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Chemistry & Biology 8 (2001) 1–7

Chemistry & Biology

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Minireview

Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA

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Received 20 October 2000; revisions requested 2 November 2000 revisions received 7 November 2000; accepted 17 November 2000
First published online 19 December 2000

Abstract

Locked nucleic acid is an RNA derivative in which the ribose ring is constrained by a methylene linkage between the 2′-oxygen and the 4′-carbon. This conformation restriction increases binding affinity for complementarity sequences and provides an exciting new chemical approach for the control of gene expression and optimization of microarrays.

Keywords: DNA recognition; Locked nucleic acid; Gene expression control; RNA recognition

1. Introduction

In 1978, Zamecnik and Stephenson [1] first focused wide attention on the potential of oligonucleotides as powerful tools for controlling gene expression. Since then, DNA and RNA analogs containing diverse chemical features have been developed in attempts to improve potency, specificity and therapeutic efficacy (Fig. 1). One of these analogs, phosphorothioate DNA, has provided the first approved oligonucleotide drug, Fomivirsen [2], and 2′-*O*-alkyl RNA–DNA chimera are being used in several ongoing clinical trials [3]. Oligonucleotides are also effective tools for functional genomics, and ISIS Pharmaceutical has developed high-throughput screening assays to rapidly identify oligonucleotides that can reduce the expression of biologically important genes and validate novel therapeutic targets (http://www.isip.com).

In spite of this progress, the successful application of oligonucleotides depends on a number of factors (Table 1) and it has recently been estimated that up to 90% of published work using oligonucleotides to inhibit gene expression may be at least partially unreliable [4]. Because oligonucleotides are not yet a routine tool for chemical genetics, attention continues to be focused on the development of additional oligonucleotide analogs [5]. The use of oligonucleotides as research tools and diagnostic agents is so pervasive that even modest improvements in their properties are likely to have a large impact. Not only would improved oligomers be more effective antisense agents, they would also provide new options for diagnostic tests, enhanced probes for basic research, and tools for gene array analysis that provide better sensitivity. Examples of promising analogs include, peptide nucleic acid (PNA) [6], phosphoramidate DNA [7], hexitol nucleic acid (HNA) [8] and morpholino [9] oligomers (Fig. 1, Table 2). This minireview will describe the properties of one new analog, locked nucleic acid (LNA), that may possess important advantages for the recognition of complementary sequences.

2. LNA

In 1998, the Wengel [10,11] and Imanishi [12] laboratories described oligomer synthesis and hybridization using a novel nucleotide termed LNA (Fig. 1), with a subsequent report from the Wang laboratory appearing in 1999 [13]. LNA nucleotides contain a methylene bridge that connects the 2′-oxygen of ribose with the 4′-carbon. This bridge results in a locked 3′-*endo* conformation, reducing the conformational flexibility of the ribose and increasing the local organization of the phosphate backbone.

LNA bases are linked by the same phosphate backbone found in DNA or RNA, allowing LNA oligomers to be

