Assessing the *in vitro* activity of NAPs in HBV: Artifacts with electroporation and transfection

Andrew Vaillant

CSO, Replicor Inc.

Introduction

Nucleic acid polymers (NAPs) are the entire genus of phosphorothioate oligonucleotides which act by a sequence independent mode action. This genus is well described in the literature[1, 2] and is protected by allowed patents worldwide. The activity of NAPs is dependent only on the length of the oligonucleotide and the presence of the phosphorothioate modification and is not altered by the presence of various base and sugar modifications[2]. The conservation of this structure activity relationship (SAR) is consistent with the interaction of NAPs with large exposed hydrophobic protein domains (such as exist in amphipathic alpha helices) which underly the broad-spectrum activity of NAPs against diverse infectious agents.

In the case of viruses with class I fusion glycoproteins[3], the antiviral activity of NAPs includes HIV, diverse members of the Herpesviridae, respiratory syncytial virus, influenza A and B, respiratory syncytial virus parainfluenza-3 virus, Ebola virus, Marburg Virus and lymphocytic choriomeningitis virus and others[1, 2, 4-8]. In each of these viruses, the target of NAPs are structurally conserved amphipathic alpha helices in the viral glycoproteins on these viruses, where the role of these proteins in viral entry or release, dictate the mechanism of action of NAPs in that particular virus (entry inhibition or inhibition or viral assembly / secretion).

For non viral infections, the antiviral activity of NAPs includes prion disease and malarial infection[1, 9], which occur via interaction with proteins encoded by these agents where amphipathic alpha-helical domains important in the conversion of prion protein from its non pathogenic to pathogenic form[9] or which are involved in the entry of the malaria parasite into the host cell[10].

In the case of DHBV and HCV, the antiviral activity of NAPs follows the same SAR as for all these other indications[11, 12], but the target is not found in any of the viral proteins[13] but in host proteins important for viral fusion (HCV) or HBsAg secretion (DHBV).

Although the ability of NAPs to selectively inhibit the release of HBsAg (likely SVP) was evident from both *in vivo* and human studies[13-17], the mechanism of action of NAPs in HBV has been a particularly important issue given the novel and unique ability of NAPs to achieve HBsAg clearance in the liver and in the blood to below the limit of detection *in vivo* and in human studies and to achieve functional cure. In these studies, the SAR of NAPs was again confirmed (Table 2), with equivalent activity observed with the NAP 2055 (having no base or sugar modifications) and REP 2139 (being fully ribose modified and extensively base modified)[15, 17].

Unique to activity of NAPs is the requirement for trafficking through the ERGIC, the location of SVP assembly[18], which has an acidified environment[19]. This was confirmed using the control NAP 2031, which by virtue its polypyrimidine sequence, tetramerizes in the acidic environment[20, 21] within the

ERGIC and becomes inactivated. REP 2031 was shown to be devoid of post-entry activity *in vitro* and to be inactive *in vivo* in DHBV[11, 22] but has activity in a variety of other viral infections *in vitro*[4, 5, 7-9, 12] and *in vivo*[5, 6, 9, 12], highlighting the importance of ERGIC trafficking in the inhibition of HBsAg release and simultaneously providing an important validated control for the presence of ERGIC-mediated SVP assembly *in vitro*.

The entry and trafficking of phosphorothioate oligonucleotides in hepatocytes *in vivo* and in humans involves endosomal uptake, followed by release from the late endosomes with trafficking to the cytoplasm, the ER-golgi intermediate compartment (ERGIC, the site of spherical SVP assembly) and the nucleus (Fig 1). While this pattern of PS-ON uptake and trafficking is preserved in duck liver co-cultures *in vitro*[11], it is absent in primary human hepatocytes and hepatocyte-derived cell lines *in vitro*, where PS-ONs remain trapped in endosomes[23-25]. This technical limitation hampered the ability of earlier mechanistic studies with NAPs to observe *in vitro* the post-entry effects of NAPs occurring *in vivo* and in human studies against HBV infection[26, 27]. The goal of this document is to provide an overview of the various approaches to overcoming this technical challenge and their suitability for examining the activity of NAPs in HBV infection *in vitro*.

Methods

All NAPs were prepared under high efficiency flow reactor conditions. NAP identity and purity were verified by LC-MS. All NAPs were > 85% pure with remaining failure species either N-1 or N+1PO. The *in vitro* activity of NAPs with validated activity *in vivo* and in humans was examined in HepG2.2.15 cells following electroporation, transfection with Oligofectamine™ or Lipofectamine® RNAiMAX or restoration of endosomal release following standard treatment using UNC 7938. Antiviral activity (HBsAg, HBeAg and HBV DNA) were assessed using ELISA and qPCR following preS1-immunoprecipation. Experiments were performed independently in four collaborating labs in Germany, France and Canada.

Results

In vivo and clinical studies have validated that NAPs selectively target the secretion of HBsAg without directly impacting HBV DNA or HBeAg secretion, indicating a selective effect on SVP assembly and or secretion (Table 1, Figure 2). Additionally, the activity of a variety of NAPs has been validated *in vivo* and in humans (Table 2) and is consistent with the sequence independent, base and sugar modification independent and ERGIC-dependent effects of NAPs.

