Quantum Mechanical Studies of DNA and LNA

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Quantum mechanical (QM) methodology has been employed to study the structure activity relations of DNA and locked nucleic acid (LNA). The QM calculations provide the basis for construction of molecular structure and electrostatic surface potentials from molecular orbitals. The topologies of the electrostatic potentials were compared among model oligonucleotides, and it was observed that small structural modifications induce global changes in the molecular structure and surface potentials. Since ligand structure and electrostatic potential complementarity with a receptor is a determinant for the bonding pattern between molecules, minor chemical modifications may have profound changes in the interaction profiles of oligonucleotides, possibly leading to changes in pharmacological properties. The QM modeling data can be used to understand earlier observations of antisense oligonucleotide properties, that is, the observation that small structural changes in oligonucleotide composition may lead to dramatic shifts in phenotypes. These observations should be taken into account in future oligonucleotide drug discovery, and by focusing more on non RNA target interactions it should be possible to utilize the exhibited property diversity of oligonucleotides to produce improved antisense drugs.

Introduction

The Watson-Crick (W/C) recognition motif, though fundamentally straightforward, leads and directs even the most complex biological processes (Watson and Crick, 1953a, 1953b). It is remarkable how this “digital” bonding motif has been a driver behind numerous applications where native nucleic acids are used as probes (Trapane and Ts’O, 1994; Dangler, 1996; Demidov and Frank-Kamenetskii, 2004). In addition, the recognition motif has served as a design basis for chemically modified nucleic acid analogs employed for highly specific purposes (Uhlmann and Peyman, 1990; Nielsen et al., 1991; Hanvey et al., 1992; Shabarova, 1994; Martin, 1995; Altmann et al., 1996; Corey, 1997; Freier and Altmann, 1997; Cook, 1999; Uhlmann, 2000; Davis et al., 2006; Debart et al., 2007; Eckstein, 2007; Koch and Ørum, 2007; Koch et al., 2008). One of the areas where nucleic acid analogs have extensively been used is for the antisense silencing of gene expression (Zamecnik and Stephenson, 1978). This therapeutic field is based on the inhibition of RNA expression by oligonucleotide analogs designed to specifically recognize, by W/C hydrogen bonding, a complementary target sequence (Zamecnik and Stephenson, 1978).

Over the past 3–4 decades, many nucleic acid analogs have been produced for antisense purposes (Zamecnik and Stephenson, 1978; Uhlmann and Peyman, 1990; Martin, 1995; Freir and Altmann, 1997; Uhlmann, 2000; Wengel, 2001; Koch and Ørum, 2007; Koch et al., 2008; Yamamoto et al., 2011). Despite this extensive work, antisense oligonucleotides have not fully realized their clinical promise. Only a small fraction of the analogs have ever been clinically evaluated, and just two antisense drugs have been approved by the United States Food and Drug Administration for marketing (Kynamro™ and Vitravene™). Enthusiasm for antisense therapeutics was renewed when the nucleic acid analog locked nucleic acid (LNA) was synthesized (Obika et al., 1997; Singh et al., 1998). LNA represents a class of bicyclic nucleic acid analogs, and in the parent LNA nucleoside, the furanose ring is chemically modified by insertion of a 2′-O-CH2-4′ linkage that transforms the furanose to the bicyclic structure. When LNA nucleosides are incorporated into oligonucleotides, the bicyclic modification induces not only substantially increased nuclease stability, but also good mismatch discrimination and high hybridization affinity. LNA nucleosides are equally well combined with native phosphodiester oligonucleotides (PO) or phosphorothioates (PS) (Cohen, 1993; Eckstein, 2007). Driven by its high affinity, LNA has enabled a platform technology for the inhibition of both coding and non-coding RNAs (Wahlestedt et al., 2000; Jepsen and Wengel, 2004; Hansen et al., 2008; Koch et al., 2008; Elmen et al., 2008a, 2008b; Seth et al., 2009; Lanford et al., 2010; Straarup et al., 2010; Lindholm et al., 2011; Obad et al., 2011).

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Material and Methods

The molecules studied were first constructed *ab initio* by adding the atoms or whole nucleotides together with a phosphor-sugar backbone using the chemical program SPARTAN ’10 (Wavefunctions, Inc.), or employing the graphics in Gauss-view. After having built the desired molecular structure, the molecule is transferred to a Quantum Mechanical program (e.g. Gaussian program package) (Frisch, 2009) on a supercomputer where the optimization procedure can be carried out.

