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Design of antisense oligonucleotides stabilized by locked nucleic acids

Jens Kurreck[1], Eliza Wyszko[1,2], Clemens Gillen[3] and Volker A. Erdmann[1,*]

[1]Freie Universität Berlin, Institut für Chemie/Biochemie, Thielallee 63, 14195 Berlin, Germany, [2]Institute of Bioorganic Chemistry of the Polish Academy of Sciences, Noskowsiego 12, 61794 Poznan, Poland and [3]Grünenthal GmbH, Molekulare Pharmakologie, Zieglerstrasse 6, 52078 Aachen, Germany

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ABSTRACT

The design of antisense oligonucleotides containing locked nucleic acids (LNA) was optimized and compared to intensively studied DNA oligonucleotides, phosphorothioates and 2′-*O*-methyl gapmers. In contradiction to the literature, a stretch of seven or eight DNA monomers in the center of a chimeric DNA/LNA oligonucleotide is necessary for full activation of RNase H to cleave the target RNA. For 2′-*O*-methyl gapmers a stretch of six DNA monomers is sufficient to recruit RNase H. Compared to the 18mer DNA the oligonucleotides containing LNA have an increased melting temperature of 1.5–4°C per LNA depending on the positions of the modified residues. 2′-*O*-methyl nucleotides increase the T_m by only <1°C per modification and the T_m of the phosphorothioate is reduced. The efficiency of an oligonucleotide in supporting RNase H cleavage correlates with its affinity for the target RNA, i.e. LNA > 2′-*O*-methyl > DNA > phosphorothioate. Three LNAs at each end of the oligonucleotide are sufficient to stabilize the oligonucleotide in human serum 10-fold compared to an unmodified oligodeoxynucleotide (from $t_{1/2}$ = ~1.5 h to $t_{1/2}$ = ~15 h). These chimeric LNA/DNA oligonucleotides are more stable than isosequential phosphorothioates and 2′-*O*-methyl gapmers, which have half-lives of 10 and 12 h, respectively.

INTRODUCTION

Antisense oligonucleotides are a rather new class of therapeutic agents that have a great potential to provide effective therapies for a wide variety of diseases, like viral infections, cancer and inflammatory and cardiovascular diseases (for reviews see 1–4). They have been successfully used to regulate gene expression through RNA targeting in cell culture and animal models. In many studies modified DNA analogs were used to increase the stability of oligonucleotides in biological fluids. The majority of experiments to date have been performed with phosphorothioate DNA analogs. Several antisense agents have reached the stage of clinical trials and in 1998 the first antisense oligonucleotide drug, Vitravene (Fomivirsen), was approved by the FDA (5).

Despite the increased resistance of phosphorothioates to nucleases compared to unmodified DNA, they exhibit several disadvantages: they have a comparably low binding capacity to complementary nucleic acids and show non-specific binding to proteins (see for example 6–8), causing toxic side-effects that limit many applications (for a review see 9). The toxicity is reduced but not absent in second generation antisense agents with mixed backbone oligonucleotides (MBOs) (10). However, it still remains a major challenge in this field to develop modified oligonucleotides that provide efficient and specific antisense activity *in vivo* without being toxic.

In recent years a variety of modified antisense oligonucleotides have been examined with respect to stability in biological media and their ability to bind specifically to a target RNA (for a review see 11). Two mechanisms are known for translational inhibition of antisense molecules: (i) block of the translational apparatus and (ii) induction of cleavage of the target RNA by RNase H, a ubiquitous enzyme cleaving the RNA part of RNA/DNA hybrids (for a review see 12). Digestion by *Escherichia coli* RNase H1 occurs in the RNA/DNA helix as well as in the single-stranded region 3′ of the heteroduplex and produces 5′-phosphate and 3′-hydroxyl termini (13). Despite many enzymatic properties shared by RNase H of bacterial and mammalian origin, some differences exist: a minimal stretch of at least four and five deoxy residues between flanking 2′-*O*-methyl nucleosides was reported to be required for efficient activation of *E.coli* and human RNase H *in vitro*, respectively (14,15). Unlike RNase H from *E.coli*, the mammalian enzyme displays a strong positional and modest sequence preference (16).