Electroporation

Electroporation involves the permeabilization of the external phospholipid bilayer of the cell using low amperage, high voltage electricity[28]. PS-ONs in the supernatant then enter the cell passively. This technique is technically difficult and must be tuned to individual cell types. While this technique is theoretically compatible with IC_{50} determination, damage to cellular membranes occurs[29, 30] and the normal route of PS-ON uptake and trafficking is bypassed (Figure 3).

Electroporation of REP 2055 and REP 2139 into HepG2.2.15 cells was accompanied by similar effects on HBsAg but in the presence of REP 2139, was also accompanied by direct inhibition of release of HBeAg and HBV DNA, inconsistent with *in vivo* and clinical data (Fig 4). The presence of these

pleiotropic effects with electroporation and the technical requirements for this method made it unsuitable for further experimentation.

Transfection

Transfection involves the use of cationic lipids to form liposomes which then fuse with the external plasma membrane[31] (Fig 5). While several different transfection reagents are available, they all employ highly related cationic lipids to form liposomes[32]. While technically the easiest of approaches, this method suffers from several shortcomings:

1. Cationic lipids alter membrane fluidity[33] and lipid metabolism which are important in the HBV life cycle[34-38].
2. PS-ON uptake is dependent on liposome formation, which is influenced by pH, buffer, lipid: PS-ON ratio and their overall concentration[39].
3. Liposome formation is enhanced with increasing hydrophobic content[40], with PO < PS << methylphosphonate backbone modifications[23].
4. Liposome formation is inhibited by the extent of PS-ON hydration[41, 42], which increases with increasing 2'O-methyl or 2'O-allyl modifications[43, 44] (e.g. REP 2055 is unhydrated while REP 2139 is fully hydrated, see Table 2).

While these limitations appear to make transfection entirely unsuitable for the *in vitro* assessment, of NAPs attempts were made to characterize the effects of NAPs *in vitro* using transfection-mediated PS-ON uptake. Following transfection of NAPs into HepG2.2.15 cells by Oligofectamine, several inconsistencies between *in vitro* effects and validated effects *in vivo* and in human studies were noted (Fig 6). Comparably active NAPs with mild secondary structure (REP 2006 and REP 2107) showed little effect. Additionally, all active 2' O methylated NAPs were inactive. Most importantly, REP 2031 (the inactive control NAP) was the most potently active NAP, indicating that normal ERGIC-dependent SVP assembly and secretion was absent following transfection. The use of RNAiMAX yielded similar results: REP 2006 was inactive, REP 2139 was active but only at very low concentrations and REP 2031 was potently active (Figure 7,8). These results indicated that the introduction of cationic lipids with transfection reagents such as Oligofectamine and RNAiMAX into cells alters the normal mechanism of SVP assembly in the ERGIC. Also, PS-ONs either with mild secondary structure or that are fully hydrated (fully 2' O-allyl modified) were inefficiently incorporated into liposomes. These observations make it clear that transfection cannot be used to reliably compare the antiviral activity of any PS-ON in HBV infection as aspects of SVP morphogenesis are altered by the presence of cationic lipids. Moreover, the activity of NAPs with different chemistries cannot be compared due to their differential inhibition of liposome formation.

Endosomal release with UNC 7938

The discovery of a small molecule which restored normal endosomal release and trafficking of PS-ONs *in vitro*[25] (Fig 9) had been previously validated with a variety of antisense PS-ONs. Results from treatment of HepG2.2.15 cells with NAPs followed by endosomal release via UNC 7938 were recently published[45] and demonstrated the following:

1. Restoration of the endosomal release and trafficking of REP 2139 similar to that observed for PS-ONs *in vivo* and observed *in vitro* in the DHBV infected liver co-cultures[11].
2. Selective effects on HBsAg and not HBeAg reduction (consistent with *in vivo* and clinical data).
3. Comparable effects of REP 2055 (unhydrated) and REP 2139 (fully hydrated).
4. Comparable effects of optimized sequences (REP 2139) and sequences with mild secondary structure (REP 2107), similar to *in vitro* and *in vivo* data (see table 2).
5. Confirmed the size and phosphorothioate dependent SAR observed for NAPs in all other infectious models and demonstrated *in vitro* in DHBV[11].
6. Confirmed the inhibition of the NAP effect with NAP analogs inhibited inside the ERGIC (at acidic pH) similar to earlier *in vitro* and *in vivo* studies in DHBV[11, 22].

Additional data presented at the 2019 HBV International Meeting also confirmed the absence of effect on HBV DNA release, consistent with *in vivo* and clinical data (Fig. 10).

Conclusions

Two validated *in vitro* models currently exist for evaluating the *in vitro* effects of NAPs against HBV:

1. DHBV infection of liver co-cultures with treatment of NAPs

2. UNC 7839-mediated endosomal release of NAPs in hepatocyte-derived cell lines.

Both electroporation and transfection are entirely unsuitable for *in vitro* evaluation as they both introduce pleiotropic effects altering aspects of HBV replication (inhibition of HBeAg and HBV DNA secretion) and SVP assembly. Additionally, transfection efficiency of NAPs is altered by 2'O-allyl modification and secondary structure, both of which inhibit liposome formation and make NAPs harbouring these modifications look artifactually ineffective.

