in these patients except for administration side-effects common for PS-ONs administered by IV infusion (Tolcher et al., 2004; Chi et al., 2005). These included shivering, fever and headache which resolved after administration but which required extended infusion times and or the use of supportive therapy during administration. To address this tolerability issue a new NAP compound, REP 2139, was designed and prepared as a calcium chelate complex (REP 2139-Ca, see above).

Overexpression of the large form of HBsAg (LHBsAg) in transgenic mice is associated with hepatotoxicity (Chisari et al., 1987) and with the observations in the duck model indicating that the post-entry activity of NAPs involves blockade of HBsAg release (Noordeen et al., 2015), there has been some debate as to the potential for liver toxicity with NAPs due to the possibility of accumulation of HBsAg within hepatocytes. The fate of intracellular HBsAg with NAP treatment is currently unknown, however in DHBV infection, NAP therapy was not accompanied by any signs of liver dysfunction and DHBsAg was ultimately absent from the livers of ducks which maintained control of infection off treatment (Noordeen et al., 2015). Moreover, reduction of serum HBsAg in human patients exposed to NAP therapy was associated with transient elevation of serum transaminases in some but not all patients (Al-Mahtab et al., 2016). The lack of a clear link between surface antigen reduction in the serum and liver toxicity with NAP therapy *in vivo* and in the clinic suggests that if surface antigen accumulation is occurring with NAP therapy that it is not causing any significant liver dysfunction.

In a second proof of concept trial conducted in patients with HBeAg positive chronic HBV infection, REP 2139-Ca monotherapy had antiviral effects comparable to REP 2055 but was not accompanied by administration tolerability issues (REP 102 study, Al-Mahtab et al., 2016). In this trial, 9/12 patients achieving reductions in serum HBsAg and HBV DNA >2 log from baseline were permitted to undergo short term combination therapy (13–26 weeks) with immunotherapies approved for use in HBV at the trial site (pegylated interferon alpha 2a or thymosin alpha 1). With either of these therapies, rapid and substantial increases (>200 mIU/ml) in serum anti-HBsAg antibody titers were observed within 5–10 weeks in all patients (9/9) receiving combination therapy, a response highly unusual with immunotherapy when used alone in patients with HBeAg + chronic HBV infection (Lau et al., 2005; Marcellin et al., 2016). After withdrawal of treatment, 8/9 patients initially achieved serum HBV DNA < LLOQ (116 copies/ml) and 3 further maintained persistent suppression of serum viremia (HBV DNA < 1000 copies/ml and HBsAg <1 IU/ml) for more than 2 years after stopping therapy.

Initial, unpublished interim results from an ongoing proof of concept trial at a second trial site in Caucasian patients with chronic HBV/HDV co-infection (Bazinet et al., 2016) have confirmed the ability of REP 2139-Ca monotherapy to achieve clearance of serum HBsAg and appearance of anti-HBsAg antibodies. Additionally, in those patients achieving HBsAg <1 IU/ml, the same synergistic

antiviral response to pegylated interferon alpha 2a was observed as in the REP 102 study. Moreover, REP 2139-Ca treatment was also accompanied by the clearance of serum HDV RNA. The mechanisms underlying this additional antiviral effect against HDV are still under investigation.

9. Future perspectives

Given the established therapeutic activity of NAPs against HBV and HDV infection *in vivo* and in several preliminary clinical investigations, the elucidation of the molecular basis for the clearance of HBsAg and HDV RNA from the blood by NAPs may lead to an improved understanding of the HBV and HDV lifecycle. Investigations into the post-entry antiviral mechanisms of NAPs in HBV and HBV/HDV infections are underway in earnest. Notwithstanding this, the current pattern of antiviral effects of NAPs *in vivo* and in patients support the general hypothesis that NAPs act to block HBsAg release from infected hepatocytes (perhaps selectively for SVPs) by some non-immunostimulatory mechanism inside infected cells and demonstrate that NAP therapy can achieve substantial reduction or clearance of serum HBsAg in chronic HBV infection.

Of particular interest is the observation that the antiviral effects of two different immunotherapies (pegylated interferon alpha 2a and thymosin alpha 1) appear to be dramatically improved after clearance of HBsAg from the blood in human subjects (Al-Mahtab et al., 2016). The HBsAg protein is the most abundant circulating viral antigen and has been linked to immunosuppression in HBV infection (Kondo et al., 2013). Its clearance from the blood during therapy is recognized as the most reliable indicator of persistence of suppression of viremia off treatment (Frenette and Gish, 2009; Moucari et al., 2009). Moreover, the HBsAg protein has been shown to directly block both adaptive and innate immune processes (Vanlandschoot et al., 2002; Cheng et al., 2005; Op den Brouw et al., 2008; Wu et al., 2009; Xu et al., 2009; Shi et al., 2012) suggesting that circulating HBsAg may not only play a role in suppression of the host immune response to HBV infection but may also interfere with its simulation by immunotherapy.

The initial *in vivo* and clinical investigations described above provide important preliminary observations of the antiviral effects of NAPs in chronic HBV infection. However additional controlled trials will be required to clearly define the full clinical impact of NAP therapy in the treatment of HBV infection and HBV/HDV co-infection. The first of these planned trials is underway and will assess the safety and antiviral activity of add-on treatment of REP 2139-Ca and pegylated interferon alpha 2a in patients with HBeAg negative chronic HBV infection already on treatment with tenofovir disoproxil fumarate (REP 401 study, NCT02565719). Additional combination therapy trials with NAPs would also be useful to examine the effects of HBsAg clearance on the efficacy of therapeutic vaccines and other therapeutic approaches currently in development as reviewed by Block et al., 2015.

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