Most of the newly developed nuclease-stable antisense oligonucleotides (like peptide nucleic acids, N3′,P5′-phosphoramidates, morpholino phosphoroamidates and 2′-*O*-methoxyethyl nucleic acids) do not induce RNase H cleavage; only 2′-fluoro-, arabino- and locked nucleic acids were reported to activate RNase H (11). Cyclohexenyl nucleic acids also recruit RNase H, but with much lower rate constants than DNA and phosphorothioates (17,18).

Locked nucleic acids (LNA) are ribonucleotides containing a methylene bridge that connects the 2′-oxygen of ribose with the 4′-carbon (Fig. 1; for reviews see 19,20). Oligomers containing LNA were first described by Wengel and co-workers

*To whom correspondence should be addressed. Tel: +49 30 83 85 6002; Fax: +49 30 83 85 6413; Email: erdmann@chemie.fu-berlin.de

The authors wish to be known that, in their opinion, the first two authors should be regarded as joint First Authors

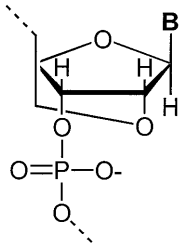

Figure 1. Chemical structure of a LNA monomer.

(21,22) and Imanishi and co-workers (23). Introduction of LNA into a DNA oligomer improves the affinity for complementary sequences and increases the melting temperature (T_m) by several degrees (19).

In the first *in vivo* study with LNA, chimeric LNA/DNA antisense oligonucleotides were used to knockdown the rat delta opioid receptor (24). Mixed LNA/DNA oligonucleotides as well as LNA/DNA/LNA gapmers with a contiguous stretch of DNA were reported to induce RNase H cleavage and LNA-containing oligonucleotides injected into the parenchyma of rat brains did not elicit a toxic action. The antisense oligonucleotides showed potent biological activities and could inhibit the spinal antinociceptive response to deltorphin II in a dose-dependent manner. LNA-containing oligonucleotides were highly efficacious, with potencies exceeding those of isosequential phosphodiesters. The authors concluded that LNA-containing oligonucleotides offer attractive properties as antisense agents: biological stability, RNase H activation, lack of detectable toxicity and potent biological activities.

However, no systematic study on the optimal design of LNA-containing antisense molecules has yet been performed. In the present paper we therefore examined stability, RNase H activation and T_m of DNA/LNA chimeras of different design and compared the results with well-characterized DNA oligonucleotides, phosphorothioates and 2′-*O*-methyl gapmers. As a target we chose the mRNA of vanilloid receptor subtype I (VR1), which is a cation channel predominantly expressed in primary sensory neurons (25). The receptor, also known as the capsaicin receptor, can be activated by capsaicin, the pungent component of chilli peppers, and noxious heat and is further regulated by pH (26). We have recently identified efficient antisense molecules against this target RNA by systematic screening of its structure with oligodeoxynucleotides in the presence of RNase H (27). The best antisense oligonucleotides found in that study have now been stabilized by the introduction of LNA.

MATERIALS AND METHODS

Oligonucleotides

Locked nucleic acids (DNA/LNA chimeras) were obtained from Proligo (Boulder, CO), while the unmodified DNA and phosphorothioate were purchased from MWG Biotech AG (Ebersberg, Germany). The sequences of the antisense oligonucleotides used in this study are shown in Table 1.

An unmodified RNA of sequence CCU AAC CGU CAU GAC AUG and 2′-*O*-methyl oligonucleotides were obtained from IBA-NAPS (Göttingen, Germany).

