LNA: a versatile tool for therapeutics and genomics

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Locked nucleic acid (LNA) is a nucleic acid analogue that displays unprecedented hybridization affinity towards complementary DNA and RNA. Structural studies have shown LNA to be an RNA mimic, fitting seamlessly into an A-type duplex geometry. Several reports have revealed LNA as a most promising molecule for the development of oligonucleotide-based therapeutics. For example, Tat-dependent transcription and telomerase activity have been efficiently suppressed by LNA oligomers, and efficient cleavage of highly structured RNA has been achieved using LNA-modified DNAzymes ('LNAzyme'). Furthermore, convincing examples of the application of LNA to nucleic acid diagnostics have been reported, including high capturing efficiencies and unambiguous scoring of single-nucleotide polymorphisms.

Nucleic acid recognition between two complementary nucleic acid strands by Watson–Crick hybridization is fundamental for a vast number of processes in molecular biology, and is a complex interaction governed by subtle enthalpic and entropic contributions. During the past decade or so, exploration and examination of nucleic acid hybridization has boomed, and a multitude of nucleic acid analogues have been synthesized. This research effort has been prompted by (1) the promise of therapeutic applications, (2) possible uses within biotechnology and (3) sheer scientific curiosity.

Modifications to native nucleic acids can be introduced in the nucleobase, the sugar ring or the phosphodiester backbone [1,2]. In an effort to increase binding affinity towards RNA by conformational restriction, many sugar-modified nucleic acids have been prepared [3]. Model building revealed an O2′- to C4′-linked analogue to be of interest, owing to its conformationally locked C3′-*endo* sugar conformation, which is supposedly ideal for recognition of RNA; a 2′-*O*,4′-*C*-methylene-linked ribonucleotide derivative was subsequently synthesized [4–10] and named LNA (locked nucleic acid) (Fig. 1) [4,5]. Indeed, an X-ray crystallographic study and NMR investigations confirmed that the furanose ring of LNA monomers is locked in a C3′-*endo* (^3E, P = 17°) conformation [4,5,11].

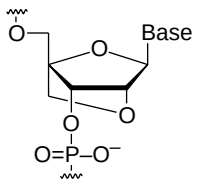
The characteristics of LNA include its close structural resemblance to native nucleic acids, which, for example, leads to very good solubility in water and easy handling. Furthermore, LNA oligonucleotides can be synthesized using conventional phosphoramidite chemistry, allowing automated synthesis of both fully modified LNA and, for example, LNA/DNA and LNA/RNA chimeric oligonucleotides [5,9]; LNA oligonucleotides [Proligo LLC (http://www.proligo.com)] and LNA phosphoramidites [Exiqon A/S (http://www.exiqon.com)] are commercially available. Finally, owing to its charged phosphate backbone, LNAs can be delivered into cells using standard protocols that use cationic transfection agents [12,13].

LNA-mediated high-affinity hybridization

Recognition of single-stranded DNA and RNA

The hybridization properties of LNA oligonucleotides have been evaluated in several different sequences, ranging from six to ~20-nucleotide long oligomers with various levels of LNA content (for reports with comprehensive tables, see for example [5,9,14,15]). Hybridization of LNA with either DNA or RNA targets has demonstrated unprecedented thermostabilities, shown by remarkable increases in melting points per LNA monomer introduced (ΔT_m values): $\Delta T_m = +1 - +8$°C against DNA [5,6,9,14,16] and $\Delta T_m = +2 - +10$°C against RNA [5,6,9,14,15,17–20]. The thermostabilities observed depend on oligomer length and composition. The impact upon introduction of LNA monomers is most pronounced for single or multiple, but isolated, modifications, and for short oligonucleotides. For instance, fully modified LNA–RNA hybrids with lengths of approximately nine base pairs have melting temperatures of nearly 80°C under standard hybridization conditions [5,18]. As such, short fully or partly modified LNA oligonucleotides combine high-affinity binding towards DNA and RNA with minimal size; it is also noteworthy that LNA displays a mismatch discrimination/selectivity equal or superior to that of native nucleic acids.

The formation of LNA–DNA duplexes has been studied using stopped-flow kinetic experiments. For three LNA octamers (LNA/DNA chimera) with one, two or three LNA



LNA definition: An oligonucleotide that contains one or more LNA monomer(s) [2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl monomer(s)]

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Fig. 1. The chemical structure and definition of locked nucleic acid (LNA).

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Box 1. The locked nucleic acid molecular family

The locked nucleic acid (LNA) molecular family (Fig. I) [a–h] shows the chemical diversity explored and the additional design freedom at hand to supplement the use of fully modified LNA, LNA/DNA and LNA/RNA chimeras.

