Molecular Therapy
Nucleic Acids
Editorial

Editorial: *In vitro* mechanistic evaluation of nucleic acid polymers: A cautionary tale

INTRODUCTION

Nucleic acid polymers (NAPs) are broad-spectrum antiviral compounds active against diverse enveloped viruses and other infectious agents.[1] The activity of all NAPs is sequence independent and driven by a length-dependent (optimal with 40-mers) and phosphorothioation (hydrophobic)-dependent interaction with the exposed hydrophobic surfaces of amphipathic alpha helices.[1,2]

Recent focus on NAPs for the treatment of chronic hepatitis B and hepatitis D infection has been driven by several phase II trials demonstrating the ability of several different clinical NAP compounds (REP 2055, REP 2139, and REP 2165) to achieve rapid hepatitis B surface antigen (HBsAg) loss and seroconversion and high rates of therapeutic transaminase flares, leading to high rates of functional cure of hepatitis B virus (HBV) and hepatitis D virus (HDV).[2–4]

Recently published *in vitro* data[5] suggest that NAPs modified with locked nucleic acids (LNAs) have substantially improved potency compared with previously published NAPs and suggested mechanisms of action and potential host targets very different from those previously published for NAPs. The even more recent announcement of the abandonment of the LNA-modified NAP ALG-10133 and the entire LNA-modified NAP platform because of a lack of antiviral activity in humans[6] raises serious questions regarding these recently published *in vitro* data. The disconnect between the efficacy of LNA-modified NAPs reported *in vitro* and in the clinic are the result of several limitations/artifacts in the experimental approaches used that are important to highlight.

UNDERSTANDING NAP STRUCTURE

REP 2055 is a 40-mer phosphorothioate oligodeoxyribonucleotide with the sequence $(AC)_{20}$. REP 2139 is an RNA derivative of REP 2055 in which each ribose is 2′O methylated and each cytosine base is 5-methylated (Figure 1). The poly AC sequence does affect the antiviral activity of NAPs but is designed to eliminate intra-/intermolecular interactions, to minimize secondary structure and off-target interactions, and to minimize recognition by pattern recognition receptors to render NAPs immunologically inert while maintaining the flexible B-form DNA structure required for optimal NAP activity.[1] Both REP 2055 and REP 2139 have been extensively evaluated against HBV infection *in vitro*, *in vivo*, and in human studies, in which they have been shown to have comparable activity[2,7] demonstrating that 2′ribose modification in the NAP polymer has no impact on the antiviral activity of NAPs. ALG-10000 is a previously described,[8] LNA-modified derivative of REP 2055 and REP 2139 (Figure 1). The implications of LNA modification on NAP activity are discussed below.

THE TARGET(S) OF NAPs RESIDE WITHIN THE LUMEN OF SECRETORY VESICLES

REP 2031 is a 40-mer poly C NAP (Figure 1) that has broad-spectrum antiviral activity *in vitro* and *in vivo* against all enveloped viruses and other infectious agents except HBV.[2] Like all polypyridines, REP 2031 forms intermolecular tetramers at pH < 6.8 (Figure 2),[9] a transformation that does not occur with purine/pyrimidine altimers such as REP 2055, REP 2139, and REP 2165[10] or degenerate NAPs such as REP 2006 and REP 2107 (Figures 1 and 2). The tetramerization of REP 2031 prevents target engagement at acidic pH and leads to the inactivity of REP 2031 against HBV infection *in vitro*[7,11] and *in vivo*.[12] These observations demonstrate that NAPs that are active against HBV infection act inside the acidified lumen of secretory vesicles. Therefore, host targets for NAPs need to satisfy two criteria: (1) be present and active inside secretory vesicles and (2) have binding interactions that are consistent with length and phosphorothioate-dependent antiviral activity conserved in all NAPs.