Table 1. List of oligonucleotides used in this study

name	sequence	DNA gap	% mRNA cleavage (normalized)	T_m °C	T_m/modification °C
DNA 1	catgtcatgacggttagg	-	100	58	-
PS	*CATGTCATGACGGTTAGG*	-	85 ± 6	49	-0.5
	mixmers				
LNA 1	catg**T**ca**T**gacgg**T**tagg	5	13 ± 2	70	4.0
LNA 2	**C**atg**T**ca**T**gacgg**T**tagg	5	12 ± 10	70	3.0
LNA 3	ca**T**g**T**ca**T**gacgg**TT**agg	5	5 ± 1	77	3.8
LNA 4	ca**T**g**T**ca**T**ga**C**gg**T**tag**G**	2	6 ± 10	80	3.7
LNA 5	**C**a**T**g**T**ca**T**ga**C**gg**TT**ag**G**	2	2 ± 3	~84	3.25
LNA 6	catg**T**cat**G**acgg**T**tagg	4	8 ± 8	70	4.0
LNA 7	**C**atg**T**cat**G**acgg**T**tagg	4	9 ± 5	70	3.0
LNA 8	catg**T**cat**G**acgg**T**tag**G**	4	10 ± 1	71	3.25
LNA 9	**C**atg**T**cat**G**acgg**T**tag**G**	4	5 ± 4	71	2.6
LNA 10	**C**atg**T**ca**T**ga**C**gg**T**tag**G**	2	7 ± 1	77	3.2
LNA 11	**C**a**T**g**T**cat**G**acgg**TT**ag**G**	8	91 ± 6	77	3.2
	gapmers				
LNA 12	**C**atg**T**ca**T**gacgg**T**tag**G**	5	7 ± 2	72	2.8
LNA 13	**C**atg**T**c**A**tgacgg**T**tag**G**	6	65 ± 13	70	2.4
LNA 14	**C**atg**TC**atgacgg**T**tag**G**	7	92 ± 5	73	3
LNA 15	**C**atg**T**catgacgg**T**tag**G**	8	99 ± 6	69	2.75
	end blocks				
LNA 16	**CATGT**catgacgg**TTAGG**	8	110 ± 10	~85	2.7
LNA 17	**CATG**tcatgacggt**TAGG**	10	122 ± 13	79	2.6
LNA 18	**CAT**gtcatgacggtt**AGG**	12	115 ± 17	73	2.5
LNA 19	**CA**tgtcatgacggtta**GG**	14	104 ± 15	66	2.0
LNA 20	**C**atgtcatgacggtta**G**	16	107 ± 9	61	1.5
	controls sequence				
DNA 2	atcttgttgacggtctca	-	100	-	-
LNA 21	**A**tct**T**gtt**G**acgg**T**ctc**A**	4	0 ± 0	-	-
LNA 22	**A**tct**T**gttgacgg**T**ctc**A**	8	97 ± 2	-	-
LNA 23	**ATCTT**gttgacgg**TCTCA**	8	99 ± 1	-	-
	2′-O-methyl				
OMe 1	catgtcatgacggttagg	5	14 ± 3	59	0.2
OMe 2	catgtcatgacggttagg	6	93 ± 4	58	0
OMe 3	catgtcatgacggttagg	8	94 ± 5	58	0
OMe 4	catgtcatgacggttagg	10	110 ± 3	64	0.75

Lower case, DNA monomers; italic, phosphorothioate; bold upper case, LNA monomers; underlined, 2′-*O*-methyl monomers. The length of the longest contiguous DNA stretch, percent mRNA cleavage in the RNase H assay normalized to the value of the control DNA oligonucleotide, T_m and ΔT_m per modification are given.

In vitro transcription of substrate RNA

The cDNA of the vanilloid receptor was cloned as described previously (27). Sequence analysis revealed the sequence of the vanilloid receptor type 1-like protein 1 (VR1L1) published in GenBank by Tsutsumi *et al.* (accession no. AB040873). The plasmid was linearized prior to *in vitro* transcription with T7 RNA polymerase, which was performed with the RiboMAX Large Scale Production System from Promega (Madison, WI).