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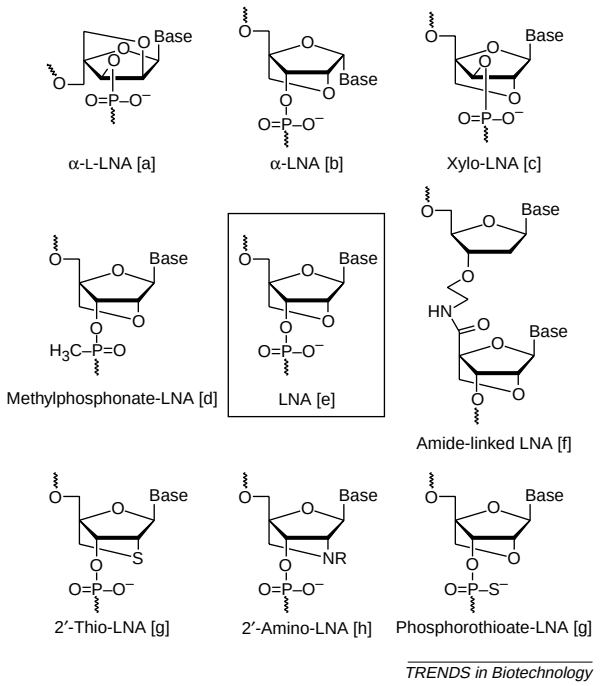

Fig. I. The LNA molecular family.

monomers, respectively, it was found that their high-affinity hybridization was a result of slower dissociation than for native DNA [21]. The rate of duplex formation was determined to be diffusion-controlled for both LNA–DNA and DNA–DNA hybridization.

LNA is a general and versatile tool for high-affinity recognition of single-stranded DNA (ssDNA) and ssRNA. A remarkable freedom exists for the design of high-affinity LNAs, both as fully modified LNA, LNA/DNA chimera and LNA/RNA chimera, and also combined with, for example, phosphorothioate linkages or 2′-*O*-Me-RNA [10,19] (Box 1). Owing to the strong base pairing in LNA–LNA duplexes, it is however, important to design LNAs without extensive self-complementary segments and/or to apply chimeric LNAs.

LNA as triplex-forming oligonucleotide

Recognition of double-stranded DNA (dsDNA) by Hoogsteen base pairing in the major groove of dsDNA is a possible avenue to gene regulation. Triplex formation is strongest at low pH, ensuring protonation of cytosine bases, and is thus relatively weak under physiological conditions [22]. A further obstacle for the generality of triplex formation is the inability to recognize non-homopurine sequences. A preliminary study involving bimolecular triple helix formation between a Y-shaped oligonucleotide with an LNA branch and a homoadenylate DNA target revealed LNA to be a promising triplex-forming oligonucleotide (TFO) [23].

Subsequently, Imanishi and co-workers have systematically studied different LNA constructs as TFOs [24–27]. Incorporation of one LNA monomer centrally in TFOs significantly increased their binding affinity ($\Delta T_m > +10°C$) [24]. Increasing the number of LNAs in the TFO induced increases in melting temperatures of +4 to +5°C per modification using 14mer TFOs at pH 6.6 [25]. Furthermore, at pH 7.2, transitions were detectable, demonstrating that triplex formation occurred even at neutral pH. A gel-retardation assay demonstrated that at pH 7.0, the binding constant of an LNA TFO was at least 300-fold higher than was that of the reference DNA TFO. These optimum results were obtained with LNA TFOs in which LNA monomers and DNA monomers were alternating in the sequence. By contrast, fully modified LNA TFOs showed no binding to dsDNA, which was attributed to their rigidity as supported by structural studies by Gotfredsen *et al.* [28]. Further studies on LNA as TFO have, for example, demonstrated decreased dissociation constant for an LNA TFO compared to the corresponding reference TFO, and improved recognition of pyrimidine nucleobases in the major groove, using various LNA monomers with unnatural aglycons [26,27].

The high-affinity hybridization properties of LNA encompass dsDNA recognition, and it appears that the optimum design of LNA TFOs is LNA/DNA chimera with alternating LNA monomers and DNA monomers. The LNA skeleton is a promising scaffold for synthesis of modified TFOs with recognition potential beyond homopurine target sequences.