Although the ligand-mediated approach used for identifying NAP targets described by Kao et al. is in principle correct, the methodology they used suffers from several critical flaws. The first is the use of ALG-10000 as the single and only NAP bait in the ligand assay. Several kinds of off-target protein interactions can occur with NAPs: off-target hydrophilic versus antiviral hydrophobic and small-target versus large-target (antiviral) interface-driven interactions.[2] These off-target interactions will be stronger with ALG-10000 because of the absence of 2′O methylation, a modification that increases the hydration of oligonucleotides and inhibits off-target interactions[13,14] and does not affect the antiviral activity of NAPs.[1,2] Additionally, LNA-modified oligonucleotides adopt a rigid A-form DNA structure,[15–17] compared with the flexible B-form DNA structure of non-LNA-modified NAPs, which will also affect binding affinity and selectivity. Although the protein targets identified by Kao et al. bind to ALG-10000 *ex vitro*, and RNAi-mediated reduction of these targets results in inhibition of HBsAg secretion, these cannot be identified as physiological NAP targets involved in HBV activity without controlling for binding driven by off-target hydrophilic interactions (i.e., by using an ALG-10000 analog without the phosphorothioate modification) or for off-target smaller interface interactions (by using an ALG-10000 analog with suboptimal size, such as a 20-mer) or for pH-dependent interactions by performing the binding at neutral and acidic pH or for the effect of the LNA structural alterations by using REP 2055. Importantly, none of the identified targets reported by Kao et al. exhibits the hallmark binding site for NAPs,[1,2] and

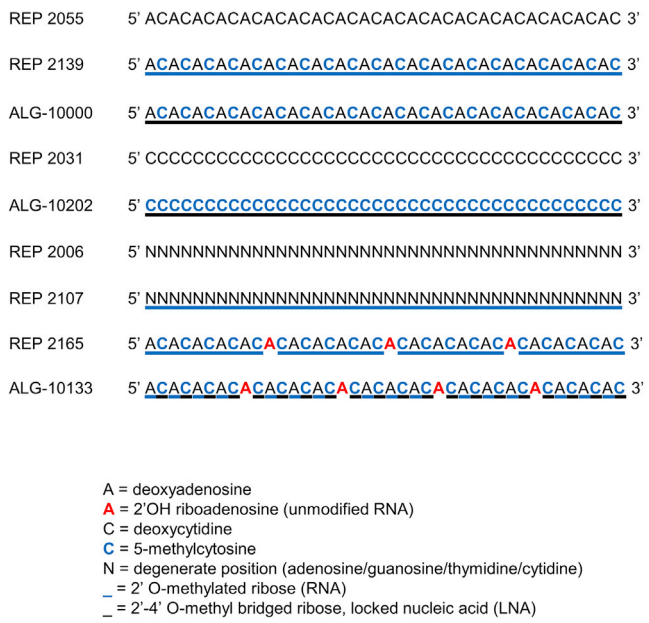

Figure 1. Sequence and chemical modifications of various NAPs. All NAPs contain phosphorothioate at each linkage

with the exception of GRP78, none is found within the lumen of acidified vesicles. Moreover, if RPLP1 and RPLP2 (60S ribosomal subunits), SRSF1 (pre-mRNA splicing factor), and HNRNPA2B1 (nuclear ribonucleoprotein) as identified by Kao et al. were indeed physiological targets for NAPs, the well-conserved nature of these housekeeping genes in all mammals would result in the antiviral activity of NAPs in rodent models of HBV infection being easily observed, which is not the case, even with prolonged NAP therapy with REP 2055 or REP 2139.[18] Additionally, prolonged interference with the housekeeping functions of these proteins with NAP therapy would be expected to result in significant toxicity in animals and humans, which has never been observed with any clinically evaluated NAP.[3,19–21]

An ongoing study to identify the physiological host target(s) of NAP interaction involved in the antiviral activity against HBV is nearing completion with REP 2139,[22] which includes controlled ligand binding done at neutral and acidic pH and comparing binding interactions observed with REP 2139 with those observed with no-PS (REP 2147) and 20-mer analogs (REP 2179) of REP 2139 and also the acid-inactivated REP 2031. Preliminary results have clearly excluded all of the targets identified by Kao et al. and identified the HSP40 chaperone DnaNAB12 as the physiological target for all NAPs acting in HBV infection.

