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Inhibition of HBsAg secretion by nucleic acid polymers in HepG2.2.15 cells

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ABSTRACT

More than 290 million people have chronic HBV infection and are at risk of developing cirrhosis and hepatocellular carcinoma. HBV subviral particles are produced in large excess over virions in infected patients and are the primary source of HBsAg, which is postulated to be important in allowing HBV to chronically persist by interfering with immune function. Nucleic acid polymers (NAPs) have been shown to result in clearance of HBsAg from the blood in pre-clinical and clinical studies. In this study, we show for the first time the recapitulation of NAP- induced inhibition of secretion of HBsAg *in vitro* using the human HepG2.2.15 cell line. With the restoration of endosomal release of NAPs *in vitro* using the UNC7938 compound, NAPs were observed to selectively impair the secretion of HBsAg without any intracellular HBsAg accumulation. Additionally, the structure-activity relationship of NAPs for this antiviral activity is similar to that previously reported in other infectious diseases and identifies an exposed hydrophobic protein domain as the target interface for this antiviral effect. The presented *in vitro* model, the first one to be based on a human derived cell line that constitutively expresses HBV, is a very promising tool for the identification of the host proteins(s) targeted by NAPs.

1. Introduction

More than 2 billion people have been infected by the hepatitis B virus (HBV). However, even with vaccine use and self resolution in the majority of cases, over 290 million people have chronic HBV infection (Polaris Observatory, 2018) and are at higher risk of developing cirrhosis and hepatocellular carcinoma. Clearance of the hepatitis B surface antigen (HBsAg) from the blood is the standard for cessation of therapy in these patients (European Association for the Study of the Liver, 2017; Terrault et al., 2018) and is the goal for new therapies attempting to achieve functional cure of HBV infection (Dusheiko et al., 2016). Currently approved treatments include nucleos(t)ide analogs (NUCs) such as tenofovir disoproxil fumarate (TDF) and entecavir (ETV), and immunotherapies such as pegylated interferons (pegIFN). NUCs inhibit the viral reverse transcriptase, leading to on-treatment suppression of HBV DNA (Marcellin et al., 2016b) and reductions in the prevalence of cccDNA (Lai et al., 2017; Lam et al., 2017; van Campenhout et al., 2016; Werle-Lapostolle et al., 2004). PegIFN acts to stimulate the host immune response to HBV infection. However, even when used in combination, these treatments rarely lead to serum HBsAg loss (Marcellin et al., 2016a).

Nucleic acid polymers (NAPs) are phosphorothioated oligonucleotides (PS-ONs) with broad spectrum antiviral activity (Vaillant, 2016). Phosphorothioation protects oligonucleotides from nuclease attack but also allows NAPs to interact with the hydrophobic surfaces of uncomplexed α-helixes present in the fusion glycoproteins of various class 1 enveloped viruses and other infectious agents (Vaillant, 2018). Importantly, the antiviral activity of NAPs toward these viruses as well as duck hepatitis B virus (DHBV) and hepatitis C virus (HCV) has been shown to be size dependent and sequence independent (Matsumura et al., 2009; Noordeen et al., 2013a; Vaillant, 2016) indicating that a similar target interface (although absent in the glycoproteins of HBV and HCV) is also involved in their antiviral activity toward these viruses. The clinically evaluated NAPs REP 2055 and REP 2139 are unique in their ability to achieve HBsAg clearance from the blood in a majority of patients in clinical studies. Additionally, REP 2139, when combined with immunotherapy, leads to the persistent control of infection after removal of therapy in the majority of patients. This activity is conserved in multiple HBV genotypes and in HBeAg positive and HBeAg negative chronic HBV infection and in HBeAg negative HBV/HDV co-infection (Al-Mahtab et al., 2016; Bazinet et al., 2017; Bazinet

Abbreviations: HBV, hepatitis B virus; DHBV, duck hepatitis B virus; HDV, hepatitis delta virus; HCV, hepatitis C virus; SVP, subviral particles; NAP, nucleic acid polymer; NUC, nucleos(t)ide analogs; TDF, tenofovir disoproxil fumarate; ETV, entecavir; pegIFN, pegylated interferons; PS-ON, phosphorothioated oligonucleotide; ASO, antisense oligonucleotide; LNA, locked nucleic acid; PHH, primary human hepatocytes

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Table 1
Sequence and chemical properties of NAPs used in this study.

NAP	Sequence	Length (nt)	PS	2′OMe	5-MeC	Note
REP 2139	$(AC)_{20}$	40	+	+	+	Clinically active lead RNA NAP
REP 2176	$(CA)_{19}C$	39	+	+	+	REP 2139 size derivative
REP 2177	$(AC)_{19}$	38	+	+	+	REP 2139 size derivative
REP 2178	$(CA)_{17}C$	35	+	+	+	REP 2139 size derivative
REP 2179	$(AC)_{10}$	20	+	+	+	REP 2139 size derivative
REP 2173	$(AC)_{20}$	40	+[a]	+	+	REP 2139 PO variant: 1 PO at position 20
REP 2174	$(AC)_{20}$	40	+[a]	+	+	REP 2139 PO variant: 2 PO at position 13 and 27
REP 2175	$(AC)_{20}$	40	+[a]	+	+	REP 2139 PO variant: 5 PO at position 7, 14, 21, 28, and 35
REP 2143	$(AC)_{20}$	40	+[a]	+	+	REP 2139 PO variant: 20 PO (every other linkage)
REP 2107	$(N)_{40}$	40	+	+		REP 2139 degenerate analog
REP 2055	$(dAdC)_{20}$	40	+			Clinically active DNA NAP
REP 2031	$(dC)_{40}$	40	+			Loss of amphipathicity at acidic pH
REP 2057	$(dAdG)_{20}$	40	+			Loss of amphipathicity at acidic pH
REP 2117	None (abasic)	40	+			Removal of bases (Noordeen et al., 2013a)
REP 2118	None (propane)	40	+			Removal of bases and ribose sugars (Noordeen et al., 2013a)

Nt; nucleotide; d, DNA; N, degenerate sequence; PS, phosphorothioate linkage; PO, phosphodiester linkage.
2′OMe, O-linked methylation at the 2′ position in ribose; 5-MeC, methylation of 5′ position in cytidine base.
[a] Specific linkage(s) are PO, see the Note column.

et al., 2018). Early *in vitro* and *in vivo* studies in the duck model identified an antiviral activity of NAPs occurring after viral entry that impaired DHBsAg secretion, consistent with the antiviral effects observed in humans (Noordeen et al., 2013a, 2015; Quinet et al., 2018). Recent studies using *in vitro* models of HBV and HDV infection observed entry inhibitory activity significantly weaker than other entry inhibitors such as myrcludex B (Schulze et al., 2010) for REP 2055 and no detectable entry inhibition with REP 2139. Moreover, these studies failed to observe any alteration in HBsAg secretion upon treatment with NAPs (Beilstein et al., 2018; Guillot et al., 2017). The difficulty in observing this effect may be due to altered PS-ON trafficking *in vitro* versus *in vivo*: PS-ONs are properly internalized and transported to the cytoplasm and nucleus *in vivo* (Agrawal, 1996; Dias and Stein, 2002; Geary et al., 2015; Koller et al., 2011) but remain sequestered in endosomes/lysosomes *in vitro* (Dias and Stein, 2002; Koller et al., 2011; Yang et al., 2015).

Here we report the observation the inhibition of HBsAg secretion by NAPs *in vitro* by sequentially treating HepG2.2.15 cells with NAPs and UNC7938, a compound known to restore the release of PS-ONs from the late endosome (Yang et al., 2015) *in vitro*. Indeed, we provide evidence of an impaired secretion of HBsAg (and not of HBeAg) in the culture supernatant accompanied by a reduction in intracellular HBsAg. Finally, we also demonstrate that this antiviral effect of NAPs is sequence independent and relies on the size and amphipathic properties of the polymer.

