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Antisense molecules and furanose conformations—is it really that simple?

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Selected nucleic acid mimics are discussed in the context of the antisense therapeutic strategy with focus on furanose conformation, RNA-binding affinity, and activation of the RNA-cleaving enzyme RNase H.

Introduction

In order to inhibit gene expression by targeting RNA with short antisense oligonucleotides, multiple chemically modified nucleoside and oligonucleotide analogues have been synthesized and evaluated during the last 15 years.[1,2] Selected examples of modified nucleic acids are described here with the emphasis on their hybridization properties and capability to activate RNase H, an RNA-degrading enzyme, in relation to their conformational and structural characteristics. The focus is on important recent developments and their prospects towards the realization of the antisense therapeutic strategy.

The antisense strategy

The carriers of all genetic information in living organisms are 2′-deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), polymers consisting of repetitive units of nucleotide monomers. Each nucleotide contains a phosphate group, a carbohydrate moiety and a nucleobase (Fig. 1). The natural nucleobases in

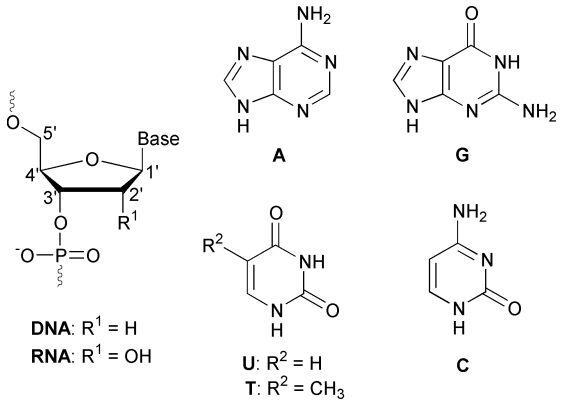

Fig. 1 Structures of a nucleotide monomer (left) and the nucleobases adenine (A), guanine (G), uracil (U), thymine (T), and cytosine (C).

DNA (R^1 = H) are A, G, T and C while U replaces T in RNA (R^1 = OH). Two complementary polymeric or oligomeric single strands hybridize in an anti-parallel fashion forming a right-handed duplex with the specific Watson–Crick base-pairing of G to C and A to T or U.

As depicted schematically in Fig. 2, the genetic information is transcribed from double-stranded DNA, located in the cell

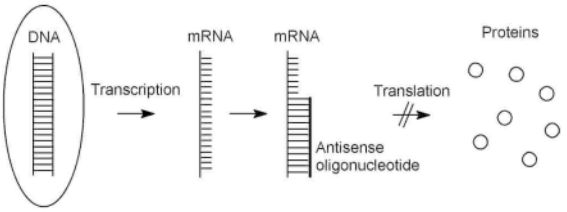

Fig. 2 Schematic overview of the antisense strategy.

nucleus, to single-stranded messenger-RNA (mRNA) which functions as a carrier of genetic information from the cell nucleus to the ribosomes in the cytoplasm. The principle of the antisense strategy is to target mRNA by duplex formation using a short (10–20 nucleotides long) antisense oligonucleotide, thereby preventing the translation of the mRNA into proteins. In principle, any genetic sequence, and thus any disease with a genetic origin, should be subject to selective targeting by varying the nucleotide sequence of an antisense oligonucleotide approximately 18 nucleotides long. This makes the antisense strategy general and attractive compared with the individual development of traditional drugs acting at the protein level. An antisense oligonucleotide has to fulfil several functional requirements, *e.g.* high binding affinity toward the RNA binding strand, resistance towards nucleases, low toxicity, and efficient delivery to the desired target site, *in vivo*, rendering

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chemical modification of the natural nucleic acid structures needed.

Nucleic acid structure and furanose conformations

The pseudorotational circle[3] depicted in Fig. 3 describes the possible furanose conformations of the nucleotide monomers.