INTRACELLULAR NAP TRAFFICKING *IN VIVO* DOES NOT OCCUR *IN VITRO*

Pharmacological effects of phosphorothioate oligonucleotides such as antisense compounds are easily achieved in the liver *in vivo* and in humans because of the natural uptake and cytoplasmic transit of these compounds in hepatocytes (Figure 3A). However, standard treatment of hepatocyte-derived cell lines with antisense compounds results in endosomal entrapment of oligonucleotides (Figure 3A), preventing cytoplasmic trafficking and pharmacological effects of this chemical class of compounds.[7,23,24] With the exception of primary co-cultures of duck liver parenchymal and non-parenchymal cells,[11] NAPs also obey this limitation, with post-entry pharmacological effects against HBV infection easily obtained *in vivo* and in humans[2] but not observed in HepG2.2.15, HepaRG, Huh-106, and primary human hepatocytes with standard treatment.[25,26]

NAP TRAFFICKING AND EFFICACY *IN VIVO* IS NOT RECAPITULATED *IN VITRO* WITH TRANSFECTION

In order to overcome this limitation with oligonucleotide treatment *in vitro*, it is tempting to use transfection. As described by Kao et al., transfection leads to the enhanced delivery of oligonucleotides to the cytoplasm; however, although this effect is useful for studying the activity of oligonucleotides that act in the cytoplasm (i.e., antisense or RNAi), it is not appropriate for the evaluation of NAPs, as the action for these compounds occurs in the secretory pathway (Figures 3A and 3B). Additionally and equally important is the unsuitability of transfection to evaluate the relative *in vitro* activity of NAPs with significant differences in hydration or secondary structure. The disconnect between the *in vitro* activity of a variety of NAPs with activity validated *in vivo* and in humans observed with transfection and in the absence of transfection/*in vivo* has been previously observed in several independent laboratories in collaborations that began in 2007 (A.Vaillant, unpublished data) and are summarized in Figure 2. Liposome formation is critical to ensure efficient oligonucleotide delivery with lipid-based transfection and is inhibited by oligonucleotide hydration[27,28] (Figure 3B). Thus, poorly hydrated NAPs such as REP 2055 and ALG-10000 perform well via transfection, whereas NAPs well hydrated with complete 2′O methylation such as REP 2139 and REP 2107 perform poorly (see Figure 2), even though they have comparable antiviral activity to REP 2055 when evaluated *in vitro* with non-transfection-dependent systems, *in vivo*, and in humans.[7,19,29,30] Thus, the differences in apparent *in vitro* activity against HBV between LNA-modified NAPs (e.g., ALG-10000) and non-LNA-modified NAPs (i.e., REP 2139) are a function of reduced transfection efficiency with REP 2139, not a lower specific antiviral activity.

Additionally, degenerate NAPs such as REP 2006 and REP 2107 adopt secondary structure at neutral pH (Figure 4A), but protonation of these NAPs at acidic pH abolishes this secondary structure (Figures 2 and 4B), which renders these NAPs active *in vitro* and *in vivo*. Although these NAPs have similar antiviral activity *in vitro* in the absence of transfection and *in vivo* compared with REP 2055 and REP 2139,[7,11,12] they have no antiviral effect *in vitro* with lipid-based transfection (Figure 2). This also demonstrates that oligonucleotides with significant secondary structure are also poorly transfected (Figure 3B).

