ACCURATELY ASSESSING *IN VITRO* ACTIVITY OF NAPS

A. Vaillant , Replicor Inc., Montreal, Canada

INTRODUCTION

Nucleic acid polymers (NAPs) are phosphorothioate oligonucleotides that interact with exposed hydrophobic surfaces of amphipathic α-helices. This interaction is driven only by phosphorothioation of the phosphodiester linkage and length of the NAP, with optimal activity observed with NAPs ≥ 40 mer in length[1]. While this interaction tolerates a variety of base and sugar modifications, they have no impact on activity of NAPs.

NAPs are active against a wide range of infectious agents including HBV, HDV, HCV, HIV, HSV, CMV, RSV, PIV-3, influenza A and B, Ebola, Marburg, LCMV, prion disease and malaria[1]. The basis for this broad-spectrum activity is the conservation of exposed amphipathic helices important for disease progression which obey a common target interface[1]. In the case of HBV and HCV, this target interface is absent in viral proteins but present in a host protein(s) important for HBV SVP assembly[2] and HCV fusion[3].

In HBV infection, the activity of a diverse range of NAPs has been validated *in vitro* in primary liver co-cultures, *in vivo* and in humans. In these systems, NAPs enter hepatocytes by uptake into endosomes followed by release into the cytoplasm and trafficking to the ERGIC and nucleus. In hepatocyte-derived cell lines, the endosomal release of NAPs does not occur[2,4-8] (Figure 1), which makes examination of their mechanisms of action *in vitro* in HBV and HDV more difficult to investigate.

Using NAPs with antiviral activity against HBV validated in these systems, various methods of restoring trafficking of NAPs in cell lines were explored to determine the appropriate *in vitro* method for examining NAP activity *in vitro*.

MATERIAL & 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 was examined in HepG2.2.15 cells via electroporation, transfection with Oligofectamine™ and Lipofectamine® RNAiMAX and restoration of endosomal release using UNC 7938[2,6]. Antiviral activity (HBsAg, HBeAg and HBV DNA) were assessed using ELISA and qPCR following preS1-immunoprecipitation. Experiments were performed independently in four labs in Germany, France and Canada.

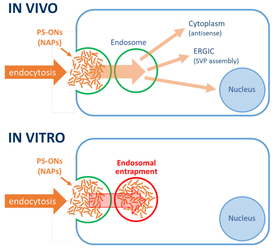


Figure 1. Uptake of phosphorothioate oligonucleotides is defective in vitro. Efficient release of PS-ONs from endosomes *in vivo* (top) does not occur *in vitro (bottom).* Entry of PS-ONs into the cells and trafficking to cytoplasm, ERGIC (site of SVP assembly) and nucleus is highly attenuated.

ANTIVIRAL EFFECTS OF NAPS VALIDATED *IN VIVO* AND IN HUMANS

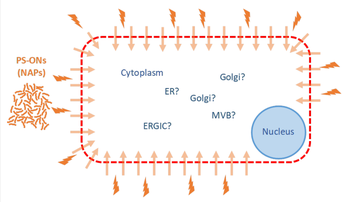
Parameter	Effect	References
HBsAg	Direct effect	9-13
HBeAg	No direct effect	Unpublished data
HBV DNA	No direct effect	10-13

NAPS WITH VALIDATED ACTIVITY

NAP	TYPE	SEQ	Base	Sugar	ACTIVITY	REFS
REP 2006	DNA	$(N)_{40}$	Unmodified	Unmodified	YES **(similar to REP 2006)**	9,14
REP 2055	DNA	$(AC)_{20}$	Unmodified	Unmodified	YES **(similar to REP 2006)**	9,10,12, 15
REP 2031	**DNA**	**$(C)_{40}$**	Unmodified	Unmodified	**NO** Inactivated by tetramerization at acidic pH inside ERGIC	9,14
REP 2107	RNA	$(N)_{40}$	Unmodified	All 2'OMe ribose	YES **(similar to REP 2006)**	14
REP 2139	RNA	$(AC)_{20}$	All 5'MeC	All 2'OMe ribose	YES **(similar to REP 2055)**	11,12,13, 15
REP 2165	RNA	$(AC)_{20}$	All 5'MeC	All 2'OMe ribose*	YES **(similar to REP 2139)**	15

* A_{11}, A_{21} and A_{31} are 2'OH

ELECTROPORATION



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.