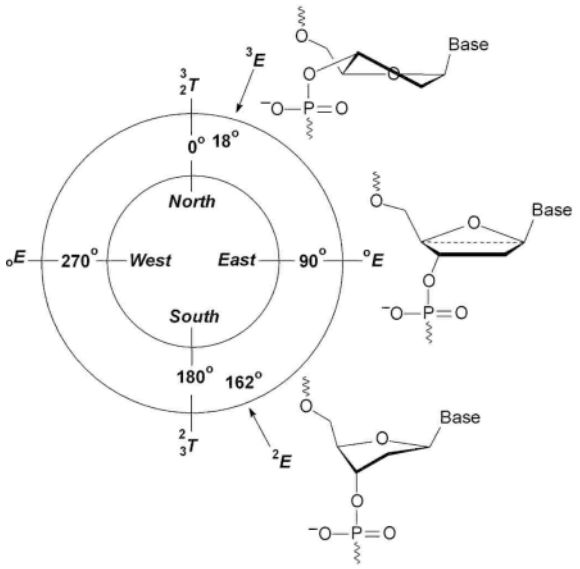

Fig. 3 The pseudorotation cycle showing the relation between the pseudorotation angle *P* (0–360°; calculated from the five torsional angles of the furanose ring[3]) and furanose ring conformations.

All distinct conformations (*i.e.* envelope, *E*, and twist, *T*, conformations) are separated by 18° with a superscript designating an atom above the plane described by the remaining three or four atoms of the furanose ring, and a subscript designating an atom below.

The two dominating furanose conformations for DNA and RNA generally give rise to two different duplex forms, as depicted in Fig. 4. Apart from having all furanose conformations in an *S*-type (*south*-type, C2′-*endo*, ^2E) conformation, the B-type helix seen in solution for DNA is characterized by 10 base-pairs almost perpendicular to the helix axis per helix turn, and a distinct difference in width of the major and the minor grooves.[4,5] In contrast, RNA adopts an A-type helix in which the furanose conformations are all *N*-type (*north*-type, C3′-*endo*, ^3E) with 11 base-pairs tilted 20° with respect to the helix axis per helix turn, and the minor and major grooves being almost equally wide.[4,5]

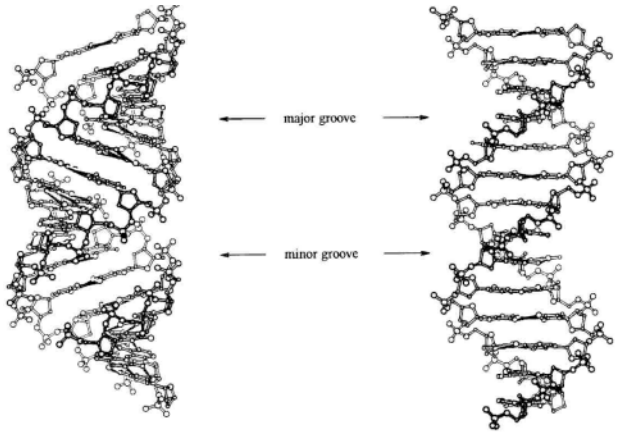

Fig. 4 Globular conformations of an A-type duplex (left) and a B-type duplex (right) generally seen for RNA:RNA and DNA:DNA duplexes, respectively.

Modes of action and conformational requirements of antisense molecules

An antisense oligonucleotide has basically two possible modes of action both depending on the duplex formation between the RNA target and the antisense oligonucleotide. One is a simple steric blocking of the mRNA, the other recognition of the RNA–antisense duplex as a substrate for the enzyme RNase H which cleaves the RNA strand of an RNA–DNA duplex. In the latter scenario, one antisense oligonucleotide is able to pacify multiple mRNA strands. A high binding affinity towards RNA is crucial, especially for the steric blocking approach. Conformational restriction of the single-stranded antisense oligonucleotides is believed to favour duplex formation entropically by diminishing the loss of conformational degrees of freedom upon duplex formation, and conformational restriction of the furanose rings of oligonucleotides has been successfully applied to the field in the recent years.[6–8] Studies of a large number of oligonucleotide analogues have been generalized to the hypothesis that oligonucleotides restricted into *N*-type furanose conformations, thus yielding A-type duplexes when hybridized to RNA, effect the highest duplex stabilities.[1,9]