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Table 1. Effects of NAPs validated in vivo and in human studies

Parameter	Effect	References
HBsAg	Direct effect	[13, 14, 16, 17, 22]
HBeAg	No direct effect	Figure 2 and [45]
HBV DNA	No direct effect	[13, 14, 16, 17]

Table 2. Validated effects of various NAP species *in vitro*, *in vivo* and in human studies.

NAP	TYPE	SEQ	Base	Sugar	ACTIVITY	REFS
REP 2006	DNA	$(N)_{40}$	Unmodified	Unmodified	YES	11, 22
REP 2055	DNA	$(AC)_{20}$	Unmodified	Unmodified	YES (similar to REP 2006)	14, 15, 17, 22
REP 2031	DNA	$(C)_{40}$	Unmodified	Unmodified	NO Inactivated by tetramerization at acidic pH inside the ERGIC	11, 22 and 45
REP 2107	RNA	$(N)_{40}$	Unmodified	All 2'OMe ribose	YES (similar to REP 2006)	11
REP 2139	RNA	$(AC)_{20}$	All 5'MeC	All 2'OMe ribose	YES (similar to REP 2055)	13, 15-17
REP 2165	RNA	$(AC)_{20}$	All 5'MeC	All 2'OMe ribose*	YES (similar to REP 2139)	15, 46

Clinically validated NAPs are indicated in green.

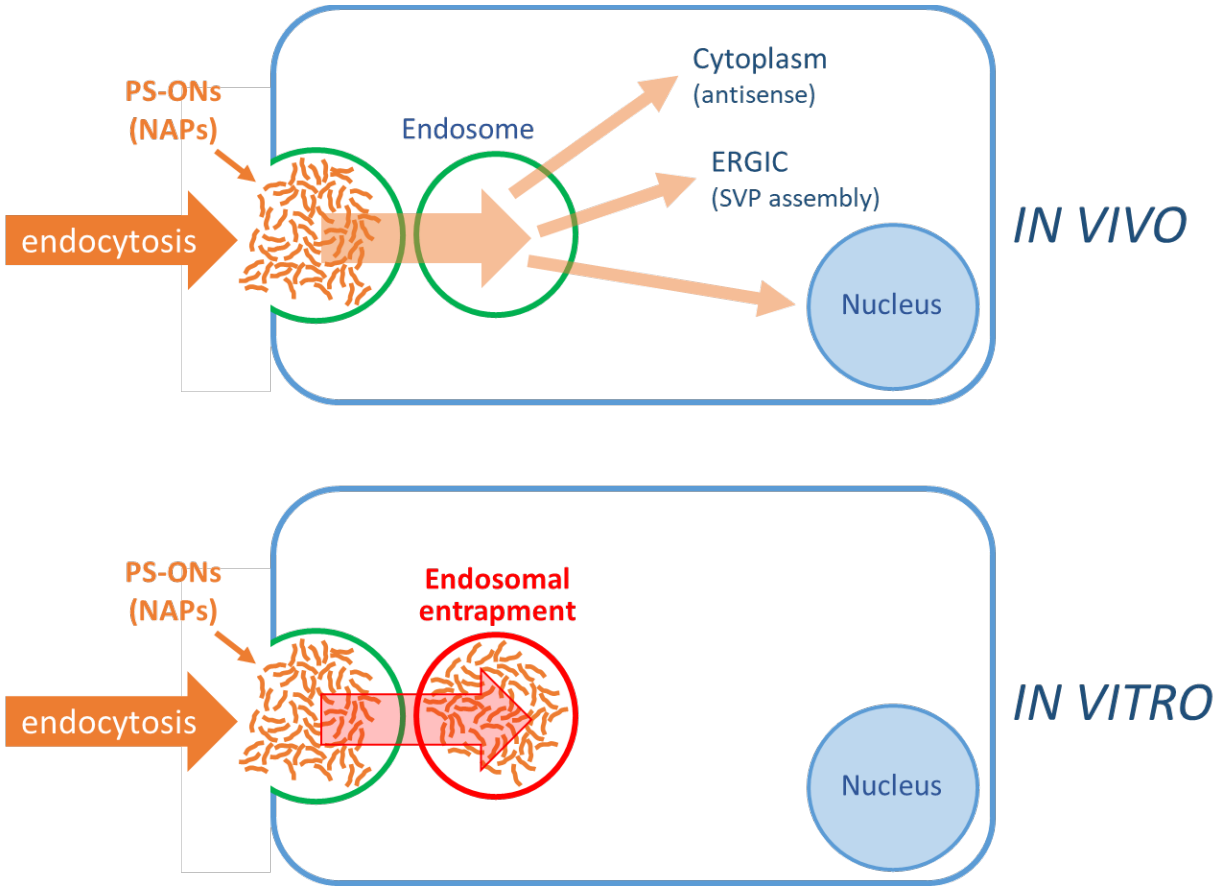


Figure 1. Dysfunctional uptake of PS-ONs in vitro in hepatocyte derived cell lines. Efficient release of PS-ONs from endosomes in vivo does not occur in vitro. Entry of PS-ONs into the cells and trafficking to cytoplasm, ERGIC (site of SVP assembly) and nucleus is highly attenuated.