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PII: S1074-5521(00)00058-2

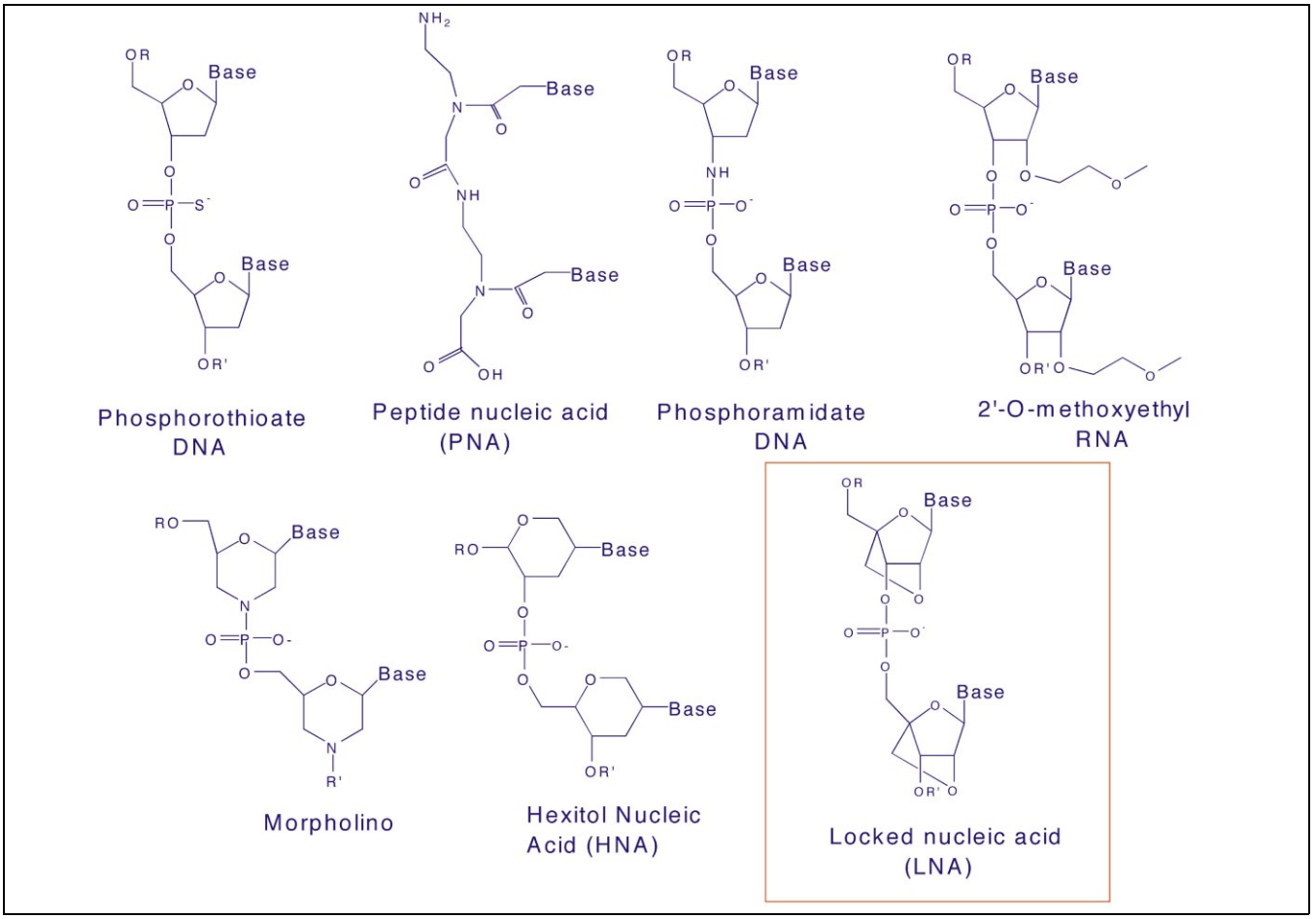

Fig. 1. Nucleic acid analogs studied for their potential as antisense agents. Phosphodiester DNA, phosphorothioate DNA, PNA, phosphoramidate DNA, morpholino and LNA.

obtained using standard reagents and automated synthesizers. The use of standard reagents and synthesizers avoids the need to develop new protocols and is an important practical advantage. The similarity of LNA to DNA/RNA synthesis also permits LNA bases to be interspersed among DNA and RNA, permitting the properties of LNA-containing oligomers to be fine-tuned. For example, as discussed below, the ability to mix DNA and RNA bases allows the affinity for complementary sequences or the susceptibility to RNase H to be optimized for individual applications. Another practical advantage is that LNAs are as soluble as DNA or RNA, facilitating their handling and simplifying experiments. Finally, because LNAs possess a charged phosphate backbone they can be delivered into cells using standard protocols that employ cationic lipid.