The electronic properties of the oligonucleotides, both with respect to their molecular orbitals and their electrostatic surfaces, were obtained by QM Hartree-Fock (HF) optimization employing the basis set of Gaussian type functions. In this approach, a large energy functional can be optimized with respect to the electrostatic energy configuration solely on the basis of the electronic density by separating electron and nuclear motions. Once the energy-minimized electronic structure is obtained (i.e., the nuclear coordinates and electron orbitals in the ground state), the electrostatic surfaces and the HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) bonding system can be calculated, thus providing information about electron donor/acceptor sites.

More precisely, we employed the Hartree-Fock self-consistent field (HF SCF) QM method with linear combination of atomic orbitals approximation applied to the usual Schrödinger electronic equation (used together with matrix equations of the Roothaan Hall type). We selected HF since it is generally acknowledged that this method provides better optimization results for large biomolecules due to non-local interactions and avoidance of empirical parametrization. The Schrödinger electronic equation can be written as:

$$ F \langle \epsilon_{\text{coef}} \rangle = E \ L \ \langle \epsilon_{\text{coef}} \rangle $$

where $\epsilon_{\text{coef}}$ are wave function orbital coefficients, $E$ are orbital energies, $L$ are overlap terms and $F$ is a so-called Fock matrix given in terms of a core-electron Hamiltonian ($H$), the Coulomb, and Exchange terms such that:

$$ F_{\mu
u} = H_{\mu
u}^{\text{core}} + J_{\mu
u} + K_{\mu
u}. $$

The core Hamiltonian is derived from the Schrödinger equation with the one electron wavefunction $\phi_{\mu}$:

$$ H_{\mu
u}^{\text{core}} = \int \varphi_{\mu}(-\hbar^2/2m)\Delta - (e^2/4\pi\varepsilon_0)\sum Z_i/r)\varphi_{\nu} \ d\tau $$

where $\Delta$ stands for the Laplace operator, $m$ is the electronic mass, $e$ is the electron charge, $Z_i$ is the charge of the nucleus $i$, and the Coulomb and Exchange potential terms are expanded on the basis functions. These are here taken as split-valence basis sets of Gaussian functions denoted as either 6-31G* or 3-21G (less accurate), which describe the number of Gaussian functions in a sum with coefficients. The energies of the molecular parts with wave functions are used to calculate the potential energy surface. This represents the electrostatic potential energy based on wave-function optimization of the orbital structure from where the effective charges can be extracted, or in other words, the electrostatic potential energy a positive test charge is experiencing when approaching the molecule. It was found convenient to describe this potential energy by an *iso*-surface of defined field strength, which was identical over the entire surface (Bohr, 2013).

In the HF calculation, the lowest energy states are found by optimization under the constraints of self-consistent (SCF) field procedures that obey the laws of electrodynamics. The HF calculations are based on independent particle models where the electrons experience a mean field from the electrons and the nuclei. In the Density Functional Theory approach, the exchange potential is based on electron densities, while the HF method uses wave functions also describing non-local exchange potentials.

The HF calculations are stepwise processes: Initially, single stranded molecules were constructed. DNA or LNA oligonucleotide (nucleobase and sugar-phosphate backbone) consists of hundreds of atoms. Next, the molecule was subjected to a basic electrostatic optimization to determine if the molecular structure is stable. Finally, the coordinate file was transferred to a high performing supercomputer that optimizes the proposed electronic structure using variational methods, and constructs the electronic orbitals that are consistent with the electrostatic functionals.

The process of proposing a system of wave functions and checking their consistency according to their electronic densities is then carried out for many iterations (ca. 1,000) and is terminated when a convergent state is reached. In a complete quantum mechanical calculation of a molecular structure, it is important to include solvent effects on the
naked (gas phase) molecule. Water molecules are here included indirectly by altering the dielectric constant (relative permittivity 75) in a cavity. From the electrostatic iso-surfaces, in addition to charge densities, differential geometric properties such as topology can also be obtained. These geometric properties appear in the connectedness of the surface and the curvature values (see Figs. 1–6).

