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Locked nucleic acid (LNA): High affinity targeting of RNA for diagnostics and therapeutics

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Locked nucleic acid (LNA) is a nucleic acid analogue containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA mimicking sugar conformation. This conformational restriction results in unprecedented hybridization affinity towards complementary single stranded RNA and thus, makes LNA uniquely suited for mimicking RNA structures and sequence specific targeting of RNA *in vitro* or *in vivo*. The focus of this paper is on LNA-antisense, LNA-modified siRNA (siLNA), and detection and analysis of microRNAs by LNA-modified oligonucleotide probes.

Introduction

The future challenges in diagnostics and treatment of diseases by siRNA and antisense technologies call for novel, improved technologies for *in vitro* and *in vivo* targeting of RNA molecules. Locked nucleic acid (LNA) comprises a class of RNA analogues in which the furanose ring of the ribose sugar is chemically locked in an RNA-mimicking conformation by the introduction of a O2',C4'-methylene linkage [1,2]. Several studies have demonstrated that LNA-modified oligonucleotides exhibit unprecedented thermal stabilities when hybridized with their RNA target molecules [1–4]. Thus, an increase in melting

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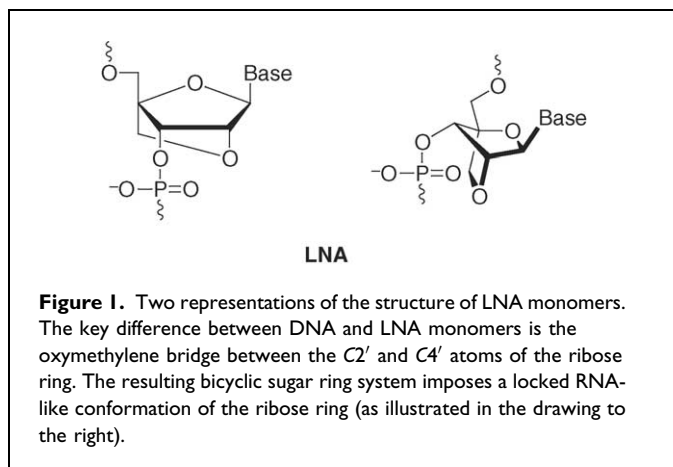
temperature (T_m value) of +2 to +10°C per LNA monomer against complementary RNA compared to unmodified duplexes has been reported. Here, we describe some basic properties of LNA followed by recent examples of applications based on LNA-mediated RNA targeting *in vitro* or *in vivo*.

Hybridization properties of LNA

We have defined LNA as an oligonucleotide containing at least one LNA monomer, that is, one 2'-O,4'-C-methylene-β-D-ribofuranosyl nucleotide (Fig. 1) [1,5]. The LNA monomers adopt an RNA mimicking N-type sugar conformation [5,6]. The majority of reports on LNA-mediated RNA targeting have utilized mixmer LNA oligonucleotides, that is, LNAs containing a limited number of LNA monomers in combination with other types of monomers, typically DNA but also RNA or 2'-O-Me-RNA monomers. The fact that LNA phosphoramidites and LNA (fully modified or mixmers) oligonucleotides are commercially available, and the compatibility of these LNA constructs with standard modifiers and modifications like phosphorothioate linkages [7], allow use of LNAs in a wide range of applications.

A key feature of oligonucleotides containing LNA nucleotides is the very high thermal stability of duplexes towards

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complementary RNA [1–3,5,7,8]. Notably, this increase in affinity goes hand in hand with preserved or even improved, Watson-Crick base pairing selectivity. Examples of melting temperatures for LNAs complexed with RNA are shown in Fig. 2. LNA:RNA base pairing is also very strong [9] (see <http://www.exiqon.com/> for LNA design tools to avoid extensive self-complementarity). The largest affinity increase per LNA monomer and the optimum mismatch discrimination is achieved for short DNA oligonucleotides with a single or several dispersed LNA monomers.

Susceptibility of LNA to nucleases

The RNA-targeting capacity of LNA makes it attractive for diagnostic and therapeutic applications, which implies contact with various nuclease-containing media. Complete stability against the 3'-exonuclease snake venom phosphodiesterase (SVPD) was reported for a fully modified LNA [10] while a significant increase in 3'-exonucleolytic stability was observed by blocking the 3'-end with two LNA monomers. In a study of oligonucleotide stability in serum, LNA mixmers were found to be very stable and LNA/DNA/LNA gapmers (see section below for definition of gapmers) to be significantly more stable than DNA alone [11]. Furthermore, Kurreck *et al.* [12] reported that DNA oligonucleotides with LNA nucleotides at the ends are more stable in human serum than the corresponding oligonucleotides composed of

phosphothioate DNA gaps with 2'-O-methyl-RNA flanks. DNase I endonuclease degradation of end-modified 30mer dsDNA showed that incorporation of one or two terminal LNA nucleotides ensures markedly increased stability [13]. From these studies it is clear that LNA nucleotides impose significant protection against nucleolytic degradation, especially if more than one LNA nucleotide is incorporated into an oligonucleotide.