However, no fully modified antisense oligonucleotide with restricted *N*-type furanose conformations has been reported to be able to activate RNase H. The RNA–DNA heteroduplexes being substrates of RNase H which has been reported to bind in the minor groove, adopt an intermediate duplex form with a minor groove width also intermediate between that of the A form and the B form.[10–14] The furanose conformations in the RNA strand are still *N*-type (3′-*endo*) while hybridization to the RNA strand causes the furanose conformations of the DNA strand to change from the typical *S*-type (C2′-*endo*) into *E*-type conformations (O4′-*endo* range, see Fig. 3).[10,11,14] Thus, the activation of RNase H proposedly requires antisense oligonucleotides with furanose rings able to adopt *E*-type (O4′-*endo*), or perhaps *S*-type (C2′-*endo*), conformations, and *not* the duplex-stabilizing *N*-type conformations.

An appealing way to circumvent the lack of RNase H activation is the introduction of chimeric structures consisting of high-affinity nucleotide modifications in the terminal regions of the antisense oligonucleotide around a ‘gap’ of RNase H-activating nucleotides in the central part. To activate the mammalian RNase H, a gap size of five[15] to seven[16] unmodified 2′-deoxynucleotides has been suggested.

Selected oligonucleotide analogues

A number of the more promising nucleic acid mimics for the development of successful antisense oligonucleotides are discussed in the following. Emphasis is given to analogues in which the furanose ring is restricted into a distinct conformation by either stereoelectronical or constitutional (sterical) means. Average duplex stabilities for hybridization towards complementary RNA targets are reported as the change in the melting temperature per modified nucleotide (°C per mod.) relative to the corresponding unmodified DNA–RNA reference duplex, the melting temperature being defined as the temperature at which half of the duplex is dissociated as measured by the associated hyperchromic shift at 260 nm.

Phosphorothioates

In 1998, the first antisense drug, Vitravene™ (ISIS-2922), was approved by the FDA for the treatment of cytomegalovirus retinitis in immunocompromised patients.[17] This antisense oligonucleotide consists of phosphorothioate (thiophosphate) nucleotides, in which one of the non-bridging phosphate oxygens is substituted by sulfur (**5**, Fig. 5) resulting in improved nuclease stability while the capability to activate RNase H remains intact. Phosphorothioates are, however, considered as

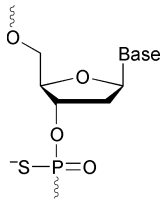

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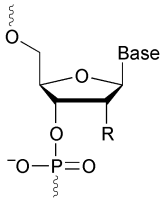
Fig. 5 Structure of phosphorothioates.

only 'first generation' antisense oligonucleotides due to less desirable features such as lowered duplex stabilities (approximately −0.8 °C per mod.), the existence of diastereomeric mixtures because of the chirality of the phosphorus atoms, and significant toxicity caused, *e.g.* by non-specific binding to proteins.[18]

Selected 2′-*O*-alkylated RNA derivatives

For 2′-*O*-alkylated RNA derivatives, the *gauche* effect between O2′ and O4′ (along the C1′–C2′ bond) induces a conformational shift towards an *N*-type (C3′-*endo*) furanose conformation resulting in A form duplexes as also seen for unmodified RNA. Provided that the 2′-*O*-alkyl group is not too sterically demanding (*i.e.* alkyls smaller than hexyl),[19] increased duplex stability is observed. A 2′-*O*-Me-RNA monomer (**6A**, Fig. 6)