Several protocols for the synthesis of LNA monomers have been published [10–14]. LNA monomers are not yet commercially available, but the custom synthesis of LNAs is offered by Proligo (Boulder, CO, USA, http://www.proligo.com). Prices for commercial LNA synthesis are high, but it is expected that they will fall as the demand for LNAs increases. Availability of custom synthesis sets LNA apart from other oligonucleotides with altered sugar backbones [5], such as pentopyranosyl [15] or HNA [8] oligomers that also bind with high affinity but that are not generally available.

3. Structures of LNA hybrids

Nuclear magnetic resonance has been used to determine solution structures of LNA–DNA chimeras bound to DNA [16] or RNA [17]. Both the RNA and DNA hybrids form right-handed helices containing normal Watson–Crick pairs with all the bases in the *anti* conformation. The LNA bases within the DNA–LNA strand of the RNA complex induce flanking DNA bases to adopt an A type conformation. This is an important observation because the ability of LNA bases to affect the conformation of the DNA strand will affect its ability to activate RNase H cleavage of bound RNA, an important consideration for antisense applications (see below). Both structures also suggest that enhanced stability of LNA is due to local organization of the phosphate backbone which increases the strength of base stacking interactions.

Table 1
Factors affecting the utility of DNA/RNA analogs and mimics

Factor	Comments
RNase H activity	Catalytic destruction of mRNA amplifies efficacy. Oligomers can be targeted through mRNA.
Affinity for target sequence	High affinity is necessary for efficient binding under physiologic conditions and may lead to more efficient gene arrays.
Clinical use	Any application aimed at therapy will benefit from prior clinical experience with oligomers that possess the same chemical features.
Nuclease resistance	The oligomer must be stable inside the cell.
Commercial availability	Necessary for wide use.
Standard synthetic procedures	Synthetic procedures adapted from DNA or peptide synthesis are familiar to many researchers and allow oligomers to be readily obtained.
Solubility	Some oligonucleotide analogs, particularly those that are uncharged, are less soluble than others.
Methods for cellular delivery	The use of cationic lipid is a standard procedure, although other methods are available. Oligomers containing phosphorothioate linkages are spontaneously taken up by some animal and human tissues.
Binding to cellular proteins	A major cause of nonspecific effects, but also a contributor to favorable pharmacokinetics. Phosphorothioate linkages contribute to protein binding, as does the potential to form secondary structure such as Q-quartets.
Toxicity	Low toxicity in clinical trials to date. Toxicity in cell culture can be unpredictable and sequence-dependent. Proper controls are a necessity.
Ability to act as a primer	Necessary for applications involving polymerase chain reaction, sequencing.

4. Affinity of LNAs for complementary sequences

Several studies have been published that demonstrate that LNAs have improved affinities for complementary DNA and RNA sequences (Table 3) (Fig. 2). Melting temperatures (T_ms) have been increased by as much as 41°C relative to DNA:DNA complexes of the same length and sequence, and addition of a single LNA base can raise the T_m value by as much as 9.6°C. As more LNA bases

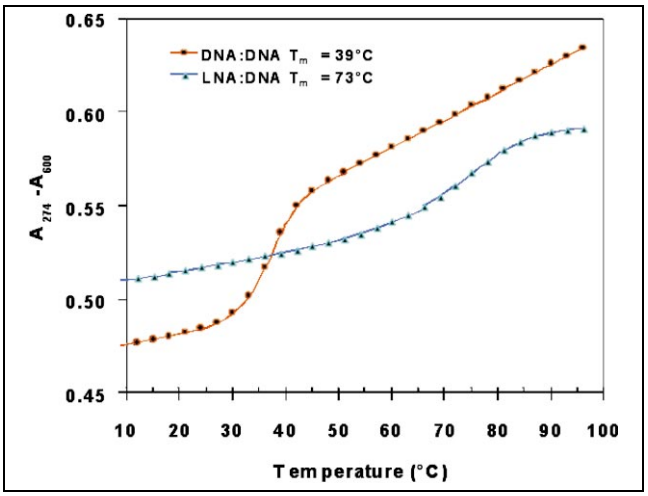

Fig. 2. LNAs bind tightly to DNA. Melting curves for an LNA oligonucleotide bound to complementary DNA compared with the melting curve for the analogous all-DNA duplex. The sequence of the LNA is 5′-AGGATCmeTAGG-3′ (Braasch, unpublished results).