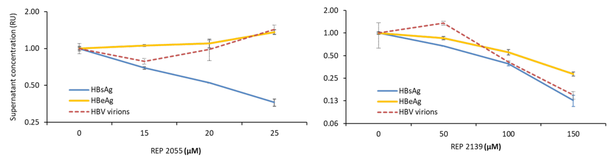
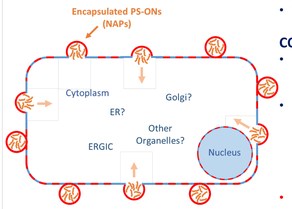


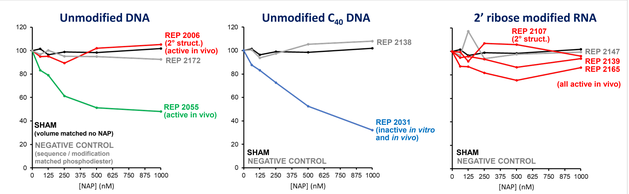
Figure 2. Effects of NAPs in HepG2.2.15 cells via electroporation. REP 2055 and REP 2139 have similar effects *in vivo* and in humans however in vitro, REP 2139 was accompanied by direct effects on HBeAg and HBV DNA secretion, which are absent *in vivo* and in humans.

TRANSFECTION



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[16-18]
  - Liposome formation inhibited by PS-ON secondary structure and 2'ribose modification due to hydration[19-22]
- **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.

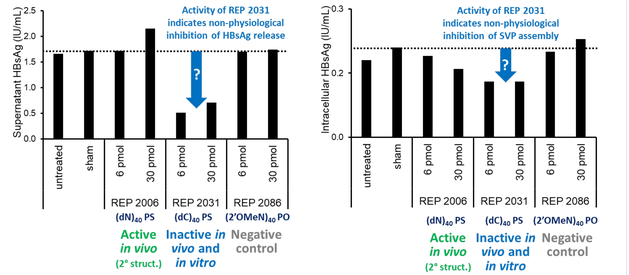


Mild secondary structure inhibits encapsulation

Strong activity of REP 2031 consistent with disruption of the ERGIC and / or non-physiological inhibition of HBsAg release in the presence of Oligofectamine.

Poor encapsulation efficiency when all ribose are 2'O methyl modified

Figure 3. Effects of NAPs in HepG2.2.15 cells via transfection with Oligofectamine. REP 2006, REP 2055 and REP 2139 have similar effects *in vivo* and in humans however only REP 2055 shows activity following transfection with Oligofectamine. REP 2031, which is inactivated *in vitro and in vivo* as it enters the ERGIC, also displays potent activity with Oligofectamine.



Activity and dose response inconsistent with efficacy *in vivo* and pharmacology of NAPs

Figure 4. Effects of NAPs in HepG2.2.15 cells via transfection with RNAiMAX. REP 2006 has no activity with RNAiMAX (similar to Oligofectamine). REP 2031, which is inactivated *in vitro and in vivo* as it enters the ERGIC and has no activity *in vitro* or *in vivo*, also displays potent activity with RNAiMAx.

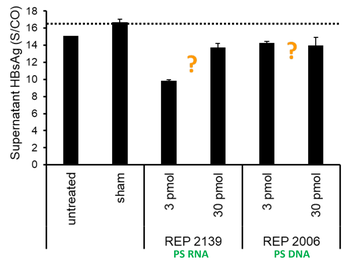
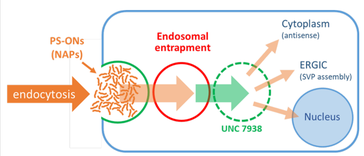


Figure 5. Effects of NAPs in HepG2.2.15 cells via transfection with RNAiMAX. REP 2006 has no activity with RNAiMAX (similar to Oligofectamine). REP 2139 is only active at very low concentrations (consistent with poor activity with Oligofectamine).

Activity inconsistent with efficacy *in vivo* and in humans and pharmacology of NAPs

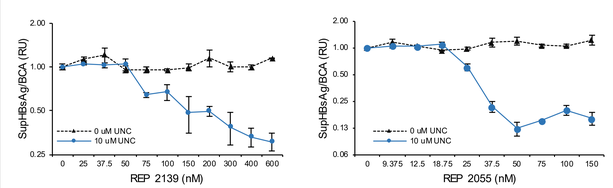
Poor encapsulation efficiency with NAPs having secondary structure (REP 2006) or complete 2'O-allyl ribose modification (REP 2139)

ENDOSOMAL RELEASE[2]



PROs:
- Preserves PS-ON uptake and trafficking that ocurrs *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[2,6].

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



Spectrum of possible NAP physiochemistry is bracketed by REP 2055 and REP 2139

Figure 5. Effects of NAPs in HepG2.2.15 cells following endosomal release. Comparable activity of REP 2055 and REP 2139 following endosomal release is consistent activity *in vivo* and in humans.

