Structural determinants of the post-entry antiviral effects of nucleic acid polymers against HBV infection *in vitro*

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INTRODUCTION

- Nucleic acid polymers (NAPs) are phosphorothioated oligonucleotides (PS-ONs) that inhibit HBV[1,2] and HDV[3] infection.
- NAPs act via a post-entry mechanism to block the assembly/release of HBV subviral particles[4,5], allowing clearance of HBsAg from the circulation[1,2], an effect which also participates in the antiviral effects of NAPs in HDV[3].
- Similar to other PS-ONs, the intracellular trafficking of NAPs *in vivo* is altered *in vitro*[6-8], preventing the observation of any post-entry effects in the absence of delivery system.
- An electroporation-based protocol to facilitate NAP transit *in vitro* resulted in a post-entry inhibition (EC50) of HBsAg secretion in the µM range[5].
- **An electroporation-free method for restoration of NAP transit *in vitro* is presented which results in a post-entry inhibition (EC50) of HBsAg secretion in the nM range.**

AIM

- To further characterize our *in vitro* model for investigating the antiviral effect of NAPs in HBV infection.
- Gain insight into the mechanism of action of NAPs:
  - Assess the effect of NAPs on the extracellular concentration of HBsAg and HBeAg.
  - Examine the levels of intracellular HBsAg when HBsAg secretion is blocked by NAPs.
  - Determine the structure-activity relationship for the inhibition of HBsAg release by NAPs

MATERIAL & METHODS

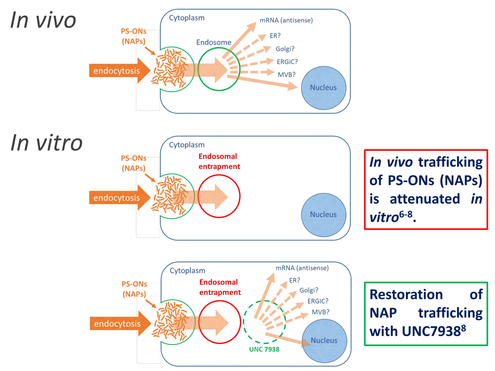
- Electroporation (Ep) parameters of HepG2.2.15 cells were as follows: 4 pulses of 99 uS at 820V with a 1.1 s interval. This method was conducted exclusively for figure 6.
- Endosomal release was achieved by adding 10uM UNC7938[7] for 2 h after overnight exposure of cells to NAPs. This method was conducted for figures 1 to 5.
- Confocal fluorescence microscopy was conducted on cells plated on glass coverslip using Cy3 labeled REP 2139. At each time point indicated in the figure, cells were washed and fixed for 10 min in 4% formaldehyde at room temperature. Photomicrographs are from optical sections taken from the bottom third of the cell.
- ELISA kits used in this study are as follows: HBsAg, Murex version 3 (Diasorin); HBeAg,ETI-EBK plus, N0140, DIASORIN; human albumin, Abcam ab179887; human apolipoprotein A1, Abcam ab189576; human transferrin, Abcam ab187391; hTIMP2, Abcam ab188395.
- Results were normalized to the concentration of total cellular protein in cell lysates, as measured by BCA (Pierce).
- Quantification of the cellular HBV RNA was performed by reverse transcription and Taqman qPCR following RNA extraction. Results were normalized to total RNA as measured on a Nanodrop.
- Selected cellular mRNA were monitored by reverse transcription and SYBR Green qPCR following RNA extraction. Results were normalized to total RNA as measured on a Nanodrop.

NAPs used in this study

Oligonucleotide	Sequence (5'-3') and chemical modifications	Length	PS	2'OMe	5-MeC
REP 2055	ACACACACACACACACACACACACACACACACACAC	40	+	-	-
REP 2150	ACACACACACACACACACACACACAC	30	+	-	-
REP 2151	ACACACACACACACAC	20	+	-	-
REP 2152	ACACACACAC	10	+	-	-
REP 2139	ACACACACACACACACACACACACACACACACACAC	40	+	+	+
REP 2169	ACACACACACACACACACACACACAC	30	+	+	+
REP 2179	ACACACACACACACAC	20	+	+	+
REP 2184	ACACACACAC	10	+	+	+
REP 2031	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	40	+	-	-

Nucleotide positions with 2'O-methyl modified ribose are underlined.
NAPs outlined in green have clinically validated antiviral activity[1-3].

Solving the problem of NAP trafficking *in vivo*



***In vivo* trafficking of PS-ONs (NAPs) is attenuated *in vitro*[6-8].**

Restoration of NAP trafficking with UNC7938[8]

Trafficking of NAPs is altered *in vitro*

Phosphorothioate oligonucleotides (including NAPs) are pharmacologically active *in vivo* (top) but are trapped in endosomes *in vitro* (middle), preventing activity in the cytoplasm and other cellular compartments. UNC7938[8] restores endosomal release (bottom), restoring pharmacological activity.