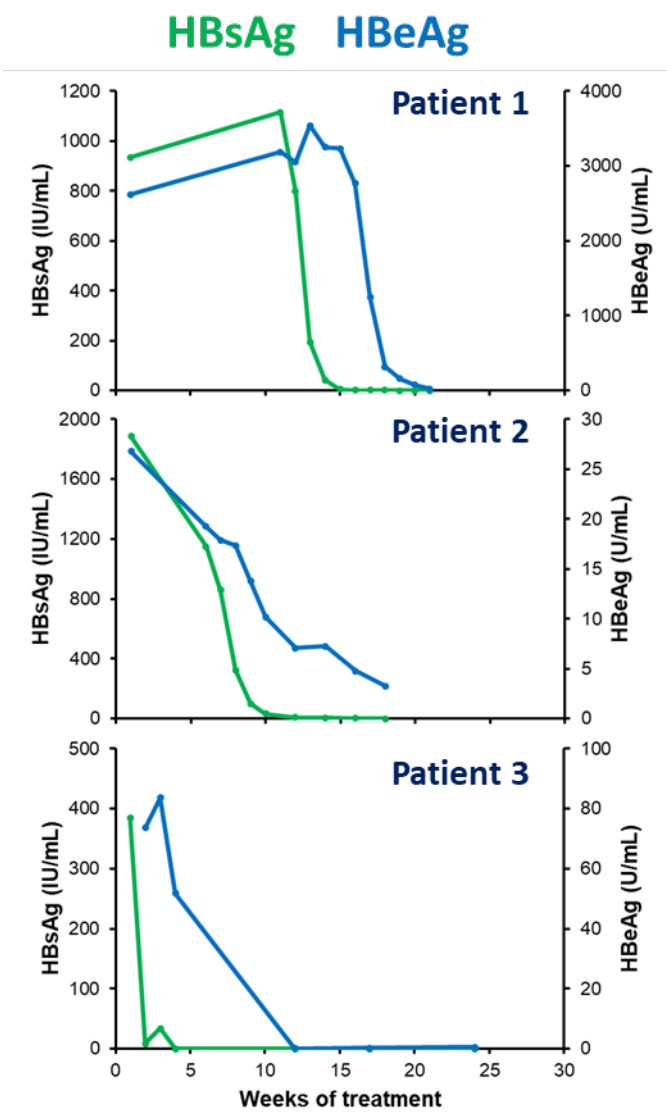
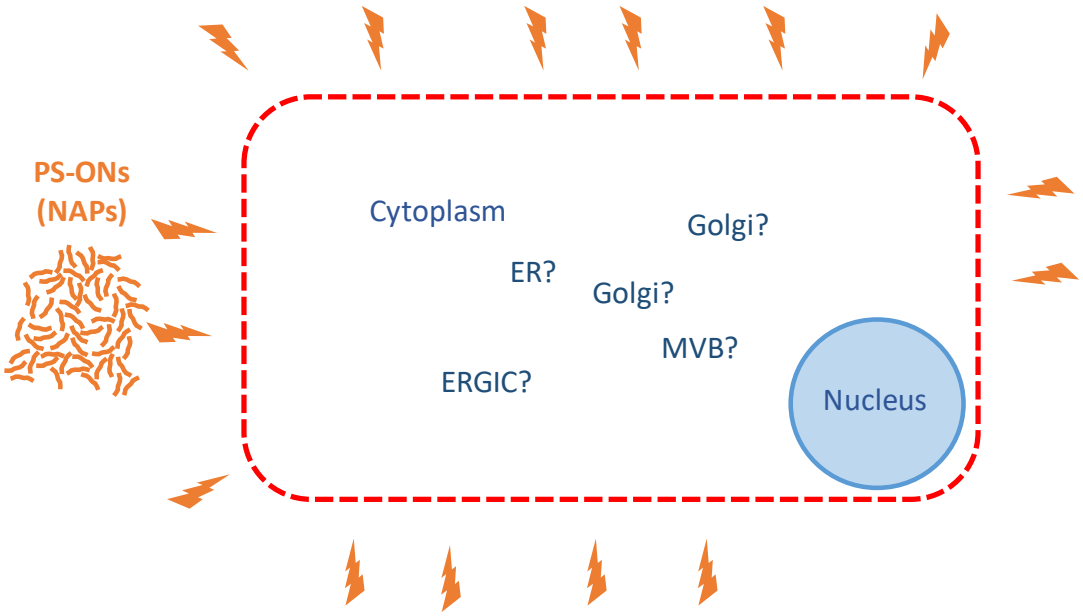


Figure 2. Disconnection of HBeAg from HBsAg response in the REP 101 study. Antigen responses from serial frozen serum samples in participants receiving REP 2055 monotherapy were measured using the Roche Impact® multiplex platform. HBeAg declines are disconnected from HBsAg response by 4-5 weeks. HBeAg decline and seroconversion likely represent the initial stages of immunological reconstitution following reduction in hepatic HBsAg and clearance of serum HBsAg during NAP monotherapy



PROs:

- PS-ON uptake is proportional to extracellular concentration (may be suitable for IC_{50} assessment).
- Uptake is independent of sequence composition or presence of other modifications (better suited for compound optimization).

CONs:

- Technically complex.
- Electroporation induces membrane damage.
- Indiscriminate PS-ON uptake does not reproduce normal PS-ON update *in vivo* ***(endosomal uptake and release is bypassed)***.
- Effects of indiscriminate PS-ON trafficking / organelle association unknown.