An important question is which role the initial conditions play on the quantum electronic structure calculation and optimization and if changed initial conditions will lead to different conformational structures. These could be purely technical initial conditions such as the choice of basis set for the quantum mechanical optimization. In order to assess the dependence of the initial conditions of the calculated optimized structures of a given molecule, we chose a DNA oligomer as a test case. Hence, we calculated optimized electronic structures of the DNA-7mer of ATGTAGC using the basis set 3-21G and 6-31G*, where the latter is the double electronic structures of the DNA-7mer of ATGTAGC using oligomer as a test case. Hence, we calculated optimized structures of a given molecule, we chose a DNA

molecules (peptides). The theoretically derived spectra are frequently included in AONs (Lebedeva et al., 2000; Uhlmann, 2000; Krutzfeldt et al., 2005; Koch et al., 2008; Lanford et al., 2010; Stein et al., 2010; Straarup et al., 2010; Koller et al., 2011; Moschos et al., 2011; Obad et al., 2011; Stanton et al., 2012).

Results

The QM calculated molecular iso-potentials were compared for either native phosphodiester oligonucleotides (PO) or chemically modified phosphorothioates (PS) (Cohen, 1993; Eckstein, 2007). The value of the iso-potentials was chosen to be an energy ranging from 10 - 20 kcal/mol. This value corresponds to the energy of 2–5 H-bonds and characterizes a potential binding to a receptor.

Initially, the effects of modifying the ribose backbone were examined. DNA-PO-AAG was compared with all LNA-PO-
AAG (Fig. 1). From the structural representation, DNA is generally more bent while LNA is more stretched. This is consistent with the more rigid bicyclic structure of LNA (Obika et al., 1997; Nielsen et al., 2000; Petersen et al., 2000, 2002; Nielsen and Spielmann, 2005; Hughesmann et al., 2011). When the iso-potential surfaces are added, the surface of LNA-PO-AAG is constricted and concentrated on the phosphate backbone face of the molecule. The HOMO orbital was shifted from the 5′ adenine in DNA to the 3′ guanine of the LNA, while the LUMO orbital resides on the central adenine in both. Interestingly, the electrostatic potential of the 3′ PO in the DNA is weak, much weaker in fact than for the other phosphate in the trimer, and exhibits a potential comparable to the lone pairs on the nucleobases—particularly the 5′ adenine.

Next, the effects of changed internucleoside linkages and PS configuration were examined. LNA-PO-AAG (Fig. 1) was compared with LNA-PS-AAG (with the PSs in the RS configuration) (Fig. 2A). The PS configuration was determined by assigning the hydrogen to sulfur (S–P single bond). The LNA-PS-AAG (RS) modification results in a large potential change and exhibits a more scattered topology compared to LNA-PO-AAG, with a shift of the electrostatic potential towards the 5′ end (Figs. 1, 2A). Interestingly, with the PS in the SR configuration the topology reverts into a coherent structure resembling the compact form of the PO congener (Figs. 1B, 2B). PS modification also induces changes in the localization of the frontier orbitals. The HOMO-LUMO orbitals are split between adenine and guanine for LNA-PO-AAG but the LUMO resides on the central adenines for both PSs, irrespective of configuration. Interestingly, the HOMO resides on sulfur on the PSs in the S configuration.

The effects of changing/mutating a nucleobase were examined. LNA-PO-AAG was compared with the mutant LNA-PO-AGG, (Fig. 3). The single nucleotide mutation causes a shift in the iso-potential from a compact surface for the AAG sequence (Fig. 3A) to a more scattered surface for the mutant, AGG sequence, placing a stronger field on 5′ adenine (Fig. 3B). In addition, the potential for the 3′ PO is weakened. The HOMO shifts from the 3′ guanine to the 5′-adenine, while the LUMO is located on the central guanine (Fig. 3A, C).

The effects of increasing the nucleotide sequence from trimer to pentamer were also examined. The potential of the LNA-PO-AAG is concentrated around the central and 5′-adenosines. Adding two nucleotides (A and C) changes the iso-potential concentration towards the 3′ end and leaves the two 5′-adenines with much less electrostatic density (Fig. 4). The frontier orbitals also shift from the central adenine and 3′ guanine in the trimer to the penultimate 5′ adenine and the 3′ adenine in the pentamer. Electrostatically, the potential of the pentamer is unique.