Molecular structures of LNA hybrids

Several LNA–RNA and LNA–DNA hybrids have been structurally characterized by NMR spectroscopy and

Box 2. Nucleic acid structure and sugar puckers

By nature, nucleic acid duplexes have two general preferred conformations – A- and B-types. The morphological difference originates in the conformation of the sugars in the nucleic acid backbone; C3′-*endo* (*N*-type) sugar puckers impel an A-type geometry whereas C2′-*endo* (*S*-type) sugar puckers yield a B-type geometry (Fig. I). Riboses (the RNA sugar) have an inherent preference for C3′-*endo* puckers, whilst deoxyriboses (the DNA sugar) possess two energetically favourable puckers, C3′-*endo* and C2′-*endo*, with an energy barrier to interconversion of ~2 kcal mol^{-1}[a]. The C2′-*endo* pucker is the low-energy conformation, being ~0.6 kcal mol^{-1} lower in energy than is the C3′-*endo* pucker. This is the rationale for the observations that dsRNA duplexes always assume A-type geometries, whilst dsDNA duplexes adopt either A- or B-type conformations, depending on the environment (e.g. ionic strength or relative humidity).

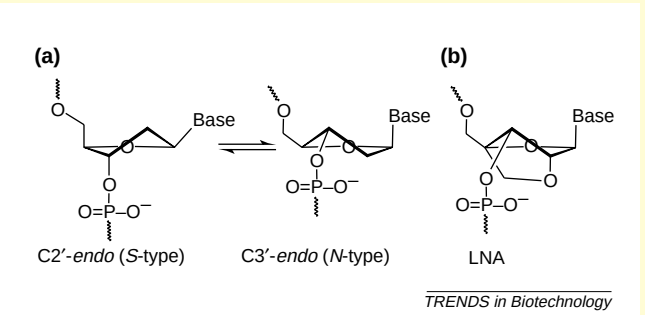

Fig. I. (a) The C2′-*endo*–C3′-*endo* sugar ring equilibrium present in nucleic acids. (b) The molecular structure of locked nucleic acid (LNA), which shows the locked C3′-*endo* sugar conformation.

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X-ray crystallography. In general, these hybrids retain the features common for native nucleic acid duplexes (i.e. usual Watson–Crick base pairing, nucleobases in the *anti* orientation, base stacking and a right-handed helical conformation).

NMR spectroscopic studies of three nonamer LNA–RNA hybrids, in which the LNA strand contained one, three and nine LNA monomers, respectively [17,29], revealed increased A-like character in the hybrids as the LNA content of the LNA strands increased. Circular dichroism (CD) spectra corroborated this observation. Notably, the hybrid with three modifications adopts a near canonical A-type duplex geometry (i.e. the LNA monomers perturb the deoxyriboses in the LNA strand so as to attain *N*-type sugar puckers, which contrasts with the equilibria between *N*- and *S*-type puckers observed in the corresponding native DNA–RNA hybrid) (Box 2). Similarly, in the LNA–RNA hybrid with one modification, the LNA nucleotide perturbs the sugar puckers of the neighbouring nucleotides, predominantly in the 3′-direction. From the hybrid with three LNA monomers, it appears that with the inclusion of three modifications in a nonamer strand a saturation level has been reached with respect to structural changes. This correlates well with the observation that the increase in helical thermostability per LNA monomer (relative to native reference duplexes) reaches a maximum for LNAs containing <50% LNA monomers [4,5,16]. This hypothesis of 'structural saturation' is validated by the structure of the fully modified nonamer LNA–RNA hybrid, as this hybrid adopts an A-type geometry (J. Rasmussen *et al.*, unpublished).

In all the LNA–RNA hybrids studied, the sugar–phosphate backbone torsion angles adopt values consistent with the A-type standard genus, and the ability of LNA to fit seamlessly into A-like duplex geometries is further indicated by the regular duplex geometry of the LNA–RNA hybrids and by an X-ray crystallographic study [30]. The structures and insignificant NMR chemical shift changes of the RNA strands in the hybrids suggest that the RNA strands are rather unperturbed by differing the number of LNA monomers in the cognate strands. This is consistent with RNA strands in duplexes usually being rigid and A-like.

When LNA monomers are incorporated into dsDNA duplexes, a similar general trend is observed, albeit with some alterations. Generally, the B-like character of the duplexes decreases as LNA monomers are incorporated [31–33]. As described above, the LNA monomers alter the sugar pucker of 3′-flanking nucleotides from a preferential *S*-type pucker in dsDNA duplexes to a mixture of *N*- and *S*-type conformations. For nucleotides located between two modifications, a further perturbation to an *N*-type sugar pucker and thus a cooperative effect is observed. In LNA–DNA hybrids, the cognate DNA strands respond to the more A-like geometry of the LNA strand by slightly increasing the population of *N*-type sugar puckers. Therefore, in a fully modified LNA–DNA nonamer hybrid, the sugar puckers of the DNA strand resemble those observed for deoxyriboses in an RNA–DNA hybrid (J. Rasmussen *et al.*, unpublished).

Changes in chemical shifts for the nuclei located in the centre of the duplex are an indicator of changes in nucleobase stacking. Such changes are more pronounced in the LNA–DNA hybrids than in the LNA–RNA hybrids studied. This indicates that a larger change in stacking interactions occurs when introducing LNA monomers in dsDNA duplexes than in DNA–RNA hybrids. However, based merely on the chemical shift observations, it cannot be concluded whether these changes correspond to more favourable stacking interactions or not.