are incorporated, the increase per LNA base declines. In some LNA–DNA chimeras the inclusion of LNA bases changes the T_m values by less than 2°C per base. Longer oligomers that would have high T_m values regardless of the presence of LNA also show smaller effects when LNA bases are included. Taken together, these data indicate that inclusion of LNA bases will have the greatest effect on oligomers less than 10 bases long. Short all-LNA oligomers combine high affinity binding with minimal size.

We have observed that LNA binding is sensitive to the presence of mismatched bases (Braasch, unpublished results). However, because LNA offers the potential to obtain oligomers that bind with exceptionally high affinity, the design of mismatch-containing oligomers will need to be carefully considered to ensure adequate discrimination between fully complementary and mismatch-containing LNAs. Homopyrimidine LNAs can also bind to duplex DNA by triple helix formation [18,19]. This triplex formation occurs at neutral pH [19] and suggests that LNA may

Table 2
Properties of chemically modified oligomers

Oligomer chemistry	RNase H activity	Elevated affinity	Clinical use	Nuclease resistant	Commercially available
PO-DNA	yes	no	no	no	yes
PO-RNA	no	no	no	no	yes
PS-DNA	yes	no	yes	yes	yes
2′-*O*-MeRNA	no	yes	no	yes	yes
2′-*O*-MOE-RNA	no	yes	no	yes	no
2′-*O*-MeRNA/DNA	yes	yes	yes	yes	yes
2′-*O*-MOE-RNA/DNA	yes	yes	yes	yes	no
PNA	no	yes	no	yes	yes
Phosphoramidate	no	yes	no	yes	yes
Morpholino	no	yes	no	yes	yes
LNA	yes[a]	yes	no	yes	yes

PO = phosphodiester; PS = phosphorothioate; Me = methyl; MOE = methoxyethoxy; PNA = peptide nucleic acid; LNA = locked nucleic acid.
[a] Especially as LNA–DNA chimera.

Table 3
Melting temperature (T_m) values for LNA and LNA/DNA

LNA or LNA/DNA	T_m	T_m(ref)	ΔT_m	ΔT_m/LNA base	Refs.
GTGATATGC	44	28	16	5.3	[10]
GTGAUATGC	44				[10]
GTGAGATGC	49	33	15	5	[10]
GTGACATGC	52				[10]
GTGAAATGC	45				[10]
GTGATATGmeC	64	28	36	4.5	[10]
GGTGGTTTGTTTG	57	47	10	2.5	[10]
GGTGGTTTGTTTG	83	47	36	3	[10]
GCGTTTTTTGCT	50	47	3	3	[12]
GCGTTTTTTGCT	51	47	4	2	[12]
GCGTTTTTTGCT	58	47	11	1.8	[12]
TCTCTCTCCCTTTT	54	46	8	1.1	[12]
TCTCTCTCCCTTTT	65	46	19	3.2	[12]
TCTCTCTCCCTTTT	74	46	28	2.2	[12]
CmeCmeTCmeGCmeCmeT	80	44	36	2.5	[29]
CmeCmeTTGCmeCmeT	71	36	35	2.45	[29]
AGGCmeAAGG	77	36	41	5	[29]
GTGTTTTGC	52	28	24	5	[28]
GTGTCCGAGA**CGTTG**	72	59	13	1.5	[27]
GTGTCCGAGACGTTG	83	59	24	3	[27]
GTGTCCGAGACGTTG	>90	59	>31	2	[27]
CACTATACG	40	29	11	3.3	[30]
CTGATATGC	36.8	27.2	9.6	9.6	[17]
CTGATATGC	51.6	27.2	24.4	8.1	[17]
ATCTCTCCGCTTCCTTTC	65	58	7	0.8	[13]
AGGGTCGCTCmeGGTGT	>96	53	43	3	[*]
AGGGTCGCTCmeAATGT	83				[*]
CmeAGTTAGGGTTAG	81	50	31	3.1	[*]
CmeAGTTAGAATTAG	65				[*]
TAGGGT	56	nd	-	-	[*]
TAGGGTTA	74	22	52	6.5	[*]
AGGATCmeTAGGTGAA	>96	71	25	2.9	[*]
AGGATCmeTAGG	73	39	34	3.4	[*]
AGGATCmeTAGGTGAA	59	53	6	0.6	[*]