RNase H assay

The standard RNase H assay was performed as described previously (27): 100 nM VR1 mRNA were incubated with a 5-fold excess of an antisense oligonucleotide in a total volume of 10 µl in RNase H buffer (40 mM Tris–HCl pH 7.2, 4 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl and 1.25 U/µl RNasin; Promega, Madison, WI) for 7.5 min at 37°C in the presence of 0.4 U *E.coli* RNase H (Promega). *Escherichia coli* RNase H was used because it is commercially available and its cleavage properties are not very different from those of the mammalian enzyme. The reaction was stopped by addition of EDTA (final concentration 83 mM). Uncleaved substrate and digestion products were separated on a 1.5% agarose gel and stained with ethidium bromide. The gels were photographed using the Gel Doc 2000 Gel Documentation System and quantitatively evaluated with the program Quantity One (Bio-Rad Laboratories, Munich, Germany). All values

given are the average and standard deviation of at least three independent experiments.

Kinetics of RNase H cleavage with different types of oligonucleotides were determined under the same conditions except that equimolar concentrations of RNAs and antisense oligonucleotides (100 nM each) were used. Aliquots of 10 µl were taken after 0.5, 1, 2, 3, 5, 10, 15 and 20 min and the reactions were stopped by addition of EDTA (final concentration 83 mM) and cooling on ice. Data were finally evaluated with Origin (Microcal Software, Northampton, MA). Reaction rates were determined by fitting to single exponential decay functions. All values given are averages including standard deviations of three independent experiments.

For RNase H assay with a short 18mer target RNA a one base ladder and RNase T1 digestion products were prepared for analysis of the cleavage products. The ladder was obtained by incubating labeled RNA (40 000 c.p.m.) in the presence of 2 µg tRNA in a total volume of 10 µl of 0.1 M NaOH and 1 mM EDTA for 1 min at 95°C. For digestion by RNase T1, which cleaves after guanosine bases, 20 000 c.p.m. of the labeled RNA were incubated in the presence of 2 µg tRNA in RNase T1 buffer (7 M urea, 50 mM natrium citrate pH 5.5) for 1 min at 95°C. Subsequently, RNase T1 (17 U) was added and the sample was incubated for 10 min at 55°C. As a control, labeled RNA (20 000 c.p.m.) which was denatured for 2 min at 86°C was used. For the RNase H assay, 1 pmol unlabeled RNA and 20 000 c.p.m. labeled RNA were incubated in RNase H buffer with a 5-fold excess of oligonucleotide (DNA 1, LNA 10, LNA 21 or PS) in the presence of 0.4 U RNase H in a total volume of 10 µl for 10 or 30 min at 37°C. Aliquots of 2 µl of 0.5 M EDTA were added to stop the reaction. The samples were denatured for 2 min at 86°C and analyzed on a 24% polyacrylamide gel with 7 M urea.

Melting temperature

Melting curves of LNA/RNA duplexes were recorded with a Hewlett Packard Diode Array spectrophotometer 8452A in medium salt buffer (10 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, pH 7.0) using the two complementary strands at 1.5 µM. Samples were denatured at 95°C for 5 min and slowly cooled to 20°C prior to measurements. $OD_{260\ nm}$ was measured as a function of temperature from 20 to 90°C with a 1°C increase per min. T_m values were obtained from the maxima of the first derivatives of the melting curves.

Serum stability assay

Aliquots of 300 pmol oligonucleotides were end-labeled with 20 µCi [γ-^{32}P]ATP using T4 polynucleotide kinase at 37°C for 45 min and purified on a 12% polyacrylamide gel with 7 M urea. Oligonucleotides were incubated at 37°C in 120 µl of human serum type AB (male) from Sigma Aldrich Chemie (Schnellendorf, Germany). Samples of 10 µl were removed after 15 and 30 min and 1, 2, 4, 6, 24 and 48 h. Reactions were terminated by addition of 10 µl of loading buffer (0.1 M Tris–HCl pH 8.4, 0.09 M boric acid, 7 M urea, 0.1% bromophenol blue, 0.01% xylene cyanol) and subsequent freezing in liquid nitrogen. Full-length and digested oligomers were separated on a denaturing 20% polyacrylamide gel with 7 M urea and autoradiographed at –80°C. Quantitative analysis was performed with the Gel Doc 2000 Gel Documentation System and Quantity One software (Bio-Rad). Data were further evaluated with Origin (Microcal Software). Average half-lives and standard deviations from at least two independent experiments for each oligonucleotide were obtained by fitting to a single exponential decay function.