LNA and therapeutic applications

The use of oligonucleotide analogues for regulation of gene expression is a concept eagerly pursued because of its enormous therapeutic potential [34,35]. In principle, an antisense oligonucleotide (AO), complementary to a given mRNA, is introduced into the cell and binds to the mRNA, thereby inhibiting translation. There are several mechanisms by which an AO might silence the message of the target mRNA (e.g. inhibition or alteration of splicing,

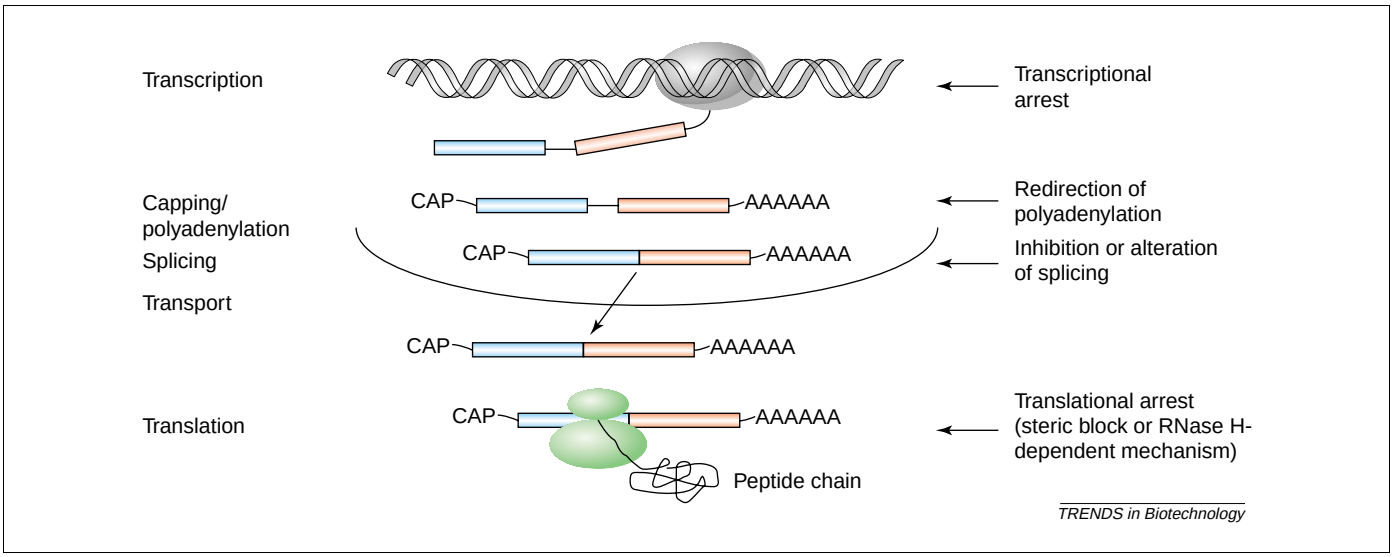

Fig. 2. The genetic flow and mRNA processing, indicating possible strategies for gene regulation.

translational arrest, redirection of polyadenylation and degradation of the mRNA, for example by ribonuclease H (RNase H; Fig. 2) [36]). In one preferred scenario, RNase H selectively cleaves the RNA strand of the AO–mRNA hybrid, thus inactivating the mRNA and rendering the AO free to interact with another cognate mRNA. However, an advantage of the steric block mechanism (strong binding leading to translational arrest) is that an AO hybridized to an incorrect RNA by virtue of partial complementarity would be less likely to lead to unwanted gene inactivation [13].

To be functional, AOs need to meet several requirements [37] (e.g. sufficient biostability and ability to penetrate cell membranes). Furthermore, it is difficult to select the base sequence of an AO because of the unknown secondary and tertiary structures of the mRNA. This is true, for example, for the phosphorothioate DNA antisense standard owing to its relatively weak RNA-binding properties. Consequently, it might be necessary to screen dozens of potential AOs before identifying one that functions adequately [38]. Finally, once a potent AO has been identified, the dose required to inhibit expression might not be much different from those causing nonselective toxicity [39]. In spite of these hurdles, it is noteworthy that >12 AOs are in various stages of clinical trails, and that one 21mer phosphorothioate-DNA is approved as a drug (Vitravene™). Stimulated by the chemical and biophysical properties of LNA, several recent studies have focused on the use of LNA for therapeutic purposes.