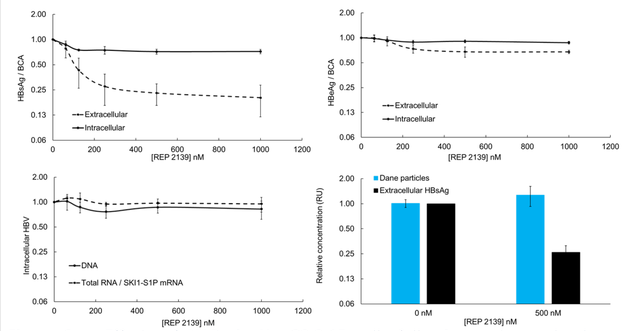


Figure 6. Effects of NAPs in HepG2.2.15 cells following endosomal release. Selective effect of REP 2139 on SVP assembly results in reduction in intracellular HBsAg and inhibition of SVP secretion (HBsAg) into the supernatant. The absence of effects on HBeAg or Dane particles is consistent with NAP effects in vivo and in humans.

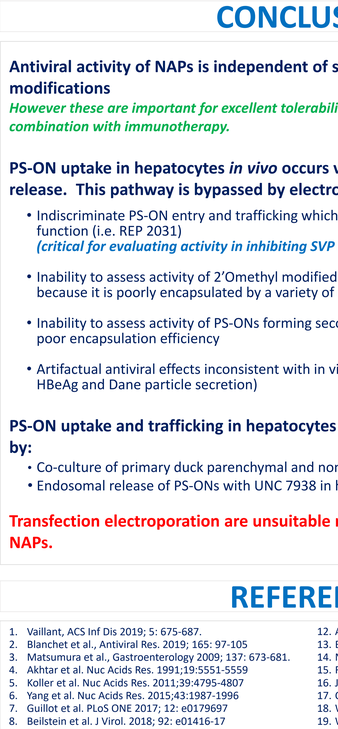


Figure 7. Effects of NAPs in HepG2.2.15 cells following endosomal release. Comparable activity of diverse 2'Ome modified NAPs is consistent with *in vitro* and *in vivo* activity.

CONCLUSIONS

Antiviral activity of NAPs is independent of sequence and base or sugar modifications

However these are important for excellent tolerability in humans, especially when being used in combination with immunotherapy.

PS-ON uptake in hepatocytes *in vivo* occurs via endosomal accumulation and release. This pathway is bypassed by electroporation and transfection leading to:

- Indiscriminate PS-ON entry and trafficking which appears to bypass the ERGIC or alter ERGIC function (i.e. REP 2031) ***(critical for evaluating activity in inhibiting SVP assembly and release)***
- Inability to assess activity of 2'Omethyl modified RNA (REP 2107, REP 2139 and REP 2165) because it is poorly encapsulated by a variety of cationic lipid-based transfection reagents
- Inability to assess activity of PS-ONs forming secondary structure (REP 2006, REP 2107) due to poor encapsulation efficiency
- Artifactual antiviral effects inconsistent with in vivo and clinical efficacy data (inhibition of HBeAg and Dane particle secretion)

PS-ON uptake and trafficking in hepatocytes is only appropriately modeled in vitro by:

- Co-culture of primary duck parenchymal and non-parenchymal liver cells
- Endosomal release of PS-ONs with UNC 7938 in human hepatocyte derived cell lines

Transfection electroporation are unsuitable methods to assess *in vitro* activity of NAPs.

REFERENCES

1. Vaillant, ACS Inf Dis 2019; 5: 675-687.
2. Blanchet et al., Antiviral Res. 2019; 165: 97-105
3. Matsumura et al., Gastroenterology 2009; 137: 673-681.
4. Akhtar et al. Nuc Acids Res. 1991;19:5551-5559
5. Koller et al. Nuc Acids Res. 2011;39:4795-4807
6. Yang et al. Nuc Acids Res. 2015;43:1987-1996
7. Guillot et al. PLoS ONE 2017; 12: e0179697
8. Beilstein et al. J Virol. 2018; 92: e01416-17
9. Noordeen et al., AAC 2013; 57: 5299-5306.
10. Noordeen et al., PLoS ONE 2015; 10: e0140909.
11. Quinet et al., Hepatology 2018; 67: 2127-2140.
12. Al-Mahtab et al., PLoS One 2016; 11: e0156667.
13. Bazinet et al., Lancet Gastro. Hepatol. 2017; 2: 877-889.
14. Noordeen et al., AAC 2013; 57: 5291-5298.
15. Roehl et al., Mol. Ther. Nuc. Acids 2017; 8: 1-12.
16. Jääskeläinen et al., Bioch Biophys Acta 1994; 1195: 115-123
17. Campbell et al., Biochim Biophys Acta 2001; 1512: 27-39
18. Wang et al., Molecules 2018; 23: 2564
19. Wan and Seth 2016 J Med Chem. 2016; 59: 9645-9667
20. Egli et al., Ann Rev Biohys Bio Struct. 2007; 36: 281-305
21. Amarzguioui et al., NAR 2003; 31: 589-595
22. Meidan et al., Biochem Biophys Acta 2000; 1464: 251-261

Contact information: availlant@replicor.com