Figure 3. Electroporation method and advantages / disadvantages.

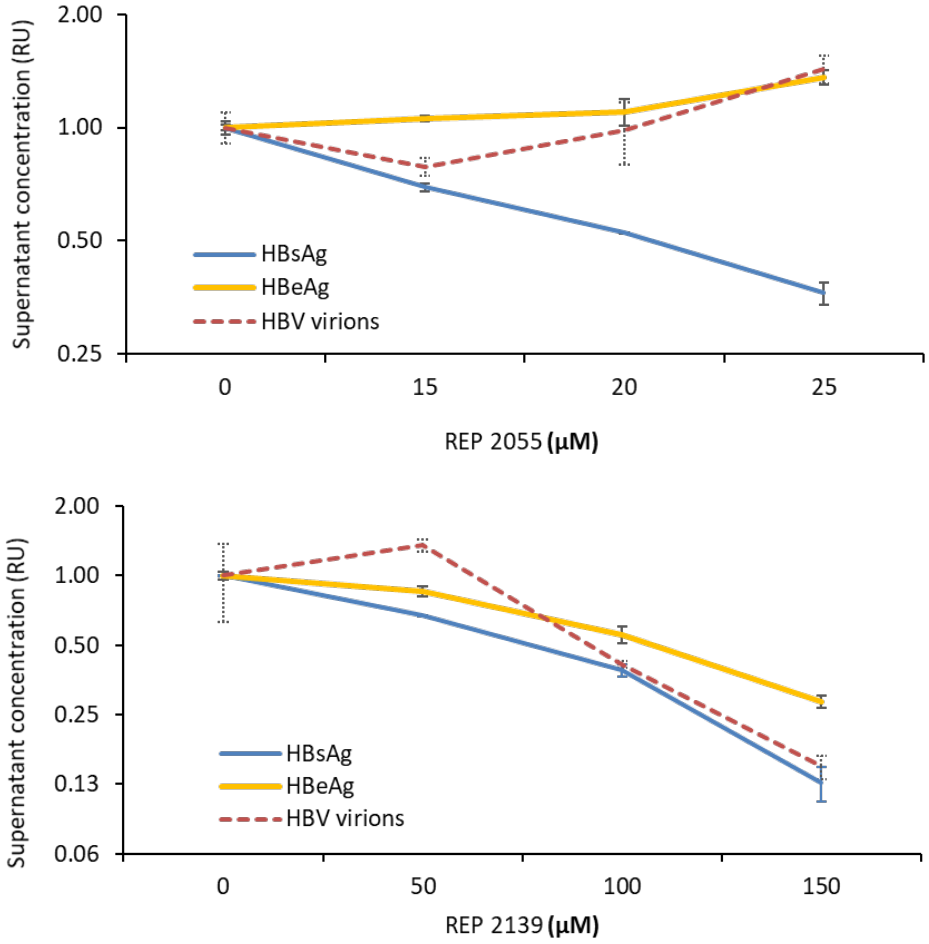
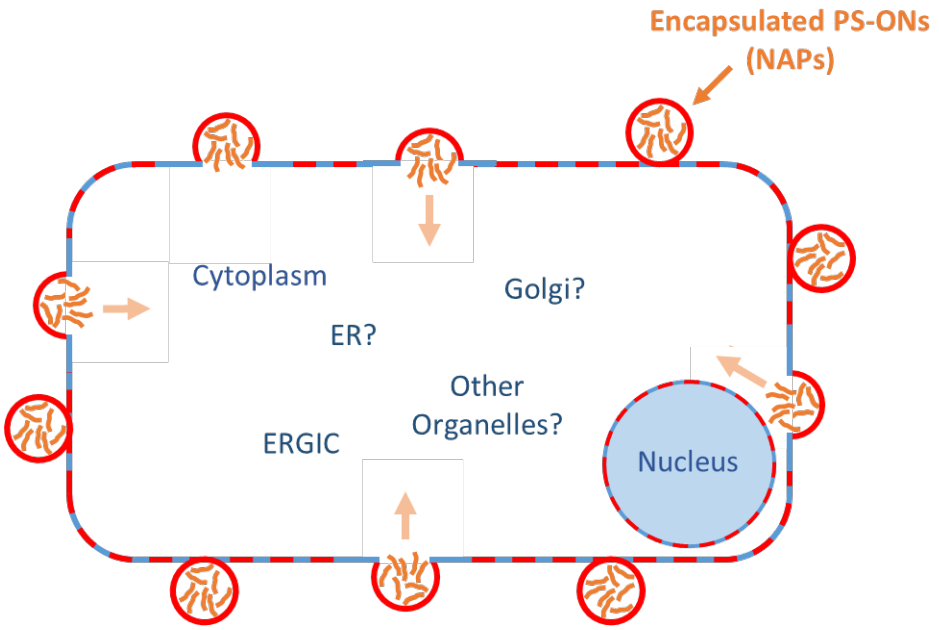


Figure 4. NAP effects in HepG2.2.15 cells following electroporation. HBeAg and HBV DNA response is not disconnected from HBsAg decline in the presence of REP 2139, in consistent with *in vivo* and human clinical data.



PROs:

- Technically simple.