Reference T_m values are for analogous DNA oligonucleotides. Boldface bases are LNA. Cme = 5-methylcytosine. All oligomers are shown from 5′ to 3′. nd: melting temperature not detected. [*]: Dwaine Braasch, unpublished results.

offer advantages for recognition of duplex DNA within cells.

5. RNase H activation by LNAs and LNA–DNA chimera

RNase H is an enzyme that normally cleaves the RNA strand of RNA–DNA hybrids formed during lagging strand synthesis. DNA oligonucleotides can bind mRNA and activate RNase H, causing the target mRNA to be degraded [20] (Fig. 3). This is a critical determinant for the efficacy of antisense oligonucleotides because the DNA is then free to bind additional mRNAs, amplifying its ability to inhibit gene expression. Oligomers that cannot activate RNase H, such as PNA [6,21,22] or 2′-*O*-alkyl RNA [23,24] can also act as antisense agents, but are most active when targeted to the terminal sequence of the 5′-untranslated region where they can block binding of the translation apparatus (Fig. 3). Oligomers that cannot activate RNase H are less active when targeted elsewhere within mRNA because they can be displaced by the ribosome as it carries out translation, but are useful for applications such as altering splicing [25] or inhibiting ribonucleoproteins such as telomerase [26] where they are less readily displaced.

Wengel et al. have found that oligonucleotides containing only LNA bases form hybrids with RNA that activate RNase H less well than do corresponding DNA oligonucleotides [27]. Taking advantage of the standard synthesis of DNA and LNA to obtain DNA–LNA chimera, Wengel et al. have observed that these chimeric oligonucleotides readily activate RNase H. The LNA bases confer enhanced binding, while DNA bases create a suitable RNA–DNA hybrid to act as a substrate for RNase H. These results demonstrate that LNAs are flexible reagents and that RNase H activation can be exploited or avoided depending on the demands of given experimental strategies and targets. It is possible that LNAs may be able to bind throughout mRNA and function effectively as antisense agents by virtue of their exceptionally tight affinities for complementary RNA sequences, but this outcome has not yet been experimentally demonstrated.