2. Material and methods

2.1. Cells and reagents

All NAPs were prepared and characterized as previously described (Roehl et al., 2017). Treatment of cells was performed either with stock solutions of NAPs prepared directly in normal saline or with chelate complex drug products (REP 2139-Ca and REP 2139-Mg) used in the REP 301 and REP 401 clinical studies (Bazinet et al., 2017, 2018). HepG2.2.15 cells were maintained in William's medium E (WME) complemented with 10% fetal calf serum (FCS) and gentamicin. A list of the NAPs used in this reported study is presented in Table 1. The PCSK9 antisense PS-ON (LNA modified) (Gupta et al., 2010) was obtained from Exiqon. The UNC7938 compound, a generous gift from Dr. Juliano (Yang et al., 2015), was resuspended in DMSO. Treatment paradigms used in experiments are described in the diagrams present in each figure.

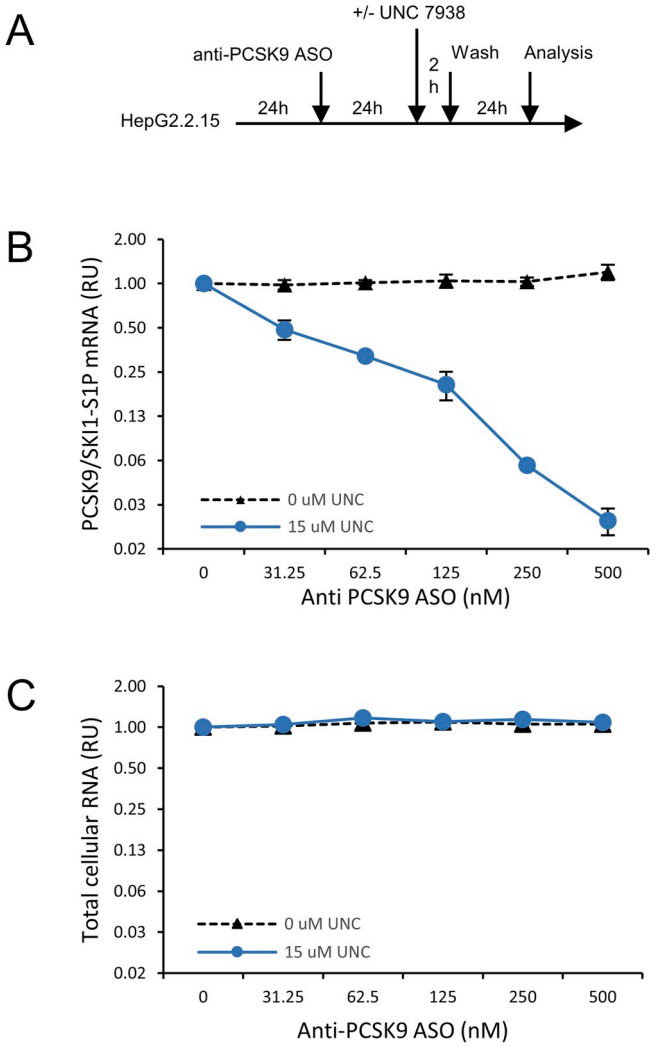

Fig. 1. Effect of endosomal release of anti-PCSK9 ASO on target mRNA. Experimental design is as indicated (A). Effect of treatment with anti-PCSK9 ASO, in the presence or absence of UNC7938, on target mRNA level relative to housekeeping gene SKI-1/S1P (B). Cell viability upon treatment was monitored (C). RU, relative unit.

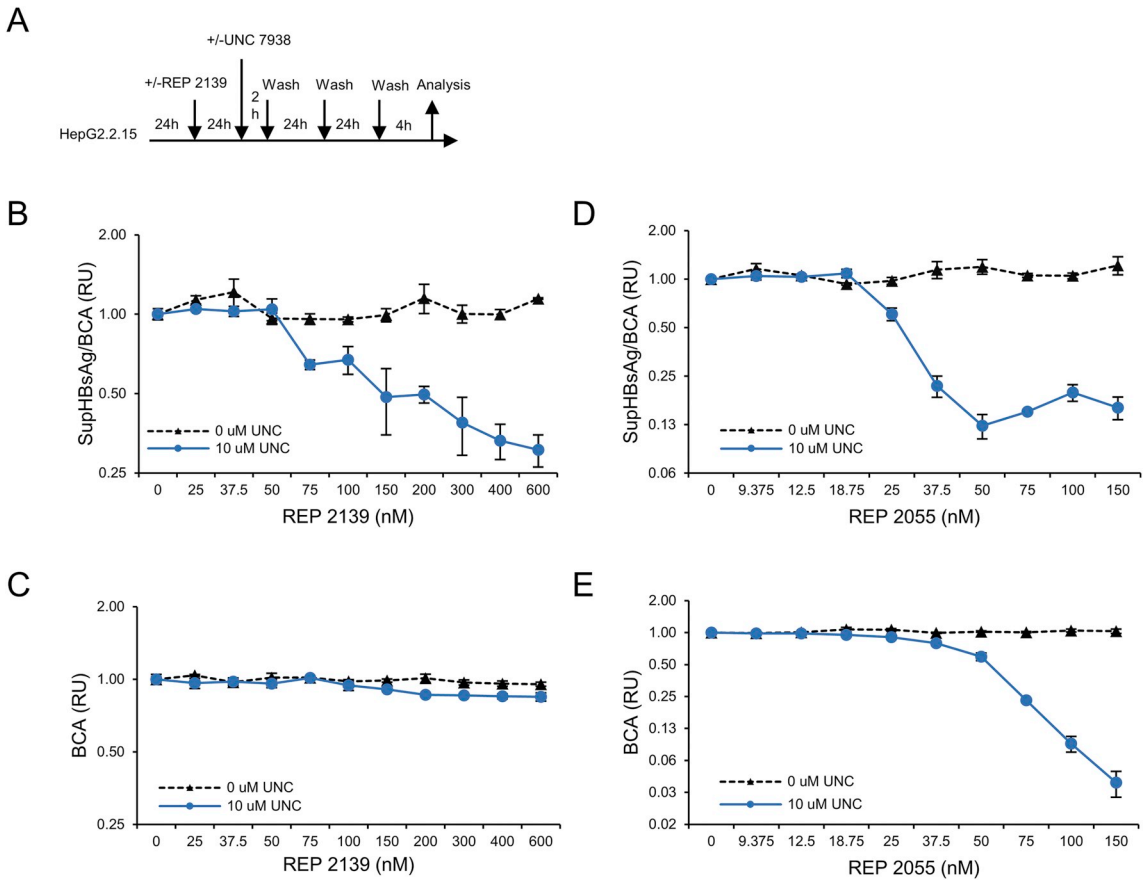

Fig. 2. Effect of endosomal release of REP 2139 and REP 2055 on secreted HBsAg. Experimental design is as indicated (A). Effect of treatment with REP 2139 (B) and REP 2055 (D), in the presence or absence of UNC7938, on HBsAg secretion. Cell viability upon treatment was monitored (C, E). RU, relative unit.

2.2. Cell viability

Cell viability was assessed using 3 different methods: i) MTS test was conducted per manufacturer instructions (Promega; Cell titer 96 ® Aqueous One solution reagent), ii) total cellular protein content after lysis in Pierce lysis buffer (25 mM Tris•HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol) was performed per the manufacturer instructions (Thermofisher Scientific; Pierce TM BCA Protein Assay Kit), iii) total cellular RNA was measured by Nanodrop following extraction using the Aurum Total RNA Mini Kit (Biorad).

2.3. HBV total RNA and PCSK9 mRNA quantifications

Cellular RNA was normalized by Nanodrop and reverse transcribed into cDNA using the iScript™ Select cDNA Synthesis Kit (Biorad).