CONs:

- Cationic lipids alter membrane fluidity throughout the cells.
  - Increased cytotoxicity
  - Altered lipid metabolism (potential to impact HBV lifecycle)
- **PS-ON uptake is dependent on formation of lipid/PS-ON micelles**
  - Liposome formation influenced by pH, buffer, ratio of lipid:PS-ON and overall concentration of lipids and PS-ONs
  - Liposome formation inhibited by PS-ON secondary structure and 2'ribose modification (due to hydration)
- **Unsuitable for determination of IC_{50} or optimization of PS-ONs.**
- Indiscriminate PS-ON uptake does not reproduce normal PS-ON uptake *in vivo* ***(endosomal uptake and release is bypassed)***.
- Effects of indiscriminate PS-ON trafficking / organelle association unknown.

Figure 5. Transfection method and its advantages / disadvantages.

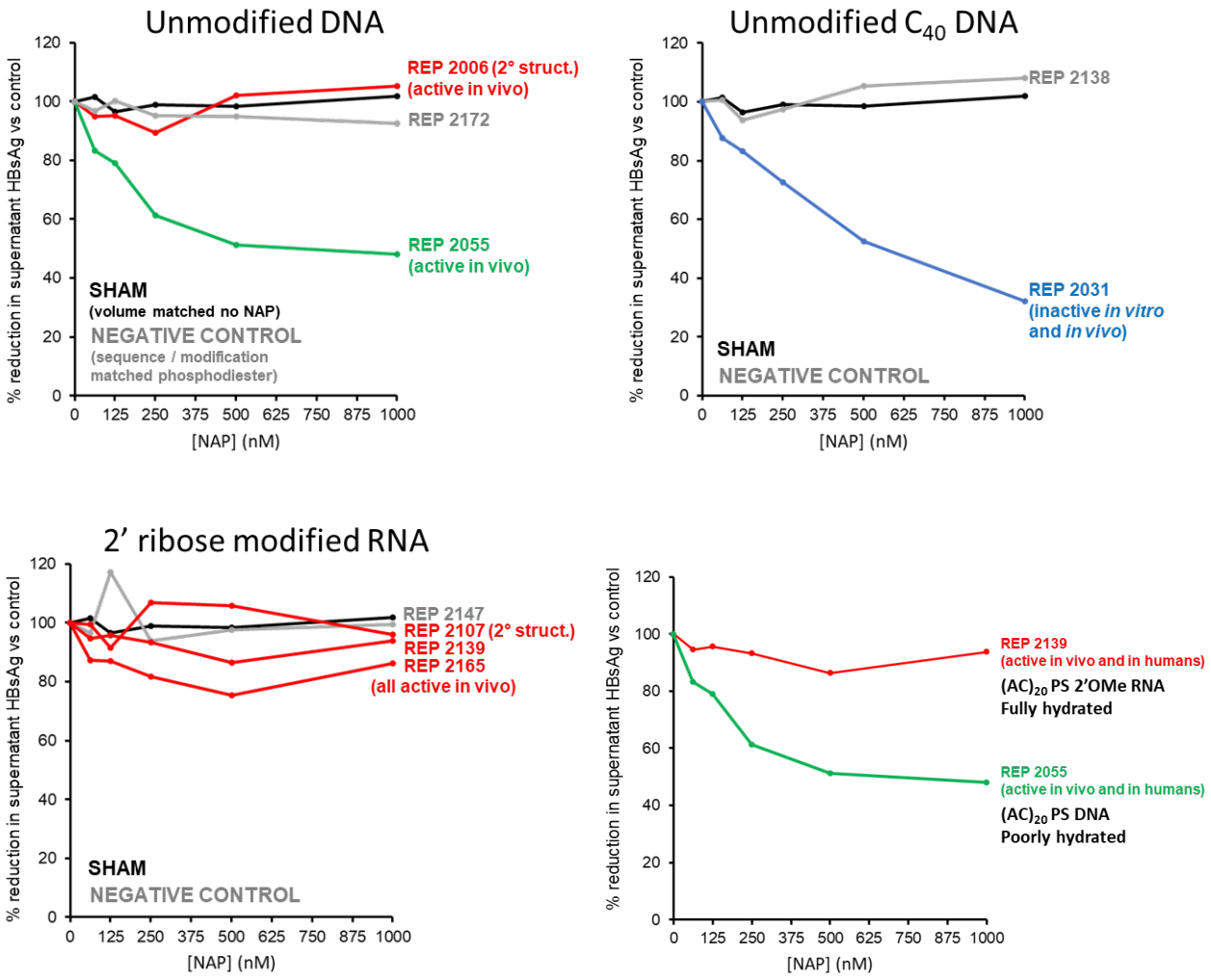


Figure 6. HBsAg response following Oligofectamine-mediated transfection of NAPs in HepG2.2.15 cells. Mild secondary structure (REP 2006, top left and REP 2107, bottom left) blocks liposomal encapsulation. Strong activity of REP 2031 (top right) indicates disruption of the ERGIC and non-physiological inhibition of HBsAg release. Poor liposome encapsulation efficiency in the presence of 2'O-methyl ribose (fully hydrated, REP 2107, REP 2139 and REP 2165, bottom left). Comparison between differential activity of two NAPs (one poorly hydrated and one fully hydrated) with equivalent activity *in vitro*, *in vivo* and in human studies (bottom right) (see Table 2).