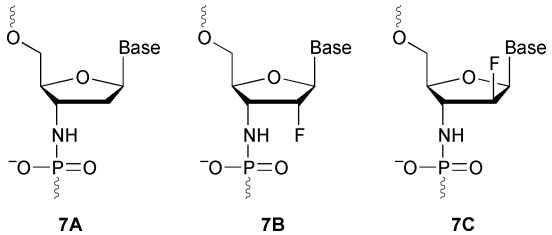
6A R = OCH_3
6B R = $OCH_2CH_2OCH_3$ (MOE)
6C R = $OCH_2CH_2CH_2NH_2$ (AP)

Fig. 6 Structures of selected 2′-*O*-alkylated RNA derivatives.

induces an increase in the melting temperature of *ca.* +0.5 to +1.0 °C per mod.,[20] while 2′-*O*-(methoxyethyl)- (MOE, **6B**) and 2′-*O*-aminopropyl- (AP, **6C**) monomers effect increases of *ca.* +1.0 to +1.5 °C per mod.[20,21] and *ca.* +1.0° C per mod.,[22] respectively. The nuclease stability of the 2′-*O*-Me derivatives is insufficient for antisense purposes,[9] which suggests the use of the more resistant MOE[21] or AP[22] modifications. Because of their preorganization into an *N*-type furanose conformation, none of the 2′-*O*-alkylated derivatives activate RNase H unless a gap-mer strategy is applied.[23]

N3′ → P5′-Phosphoramidates

Replacing the 3′-hydroxy group with an amino group introduces a non-chiral nuclease-resistant phosphoramidate internucleoside linkage. Such a modification (**7A**, Fig. 7) increases duplex



7A **7B** **7C**

Fig. 7 Structures of N3′ → P5′-phosphoramidates.

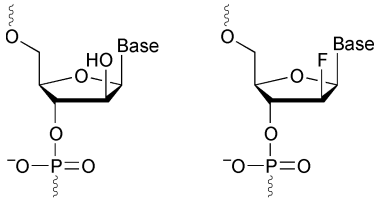
stabilities by +2.3 to +2.6 °C per mod.[24–26] which has been explained by the diminished *gauche* effect from the 3′-nitrogen atom preorganizing the furanose ring into an *N*-type (C3′-*endo*) conformation thus furnishing A-type duplexes with RNA targets as also shown by NMR[27,28] and X-ray studies.[29] Hybridized to RNA, fully modified phosphoramidates do not activate RNase H,[30] but this problem can be circumvented by the use of a gap-mer sequence with unmodified DNA monomers in the central gap[31] and also of a phosphoramidate mix-mer with alternating phosphoramidate and unmodified phosphordiester nucleotides.[32] The gap-mer phosphoramidates showed a lack of antisense activity which might be ascribed to the endonucleolytic degradation of the deoxynucleotide gap[9] while only fully modified phosphoramidates showed an RNase H-independent antisense activity.[31]

2′-Substitution with a *ribo*-configured fluorine atom (**7B**, *ribo*-configuration, Fig. 7) constrains the furanose ring entirely to a C3′-*endo* conformation due to the strong *gauche* effect from fluorine and induces increased duplex stabilities with as much as +4 to +5 °C per mod.[33] The RNA analogue of **7B** (a 2′-OH group instead of the fluorine atom) likewise afforded increased duplex stabilities (*ca.* +0.5 °C per mod.) relative to the parent phosphoramidates **7A**.[34] The *arabino*-configured 2′-fluoro phosphoramidate **7C**, too, displayed increased duplex stabilities (*ca.* +0.5 °C per mod.) relative to phosphoramidates **7A**, and, despite the opposing *gauche* effect from the 2′-fluoro substituent, a preorganized C3′-*endo* conformation, as shown by the analysis of coupling constants for trimers, still accounts for the improved hybridization properties.[35]

The results obtained for the various phosphoramidates convincingly demonstrate that stereoelectronic preorganization into a C3′-*endo* furanose conformation can be applied to induce increased duplex stabilities. It should be mentioned, though, that a considerable sequence-variation has been observed and that, *e.g.* increases as small as +0.6 °C per mod. have been reported for fully modified unsubstituted phosphoramidates **7A**.[36] A point of concern with phosphoramidates is their more difficult incorporation into oligonucleotides with step-wise coupling yields in the range of 94–97%.[25,33,35]