Quantification of specific mRNAs was following reverse transcription using the SsoFast™ EvaGreen® Supermix (Biorad). Primers used for the quantification of PCSK9 and SKI-1-S1P housekeeping gene mRNAs are described elsewhere (Blanchet et al., 2016; Gupta et al., 2010). The quantification of total cellular HBV RNA was performed using the SsoFast™ Probes Supermix (Biorad) with primers and probes used for detection as described previously (Zhao et al., 2005). PCSK9 and HBV RNA concentration were normalized to the housekeeping gene using the ΔΔct method.

2.4. ELISA

HBsAg quantification in supernatants and cell lysates (obtained in Pierce lysis buffer as described above) was conducted using the Murex HBsAg Version 3 Kit (Diasorin) employing a standard curve from dilution of HepG2.2.15 supernatant. HBeAg quantification was conducted using the ETI-EBK PLUS Kit (Diasorin) with standard cure calibration as described for HBsAg. Presented results are normalized to total intracellular protein content (BCA).

2.5. Confocal fluorescence microscopy

Cells were cultured on glass coverslips and following treatment, were fixed for 10 min in 4% formaldehyde, followed by DAPI staining. Coverslips were then mounted on microscope slides using Prolong antifade reagent (Thermo Scientific). Cells were analysed using a confocal microscope (Zeiss LSM 780). Detector sensitivity was constant for all samples. The presence of Cy3-REP 2139 in cell nuclei was quantified using ZEN 2.3 software (Zeiss) by measuring the mean of fluorescence in the nucleus of 70 cells at each indicated step of the treatment. Data is presented as a dot plot graph.

3. Results

3.1. Intracellular activity of PS-ONs (including NAPs), is restored upon release from late endosomes

Given the known endosomal sequestration of PS-ONs in hepatocytes *in vitro* (Dias and Stein, 2002; Koller et al., 2011; Yang et al., 2015), we employed the previously described compound UNC7938 to attempt to overcome this defect in HepG2.2.15 cells. Cells were treated with an anti-PCSK9 antisense oligonucleotide (ASO) for 24 h followed by addition of UNC7938 for 2 h (Fig. 1A). RT-qPCR analysis revealed that PCSK9 mRNA concentration decreased in a dose dependent manner only when cells were treated with UNC7938 (Fig. 1B) without affecting overall cellular viability (Fig. 1C and Fig. S1B).

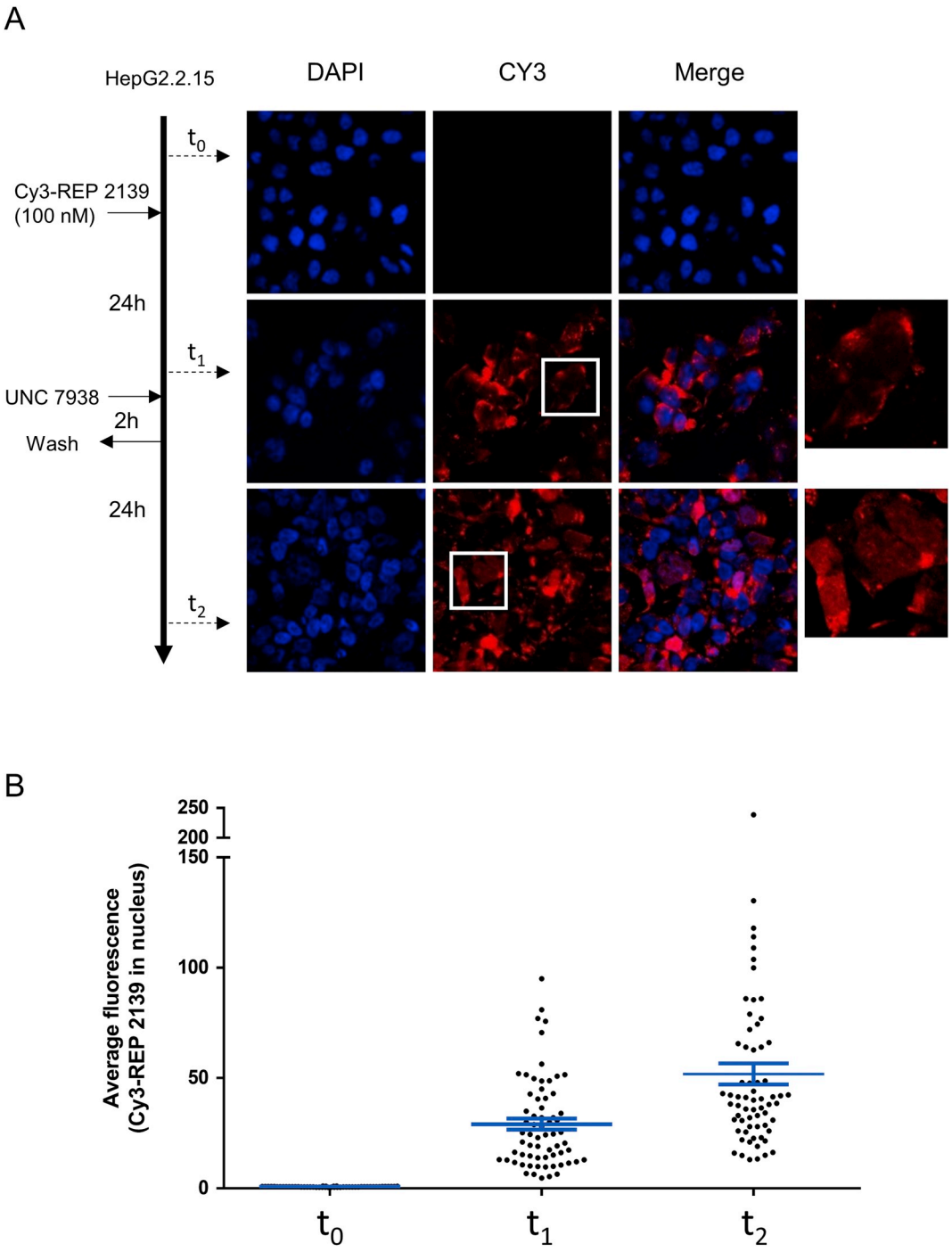

Fig. 3. Effect of UNC7938 on Cy3-REP 2139 cellular distribution. Experimental design is as indicated. Analysis was conducted by confocal microscopy. (A) Qualitative depiction of NAPs cellular distribution at successive steps of treatment. (B) Quantitative analysis of nuclear staining at the different steps. Each dot represents the mean fluorescence for the nucleus of one cell. Overall average and standard error of the mean (SEM) are indicated.

We then sought to examine if the restoration of functional trafficking of NAPs by UNC7938 would result in an observable antiviral activity in HepG2.2.15 cells. Cells were exposed to REP 2139 for 24 h, followed by UNC7938 treatment for 2 h (Fig. 2A). A significant, dose dependent reduction of HBsAg in the supernatant was observed with REP 2139 only in the presence of UNC7938, with an EC_{50} ~150 nM (Fig. 2B) during which only minor alterations in cellular viability were observed (Fig. 2C and Fig. S1D). The antiviral effect of the first clinical NAP candidate (REP 2055) was also assessed in this model. Reductions of HBsAg in the supernatant were also observed with REP 2055 (Fig. 2D). While occurring at lower concentrations than REP 2139, the reduction in HBsAg in the supernatant with REP 2055 was accompanied with a higher toxicity profile (Fig. 2E). It should however be noted that at the EC_{50}, no significant toxicity was observed.