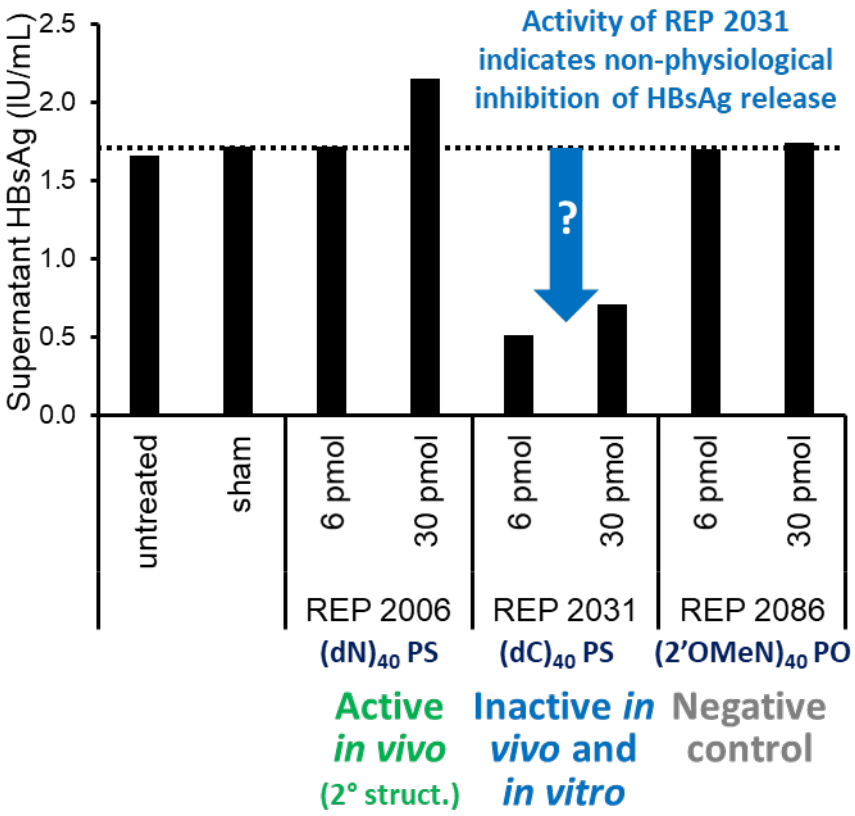


Figure 7. HBsAg response following RNAiMAX-mediated transfection of NAPs in HepG2.2.15 cells. Mild secondary structure (REP 2006) blocks liposomal encapsulation. Strong activity of REP 2031 indicates disruption of the ERGIC and non-physiological inhibition of HBsAg release.

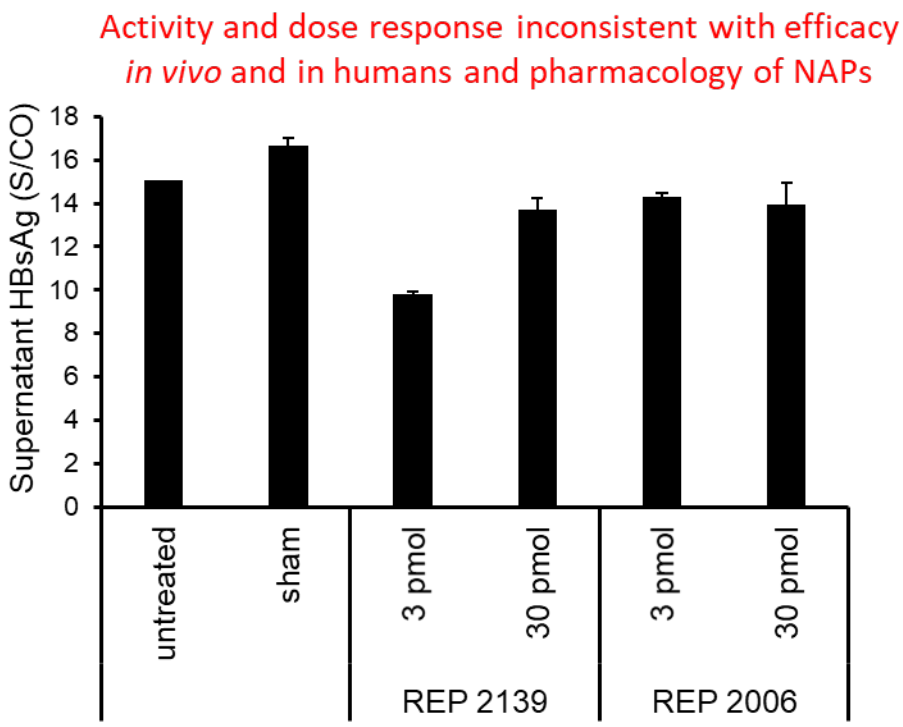
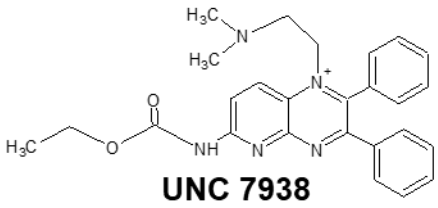
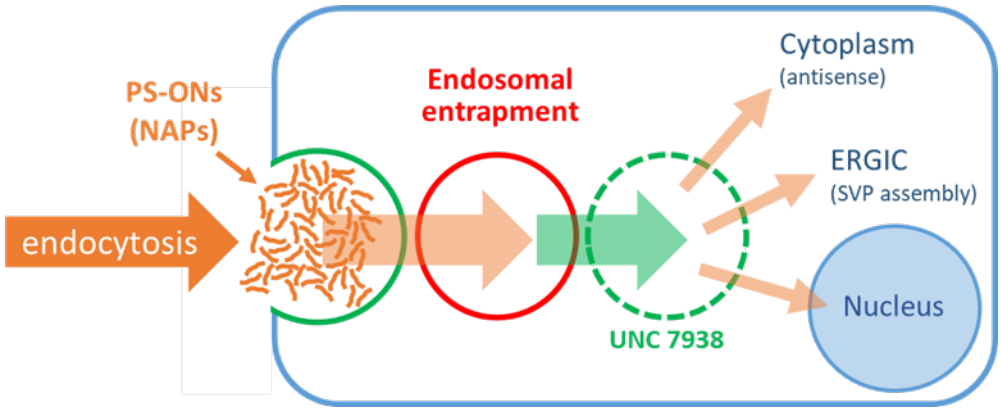