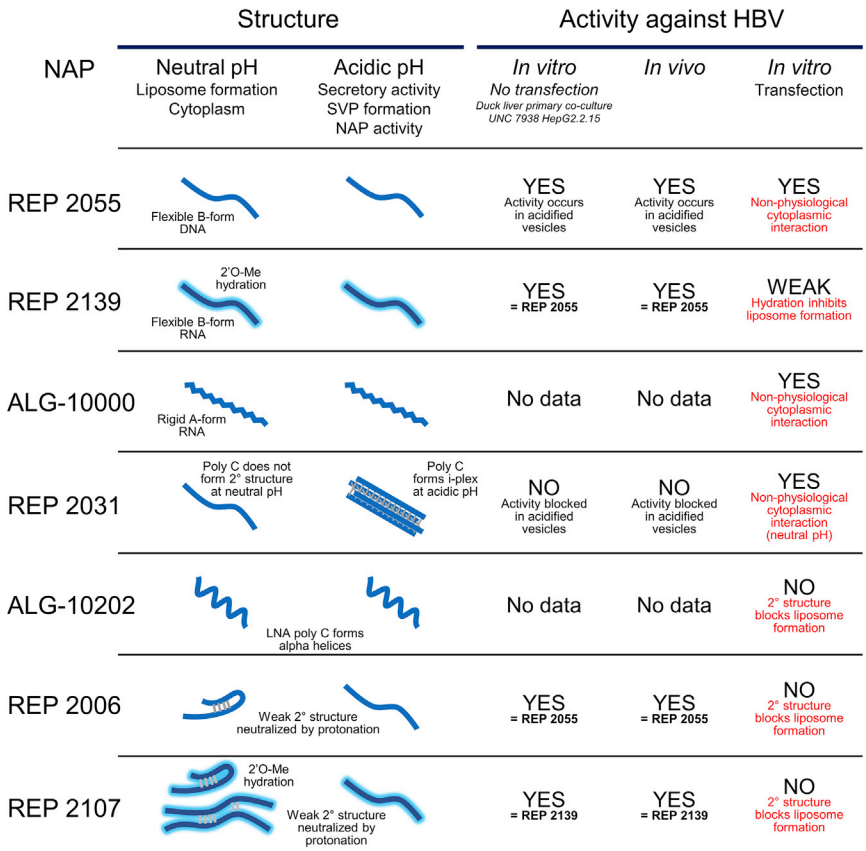

Figure 2. Effects of pH on NAP structure and antiviral activity against HBV *in vitro* with and without transfection and *in vivo*

The use of the *in vivo*-validated positive NAP controls REP 2006, REP 2107, REP 2055, and REP 2139 and the validated negative controls REP 2031 and phosphodiester versions of REP 2107 (REP 2086) and REP 2139 (REP 2147) are critical to ensure that any *in vitro* system is appropriate for evaluating NAP activity.[7] These important controls are missing from transfection studies described in Kao et al. and would have revealed these defects in transfection-based evaluation of NAP activity.

A key observation illustrating altered NAP trafficking with transfection is the observation of apparent antiviral activity of REP 2031 against HBV infection when transfected *in vitro* (Figure 2). This observation indicates non-physiological cytoplasmic target interactions occurring under these experimental conditions. When used in *in vitro* models in which normal oligonucleotide trafficking occurs in the absence of transfection[11] or when endosomal entrapment is removed,[7] REP 2031 activity is absent or significantly reduced compared with REP 2006, REP 2055, or REP 2139. In our ongoing studies,[22] we have identified casein kinase 1 delta as a non-physiological target for NAPs in the cytoplasm.[22] Engagement of this target appears to universally inhibit retrograde vesical transport, yielding inhibition of both HBsAg and hepatitis B e-antigen (HBeAg) secretion.[22] It is important to note that NAPs do not affect HBeAg secretion *in vitro*[7] or in humans (A. Vaillant, personal communication). Although not reported by Kao et al. in their publication, the authors also performed transfection experiments with an LNA-modified version of REP 2031 (ALG-10202; Figure 1),[31] in which they observed no activity against HBV *in vitro*. This is easily explained by the spontaneous formation of highly stable alpha-helical structures of LNA-modified polycytidine oligonucleotides at neutral pH[17,32] (Figure 2), which most likely block liposome formation.