3.2. Effect of UNC7938 on intracellular localization of REP 2139 in HepG2.2.15 cells

Given the restoration of the post-entry effect of REP 2139 on lowering HBsAg in the supernatant, we sought to correlate this recovery with an expected relocalization of REP 2139 upon cell treatment with UNC7938. To this end, cells treated with 100 nM Cy3-REP 2139 were

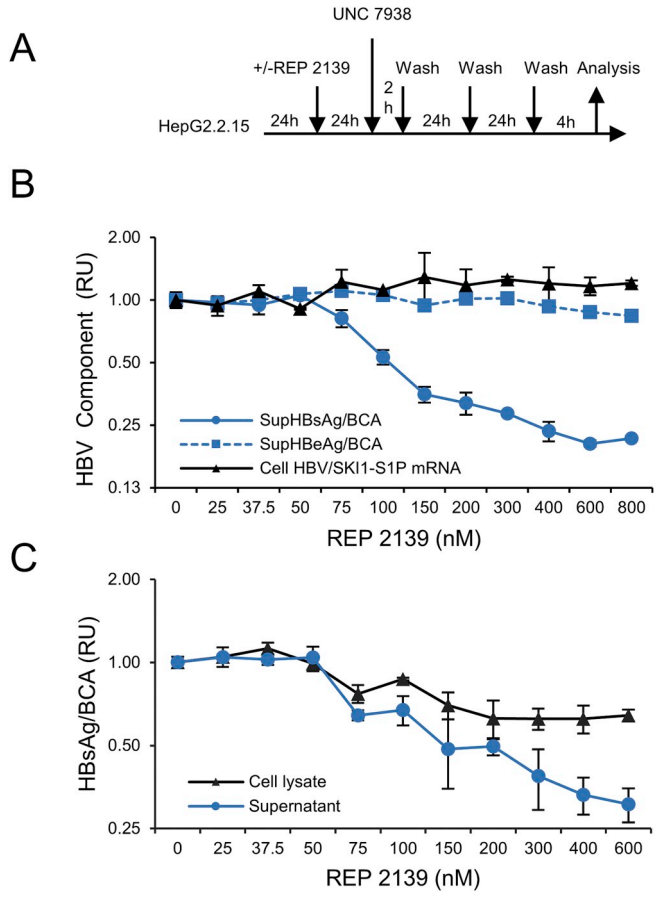

Fig. 4. Specificity of REP 2139 antiviral effect toward HBsAg secretion, and resulting intracellular concentration. Experimental design is as indicated (A). Comparative effect of treatment with REP 2139 on HBsAg and HBeAg secretion, and on total HBV RNA intracellular concentration. (B) Comparative effect of treatment with REP 2139 on HBsAg concentration in culture medium and cell lysates (C). RU, relative unit.

fixed before or after exposure to UNC 7938, and the localization of REP 2139 was monitored qualitatively and quantitatively by fluorescence confocal microscopy (Fig. 3A and B, respectively). While changes in the overall cytoplasmic distribution of Cy3-REP 2139 could not be discerned in the presence of UNC7938, a marked increase in REP 2139 distribution to the nucleus was observed.

3.3. Selectivity of antiviral effect of NAPs towards HBsAg secretion

To gain an initial insight into the specificity of the antiviral effect of NAPs towards HBsAg in HepG2.2.15 cells, we monitored changes in intracellular HBV mRNA and secreted HBeAg and both secreted and intracellular HBsAg (Fig. 4). Neither intracellular total HBV RNA nor secreted HBeAg were altered in the presence of REP 2139 (Fig. 4B). Interestingly, a smaller but still significant reduction of intracellular HBsAg accompanied declines in HBsAg secretion into the supernatant (Fig. 4C).

3.4. NAPs inhibition of HBsAg secretion is sequence independent and requires continuous stretch of amphipathic polymer

In all previous studies on NAPs, their pharmacological effects have been shown to be sequence independent and to rely on size and amphipathicity (conferred by phosphorothioate linkages). The conservation of this structure activity relationship in the antiviral activity of NAPs in HepG2.2.15 cells was therefore examined.

We first compared the antiviral activities of REP 2139 and its degenerate counterpart REP 2107 and observed that both compounds exerted very similar activity, ruling out the need for a specific sequence (Fig. 5B). Results from cell treatment with REP 2139 size derivatives demonstrated that the post-entry antiviral effect was clearly dependent on the increased length of NAPs and was largely absent with NAPs smaller than 35 nucleotides in length (Fig. 5C). The requirement of amphipathicity was first assessed through treatment with REP 2139 and derivatives in which phosphorothioate linkages were replaced with an increasing number of phosphodiester linkages, thereby increasingly disrupting the amphipathic character along the length of the NAP. Results revealed that the post-entry antiviral effect was increasingly inhibited with increasing numbers of phosphodiester linkages and abolished when 5 or more phosphodiester linkages were present (Fig. 5D). Cells were then treated with 40-mer NAP analogs lacking either the base (abasic PS-ON, REP 2117) or the base and the ribose sugar (propane phosphorothioate, REP 2118), which reduce the hydrophilic properties of NAPs (see Table 1). Neither of these compounds were able to trigger any antiviral effect even when used at concentrations up to 1 μM (Fig. 5E). Altogether, the results are in line with a conserved structure/activity relationship of NAPs, relying on size and amphipathicity of the polymer, independent of the sequence present in the NAP.

3.5. REP 2139 formulation as chelate complexes does not affect its antiviral activity in vitro

In clinical studies, the initial formulation of NAPs in normal saline was accompanied by significant side effects during intravenous (IV) infusion (shivering, fever, and headaches (Al-Mahtab et al., 2016), which are common with IV infusion of PS-ONs (Chi et al., 2005; Tolcher et al., 2004). To improve tolerability during infusion of the current lead NAP, REP 2139 was subsequently formulated as calcium (REP 2139-Ca) or magnesium (REP 2139-Mg) chelate complex, which neutralized the IV infusion reactivity (Al-Mahtab et al., 2016; Bazinet et al., 2017; Bazinet et al., 2018). In comparing the post-entry activity of REP 2139 versus REP 2139-Ca or REP 2139-Mg (Fig. 6A), no meaningful difference in dose-dependent inhibition of HBsAg secretion was observed (Fig. 6B).

3.6. NAP activity seems to occur at least in part in an acidified compartment

REP 2031 is a poly-dC based 40-mer NAP which forms tetramers at mildly acidic pH, resulting in loss of amphipathicity (Kanehara et al., 1997; Leroy et al., 1994; Manzini et al., 1994). This feature is common to polypyrimidine tracts of oligonucleotides and is prevented by the presence of purines (Geinguenaud et al., 2000) such as in the degenerate NAP REP 2006 and in REP 2055. Interestingly in both *in vitro* and *in vivo* DHBV-based models, REP 2031 was unable to elicit any post-entry effect (Noordeen et al., 2013a,b) as opposed to REP 2006 and REP 2055. These results strongly suggested that in DHBV, NAP antiviral activity was occurring in a subcellular acidified compartment.

In an attempt to verify if this phenomena was transposable to our HBV *in vitro* model, HepG2.2.15 cells were treated with REP 2031 and REP 2057, which also loses amphipathicity at acidic pH (Dolinnaya and Fresco, 1992), and REP 2055 (Fig. 7A). We observed a significant loss of antiviral activity in cells treated with 40 nM of both pH sensitive NAPs, as compared with REP 2055 (Fig. 7B). However, this gap in antiviral activity is reduced at higher NAPs concentration (~80 nM). This set of data is in line with observations made in the DHBV model, and supports the hypothesis that inhibition of HBsAg secretion by NAPs occurs at least in part in an acidified compartment.