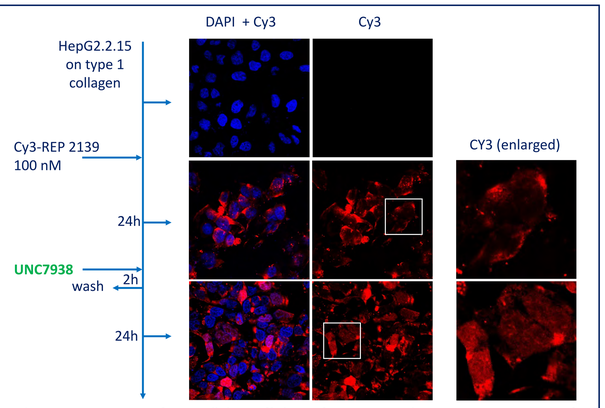


Figure 1. Restoration of intracellular trafficking of REP 2139 with UNC7938. Treatment of HepG2.2.15 cells for 24h with CY3-REP 2139 reveals endosomal entrapment (middle). A 2 hour pulse of UNC7938 restores trafficking of NAPs into the cytoplasm and other intracellular compartments 24 hours later. Detail of intracellular accumulation of CY3-REP 2139 is provided in enlarged pictures (right column).

RESULTS

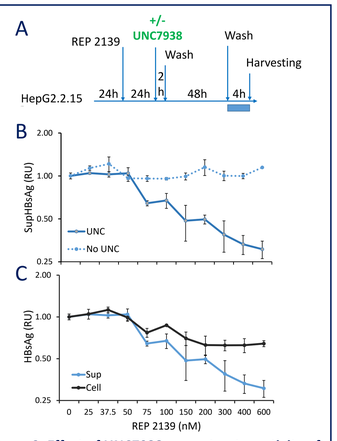


Figure 2. Effect of UNC7938 on post-entry activity of REP 2139. NAPs were allowed to accumulate in endosomes for 24h followed by endosomal release by UNC7938 (A). Supernatant was harvested 4h after an additional wash (A). UNC treatment is mandatory for the inhibition of HBsAg secretion by REP 2139 (B). Inhibition of HBsAg secretion by REP 2139 does not result in intracellular accumulation of HBsAg (C).

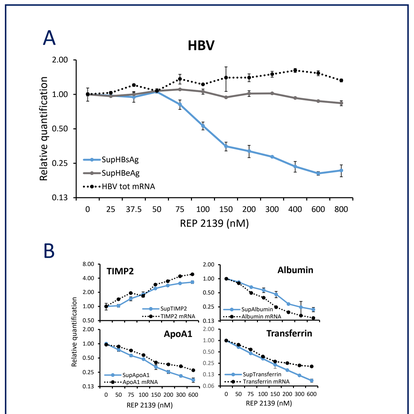


Figure 3. NAPs selectively inhibit secretion of HBsAg. In the same experimental paradigm as in Figure 2A, REP 2139 exerts a specific antiviral effect in HepG2.2.15 cells on the secretion of HBsAg (but not HBeAg), without impairing synthesis and stability of HBV total mRNA (A). In this experimental design, the reduction of mRNA and protein for the liver specific markers albumin, apolipoprotein A1 and transferrin are reduced but the expression of TIMP2 mRNA and the secretion of TIMP2 are unaltered, indicating that the secretory capacity of cells during treatment is unimpaired.

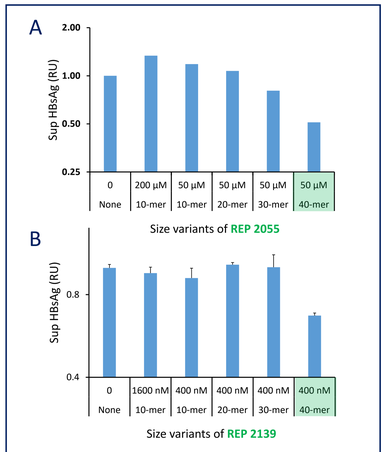
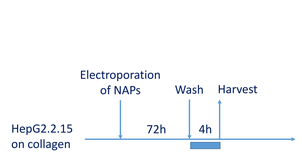


Figure 3. NAP-mediated inhibition of HBsAg secretion is size dependent. Size analogs of the clinically active NAPs REP 2055 (A) and REP 2139 (B) (in green) were tested in the same experimental paradigm as in Figure 2(A). Inhibition of HBsAg secretion was only observed for NAPs 30mer in length or longer. Quadrupling of the concentration of the smallest NAP analog (10mer) had no impact on HBsAg secretion.



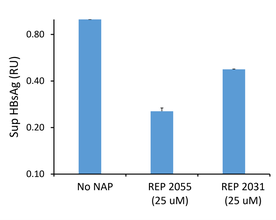


Figure 6: Inhibition of HBsAg release by NAPs occurs in an acidified intracellular compartment. REP 2031 (table left) is a NAP with entry inhibitory activity *in vitro*[9,10] but no post entry activity[10]. This NAP has negligible antiviral effect *in vivo* due to its inactivation at acidic pH[11]. Using an electroporation based method previously described for NAPs[5] (A), the inhibition of HBsAg secretion by REP 2031 was attenuated compared to REP 2055, which is not inactivated at acidic pH.

CONCLUSIONS

- **The use of UNC7938 restores the intracellular trafficking of NAPs normally sequestered in endosomes *in vitro*.**
- **The inhibition of HBsAg secretion only occurs with endosomal release.**
- **NAPs specifically inhibit HBsAg secretion without triggering intracellular HBsAg accumulation.**
- **NAP inhibition of HBsAg secretion occurs via a post-translational mechanism.**
- **Inhibition of HBsAg secretion by NAPs is size dependent, indicating a large target interface is involved.**
- **In HepG2.2.15 cells, the inhibition of HBsAg is at least in part occurring in a acidified intracellular compartment, similar to the effects of NAPs *in vivo*[11].**

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DISCLOSURES

MB and AV are employed by Replicor Inc. AV is a shareholder in Replicor Inc.

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