RESTORING CORRECT OLIGONUCLEOTIDE TRAFFICKING *IN VITRO*

UNC 7938 is a small molecule that restores endosomal release of oligonucleotides *in vitro*.[23] Brief exposure of HepG2.2.15 cells to UNC 7938 following standard NAP treatment led to evacuation of NAPs from endosomes, with subsequent cytoplasmic trafficking.[7] Using this approach, post-entry activity of NAPs could be observed that recapitulated the antiviral effects of NAPs previously observed *in vivo* and in humans.[7,33] These effects included (1) size and phosphorothioate-dependent antiviral activity; (2) comparable antiviral activity of non-hydrated (REP 2055) and hydrated (REP 2139) NAPs, recapitulating the sugar modification-independent activity observed *in vivo* and in humans; and (3) comparable activity of

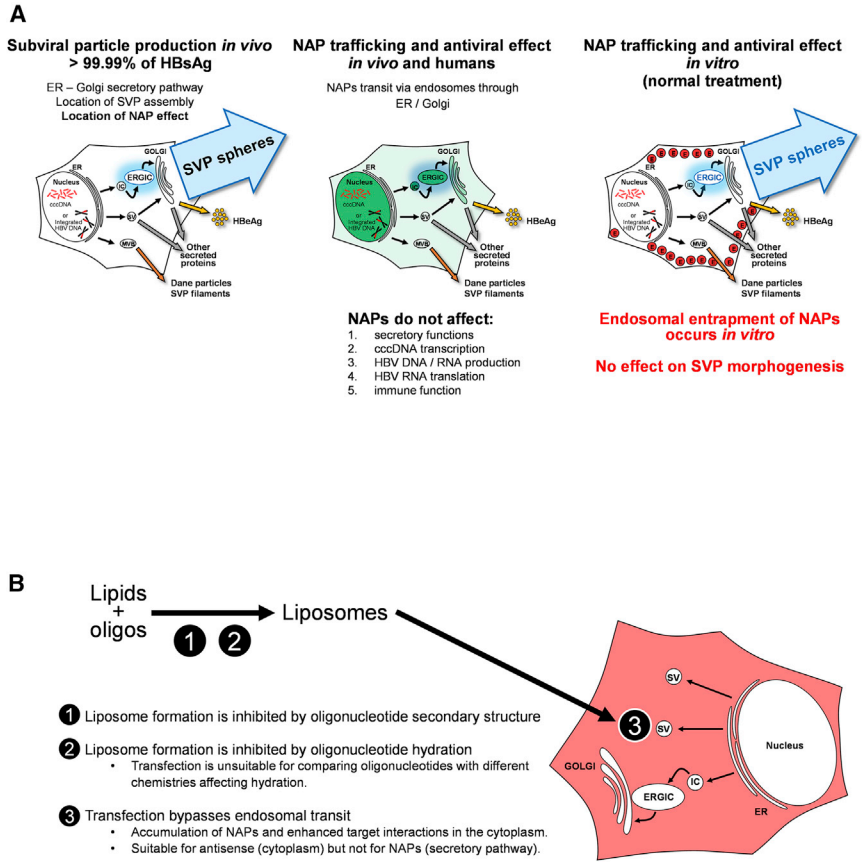

Figure 3. NAP trafficking and effects on antiviral activity

(A) Comparison of oligonucleotide trafficking occurring *in vivo* and in humans with that occurring *in vitro*. (B) Artifacts associated with transfection-based approaches with NAPs.