RNase H activity of LNAs

RNases H are enzymes ubiquitous in human, animal and bacterial cells. RNases H recognize DNA–RNA hybrids and perform endonucleolytic cleavage of the RNA strand, yielding a 3′-hydroxyl and a 5′-phosphate at the hydrolysis site. RNases H also bind dsRNA duplexes, but without inducing cleavage, whilst dsDNA duplexes are not recognized by the enzyme. It is conceivable that the differences in minor groove width between dsDNA, DNA–RNA and dsRNA duplexes is one of the key elements that determines RNase H recognition and subsequent scission of the RNA strand [40,41]. As it happens, only few modified AOs allow RNase H-mediated cleavage of a cognate mRNA. This has stimulated the development of so-called gapmer AOs, comprising two modified nucleolytically stable segments flanking a central DNA (or phosphorothioate DNA) segment, which ensures RNase H degradation of the mRNA strand [42].

In a few studies, giving somewhat contradictory results, the RNase H activity of various LNA constructs has been investigated. Wahlestedt *et al.* [12] examined two 15mer LNAs, and found that both an LNA/DNA/LNA gapmer (with a six-nucleotide DNA gap) and a so-called LNA/DNA mixmer (with six DNA and nine LNA monomers interspersed) elicited RNase H activity, albeit at a slow rate for the mixmer. By contrast, Sørensen *et al.* [43] did not observe RNase H-mediated cleavage with either a fully modified 11mer LNA or an 11mer LNA/DNA mixmer. Recently, a rigorous RNase H investigation of various LNA/DNA mixmers and LNA/DNA/LNA gapmers found that a gap of six DNA monomers is necessary to restore 65% RNase H activity (relative to the corresponding DNA reference), and that a gap of seven DNA monomers restores complete RNase H activity [15]. Interestingly, a gap of just six DNA monomers restores full RNase H activity for 2′-*O*-Me-RNA-modified gapmers. Once RNase H activity is restored, the efficiency of an oligonucleotide to elicit RNase H cleavage correlates with its affinity for the target RNA (i.e. LNA > 2′-*O*-Me-RNA > DNA > phosphorothioate DNA [15]).

Antisense studies with LNAs

For antisense applications of LNA (Table 1) it is important to note that the introduction of just two or three terminal LNA monomers in a DNA strand, or the use of LNA/DNA mixmers, ensures substantial serum stability [15] – one of the prerequisites for therapeutic use of oligonucleotides.

Wahlestedt *et al.* [12] have reported promising *in vivo* antisense activities of two different LNA sequences targeting the mRNA encoding a G-protein-coupled receptor, δ-type opioid receptor (DOR), in the rat central nervous system. First, it was observed that when injected directly into the brain of living rats, LNAs did not convey

Table 1. Selected LNAs used in gene regulation experiments[a]

Sequence[b]	Active against	Mode of action	Refs
5′-(G**T**^{L}G**T**^{L}**C**^{L}**C**^{L}G**A**^{L}G**A**^{L}**C**^{L}G**T**^{L}**T**^{L}G)	DOR-AS-1 (*in vivo*)	RNase H	[12]
5′-(**G**^{L}**T**^{L}**G**^{L}**T**^{L}CCGAGA**C**^{L}**G**^{L}**T**^{L}**T**^{L}**G**^{L})	DOR-AS-1 (*in vivo*)	RNase H	[12]
5′-(G**C**^{L}**C**^{L}**C**^{L}AAGCTGGCA**T**^{L}**C**^{L}**C**^{L}G**T**^{L}**C**^{L}A)	ICAM-1 (*in vitro*)	RNase H	[20]
5′-(G**C**^{L}**C**^{L}**C**^{L}AAGC**T**^{L}GGCA**T**^{L}**C**^{L}**C**^{L}G**T**^{L}**C**^{L}A)	ICAM-1 (*in vitro*)	RNase H	[20]
5′-(**C**^{L}**U**^{L}**C**^{L}**C**^{L}AGG**C**^{L}**U**^{L}**C**^{L}A)[c]	HIV-1 Tat (in cells)	Steric block	[13]
5′-(**C**^{L}$_{PS}$**A**^{L}**G**^{L}TT**A**^{L}**G**^{L}**G**^{L}**G**^{L}**T**^{L}TA^{L}$_{PS}$**G**^{L})	Telomerase (in cells)	Steric block	[18]
5′-(**T**$_{PS}$**AGGGTT**$_{PS}$**A**)^{L}	Telomerase (in cells)	Steric block	[18]
5′-(T**C**^{L}T**A**^{L}**C**^{L}**G**^{L}A**C**^{L}**G**^{L}**G**^{L}C**C**^{L})	*Candida ablicans* (*in vitro*)	Inhibition of splicing	[47]
5′-(**TACCTTTC**)^{L}	*Candida ablicans* (*in vitro*)	Inhibition of splicing	[47]

[a]Abbreviation: LNA, locked nucleic acid.
[b]**X**^{L} denotes LNA monomers and **C**^{L} denotes 5-methyl cytosine LNA monomers.
[c]This sequence is a 2′-*O*-Me-RNA/LNA chimera.

tissue damage. It was therefore suggested that acute LNA toxicity is minimal in the living rat brain. Second, a dose-dependent knockdown of DOR was observed with both an LNA/DNA mixmer and an LNA/DNA/LNA gapmer. Both AOs were highly efficacious, with potencies exceeding that of the isosequential phosphodiester.