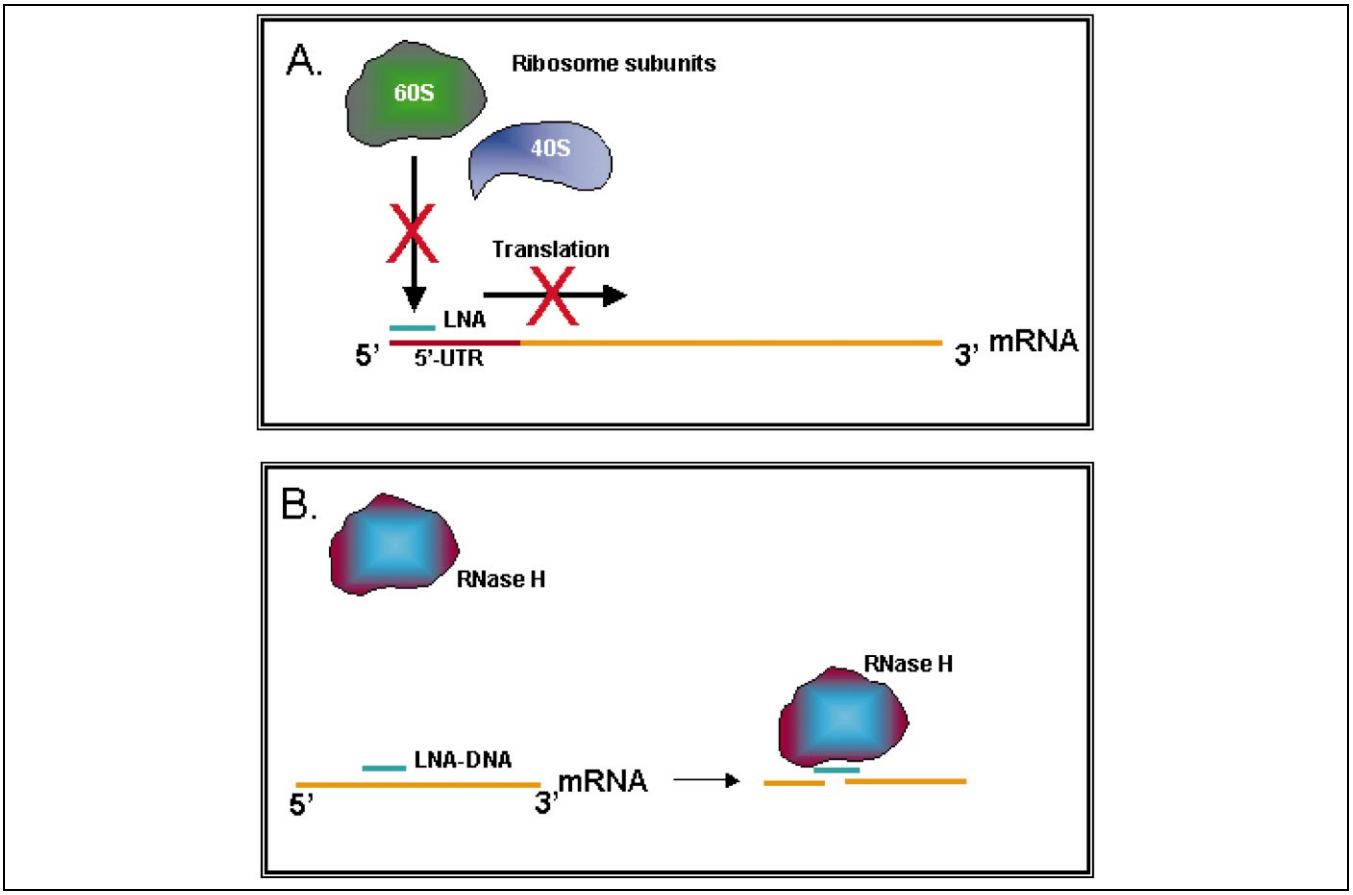

Fig. 3. Mechanisms for inhibition of gene. (A) Inhibition of gene expression by an antisense oligonucleotide that binds to the 5′-untranslated region and blocks binding of the translation apparatus. (B) Promotion of RNA cleavage and inhibition of gene expression by an antisense oligonucleotide that can activate RNase H.