Figure 8. HBsAg response following RNAiMAX-mediated transfection of NAPs in HepG2.2.15 cells. Mild secondary structure (REP 2006) blocks liposomal encapsulation. REP 2139 inhibits HBsAg secretion following transfection with RNAiMAX but only at low concentrations. Inefficient liposome incorporation with increasing concentration of REP 2139 due to its hydrated nature results in reduced delivery into cells resulting in weaker effect (similar to effects with Oligofectamine).





PROs:

- Preserves PS-ON uptake and trafficking observed *in vivo*.
- Uptake is proportional to extracellular PS-ON concentration.
- Uptake is independent of PS-ON sequence and modifications.
- **Well suited for PS-ON IC_{50} determination and optimization.**
- Does not alter HBV life cycle.
- Validated with NAPs and a variety of other antisense PS-ONs.

CONs:

- Requires two step process of NAP treatment (endosomal uptake) followed by short UNC 7938 treatment (endosomal release).

Figure 9. Endosomal release method[25] following treatment and its advantages / disadvantages.

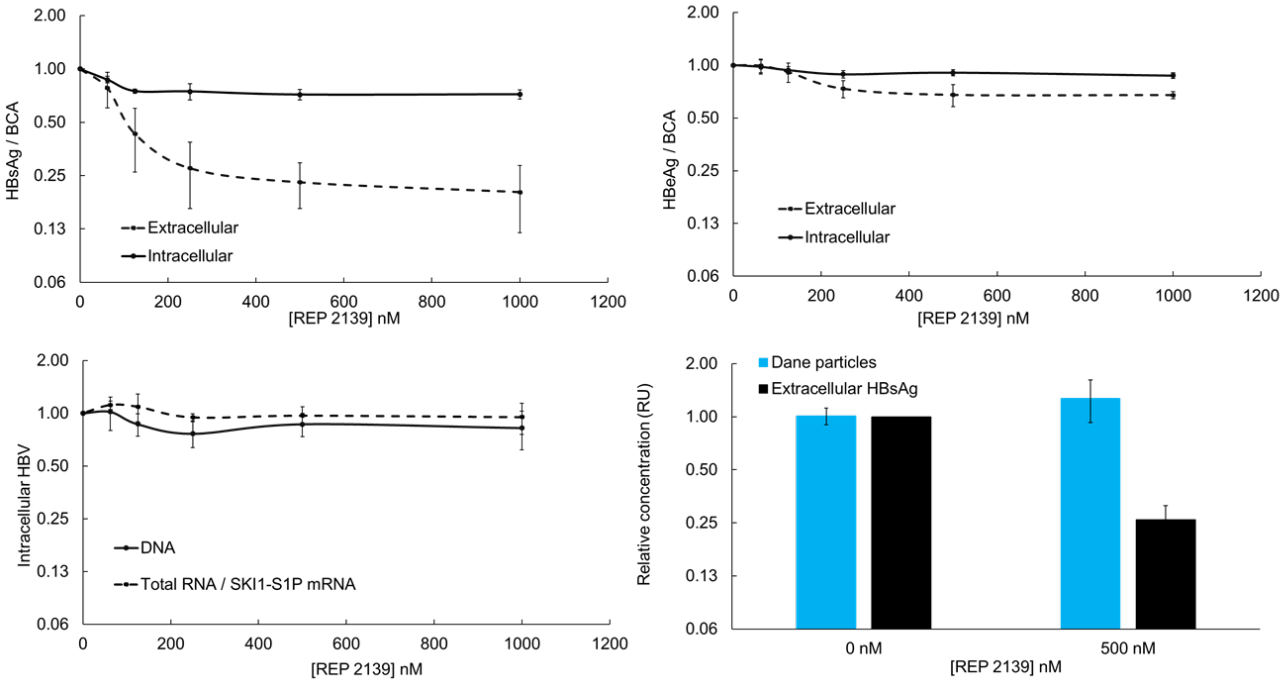


Figure 10. Evaluation of antiviral responses of REP 2139 following endosomal release in HepG2.2.15 cells (presented at 2019 International HBV meeting). No effects on intracellular HBV RNA / DNA, HBeAg secretion or HBV DNA release are observed.