Obika *et al.* [20] have targeted expression of intercellular adhesion molecule-1 (ICAM-1) with three different LNA oligomers. An LNA/DNA/LNA gapmer containing a nine-nucleotide DNA gap was a potent inhibitor of ICAM-1 expression in a dose-dependent manner in HUVEC cells. Only slightly less potent was an LNA/DNA/LNA gapmer in which the gap had the constitution d_4Ld_4, where d denotes DNA monomers and L an LNA monomer. These LNAs induced RNase H activity in assays, which suggests that the antisense activity observed was RNase H-dependent.

In two reports, LNA oligomers have been applied as steric block antisense molecules. The HIV-1 *trans*-activation responsive element (TAR) is a 59-nucleotide stem–loop RNA that interacts with the HIV *trans*-activator protein Tat and other cellular factors to stimulate transcriptional elongation from the viral long-terminal repeat (LTR) [44]. Inhibition of these interactions blocks full-length HIV transcription and hence viral replication. In addition, there is a high degree of sequence conservation in this region of HIV RNA, making it an attractive target for drug design [45].

Arzumanov *et al.* [13] have applied two 12mer-LNA oligomers as steric blockers of the HIV-1 TAR domain, one a chimeric LNA consisting of seven LNA monomers and five 2′-*O*-Me-RNA nucleotides (with the LNA monomers interspersed between the 2′-*O*-Me-RNA residues) and the other an LNA consisting of 11 contiguous LNA residues and a terminal 2′-*O*-Me-RNA residue [13]. Under the ionic conditions of *in vitro* transcription, both LNAs, in addition to isosequential peptide nucleic acid (PNA) and 2′-*O*-Me-RNA oligomers, inhibit Tat-dependent transcription. However, when introduced into HeLa cells, only the LNA composed of seven LNA monomers and five 2′-*O*-Me-RNA monomers showed dose-dependent sequence-specific inhibition of Tat-dependent HIV-1 LTR *trans*-activation in a stably integrated plasmid system involving double luciferase reporters [13].

Telomerase is a ribonucleoprotein containing an RNA domain that binds to telomere ends and a protein domain responsible for maintaining telomere length from one generation to the next [46]. Telomerase is expressed in cancer cells but not in adjoining normal tissue, leading to the hypothesis that telomerase inhibition leads to reduced tumour-cell proliferation. The RNA component of telomerase contains an 11mer sequence that binds telomeric DNA and guides the addition of telomeric repeats. This template sequence is an attractive target for oligonucleotide-induced inhibition because its role in telomerase activity depends on it being accessible to hybridization by a cognate nucleic acid [46]. Elayadi *et al.* [18] have targeted the telomerase template with LNA oligonucleotides, and found them to act as potent and selective inhibitors. 13mer LNAs with terminal phsophorothioate linkages were active with IC_{50} values down to 1 nM, and even short 8mer LNAs were potent inhibitors, with IC_{50} values down to 2 nM. Because LNAs containing mis-matches to the telomerase template RNA showed substantial deterioration in potency, the mechanism of inhibition is probably competitive sterical blocking of the template RNA. It is noteworthy that the 8mer LNA was a 200-fold better inhibitor than was the analogous PNA [18].

When the LNA oligomers directed against the template region of telomerase were transfected into cells, >80% of telomerase activity was inhibited [18]. There was no apparent alteration of cell morphology within seven days after transfection of 8mer LNAs. Thus, these results suggest that short LNAs might not only possess adequate affinity but also sufficient selectivity for targets inside cells.

Non-coding RNA as target for LNAs

Targeting non-coding regions of RNA is emerging as a promising therapeutic strategy, and recently, LNA has shown sequence-specific inhibition of *Candida albicans* group I intron splicing *in vitro* by stabilizing an inactive RNA structure [47]. In this study an 8mer fully modified LNA and a 12mer LNA (LNA/DNA chimera with six LNA monomers) displayed 50% inhibition at concentrations of 150 nM and 30 nM, respectively, whereas higher concentrations were needed for the analogous DNA and 2′-*O*-Me-RNA oligomers to obtain similar inhibition [47]. These results reveal a therapeutic strategy in which LNA has tremendous potential, owing to its superior RNA-binding properties that allow invasion into structured regions of RNA.