6. Stability of LNAs to cleavage by nucleases

To be effective inside cells or in cell extract antisense oligomers must be stable towards digestion by nucleases. Wengel et al. have reported that oligomers that contain mixtures of LNA and DNA bases are much more resistant to degradation in rat blood serum than the analogous DNA oligonucleotide [27]. The degree of nuclease resistance varies depending on the location of DNA and LNA bases, with placement of LNA bases at the 3′ to 5′ termini being an effective strategy to lessen hydrolysis by exonucleases. As with affinity and RNase H activation, it is likely that nuclease sensitivity can be fine-tuned by judicious placement of LNA and DNA bases. Another standard approach to improving stability of antisense DNA and RNA oligomers is to incorporate phosphorothioate linkages. Wengel et al. have shown that this is possible and that the resulting phosphorothioate LNAs were able to recognize complementary sequences with affinities comparable to analogous phosphodiester LNAs [28]. Use of phosphorothioate linkages can reduce the target selectivity of oligonucleotides by increasing binding to proteins, but this enhanced binding also improves pharmacokinetic properties and may be a prerequisite for successful use of LNAs to inhibit gene expression in animals.

7. LNA and PNA

Like LNA, PNA [6] is another emerging DNA/RNA mimic and comparison of LNA and PNA is a useful exercise for putting the properties of LNA into perspective (Fig. 4). LNA and PNA both bind with high affinities to complementary sequences, although the limited set of comparisons that we have performed indicates that binding by LNAs is often even stronger than that of analogous PNAs (D. Braasch, unpublished results). Both are commercially available and both cannot activate RNase H.

There are also important differences. PNAs are uncharged, whereas LNA has a normal phosphate backbone. Because the LNA backbone is charged, we do not expect that LNAs will have the remarkable strand invasion properties displayed by PNAs, although additional research will be necessary before a final determination of the strand invasion properties of can be made. Their uncharged backbone also lowers the solubility of PNAs, and while this is