Sequence/topology relations were examined for larger oligonucleotides. The biologically active LNA-PO-7-mer (5′-ATGTAGC-3′) (Fig. 5A, C) was compared with the single base pair mutant (5′-ATGAAGC-3′) (Fig. 5B, D) and with the DNA-PO-ATGTAGC (not shown). The LNA-PO-7-mer (5′-ATGTAGC-3′) (Fig. 5A, C) was originally designed by Obad et al. (as fully PS modified) to target the seed sequence of microRNA (miR)-221 and miR-222 and accordingly, was able to inhibit both microRNAs due to seed sequence homology (Obad et al., 2011).

The interchange of the central thymine with adenine does not cause a change in the position of the frontier orbitals. In both cases, the HOMO orbital is found on the adenine closest to the 3′ end and the LUMO is found on the 5′ adenine. However, changing the backbone to DNA causes a shift in both the position and energy of the frontier orbitals. Thus, in
the DNA oligonucleotide, the HOMO orbital is on the 3’
cytosine \( (E = -8.38 \text{ eV}) \), and the LUMO orbital is on the 5’
adine \( (E = -1.29 \text{ eV}) \) (data not shown). The LNA (5’-
ATGTAGC-3’) has the HOMO orbital on the adenine closest
to the 3’ end \( (E = -8.04 \text{ eV}) \) and the LUMO orbital on 5’
adine \( (E = -4.55 \text{ eV}) \). Thus, the gap is reduced for the
LNA oligonucleotide \( (\Delta E = -3.49 \text{ eV}) \) compared with the
iso-sequential DNA oligonucleotide \( (\Delta E = -7.09 \text{ eV}) \).
The molecular structure of the DNA-PO and LNA-PO oli-
gonucleotide with same sequence (5’-ATGTAGC-3’) are
very different, with the DNA oligonucleotide being bent
(ca. 90°), and the LNA oligonucleotide with a more straight
structure. Correspondingly, the topologies of the electrostatic
potentials are also very different and each unique.
Interestingly, the central thymidine/adenosine mutation causes also a
global change in topologies of the two LNA oligonucleotides
(Fig. 5C, D). The topology of the electrostatic potential of the
mutant is more evenly distributed over the molecule with a
weaker negative potential (red). The central mutation also
causes a 3’ end weakening of the positive potential (yellow)
(Fig. 5C, D).

Finally, a study was also carried out on a LNA-DNA PO 7-
gapmer. The compound has the same nucleotide sequence as
in the parent LNA compound mentioned above (5’-ATG
TAGC-3’), but the middle three nucleotides were substituted
with DNA PO nucleotides. The resulting optimized structure
(Fig. 6A) was more compact and bend—almost globular—
and the increased flexibility converged by the DNA nucleo-
tides brought the two LNA ends close to each other. The
HOMO-LUMO gap was \( \Delta E = -4.05 \text{ eV} \), and the established
iso-surface had a disrupted or scattered potential surface very
different from the potential of the iso-sequential full LNA 7-
mer (Fig. 6B). The electronic data on the 7-mers confirm that
there are no particular boundary effects in this study, al-
though it could be anticipated that special boundary effects
on the 3-mers were dominating.

**Discussion**

The QM data illustrate that small modifications can
profoundly change the structure, polarity and topology of
the molecular electrostatic potential (MEP) of oligonucle-
otides. Introduction of a single base mutation (Figs. 3, 5)
induces global structural and MEP topology changes, as do
oligonucleotide design changes for iso-sequential com-
ponds (Figs. 5, 6). Changing the sugar backbone from
DNA to LNA can lead to strong polarity changes in the
backbone phosphates (Fig. 1). The molecular structure and
MEP may also be different among diastereoisomers of the
same sequence (Fig. 2). As further illustrated in Fig. 4,
structure and MEP topology for any given compound is
unique; structural modifications lead to topologies that
cannot be viewed simply as additions to a precursor struc-
ture. The wave-function representation illustrates that
chemical modifications influence both structure and MEP, not
only at the site of incorporation, but throughout the entire
molecule.