Status on LNA as antisense molecule

Encouraging reports on the therapeutic potential of LNA oligomers have appeared recently (Table 1). For general recruitment of RNase H, it appears that the use of LNA/DNA/LNA gapmers should be the strategy of choice. Combining the results of Kurreck *et al.* [15] and Elayadi

et al. [18], one might speculate that an LNA gapmer constitution with a gap of seven or eight DNA monomers flanked by LNA segments possibly containing some phosphorothioate linkages and some DNA monomers would be the optimum design. In a steric block approach, the overriding feature of LNA is the high-affinity binding of cognate RNA. This feature enables the design of ultra-short LNAs with high therapeutic potency, as demonstrated for fully modified LNA [18,47], for LNA/2′-*O*-Me-RNA chimera [13] and for LNA/DNA chimera [47].

LNA in diagnostics

Single-nucleotide mutations in the genetic code are the cause of several ailments. The traditional methods of scanning for single-nucleotide polymorphisms (SNPs) are generally time-consuming and difficult to automate because enzymatic digestion and/or gel electrophoresis are required. Easy-to-use SNP assays based on the LNA technology have been designed and implemented. These assays rely on the observation that single-nucleotide mismatch discrimination is better for LNA than for DNA (i.e. the difference in melting temperature for a perfect match and a single-nucleotide mismatch is larger for LNA oligomers than it is for DNA oligomers [48]).

Currently, three LNA genotyping assays have been reported, screening for the factor V Leiden mutation, an apolipoprotein B (apoB) R3500Q mutation and two mutations in apolipoprotein E [48–50]. In these assays, 8mer LNA-capture probes (complementary to either the wild-type or the mutated genomic sequence) were covalently attached to individual wells of a microtiter plate [48–50]. Subsequently, hybridization of PCR amplicons was scored colourimetrically with an ELISA-like technique. The assays have been carefully evaluated and give results in full agreement with DNA sequencing.

Likewise, short LNAs have been applied for efficient SNP scoring using fluorescence polarization detection [51]. LNA probes were labelled with either rhodamine or hexachlorofluorescein, and hybridization to complementary target DNAs caused significant fluorescence polarization, whereas no change in fluorescence polarization was observed with targets containing a single mismatch. This homogenous assay can be multiplexed, and holds promise for the generation of a universal set of genotyping reagents.

Immobilized LNA probes also have been successfully used in a multiplex SNP genotyping assay based solely on hybridization between capture probe and target on a microarray platform [52].

It is evident that LNA, owing to its high affinity and efficient mismatch discrimination, should find significant use within diagnostics based either on more traditional assays or on DNA microarrays.

Exploratory applications of LNA

LNA as aptamers (decoys)

Aberrant activation and expression of genes, whose products are involved in initiation and progression of pathogenesis, cause several diseases. In recent years, strategies targeting transcription-activating proteins have emerged. One such strategy is the use of dsDNAs bearing the consensus binding sequence of a specific transcription factor. Once transfected into cells, these dsDNA aptamers, dubbed decoys, interact with the target factor. Interaction with the decoy results in incapacitation of the protein for subsequent binding to the promoter regions of target genes, hence causing reduction in, or at best, blockage of transcriptional activation [53]. Crinelli *et al.* [54] have applied various LNA oligomers as decoys for transcription factor κB (NF-κB). Inclusion of one or two terminal LNA monomers outside the κB sequence increased the protection towards nuclease digestion appreciably, without interfering with transcription-factor binding. When including LNA monomers in the κB sequence, further stabilization towards degradation was observed. However, these modifications also interfered with NF-κB binding, although in a manner dependent not only on the extent but also on the positioning of the internal LNA substitutions. These sequence-specific results are hardly surprising considering the structural implications of the inclusion of LNA monomers as described above and that NF-κB makes extensive contacts with both the nucleobases and the phosphate backbone of the recognized dsDNA. From X-ray crystallographic studies of NF-κB bound to dsDNA and the structural properties of LNAs, it might be possible to design NF-κB LNA decoys, with maximal nuclease stability, that are suitable for efficient recognition by the protein.