NAPs with a degenerate sequence (REP 2107). Importantly, REP 2031 demonstrated reduced activity in this model system,[7] consistent with the absence of antiviral activity of this NAP *in vivo*, demonstrating that trafficking of NAPs through the acidified secretory pathway was occurring. Additional validation of this system included the observation of selective effects on the assembly and secretion or subviral particles without affecting the secretion of HBeAg or virions,[33] consistent with the antiviral effects of NAPs observed *in vivo* and in humans.

INSIGHTS INTO THE FAILURE OF ALG-10133

REP 2165 is an analog of REP 2139 that is designed to be more susceptible to endonuclease attack at three unmodified riboadenosines[19] (Figure 1). ALG-10133 is an LNA-modified analog of REP 2165 in which every other 2′O methyl ribose 5-methylcytidine is replaced by LNA 5-methylcytidine. Additionally, ALG-10133 contains four unmodified riboadenosine breaks compared with the three present in REP 2165 (Figure 1). Although REP 2165 is comparable in activity to REP 2055 and REP 2139,[1,3,19] ALG-10133 had no significant effect on HBsAg reduction in human HBV infection at doses at which REP 2055 and REP 2139 yield 4–7 log reductions from baseline in HBsAg.[6]

The reasons the failure of ALG-10133 are threefold: first, the structural alterations that occur in LNA-modified oligonucleotides have the effect of reducing their hydration.[34,35] As such, in ALG-10133, the doping of the uniform 2′O-methylation present in REP 2165 (Figure 1) substantially reduced the hydration of this NAP and allowed it to be efficiently transfected, yielding apparent (but artifactually) increased antiviral activity by transfection relative to REP 2139,[31] similar to ALG-10000.[5] The second reason for the failure of ALG-10133 is the structural rigidity LNA imparts to the NAP. The unstructured and flexible nature of NAPs is essential for their optimal antiviral effect[1,2,7] because this flexibility allows optimal interaction with uncomplexed amphipathic alpha helices, for example in the J-domain of DNAJB12 (Figure 5). As such, LNA-modified NAPs such as ALG-10000 and ALG-10133, although very structurally similar to REP 2055 and REP 2139, will have drastically reduced target engagement because of their rigidity[15–17] (Figure 5). Finally, early preclinical data showed that the hepatic