RNase H-mediated cleavage of the RNA target strand of a heteroduplex formed with an antisense oligonucleotide is considered beneficial towards efficient gene silencing. Because only very few fully modified oligonucleotide analogues support RNase H-mediated cleavage, so-called gapmers, that is, oligonucleotides composed of modified segments flanking a central DNA (or phosphorothioate DNA) segment that is compatible with RNase H activity, is often used. Wahlestedt *et al.* [11] found that an LNA/DNA/LNA gapmer, with a six-nucleotide DNA gap, elicited substantial RNase H activity. However, no RNase H-mediated cleavage was observed with a fully modified 11mer LNA or with an 11mer LNA/DNA mixmer [14]. Kurreck *et al.* [12] and Elmén *et al.* [15] have investigated various LNA/DNA mixmers and gapmers and found that a gap of six DNA nucleotides is necessary for noteworthy RNase H activity, and that a gap of seven DNA nucleotides allows complete RNase H activity. In accordance herewith, Frieden *et al.* [16] concluded that a DNA gap size between 7 and 10 nucleotides is optimal for LNA/DNA/LNA antisense gapmers. The overall conclusion is that antisense LNA oligonucleotides can be designed to elicit RNase H activity while still containing LNA monomers for improved binding and target accessibility [17].

LNA-antisense for disease intervention

The term 'antisense' is generally used for nucleic acid based approaches that interferes, in a sequence selective way, with the processing of RNA from its transcription via mRNA to protein or with the effects of other forms of functional RNA. By virtue of their unprecedented RNA binding characteristics, oligonucleotides containing LNA nucleotides are obvious candidates for antisense-based gene silencing, and many previous successful LNA antisense studies have already been the subject of detailed reviews [4,17].

The first *in vivo* antisense experiment with LNA-antisense involved two different LNA sequences targeting delta opioid receptor (DOR) mRNA in the central nervous system of living rats [11]. Upon direct injection of the antisense LNA oligonucleotides into the brain a dose-dependent and highly efficacious knockdown of DOR was induced both for an LNA/DNA mixmer and an LNA/DNA/LNA gapmer. In another *in vivo* study, a fully modified LNA, targeting the RNA polymerase II gene product, inhibited tumor growth in mice and appeared nontoxic at doses less than 5 mg/kg/day [18]. These studies indicate LNA to be a much more potent

5' -TTTTTT: AAAAAA	$T_m = <10^\circ\text{C}$
5' -TTTTTT: AAAAAA	$T_m = 40^\circ\text{C}$
5' -GTGATATGC: 3' -CACUAUACG	$T_m = 28^\circ\text{C}$
5' -GTGATATGC: 3' -CACUAUACG	$T_m = 58^\circ\text{C}$
5' -GTGATATGC: 3' -CACUAUACG	$T_m = 74^\circ\text{C}$
5' -Biotin- T_{20} (DNA oligo): A_{20}	$T_m = 40^\circ\text{C}$
5' -Biotin-(TT) $_{10}$ (LNA 2.T): A_{20}	$T_m = 71^\circ\text{C}$
5' -Biotin- T_{20} (LNA full): A_{20}	$T_m = >95^\circ\text{C}$

Drug Discovery Today: Technologies

Figure 2. Some examples of melting temperatures (T_m values) for hybridization of LNA and DNA oligonucleotides to complementary RNA sequences [5,33]. LNA monomers are red, DNA monomers are black and RNA monomers are green.

class of antisense agents than the corresponding phosphorothioate DNA oligomers.

Several studies *in vitro* or in cells support the usefulness of LNA-antisense for gene silencing (e.g. [19,20]). LNA-antisense constructs targeting the RNA moiety of telomerase are potent and selective inhibitors [20] with IC_{50} values of 10 nM for a 13mer fully modified LNA and a 13mer LNA/DNA mixmer. By the introduction of two terminal phosphorothioate linkages, the potency was increased further by tenfold but this was accompanied by a decreased match versus mismatch discrimination. Notably, even very short 8mer fully modified LNAs are potent inhibitors (IC_{50} values of 2 and 25 nM with and without terminal phosphorothioate linkages, respectively). Also in cells upon employing transfection, the 13mer LNA oligomers induced inhibition of more than 80% of the telomerase activity. No alteration of cell morphology was observed seven days after transfection of 8mer LNAs [20]. These results suggest that short LNAs may provide adequate affinity and selectivity for biologically relevant RNA targeting in cells.