RESULTS

Induction of RNase H cleavage

Antisense oligodeoxynucleotides (AS ODNs) induce RNase H cleavage of the target RNA. In a previous study we selected highly efficient AS ODNs against VR1 mRNA by messenger walk screening (27). The design of these oligonucleotides stabilized by the introduction of LNA have now been tested and optimized.

Wahlestedt *et al*. (24) reported that LNA/DNA mixmers as well as LNA/DNA/LNA gapmers were able to recruit RNase H. We therefore investigated the ability of LNA/DNA chimeras of different design to activate RNase H to cleave the full-length transcript of VR1 cDNA (2614 nt). In a first set of experiments we used an all DNA oligodeoxynucleotide (DNA 1), a phosphorothioate and LNA/DNA mixmers (LNA 1–10) as well as one oligonucleotide with a contiguous DNA stretch (LNA 11). The DNA oligonucleotide induced cleavage of almost 90% of the VR1 mRNA. In the presence of the phosphorothioate slightly less of the target RNA was cleaved (85% compared to the DNA oligonucleotide). Interestingly, none of the LNA/DNA mixmers (LNA 1–10) induced significant cleavage of the target RNA by RNase H (see Table 1). Only LNA 11, which contains a stretch of eight DNA monomers, induced cleavage of ~90% of the target RNA by RNase H (normalized to the value obtained with the DNA oligonucleotide). We therefore conclude that a DNA gap in a chimeric LNA/DNA oligonucleotide is needed to recruit RNase H.

Our next goal was to determine the length of the DNA stretch needed for full activation of RNase H. Therefore, LNA/DNA chimeras with a contiguous DNA stretch of 4–8 nt (LNA 9 and 12–15) were investigated and compared to gapmers with flanking 2′-*O*-methyl residues. The results are shown in Figure 2 (top). Cleavage of substrate RNA (S) leads to two products of equal length (P1 + P2). Quantitative evaluation of the gel is presented in Figure 2 (bottom). LNA/DNA/LNA gapmers with DNA stretches of only 4–5 nt do not induce significant cleavage of the target RNA, whereas a stretch of six DNA nucleotides partially recruits RNase H. Obviously, a DNA stretch of 7–8 nt in LNA gapmers is needed for full activation of RNase H. For gapmers with 2′-*O*-methyl modifications a shorter stretch of only six deoxy monomers is sufficient to induce efficient RNase H cleavage.

For stability experiments (see below) end-block oligonucleotides with between one and five LNA at each end should be used (LNA 16–20). As expected, all of them induce RNase H cleavage of the target RNA (Table 1) since they all contain a DNA stretch of at least 8 nt.

To rule out that the inability of LNA/DNA mixmers to activate RNase H is specific for the sequence used, RNase H assays were performed with antisense oligonucleotides against a second target site of the same mRNA previously found to be highly accessible (27). Again, the all DNA oligonucleotide (DNA 2) induced almost complete cleavage of the mRNA. In contrast, no cleavage products were observed when the