4. Discussion

The present study is the first to demonstrate the impairment of HBsAg secretion upon treatment with NAPs in an *in vitro* model. This

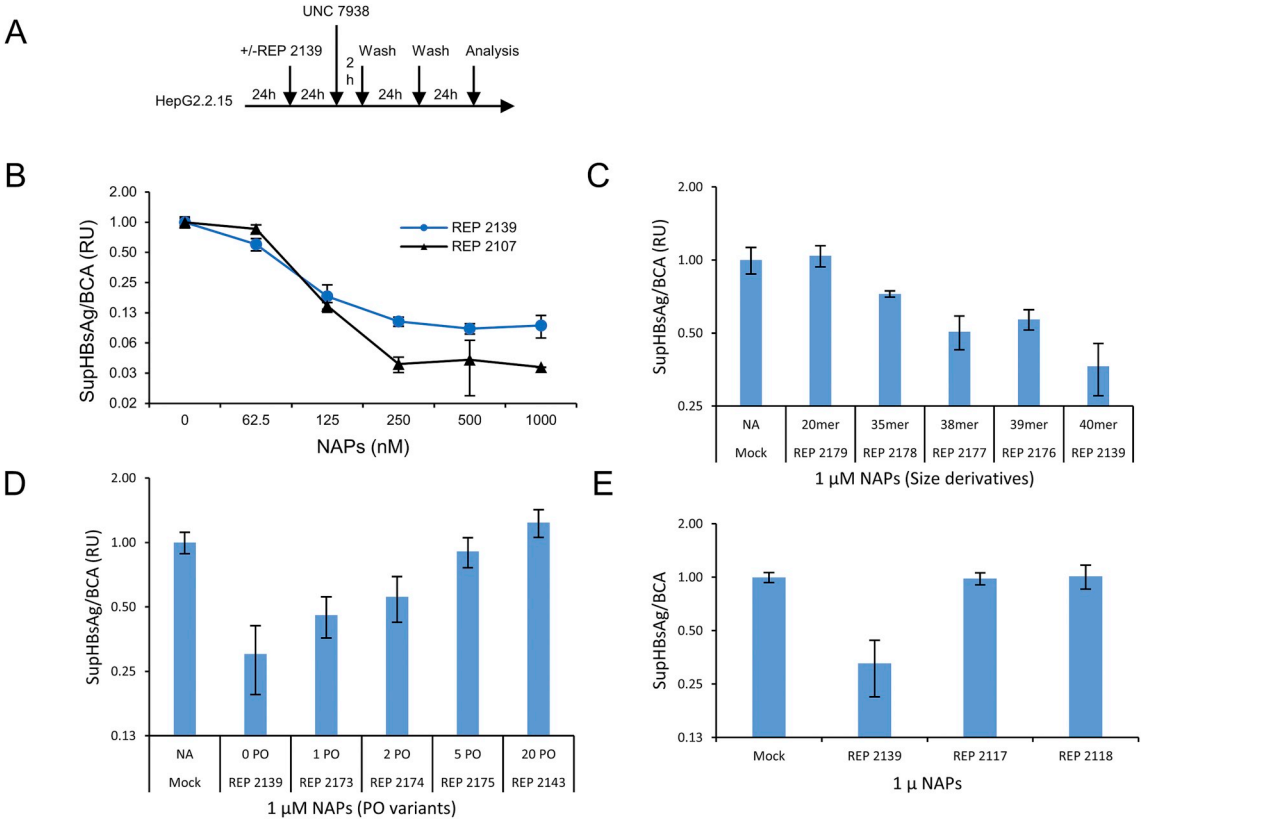

Fig. 5. Structure-activity relationship of NAPs for post-entry antiviral effect. Experimental design is as indicated. (A) Comparative antiviral effect of REP 2139 and its degenerate counterpart (REP 2107) on HBsAg secretion (B). Comparative antiviral effect of REP 2139 and size derivatives on HBsAg secretion (C). Effect of introduction of phosphodiester (PO) bonds in REP 2139 on HBsAg secretion (D). Comparative effect of REP 2139, and Abasic (REP 2117) and propane (REP 2118) NAP analogs on the secretion of HBsAg (E). RU, relative unit.

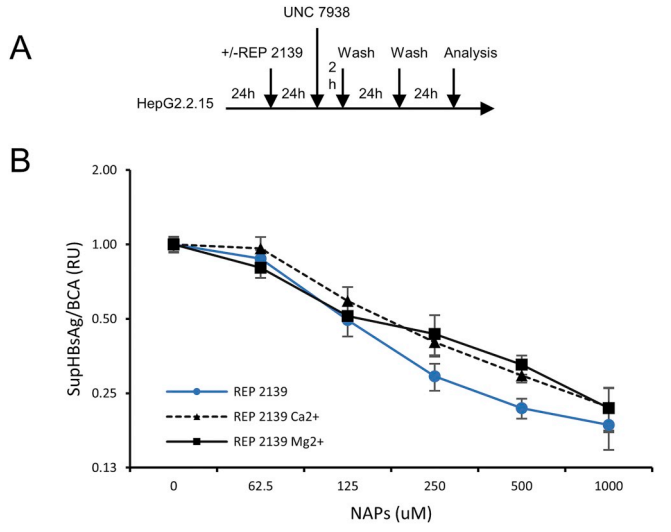

Fig. 6. Antiviral effect of chelate complex formulation of NAPs. Experimental design is as indicated (A). Comparative antiviral effect of REP 2139 sodium salt, and chelate complex formulations used *in vivo,* on HBsAg secretion (B). RU, relative unit.

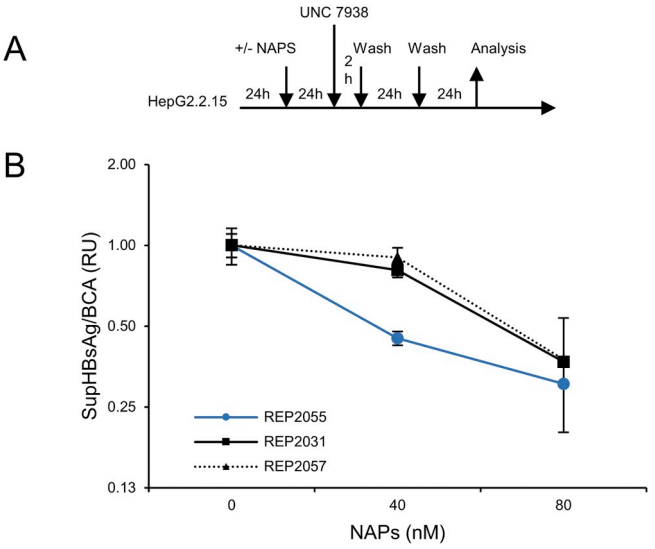

Fig. 7. Antiviral effect of pH sensitive NAPs. Cells were treated with REP 2139 or NAPs known to lose their amphipathic feature at acidic pH, as indicated (A). The antiviral effect on HBsAg secretion was monitored (B). RU, relative unit.

activity required the restoration of endosomal release and intracellular trafficking of NAPs, through the use of UNC7938. The recovery of this antiviral effect occurred concomitantly with the increased nuclear localization of NAPs. Although alterations in cytoplasmic staining were not observed, the enhanced prevalence of NAPs in the nucleus is consistent with their release from endosomes into the cytoplasm, and in line with previous observations with ASOs (Yang et al., 2015).