Molecular structure, MEP strength, and topology are de-
terminants for the interaction with and the binding pattern
between molecules. Generally, ligand-receptor binding pro-
cesses are very complex, the net binding energy is a balance
between molecular structure and MEP on one side and
counteracting desolvation effects on the other (Chong et al.,
1998; Kangas and Tidor, 1999; Sheinerman et al., 2000;
Muzet et al., 2003; Sulea and Purisima, 2003; Burgoyne and
Jackson, 2006; Kraut et al., 2006; Dhimoy et al., 2008, 2013;
Kenny, 2009). Thus, binding cannot be automatically as-
sumed to take place even if perfect structure and MEP
complementarity exists between ligand and receptor. How-
ever, in some systems, ligand-receptor MEP complementar-
ity appears to play a more determinant role for binding to take
place. These receptor-ligand systems are characterized by
ligand binding to concave pockets of a receptor where binding is driven thermodynamically by hydrogen bonding (Kangas and Tidor, 1999; Muzet et al., 2003). This has importance for oligonucleotide–protein binding. Oligonucleotide binding proteins usually share a common concave structure called the OB (oligonucleotide binding) fold (Sun and Shamoo, 2003; Harris et al., 2012). Oligonucleotide binding to the fold is driven by hydrogen (H)-bonding which is further stabilized by hydrophobic interactions and stacking between nucleobases and aromatic amino acids. Interestingly, strong binding is also observed to negatively charged proteins, and the charged backbone may also interact either via H-bonding or salt bridge formation. Notably, however, the phosphates are predominately exposed to the highly dielectric solvent (Max et al., 2006; Zeeb et al., 2006). The fact that any energy strength can be selected as an iso-value for the electrostatic potentials (e.g., fixed to H-bonding energies) means that the MEPs are informative about how well H-bonding may occur locally. Altogether, it can be assumed that electrostatic complementarity plays a more determinant role for single stranded oligonucleotide–protein interactions.