Arabino nucleic acids and 2′-fluoroarabino nucleic acids (ANA and 2′-F-ANA)

The inversion of the configuration around C2′ of natural RNA gives arabino nucleic acids (**8A**, Fig. 8) which show decreased



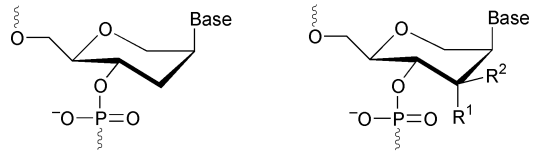
8A **8B**

Fig. 8 Structures of arabino nucleic acids.

duplex stabilities when hybridized to RNA while the corresponding 2′-fluoroarabino nucleic acids (**8B**), by contrast, enhance duplex stabilities by *ca.* +1.0 °C per mod.[37,38] The very interesting feature of both these modifications is that they were the first fully modified oligonucleotides with a chemically modified carbohydrate moiety to act as substrates for RNase H.[37–39] Their conformational similarity with natural DNA might account for this since duplexes, when hybridized to RNA, resemble an intermediate DNA–RNA duplex form as shown by CD spectroscopy.[38] The 2′-fluoro analogue **8B** adopts the O4′-*endo* furanose conformation when incorporated into DNA as shown by X-ray structure analysis.[40,41] In addition, molecular modelling[39,41] and NMR studies[39] of a fully modified 2′-F-ANA strand hybridized to RNA have confirmed an intermediate duplex form with a minor groove width intermediate between the minor groove widths of the standard A- and B-forms. The nuclease stability of the ANAs is improved compared with unmodified DNA though not as much as for the phosphorothioates.[37,39]

Hexitol nucleic acids (HNA)

Pyranose derivatives in general adopt one predominant conformation due to higher energy barriers for interconversion of ring conformers relative to furanose derivatives. To date, the 1′,5′-anhydrohexitol nucleic acids (HNA, **9A**, Fig. 9) and



9A **9B** R^1 = OH, R^2 = H
9C R^1 = H, R^2 = OH

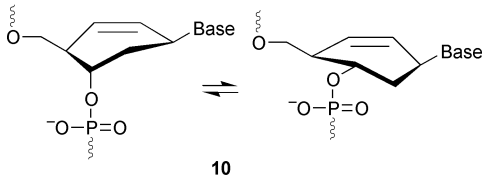
Fig. 9 Structures of hexitol nucleic acids.

derivatives constitute the most promising antisense candidates based on a pyranose moiety. Structurally, their substitution pattern makes them very good mimics of nucleotides preorganized into an *N*-type (C3′-*endo*) furanose conformation thus yielding A-type duplexes when hybridized to RNA as shown by CD spectroscopy,[42] molecular dynamics simulations,[43] and NMR studies.[44] Fully modified HNA displays enhanced duplex stabilities (typically +3.0 °C per mod.)[45] while sequence-specific variations of increases of duplex stabilities ranging between +0.9[45] and +5.8[42] °C per mod. have been observed. HNA acts only as a very poor substrate for RNase H,[42] while its nuclease-resistance[45] still allows it to display antisense effects ascribed to a mechanism of steric blocking.[46]

Interesting derivatives of HNA are the 1,5-anhydro-2-deoxy-D-altritol nucleic acids with an additional axial hydroxy group in the 3′-position (**9B**, Fig. 9) which show slightly enhanced duplex stabilities relative to HNA.[47] The altritol nucleic acids were designed to increase the surface hydrophilicity of the duplex thereby favouring duplex formation by improved hydratization.[8] In contrast, the inversion of configuration at the 3′-position, affording the corresponding 1,5-anhydro-2-deoxy-D-mannitol nucleic acids, leads to significantly reduced stability of duplexes with RNA[48] and obviously imposes a restricted conformation unfavourable for duplex formation.