In agreement with previous *in vivo* and clinical studies (Al-Mahtab et al., 2016; Bazinet et al., 2017), NAPs result in the inhibition of secretion of HBsAg with an EC_{50} in the nanomolar range. This effect appears to be post-translational and selective for HBsAg since it was not accompanied by any significant alteration in the secretion of HBeAg or in HBV transcriptional activity. NAP monotherapy in the *in vivo* duck

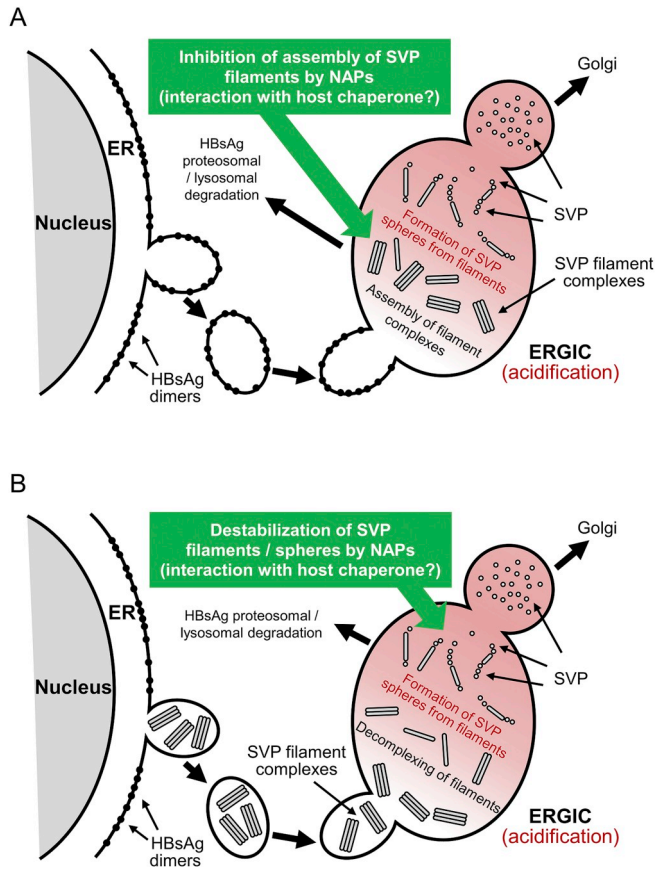

Fig. 8. Proposed models for the mechanism of action of NAPs. Current proposed mechanism of NAPs is presented in SVP assembly models from Huovila et al. (1992) (A), and Patient et al. (2007) (B). Following endosomal release, NAPs are trafficked to the ERGIC where they either inhibit the assembly of HBsAg dimers into filaments (according to the model in [A]) or destabilize filaments and/or forming spherical SVP (according to the model in [B]). In each case, this inhibitory activity is a result of the interaction of NAPs with an as yet uncharacterized host protein required either for the assembly of filaments (according to [A]) or for the morphogenesis of spherical SVP (according to [B]).

model and in clinical studies results in the clearance of HBsAg/DHBsAg and does not systematically result in HBV DNA/DHBV DNA removal from the blood (Al-Mahtab et al., 2016; Noordeen et al., 2015), suggesting the selective targeting of SVP. The effect of NAPs on the secretion of Dane particles was not addressed in the current study but is being examined in a more detailed follow-up analysis currently underway. Since HBeAg secretion appears to be only marginally affected by NAPs, the HBeAg seroconversion accompanying NAP therapy (Al-Mahtab et al., 2016) in HBeAg positive patients is likely not due to a direct effect on HBeAg by NAPs but rather a result of the recovery of host immune function.

Importantly, the inhibition of HBsAg secretion *in vitro* did not trigger any reactive intracellular accumulation but actually resulted in a concomitant decrease in intracellular HBsAg, although milder than observed for reduction of HBsAg in the supernatant. These results are in line with the clearance of DHBsAg from the livers of ducks with NAP treatment, as monitored by immunocytochemistry (Noordeen et al., 2015; Quinet et al., 2018). Although the mild declines in intracellular HBsAg may appear at odds with the inhibition of HBsAg secretion, they may reflect the mechanism of action of NAPs. Intracellular HBsAg is continually degraded through proteasomal (Liu et al., 2007) and/or autophagy related pathways (Lazar et al., 2012) and the mild reduction in intracellular HBsAg suggests that HBsAg is increasingly exposed to degradation. This further suggests that NAPs block the assembly of subviral particles rather than inhibiting their transit through the secretory pathway, in which case accumulation of intracellular HBsAg would be expected (Chua et al., 2005).

Total intracellular HBV RNA was unaffected and since mRNAs coding for HBsAg isoforms (namely the 2.1 and 2.5 kb HBV RNAs) account for ≥50% of total HBV RNA (Yang et al., 2014), it seems highly unlikely that the observed reductions in intracellular and secreted HBsAg are the result of an altered HBV transcriptional activity upon treatment with NAPs. The lack of reduction of total HBV RNA is in line with the lack of direct immunostimulatory activity of NAPs (Real et al., 2017) as stimulation of PRRs are known to be able to stimulate the intracellular degradation of viral RNA (Rigby and Rehwinkel, 2015).

The activity of NAPs on HBsAg secretion was sequence independent but required a minimum NAP length above 30 nucleotides. Additionally, loss of activity with the reduced ability of NAPs to adopt hydrophobic interactions (by removal of phosphorothioate linkages) or reduced solubility in aqueous environment (REP 2117 and REP 2118) indicate that NAPs interact with an exposed hydrophobic protein domain. The size and phosphorothioation-dependent activity of NAP also exclude any immunomodulatory component as the recognition of oligonucleotides by pattern recognition receptors (PRRs) is neither size or phosphorothioate-dependent (Bauer and Hartmann, 2008; Kawai and Akira, 2009; Krieg, 2002). Additionally, this antiviral effect of NAPs persists with 2′O-methylation of all ribose sugars (in REP 2107 and REP 2139) and 5-methylation of all cytidine bases (in REP 2139), modifications which also block recognition of oligonucleotides by PRRs (Kariko et al., 2005; Zust et al., 2011). Finally, both REP 2031 and REP 2055 are similarly immunologically inert (Cardin et al., 2009; Roehl et al., 2017) but REP 2055 is active at lower concentrations than REP 2031 both *in vitro* in this study and substantially more active with equivalent dosing *in vivo* in DHBV infected ducks (Noordeen et al., 2013b).

The nature of the putative target interface identified in the present model is highly similar to that driving NAP activity in HIV-1, HSV-1, HSV-2, CMV, HCV, prion disease and malarial entry (Vaillant, 2016). This strongly suggests that an exposed hydrophobic surface (potentially an amphipathic alpha helix) may be important for the assembly of SVP.

Finally, REP 2031 and REP 2057, two NAPs derivatives known to lose their amphipathic properties at acidic pH (Dolinnaya and Fresco, 1992; Kanehara et al., 1997; Leroy et al., 1994), showed lowered abilities to inhibit HBsAg secretion. These observations are in line with similar results obtained in both *in vitro* and *in vivo* duck models (Noordeen et al., 2013a,b), and suggest that the antiviral activity of NAPs is occurring in a subcellular compartment with an acidified lumen, consistent with known location for SVP assembly (ERGIC) (Huovila et al., 1992; Patient et al., 2007).

5. Conclusions

While the SAR for the antiviral activity of NAPs has been examined against DHBV/HBV in other duck and human *in vitro* systems, this study is the first to formally observe the inhibition of HBsAg secretion by NAPs suggested by previous *in vivo* and clinical studies and to link the SAR previously observed for antiviral activity *in vitro* in the duck system to the inhibition of HBsAg secretion in a human-derived model.

Recovery of antiviral effect of NAPs *in vitro* in HepG2.2.15 cells demonstrates a selective targeting of the release of HBsAg concomitant with milder reduction in intracellular HBsAg suggesting that NAPs block SVP assembly prior to their secretion, as presented in Fig. 8. Taken together, current and previous data suggest that NAPs to target a host protein involved in SVP assembly in an acidified subcellular compartment (i.e. the ERGIC) which contains an exposed hydrophobic domain. Importantly, the antiviral effects on HBsAg secretion and SAR for these effects are consistent with those observed with NAPs *in vitro* and *in vivo* in the duck model as well as in clinical studies, indicating that the SVP assembly in this *in vitro* system provides a reasonable modeling of SVP assembly in HBV infection *in vivo* and in humans.

This model forms the basis for ongoing investigations on the impact of NAPs on the entire HBV life cycle including Dane particle maturation and secretion. This human-derived, cell line-based model will also be central to the identification of the cellular target protein(s) responsible for the observed antiviral effect(s) and to further understand the molecular mechanisms involved in the assembly of SVP.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.02.009.