The interaction/binding of oligonucleotides to proteins is an important parameter influencing toxicity, plasma half-life, tissue accumulation, cellular uptake, and activity of antisense oligonucleotides (Beltinger et al., 1995; Prakash et al., 2002; Watanabe et al., 2006; Lai et al., 2007a, 2007b; Stein et al., 2009). Since H-bonding to folded protein structures (e.g., OB fold) is important for oligonucleotide–protein binding we can assume that structural and MEP complementarity are determinants for binding/interaction to take place. Consequently, oligonucleotides with marked differences in MEP are likely to exhibit differences in protein binding. Assisted by the QM data, it is evident that small structural modifications may lead to profound changes in the binding/interaction profile (i.e., the phenotypic profile). As an example, a single nucleotide modification (e.g., perfect match vs. one base pair mismatch) may lead to major, or “all or none,” phenotypic changes. The QM modeling data explains and illustrates reasons underlying some of the unexpected observations relating to major phenotypic changes observed for otherwise “closely related” molecules (vide infra) (Seth et al., 2009; Straarup et al., 2010; Laxton et al., 2011; Stanton et al., 2012; Hagedorn et al., 2013). The data also underlines that caution should be taken in making broad predictions about the effects of particular chemical modifications. For instance, in nearly all cases, incorporation of LNA nucleotides will lead to higher affinity and nuclease stability. However, how a single LNA modification, or other structural units for that matter, will influence the global effects, on a cellular or systemic level is more complex. As an example of how the global bioproperties of LNA gapmer phosphorothioate oligonucleotides can be affected by seemingly minimal changes, a series of these 16-mer oligonucleotides, targeted to Bcl-2, were synthesized that differed only by a single base in the gap. After gymnastic delivery of the oligos to 518A2 cells (Soifer et al., 2012; Stein et al., 2010), the phosphorylation patterns of the PI3K/Akt/mTor pathway were examined. Each oligo silenced Bcl-2 protein expression to the same extent. However, the levels of phosphorylation of PI3K, Akt, and mTOR were different for every oligo (Cy A. Stein, personal communication). Another example was recently reported where a specific LNA 13-mer was hepatotoxic. However, adding just a single additional LNA nt rendered the resulting 14-mer non-hepatotoxic (Hagedorn et al., 2013). This demonstrates that single-nucleotide substitutions may induce major phenotypic changes—changes that are not “inherited” from the single nucleotide per se, but result more likely from the nucleotide insertion/substitution in a specific sequence/compound context. At this point in time, we can predict some effects of single-nucleotide substitutions, but not how single-nucleotide modifications will alter the global interactions. In practice, this also means that caution should be taken in over-generalizing about data arising from comparative studies using single base pair mismatch control oligonucleotides. The potential to produce very different phenotype, induced by the single-nucleotide modification compared to the “full match” oligonucleotide, may be so great that any observed differences in effects may be caused by the altered global interaction profile, and only minimally resulting from the mismatch hybridization effect with the RNA target. Anti-sense oligonucleotides are composed of many discrete structural units each bringing unique properties (e.g., nucleobases, PO/PS internucleotide linkages, DNA/RNA/LNA backbones, base sequence motifs, etc.). These units collectively engender, in a sequence context, the properties of the
The question is then how large should the oligonucleotide pool be and how many structural units—pharmacophores—should be combined? We are not able to answer that question now, and numbers becomes quickly a limiting factor. For example, the number of unique 14-mers constructed from 4 nucleotide building blocks is $4^{14}$ ($2.7 \times 10^5$). If the number of building blocks is increased by adding 4 extra nucleotides (e.g., four LNA and four DNA nucleotides) an expanded property space will be formed. However, in order to explore all combinations $8^{14} = 4.4 \times 10^{12}$ compounds would have to be tested. Numbers like these are known issues from classical small molecule drug discovery and cannot be managed in practice. Nevertheless, in contrast to small molecule drug discovery, antisense offers a great advantage: The RNA-target binding motif is known! Due to the requirement of complementary base pairing to the target, the relevant compound number is immediately reduced by $\sim 6$ orders of magnitude (depending on the length and number of modifications considered). This reduces but still leaves too many compounds to handle, further reduction required. In this case, property prediction algorithms are the preferred tools, and although the QM data illustrate a more intricate structure/activity relationship of oligonucleotides, it is far from chaotic. However, new and improved prediction algorithms must be constructed from larger training data sets, where the property of interest has been measured experimentally, and correlated with several structural entities. These include nucleobase sequence, backbone, inter-nucleotide linkages, or any modification of these in combination and related to a specific molecular context. We are currently establishing such large databases of thousands of oligonucleotides tested both in vitro and in vivo. A simplified decomposition of oligonucleotides has been developed, combined with a random forest classification that with $\sim 80\%$ significance can predict the hepatotoxic potential of LNA/DNA-gapmer oligonucleotides (Hagedorn et al., 2013). Predicted experimental outcome by in silico algorithms is linked with uncertainty. Nevertheless, they offer a knowledge based strategy to reduce the relevant compound number, by zooming in on the selection of oligonucleotides where the probability is highest for finding unique/extreme properties (Fig. 7). It is therefore not necessary to test all combinations of a structural unit selection. Testing much smaller numbers (e.g., $10^2$–$10^4$), may be sufficient for a thorough investigation of the property space.

The data presented here are based on QM calculations for 3-, 5-, and 7-mer oligonucleotides; these lengths are significantly shorter than the ones used for antisense purposes (12-to 16-mers). It is therefore relevant to ask if the structural and MEP sensitivity to the chemical modifications observed here also is the case for the 12- to 16-mers. We have not yet been able to complete QM calculations on longer oligonucleotides because such calculations are very CPU demanding. However, experimental observations of large property diversity and the “all or none” phenotypes of closely related antisense compounds (vide supra) could indicate that single modifications may affect not only the site of substitution, but can affect the entire molecule as well. In that case, a wave function representation could be useful, and accordingly large variations in structure and MEP would also be expected for longer oligonucleotides.

**Conclusion**

The QM oligonucleotide modeling illustrates that structure activity relations are complex interrelations of individual chemical components and their specific structural position. Property diversity is mechanistically driven by the molecular structure and the derived molecular electrostatic potentials. Nucleobase sequence is not the only determinant for oligonucleotide properties; the global properties are derived from each structural unit in concert with the molecular composition. The diversified recognition potential between oligonucleotides and non-target biomolecules should be exploited. This could be done by testing more combinations of selected structural units. In a targeted approach, the binding/interactions between lead compounds and non-target biomolecules should be optimized together with target RNA inhibition.
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Author Disclosure Statement

This work is made by authors employed full-time by Santaris Pharma A/S (TK, ML, and HØ) and by authors (HGB and IS) that are employed by the Technical University of Denmark (DTU).

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