Cyclohexene nucleic acids (CeNA)

Noteworthy results have been obtained for the conformationally rather flexible cyclohexene nucleic acids (CeNA, **10**, Fig. 10)



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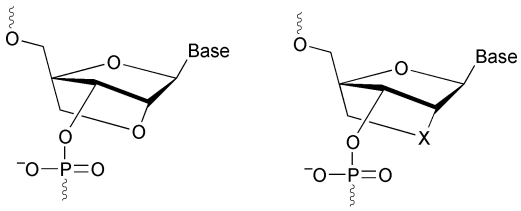
(*S*-type mimic) (*N*-type mimic)

Fig 10 Structure and conformational equilibrium of cyclohexene nucleic acids.

with a conformational equilibrium shifted towards *N*-type at the nucleoside level as shown by NMR and modelling studies (Fig. 10).[49] When incorporated into DNA strands, cyclohexene nucleotide monomers **10** induce increased duplex stabilities (+0.8 to +1.7 °C per mod.). Fully modified CeNA are nuclease-resistant and activate RNase H. The latter property establishes CeNA, next to ANA (Fig. 8), as the second fully modified oligonucleotide analogue with an altered carbohydrate part able to induce an RNase H cleavage of the target RNA strand.[50] This has been explained by the flexible conformational behaviour of the cyclohexane nucleotides allowing a $^2H_3$ (*S*-type mimic) conformation when hybridized to DNA and a $^3H_2$ (*N*-type mimic) conformation when hybridized to RNA as supported by CD spectroscopy, NMR studies of DNA duplexes with one cyclohexene nucleotide in each strand, and molecular dynamics simulations.[50]

Locked nucleic acids (LNA)

The synthesis of nucleosides and oligonucleotides containing furanose rings efficiently locked into a C3′-*endo* conformation has been accomplished by the introduction of an oxymethylene linkage between the C2′ and C4′ atoms generating the bicyclic locked nucleic acid (LNA, **11A**, Fig. 11). The incorporation of



11A **11B** X = NH
11C X = S

Fig. 11 Structures of locked nucleic acids (LNA), 2′-amino LNA and 2′-thio LNA.

one or more LNA monomers, or the use of fully modified LNA, induces unprecedented increases in duplex stabilities (typically +3.0 to +11.0 °C per mod.).[51–54] The preorganization into a C3′-*endo* furanose conformation has been demonstrated at the nucleoside level by X-ray crystallography[55] as well as NMR studies[51,52] and also by NMR studies at the oligonucleotide level both when hybridized to DNA[56–58] and RNA, the latter yielding the expected A-type duplexes.[59] LNA monomers in partly modified strands were shown to strongly preorganize flanking unmodified DNA nucleotides into *N*-type–C3′-*endo* furanose conformations and single-stranded LNA into an RNA-like conformation.[58,59] Fully modified[51] and mix-mer LNA consisting of alternating LNA and DNA monomers[60] have proved to be nuclease-resistant, and an antisense effect has been demonstrated in living rats.[60] LNA activates RNase H as gapmers[60] but not as fully modified strands.[60,61]

The LNA-type constitution leads in general to efficient C3′-*endo* preorganization and favoured duplex formation which has been demonstrated by the hybridization properties (+3.0 to +8.0 °C per mod.) obtained for the heteroatom derivatives 2′-amino-LNA[62] (**11B**, Fig. 11) and 2′-thio-LNA[63] (**11C**). A related C2′-methylene extended bicyclic nucleoside (thus containing a 2-oxapropylene linker between the O2′ and O4′ atoms), shown by X-ray crystallography and NMR studies at the nucleoside level likewise to be restricted into a C3′-*endo* furanose conformation,[64] induced less dramatic but still significantly increased duplex stabilities (+1.9 to +3.3 °C per mod.).[65]