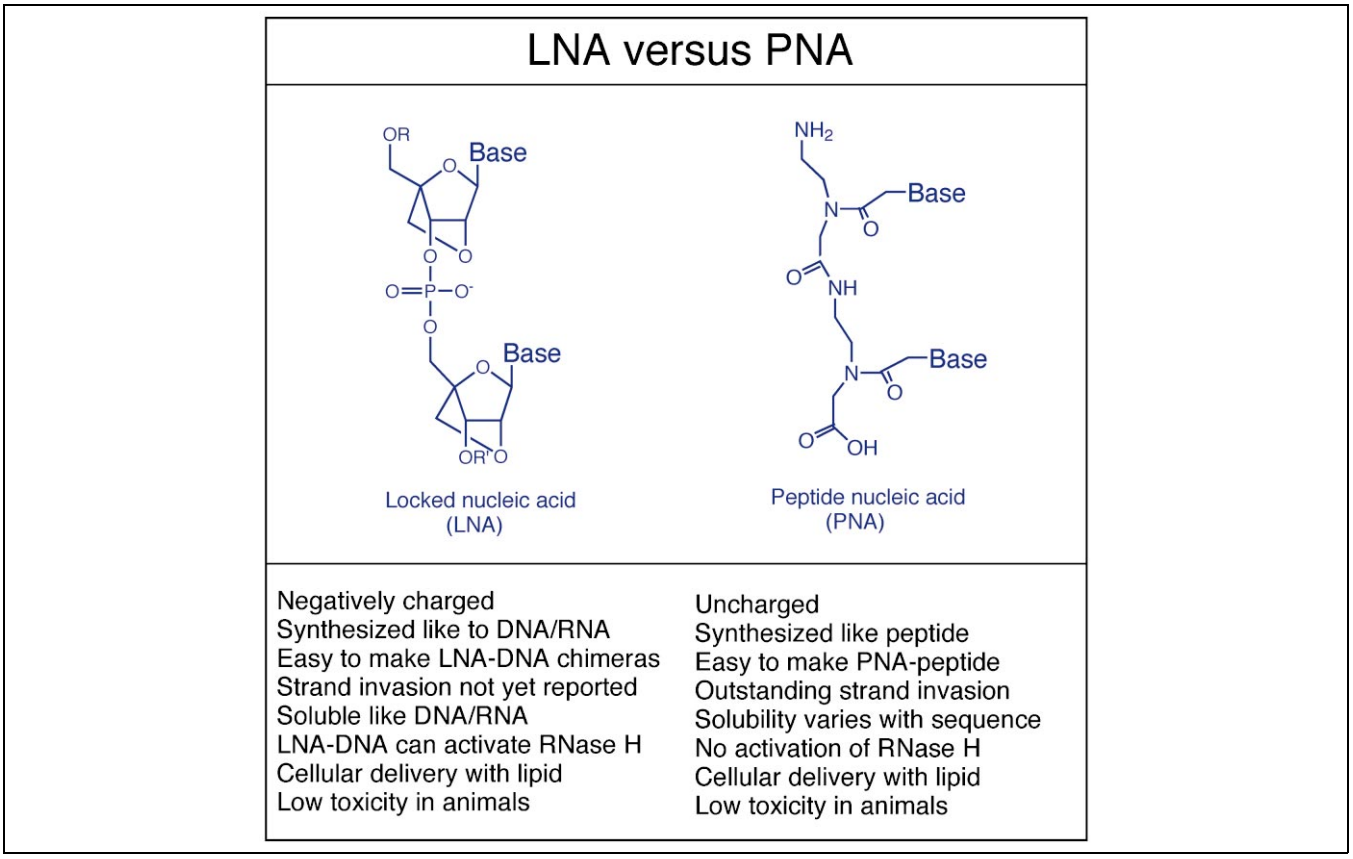

Fig. 4. Comparison of the properties of LNA and PNA.

not a large obstacle for researchers with experience handling PNA, the ready solubility of LNA will be a useful feature for new users.

Another critical distinction between LNA and PNA is that PNAs are assembled using standard peptide synthesis protocols, so it is easier to append peptide motifs onto PNAs than onto LNAs. Conversely, LNAs are assembled using standard DNA synthesis techniques, so it will be easier to make LNA–DNA hybrids that possess tailored binding affinities and the ability to activate RNase H. It will also be possible to use LNA bases to optimize existing antisense oligonucleotides, potentially leading to the immediate development of a wide range of improved compounds, including ones already in clinical development. Thus, while PNA and LNA share some similarities, there are also important differences. It is likely that PNAs will be preferred for some applications, while LNAs will be preferred for others.

8. Applications of LNAs

Given that the first description of LNA was in 1998 it is not surprising that there have been relatively few published applications. A hint of the promise of LNAs as antisense agents is the observation by Wengel et al. that LNA–DNA chimera targeted to DOR, a G-protein coupled receptor, produce a physiologic response in mice when injected directly into the brain [27]. Injections of LNA were well tolerated by the mice, suggesting that their in vivo toxicity will be mild.

The high affinity of hybridization demonstrated by pure LNA oligomers may allow improvement of DNA array technology and DNA diagnostics. Not only do LNAs offer the potential for high affinity binding, but the fact that their synthesis and physical properties closely resemble those for DNA means that existing protocols for creating arrays will need only minimal adjustment. In the only study reported to date, Orum et al. have immobilized LNAs complementary to the Factor V Leiden mutation to individual wells of a microtiter plate [29]. They then developed a 1-h enzyme-linked immunosorbent-type assay capable of discriminating between amplified DNA from Factor V homozygotes, heterozygotes and unaffected individuals.

9. Conclusion

The initial results on the properties and applications of LNAs make it clear that LNAs possess extraordinarily high affinities for complementary sequences and forcefully suggest that LNAs have the potential to be improved agents for oligonucleotide arrays and in vivo repression

of gene expression. Advantages include low toxicity, the ability to make LNA–DNA chimeras, nuclease resistance, synthesis by standard methods, and availability from a commercial supplier. These advantages, combined with the favorable data generated since the discovery of LNA, suggest that LNA will be a valuable addition to the chemical tool kit available for nucleic acid recognition and chemical genetics.

Acknowledgements

This work was supported by grants from the National Institutes of Health R01GM 60624 and the Robert A. Welch Foundation (I-1244).

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