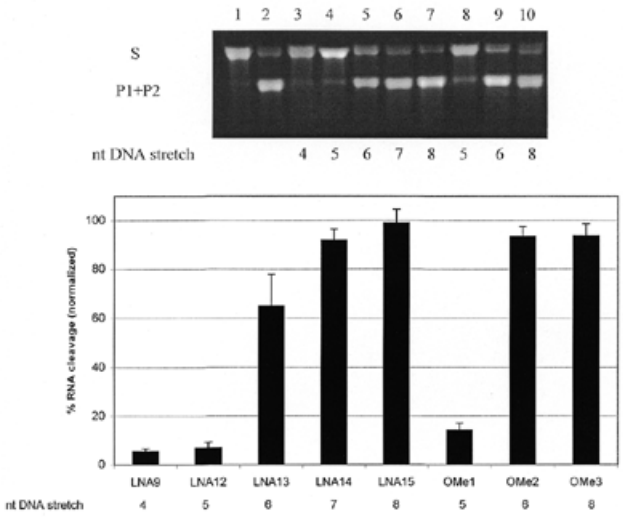

Figure 2. RNase H activation by LNA/DNA and 2′-*O*-methyl gapmers. (Top) Results of RNase H assay. Full-length VR1 mRNA was incubated with a 5-fold excess of oligonucleotides in the presence of RNase H for 7.5 min at 37°C. Substrate (S) and cleavage products (P1 + P2) were separated on a 1.5% agarose gel and stained with ethidium bromide. Lane 1, control; lane 2, DNA 1; lane 3, LNA 9; lane 4, LNA 12; lane 5, LNA 13; lane 6, LNA 14; lane 7, LNA 15; lane 8, OMe 1; Lane 9, OMe 2; lane 10, OMe 3. (Bottom) Quantitative evaluation of the gel. All values are averages and standard deviations of at least three independent experiments and were normalized.

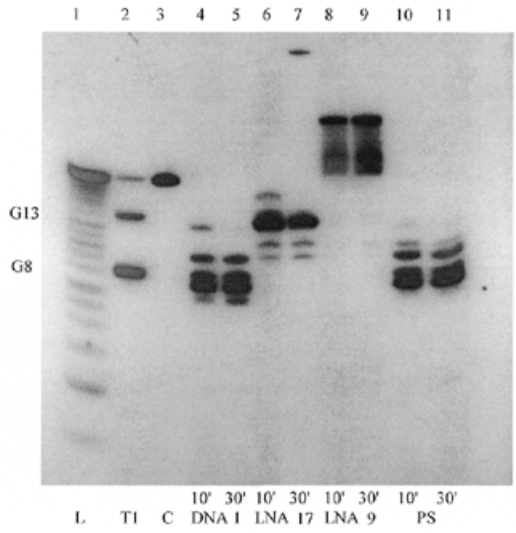

Figure 3. RNase H assays with a short RNA target. Lane 1, one base ladder produced by alkaline hydrolysis; lane 2, cleavage products after incubation with RNase T1; lane 3, 18mer RNA; lanes 4 and 5, products after incubation of target RNA with all DNA antisense oligonucleotide (DNA 1) in the presence of RNase H after 10 and 30 min, respectively; lanes 6 and 7, products after incubation of target RNA with end-block LNA/DNA/LNA oligonucleotide (LNA 17) in the presence of RNase H after 10 and 30 min; lanes 8 and 9, products after incubation of target RNA with LNA/DNA mixmer (LNA 9) in the presence of RNase H after 10 and 30 min; lanes 10 and 11, products after incubation of target RNA with phosphorothioate in the presence of RNase H after 10 and 30 min. For further details see text.

LNA/DNA mixmer LNA 21 was used in the RNase H assay (Table 1). The LNA/DNA/LNA gapmer (LNA 22) as well as the end-block oligonucleotide (LNA 23) induced full cleavage of the target RNA since they included a DNA stretch of 8 nt. These experiments support our first results that LNA/DNA mixmers do not induce RNase H cleavage of a target RNA.

This finding does not confirm results of a previous study, in which Wahlestedt *et al*. (24) reported that mixed LNA/DNA oligonucleotides induce cleavage of the complementary RNA. Since they used a short target RNA of 24 nt, we have performed additional experiments with an 18mer RNA complementary to the antisense oligonucleotides. Results of RNase H assays with the labeled short target RNA in the presence of RNase H and an all DNA oligonucleotide (DNA 1), an end-block oligonucleotide (LNA 17), a LNA/DNA mixmer (LNA 9) and a phosphorothioate, respectively, are shown in Figure 3. For comparison a one base ladder produced by alkaline hydrolysis (lane 1) and fragments of the short RNA after digestion with RNase T1 (lane 2) are shown. RNase T1 cleaves after guanosines, i.e. after bases 8 and 13 of the 18mer RNA.