α-L-LNA—a selected LNA stereoisomer

The inversion of the configuration around C3′ and/or C2′ and C4′ of LNA afforded three LNA stereoisomers which have been evaluated with respect to RNA binding.[66] Of these, 'α-L-LNA' (α-L-*ribo* configured LNA, **12**, Fig. 12) with inverted config-

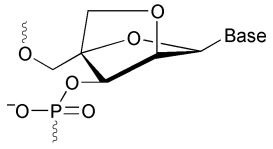


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Fig. 12 Structure of α-L-LNA.

uration around C2′, C3′ and C4′ compared to LNA, showed the most promising properties with respect to duplex formation as

shown by increased duplex stabilities of +4.3 to +5.7 °C per mod. for both partly and fully modified strands.[66–68] NMR and molecular modelling studies on duplexes of partly or fully modified α-L-LNA hybridized to DNA or RNA have established α-L-LNA as a DNA mimic and, in addition, that an α-L-LNA–RNA duplex adopts an overall conformation intermediate between the A and B form.[69–71] The possibility of RNase H activity has therefore been evaluated. Fully modified and mixmeric (consisting of alternating α-L-LNA and unmodified DNA nucleotides) α-L-LNA showed cleavage, albeit very slow, of the RNA targets at high enzyme concentrations.[61]

Summary and outlook

For efficient antisense action by the steric blocking mechanism, the use of antisense oligonucleotides containing preorganized furanose rings with *N*-type (C3′-*endo*, RNA-like) conformations and high binding affinities towards RNA target strands appears optimal. In contrast, the mechanism involving activation of RNase H demands analogues adopting *S*-type (C2′-*endo* or O4′-*endo*, DNA-like) furanose conformations.[2,41] It therefore appears impossible to take advantage of both mechanisms effectively with only one single nucleotide modification.

Despite the above points, the necessary armamentarium of chemically modified building blocks for development of efficient antisense therapeutics appears at hand. LNA, α-L-LNA and phosphoramidates, among others, offer excellent binding affinities towards RNA targets. In addition, as shorter strands (*e.g.* 10–14 nucleotides long) can be applied, good to excellent pairing selectivities are in general obtained. The binding affinity and pharmacokinetic properties are tuneable by combining these affinity-enhancing key nucleotide building blocks with, *e.g.* DNA, RNA, phosphorothioate-DNA, phosphorothioate-LNA, 2′-amino-LNA (or conjugated derivatives thereof), and 2′-*O*-alkyl-RNA nucleotides which have all been demonstrated to be applicable in DNA–LNA duplexes.[62,63,69] Whether the recruitment of RNase H is essential for efficient and general antisense action with these strongly RNA-binding nucleic acid mimics is currently not established. If RNase H activity turns out to be needed or beneficial, it is foreseen that the combination of one or more segment(s) of conformationally restricted or conformationally locked high-affinity *N*-type oligonucleotide(s) with a segment of nuclease-resistant, RNase H-activating *S*-type nucleotides with lower affinities should be ideal. A gap-mer structure is of course one possibility, but with the discovery of LNA and other extreme RNA-binders alleviating the need for binding cooperativity between the different segments, many other architectures appear possible. However, biological studies and pharmacological developments are needed to confirm these predictions.

The results obtained with ANA (**8**, Fig. 8), HNA (**9**, Fig. 9) and α-L-LNA (**12**, Fig. 12) are remarkable and need consideration. Firstly, very efficient RNA-binding is evidently possible for analogues based on a furanose skeleton with a non-natural stereochemical structure, or on an RNA-mimicking hexitol scaffold. Especially noteworthy is the dramatic RNA-binding of the α-L-*ribo* configured α-L-LNA in which the configuration at three out of the four chirality centers is inverted compared with RNA (or LNA). In addition, fully modified HNA and fully modified or mix-meric α-L-LNA have been demonstrated to induce very weak, but significant, RNase H activity. Results like these leave ample room for further curiosity-driven chemistry-based research on modified nucleic acids.

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