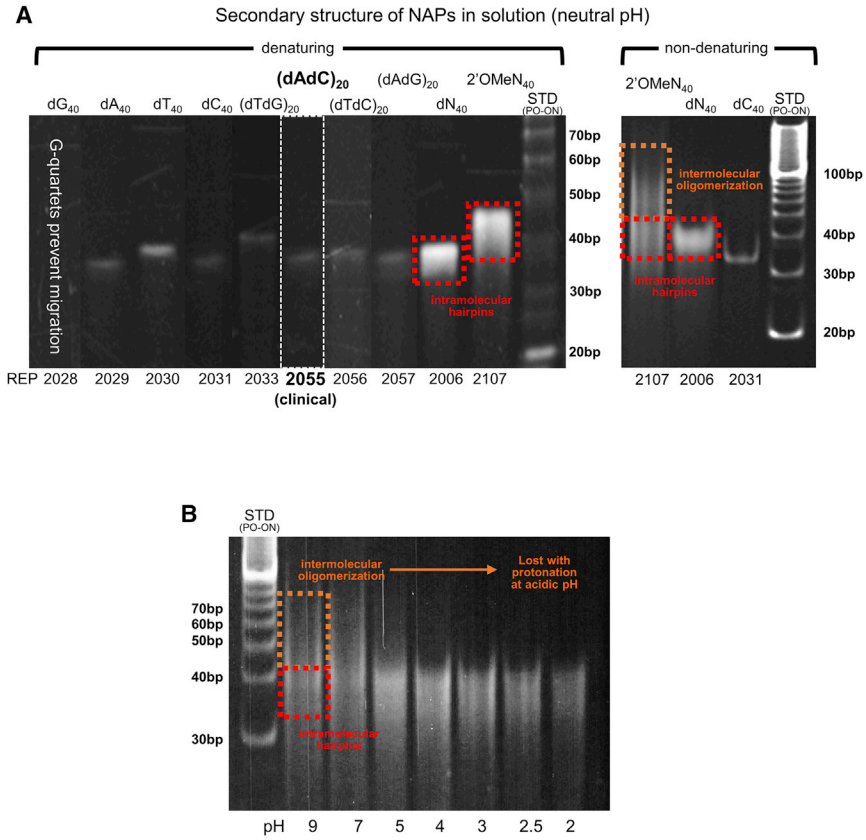

Figure 4. pH can influence the secondary structure of NAPs

(A) Secondary structure of NAPs with various sequence compositions under non-denaturing and denaturing gel electrophoresis. (B) Effect of pH on the secondary structure of REP 2107. All NAPs contain phosphorothioate at each linkage. A, adenosine; G, guanosine; T, thymidine; C, cytidine; d, deoxyribose (DNA); 2′OMe, 2′O-methyl-modified ribose (RNA).

accumulation of ALG-10133 was approximately 5-fold lower than REP 2165 at equivalent dose[19,36] which in the case of ALG-10133 was below the liver accumulation threshold required for antiviral activity of NAPs against HBV.[19] Although the reason for this suboptimal accumulation is unclear, it may be due to decreased nuclease stability or increased off-target interactions driven by the LNA modification.

PERSPECTIVES

The correct identification of the mechanism of action of NAPs as well as their host target(s) is important given their potent antiviral effects in HBV and HDV infection in humans. However, *in vitro* evaluation of NAPs cannot be conducted with transfection-based approaches, because of the impact of oligonucleotide structure and hydration on liposome formation and the non-physiological accumulation of NAPs in the cytoplasm. These artifacts prevent the correct comparison of relative antiviral activity between NAPs with different modifications and result in the observation of many inhibitory effects *in vitro* that do not occur *in vivo* or in humans. The disconnect between transfection data presented with NAPs *in vitro*[5,31,36] and in humans[3,6,20] is a clear validation of these artifacts. Future evaluations of NAPs *in vitro* must use suitable systems as described above, which have been validated with known control NAPs,[7,33] and target identification must be performed under well-controlled conditions.[22]

The LNA modification of NAPs was first described in 2003[8] but discarded early in development for its negative impact on NAP flexibility and well-documented hepatotoxicity.[37–39] To date, REP 2139 represents the fully optimized potential of this class of compounds, being highly stable, composed only of naturally occurring sugar and base modifications, and devoid of immunostimulatory properties, all while retaining its antiviral activity.

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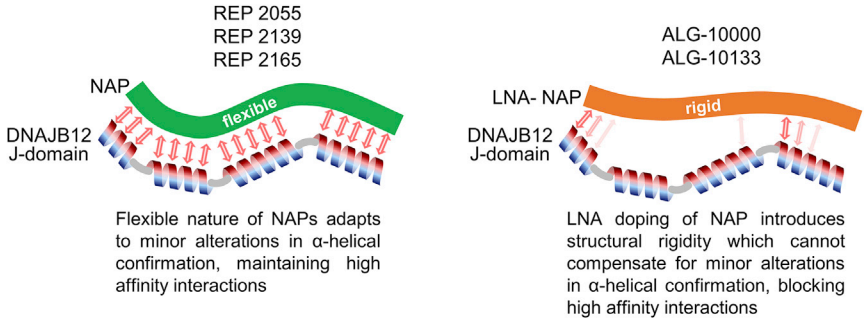

Figure 5. Model for the impact of LNA modification on the antiviral interactions of NAPs

Hydrophilic regions of target alpha helices for NAPs are indicated in blue and hydrophobic regions in red. Hydrophobic interactions and their relative strength are indicated by arrows.

DECLARATION OF INTERESTS

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