Viral RNA is another important target for LNA-antisense. The effects of different LNAs on the interactions of the HIV-1 *trans*-activation responsive element (TAR) have recently been published [21]. Binding of oligonucleotides to TAR can inhibit Tat-dependent transcription thereby blocking full-length HIV transcription and hence viral replication, and various LNA oligonucleotides were transfected into HeLa cells and derivatives with a minimum length of 12 residues showed 50% inhibition using nanomolar concentration of LNAs [21]. This study underlines the potential of LNA-antisense oligonucleotides for *in vivo* targeting of RNA using non-RNaseH dependent approaches. Elmén *et al.* [15] have demonstrated that LNA/DNA mixmers enhance the inhibition of HIV-1 genome dimerization relative to DNA oligonucleotides and have shown efficient uptake of the LNA/DNA mixmers in a T-cell line and that they can inhibit replication of a clinical HIV-1 isolate.

It is clear that LNA-antisense, either LNA/DNA/LNA gapmers for RNase H activation or LNA mixmers for RNase H independent activity, represents a favorable approach for gene silencing *in vitro* or *in vivo*, and reports indicate that LNA-antisense rivals siRNA as the method of choice [22]. Recently, a LNA-antisense drug directed against *Bcl-2* expression to treat chronic lymphocytic leukemia has entered Phase I/II clinical trials (<http://www.santaris.com/>).

LNA-modified siRNA (siLNA)

The discovery of RNA interference (RNAi), in which double-stranded RNA leads to the degradation of homologous RNA [23], has drawn much attention because it mediates potent gene silencing. RNAi relies on a complex cellular mechanism that has probably evolved for protection against viral attack and mobile genetic elements. A crucial step in the RNAi

mechanism is the generation of short interfering RNAs (siRNAs; double-stranded RNAs that are about 22 nt long). One study has directly compared the inhibitory effect of siRNA, LNA/DNA/LNA gapmers, phosphorothioate-DNA and 2'-O-methyl-RNA on the expression of vanilloid receptor subtype 1 in cells [22]. Both siRNA and LNA/DNA/LNA gapmers were found to be very efficient with the siRNA slightly more potent than the LNA/DNA/LNA gapmers.

siRNAs are themselves candidate molecules for incorporation of modified nucleotides for improved biostability and RNA targeting. LNA monomers have been shown to be tolerated by the RNAi machinery and to provide thermal stability [24]. The exact positioning and the overall number of LNA monomers will be very crucial for optimizing LNA-containing siRNA. To address this issue, Elmén *et al.* [25] systematically modified siRNA duplexes with LNA monomers and showed that siLNAs have substantially enhanced serum half-life compared to the corresponding siRNAs. Moreover, they provided evidence that the use of siLNAs reduce sequence-related off-target effects. In their study, Elmén *et al.* [25] reported on improved efficacy of siLNAs on certain RNA motifs relevant to targeting the SARS-CoV virus. These results emphasize the promise of siLNA in converting siRNA from a functional genomics technology into a therapeutic platform.

MicroRNA detection using LNA probes

MicroRNAs (miRNAs) are a novel class of short endogenous non-coding RNAs that act as post-transcriptional modulators of gene expression [26,27]. To date more than 1650 miRNAs have been identified in invertebrates, vertebrates and plants. Growing evidence shows that miRNAs exhibit a wide variety of regulatory functions and exert significant effects on cell growth, development and differentiation.

Recent studies have shown that human miRNA genes are frequently located in cancer-associated genomic regions, and perturbed miRNA expression patterns have been observed in many human cancers [28–30]. These findings have emphasized the potential of miRNA detection and expression profiling in cancer diagnostics. However, the detection of mature miRNAs has been technically challenging because of their small size and therefore calls for robust and improved technologies. In a recent paper, Valoczi *et al.* [31] describe highly efficient detection of miRNAs by northern blot analysis using LNA-modified oligonucleotide probes and demonstrate their significantly improved sensitivity by detecting different miRNAs in animals and plants. Besides being efficient as northern probes, LNA-modified oligonucleotide probes have proven highly useful for *in situ* localization of miRNAs in cells and tissues. In a recent study, the temporal and spatial expression patterns of 115 conserved vertebrate miRNAs were determined in zebrafish embryos by *in situ* hybridizations using LNA-modified oligonucleotide probes [32]. Most miRNAs

were expressed in a highly tissue-specific manner during segmentation and later stages, but not early in development. Thus these miRNAs may not be essential for tissue establishment but rather play crucial roles in the maintenance of tissue identity or in differentiation [32].

Conclusion

The remarkable hybridization properties of LNA, both with respect to affinity and specificity, position LNA as an enabling molecule for molecular biology research, biotechnology and RNA targeting *in vivo*. The facts that LNA phosphoramidites and LNA oligomers are commercially available and that LNA nucleotides can be freely mixed with, for example, DNA, RNA and 2'-O-Me-RNA monomers and standard probes, make LNA a highly flexible tool. LNA-antisense rivals siRNA for gene silencing, and targeting of non-coding RNAs, for example, microRNAs can be achieved very efficiently using LNA probes.

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