LNAzymes

Deoxyribozymes (DNAzymes) are catalytically active DNA molecules that can function as specific RNA endonucleases by binding to predetermined sequences in an RNA, with subsequent cleavage of the RNA phosphodiester backbone in the presence of divalent ions [55]. The '10–23' DNAzyme is a 31-nucleotide oligomer, which consists of a 15-nucleotide catalytic core flanked by two binding arms. The binding arms control the specificity of the DNAzyme by hybridization to an RNA substrate adjacent to the scissile phosphodiester linkage. Incorporation of LNA monomers into the binding arms of the DNAzyme yielded an LNAzyme (Fig. 3), which showed strongly enhanced efficiency of RNA cleavage, both with a 58-nucleotide RNA target and with a naturally occurring 23S-rRNA target [56]. As the cleavage site for both substrates is within a highly structured region of the RNA, these results clearly show that compared to a DNAzyme, an LNAzyme gains significantly improved access to an RNA target. This has important implications for the applicability of LNAzymes, but it also holds promise for the generality of LNA as a tool for gene regulation. Because distinctly increased rates of RNA cleavage were observed with the LNAzyme under multiple turnover conditions, the introduction of LNAzymes could be a significant step towards the realization of oligonucleotide-based therapeutics with intrinsic endonucleolytic activity [56].

Conclusions and outlook

LNA is a general and versatile tool for high-affinity recognition of ssDNA, ssRNA and dsDNA. Not only can fully modified LNA be applied but so can, for example, LNA/DNA, LNA/RNA, LNA/2′-OMe-RNA and LNA/phosphorothioate-DNA chimeras. This unique freedom of LNA

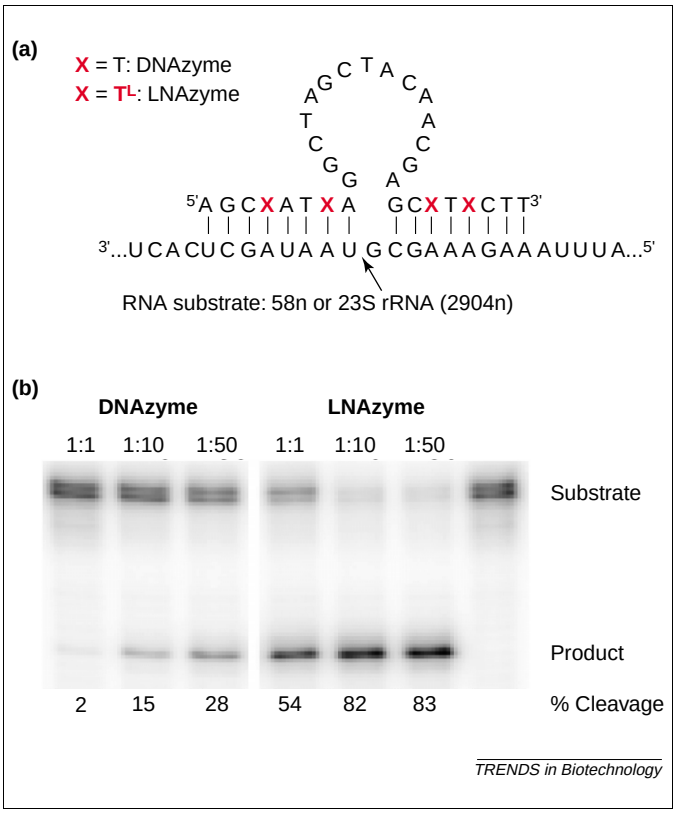

Fig. 3. (a) Sequences of a DNAzyme and the corresponding LNAzyme, and hybridization to the RNA target. The cleavage site of the target RNA is indicated by an arrow. (b) Denaturing gel electrophoresis after cleavage (60 min incubation) of a structured 58n-RNA substrate under single turnover conditions ('1:1', '1:10' and '1:50' denote substrate:DNAzyme/LNAzyme ratios used).

design offers the possibility of fine-tuning the hybridization of virtually any oligonucleotide and also the use of known oligonucleotide modifications and technologies in combination with LNA.

Several reports have recently appeared on the successful employment of LNA oligomers for gene regulation. Thus, potent and selective LNA oligomers have been identified in antisense studies *in vitro* and *in vivo*, and even LNAs shorter than ten nucleotides have shown low-nM IC_{50} values without significant toxicity. In addition, targeting LNA to noncoding RNA segments has been shown to interfere with gene regulation.

LNA is also useful in nucleic acid diagnostics, where it offers high capturing efficiencies and unambiguous SNP scoring, both using more traditional assays and DNA microarrays.

Compared to unmodified DNAzymes, LNAzymes exhibit distinctly increased rates of RNA cleavage (under both single- and multiple-turnover conditions) at a site within a highly structured region of the target RNA. The improved target RNA accessibility by LNA hybridization has important implications for the applicability of LNAzymes and also for the use of LNA in general as an RNA-binding tool for gene regulation.

Numerous other exciting experiments and applications based on LNA can be foreseen, including probing the interactions between RNA and other biomolecules, studying RNAi processes, evaluating the general applicability of LNA within molecular biology, improving aptameric structures or building complex molecular architectures within nanobiotechnology. LNA is therefore likely to have a significant impact on future developments within many areas of biotechnology and medicine (Boxes 1 and 2).

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