References

Agrawal, S., 1996. Antisense oligonucleotides: towards clinical trials. Trends Biotechnol. 14, 376–387.
Al-Mahtab, M., Bazinet, M., Vaillant, A., 2016. Safety and Efficacy of Nucleic Acid Polymers in Monotherapy and Combined with Immunotherapy in Treatment-Naive Bangladeshi Patients with HBeAg+ Chronic Hepatitis B Infection. PLoS One 11, e0156667.
Bauer, S., Hartmann, G., 2008. Toll-Like Receptors (TLRs) and Innate Immunity, 1 ed. Springer-Verlag Berlin Heidelberg.
Bazinet, M., Pantea, V., Cebotarescu, V., Cojuhari, L., Jimbei, P., Albrecht, J., Schmid, P., Le Gal, F., Gordien, E., Krawczyk, A., Mijocevic, H., Karimzadeh, H., Roggendorf, M., Vaillant, A., 2017. Safety and efficacy of REP 2139 and pegylated interferon alfa-2a for treatment-naive patients with chronic hepatitis B virus and hepatitis D virus co-infection (REP 301 and REP 301-LTF): a non-randomised, open-label, phase 2 trial. Lancet Gastroenterol. Hepatol. 2, 877–889.
Bazinet, M.P., Plancinta, G., Moscalu, I., Cebotarescu, V., Coujuhari, L., Jimbei, P., Iarovoi, L., Smesnoi, V., Musteata, T., Jucov, A., Krawczyk, A., Vaillant, A., 2018. Updated follow-up analysis in the REP 401 protocol: treatment HBeAg negative chronic hepatitis B infection with REP 2139 or REP 2165, tenofovir disoproxil fumarate and pegylated interferon alfa-2a. J. Hepatol. 68, S517.
Beilstein, F., Blanchet, M., Vaillant, A., Sureau, C., 2018. Nucleic acid polymers are active against hepatitis delta virus infection in vitro. J. Virol. 92.
Blanchet, M., Le, Q.T., Seidah, N.G., Labonte, P., 2016. Statins can exert dual, concentration dependent effects on HCV entry in vitro. Antivir. Res. 128, 43–48.
Cardin, R.D., Bravo, F.J., Sewell, A.P., Cummins, J., Flamand, L., Juteau, J.M., Bernstein, D.I., Vaillant, A., 2009. Amphipathic DNA polymers exhibit antiviral activity against systemic murine cytomegalovirus infection. Virol. J. 6, 214.
Chi, K.N., Eisenhauer, E., Fazli, L., Jones, E.C., Goldenberg, S.L., Powers, J., Tu, D., Gleave, M.E., 2005. A phase I pharmacokinetic and pharmacodynamic study of OGX-011, a 2'-methoxyethyl antisense oligonucleotide to clusterin, in patients with localized prostate cancer. J. Natl. Cancer Inst. 97, 1287–1296.
Chua, P.K., Wang, R.Y., Lin, M.H., Masuda, T., Suk, F.M., Shih, C., 2005. Reduced secretion of virions and hepatitis B virus (HBV) surface antigen of a naturally occurring HBV variant correlates with the accumulation of the small S envelope protein in the endoplasmic reticulum and Golgi apparatus. J. Virol. 79, 13483–13496.
Dias, N., Stein, C.A., 2002. Antisense oligonucleotides: basic concepts and mechanisms. Mol. Cancer Ther. 1, 347–355.
Dolinnaya, N.G., Fresco, J.R., 1992. Single-stranded nucleic acid helical secondary structure stabilized by ionic bonds: d(A(+)-G)10. Proc. Natl. Acad. Sci. U. S. A. 89, 9242–9246.
Dusheiko, G., Wang, B., Carey, I., 2016. HBsAg loss in chronic hepatitis B: pointers to the benefits of curative therapy. Hepatol. Int. 10, 727–729.
European Association for the Study of the Liver, Electronic address, e.e.e., European Association for the Study of the, L., 2017. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J. Hepatol. 67, 370–398.
Geary, R.S., Norris, D., Yu, R., Bennett, C.F., 2015. Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. Adv. Drug Deliv. Rev. 87, 46–51.
Geinguenaud, F., Liquier, J., Brevnov, M.G., Petrauskene, O.V., Alexeev, Y.I., Gromova, E.S., Taillandier, E., 2000. Parallel self-associated structures formed by T,C-rich sequences at acidic pH. Biochemistry 39, 12650–12658.
Guillot, C., Martel, N., Berby, F., Bordes, I., Hantz, O., Blanchet, M., Sureau, C., Vaillant, A., Chemin, I., 2017. Inhibition of hepatitis B viral entry by nucleic acid polymers in HepaRG cells and primary human hepatocytes. PLoS One 12, e0179697.
Gupta, N., Fisker, N., Asselin, M.C., Lindholm, M., Rosenbohm, C., Orum, H., Elmen, J., Seidah, N.G., Straarup, E.M., 2010. A locked nucleic acid antisense oligonucleotide (LNA) silences PCSK9 and enhances LDLR expression in vitro and in vivo. PLoS One 5, e10682.
Huovila, A.P., Eder, A.M., Fuller, S.D., 1992. Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. J. Cell Biol. 118, 1305–1320.
Kanehara, H., Mizuguchi, M., Tajima, K., Kanaori, K., Makino, K., 1997. Spectroscopic evidence for the formation of four-stranded solution structure of oligodeoxycytidine phosphorothioate. Biochemistry 36, 1790–1797.
Kariko, K., Buckstein, M., Ni, H., Weissman, D., 2005. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23, 165–175.
Kawai, T., Akira, S., 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int. Immunol. 21, 317–337.
Koller, E., Vincent, T.M., Chappell, A., De, S., Manoharan, M., Bennett, C.F., 2011. Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. Nucleic Acids Res. 39, 4795–4807.
Krieg, A.M., 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 20, 709–760.
Lai, C.L., Wong, D., Ip, P., Kopaniszen, M., Seto, W.K., Fung, J., Huang, F.Y., Lee, B., Cullaro, G., Chong, C.K., Wu, R., Cheng, C., Yuen, J., Ngai, V., Yuen, M.F., 2017. Reduction of covalently closed circular DNA with long-term nucleos(t)ide analogue treatment in chronic hepatitis B. J. Hepatol. 66, 275–281.
Lam, Y.F., Seto, W.K., Wong, D., Cheung, K.S., Fung, J., Mak, L.Y., Yuen, J., Chong, C.K., Lai, C.L., Yuen, M.F., 2017. Seven-year treatment outcome of entecavir in a real-world cohort: effects on clinical parameters, HBsAg and HBcrAg levels. Clin. Transl. Gastroenterol. 8, e125.
Lazar, C., Macovei, A., Petrescu, S., Branza-Nichita, N., 2012. Activation of ERAD pathway by human hepatitis B virus modulates viral and subviral particle production. PLoS One 7, e34169.
Leroy, J.L., Gueron, M., Mergny, J.L., Helene, C., 1994. Intramolecular folding of a fragment of the cytosine-rich strand of telomeric DNA into an i-motif. Nucleic Acids Res. 22, 1600–1606.
Liu, Y., Zhou, T., Simsek, E., Block, T., Mehta, A., 2007. The degradation pathway for the HBV envelope proteins involves proteolysis prior to degradation via the cytosolic proteasome. Virology 369, 69–77.
Manzini, G., Yathindra, N., Xodo, L.E., 1994. Evidence for intramolecularly folded i-DNA structures in biologically relevant CCC-repeat sequences. Nucleic Acids Res. 22, 4634–4640.
Marcellin, P., Ahn, S.H., Ma, X., Caruntu, F.A., Tak, W.Y., Elkashab, M., Chuang, W.L., Lim, S.G., Tabak, F., Mehta, R., Petersen, J., Foster, G.R., Lou, L., Martins, E.B., Dinh, P., Lin, L., Corsa, A., Charuworn, P., Subramanian, G.M., Reiser, H., Reesink, H.W., Fung, S., Strasser, S.I., Trinh, H., Buti, M., Gaeta, G.B., Hui, A.J., Papatheodoridis, G., Flisiak, R., Chan, H.L., Study, I., 2016a. Combination of tenofovir disoproxil fumarate and peginterferon alpha-2a increases loss of hepatitis B surface antigen in patients with chronic hepatitis B. Gastroenterology 150, 134–144 e110.
Marcellin, P., Zoulim, F., Hezode, C., Causse, X., Roche, B., Truchi, R., Pauwels, A., Ouzan, D., Dumortier, J., Pageaux, G.P., Bourliere, M., Riachi, G., Zarski, J.P., Cadranel, J.F., Tilliet, V., Stern, C., Petour, P., Libert, O., Consoli, S.M., Larrey, D., 2016b. Effectiveness and safety of tenofovir disoproxil fumarate in chronic hepatitis B: a 3-year, prospective, real-world study in France. Dig. Dis. Sci. 61, 3072–3083.
Matsumura, T., Hu, Z., Kato, T., Dreux, M., Zhang, Y.Y., Imamura, M., Hiraga, N., Juteau, J.M., Cosset, F.L., Chayama, K., Vaillant, A., Liang, T.J., 2009. Amphipathic DNA polymers inhibit hepatitis C virus infection by blocking viral entry. Gastroenterology 137, 673–681.
Noordeen, F., Scougall, C.A., Grosse, A., Qiao, Q., Ajilian, B.B., Reaiche-Miller, G., Finnie, J., Werner, M., Broering, R., Schlaak, J.F., Vaillant, A., Jilbert, A.R., 2015. Therapeutic Antiviral Effect of the Nucleic Acid Polymer REP 2055 against Persistent Duck Hepatitis B Virus Infection. PLoS One 10, e0140909.
Noordeen, F., Vaillant, A., Jilbert, A.R., 2013a. Nucleic acid polymers inhibit duck hepatitis B virus infection in vitro. Antimicrob. Agents Chemother. 57, 5291–5298.
Noordeen, F., Vaillant, A., Jilbert, A.R., 2013b. Nucleic acid polymers prevent the establishment of duck hepatitis B virus infection in vivo. Antimicrob. Agents Chemother. 57, 5299–5306.
Patient, R., Hourioux, C., Sizaret, P.Y., Trassard, S., Sureau, C., Roingeard, P., 2007. Hepatitis B virus subviral envelope particle morphogenesis and intracellular trafficking. J. Virol. 81, 3842–3851.
Polaris Observatory, C., 2018. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. Lancet Gastroenterol. Hepatol. 3, 383–403.
Quinet, J., Jamard, C., Burtin, M., Lemasson, M., Guerret, S., Sureau, C., Vaillant, A., Cova, L., 2018. Nucleic acid polymer REP 2139 and nucleos(T)ide analogues act synergistically against chronic hepadnaviral infection in vivo in Pekin ducks. Hepatology 67, 2127–2140.
Real, C.I., Werner, M., Paul, A., Gerken, G., Schlaak, J.F., Vaillant, A., Broering, R., 2017. Nucleic acid-based polymers effective against hepatitis B Virus infection in patients don't harbor immunostimulatory properties in primary isolated liver cells. Sci. Rep. 7, 43838.
Rigby, R.E., Rehwinkel, J., 2015. RNA degradation in antiviral immunity and autoimmunity. Trends Immunol. 36, 179–188.
Roehl, I., Seiffert, S., Brikh, C., Quinet, J., Jamard, C., Dorfler, N., Lockridge, J.A., Cova, L., Vaillant, A., 2017. Nucleic acid polymers with accelerated plasma and tissue clearance for chronic hepatitis B therapy. Mol. Ther. Nucleic Acids 8, 1–12.
Schulze, A., Schieck, A., Ni, Y., Mier, W., Urban, S., 2010. Fine mapping of pre-S sequence requirements for hepatitis B virus large envelope protein-mediated receptor interaction. J. Virol. 84, 1989–2000.
Terrault, N.A., Lok, A.S.F., McMahon, B.J., Chang, K.M., Hwang, J.P., Jonas, M.M., Brown Jr., R.S., Bzowej, N.H., Wong, J.B., 2018. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. Hepatology 67, 1560–1599.

Tolcher, A.W., Kuhn, J., Schwartz, G., Patnaik, A., Hammond, L.A., Thompson, I., Fingert, H., Bushnell, D., Malik, S., Kreisberg, J., Izbicka, E., Smetzer, L., Rowinsky, E.K., 2004. A Phase I pharmacokinetic and biological correlative study of oblimersen sodium (genasense, g3139), an antisense oligonucleotide to the bcl-2 mRNA, and of docetaxel in patients with hormone-refractory prostate cancer. Clin. Cancer Res. 10, 5048–5057.

Vaillant, A., 2016. Nucleic acid polymers: broad spectrum antiviral activity, antiviral mechanisms and optimization for the treatment of hepatitis B and hepatitis D infection. Antivir. Res. 133, 32–40.

Vaillant, A., 2018. REP 2139: antiviral mechanisms and applications in achieving functional control of HBV and HDV infection. ACS Infect. Dis.

van Campenhout, M.J., Brouwer, W.P., van Oord, G.W., Xie, Q., Zhang, Q., Zhang, N., Guo, S., Tabak, F., Streinu-Cercel, A., Wang, J., Pas, S.D., Sonneveld, M.J., de Knegt, R.J., Boonstra, A., Hansen, B.E., Janssen, H.L., 2016. Hepatitis B core-related antigen levels are associated with response to entecavir and peginterferon add-on therapy in hepatitis B e antigen-positive chronic hepatitis B patients. Clin. Microbiol. Infect. 22 571 e575-579.

Werle-Lapostolle, B., Bowden, S., Locarnini, S., Wursthorn, K., Petersen, J., Lau, G., Trepo, C., Marcellin, P., Goodman, Z., Delaney, W.E.t., Xiong, S., Brosgart, C.L., Chen, S.S., Gibbs, C.S., Zoulim, F., 2004. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology 126, 1750–1758.

Yang, B., Ming, X., Cao, C., Laing, B., Yuan, A., Porter, M.A., Hull-Ryde, E.A., Maddry, J., Suto, M., Janzen, W.P., Juliano, R.L., 2015. High-throughput screening identifies small molecules that enhance the pharmacological effects of oligonucleotides. Nucleic Acids Res. 43, 1987–1996.

Yang, L., Shi, L.P., Chen, H.J., Tong, X.K., Wang, G.F., Zhang, Y.M., Wang, W.L., Feng, C.L., He, P.L., Zhu, F.H., Hao, Y.H., Wang, B.J., Yang, D.L., Tang, W., Nan, F.J., Zuo, J.P., 2014. Isothiafludine, a novel non-nucleoside compound, inhibits hepatitis B virus replication through blocking pregenomic RNA encapsidation. Acta Pharmacol. Sin. 35, 410–418.

Zhao, J.R., Bai, Y.J., Zhang, Q.H., Wan, Y., Li, D., Yan, X.J., 2005. Detection of hepatitis B virus DNA by real-time PCR using TaqMan-MGB probe technology. World J. Gastroenterol. 11, 508–510.

Zust, R., Cervantes-Barragan, L., Habjan, M., Maier, R., Neuman, B.W., Ziebuhr, J., Szretter, K.J., Baker, S.C., Barchet, W., Diamond, M.S., Siddell, S.G., Ludewig, B., Thiel, V., 2011. Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. Nat. Immunol. 12, 137–143.