For the all DNA oligonucleotide (DNA 1, lanes 4 and 5) complete degradation of the target RNA by RNase H was already observed after 10 min incubation at 37°C. Several short degradation products could be observed. In the presence of the end-block LNA/DNA/LNA oligonucleotide (LNA 17) the target RNA was also degraded very quickly (lanes 6–7): after 10 min incubation in the presence of RNase H full-length targets were no longer detectable. Comparison with the fragments produced by RNase T1 cleavage revealed that the major product of RNase H cleavage was 12 nt long, i.e. the target RNA was cleaved between nt 8 and 9 of the DNA stretch. For the LNA/DNA mixmer (LNA 9, lanes 8 and 9) hardly any cleavage products could be observed. Due to high affinity of the mixmer for its complementary RNA, the hybrid was not completely denatured. Even after 60 min no significant degradation of the RNA target was observed (data not shown). These results confirm our findings with the full-length mRNA that LNA/DNA mixmers do not induce significant RNase H cleavage. The phosphorothioate (lanes 10 and 11) led to complete degradation of the target RNA into small fragments, as did the DNA oligodeoxynucleotide.

Melting temperature

It was shown that introduction of LNA raises the T_m of the oligonucleotide and a complementary DNA or RNA by as much as 9.6°C per LNA (28). For longer oligomers smaller effects are expected when LNA bases are included. We therefore determined T_ms of DNA, phosphorothioate, 2′-OMe/DNA and LNA/DNA oligonucleotides with their complementary RNA. Compared to the duplex with the all DNA oligonucleotide, the transition was shifted towards a lower temperature for the phosphorothioate and towards higher temperatures with increasing numbers of LNA included in the oligonucleotide. 2′-*O*-methyl gapmers elicited only small effects on the melting curve. The T_m given in Table 1 were obtained from the maxima of the first derivatives of the melting curves. T_m of the phosphorothioate was 9°C below that of the DNA oligonucleotide. In contrast, the T_m was raised by 2.4–4°C per LNA for mixmers with isolated LNA residues and by 1.5–2.7°C per LNA for oligonucleotides with segments of LNA. The terminal nucleotides especially had only small effects on the T_m. For 2′-*O*-methyl oligonucleotides the T_m was increased by <1°C per modified nucleotide.

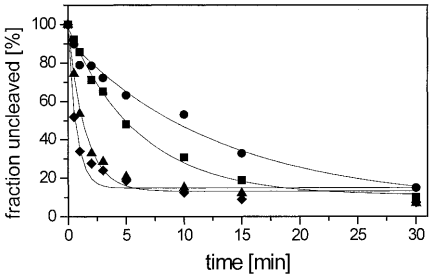

Figure 4. Kinetics of RNase H cleavage. Target mRNA and equimolar amounts of all DNA (squares), phosphorothioate (circles) and chimeric LNA/DNA (LNA 17, diamonds) and 2′-*O*-methyl/DNA (OMe 4, triangles) oligonucleotides, respectively, were incubated in the presence of RNase H. At appropriate time points aliquots were taken from the sample. The fraction remaining uncleaved is shown as a function of time.

Table 2. Observed reaction rates and relative rates normalized to the value for the control DNA oligonucleotide for RNase H cleavage of full-length VR1 mRNA induced by different types of antisense oligonucleotides

Oligonucleotide	k (per min)	k_{rel}
DNA 1	0.17 ± 0.01	1
LNA 17	1.4 ± 0.1	8
OMe 4	0.7 ± 0.1	4
PS	0.07 ± 0.01	0.4

Kinetics of RNase H cleavage

In order to investigate the influence of T_m on the RNase H activity, we performed kinetic experiments. Therefore, equimolar amounts of the target RNA and the DNA, phosphorothioate, LNA/DNA (LNA 17) or 2′-*O*-methyl/DNA (OMe 4) oligonucleotides, respectively, were incubated in the presence of RNase H. At appropriate time points aliquots were removed from the sample. The fraction of uncleaved mRNA was determined and plotted as a function of time (Fig. 4). Cleavage of the target RNA was decelerated in the presence of the phosphorothioate (circles) compared to the all DNA oligonucleotide (squares). In contrast, RNase H activity was higher in the presence of the LNA (diamonds) or 2′-*O*-methyl gapmers (triangles), the acceleration being more pronounced in the former case. Kinetic evaluation with a mono-exponential decay function (Table 2) revealed that the observed reaction correlates with the T_m. The chimeric LNA/DNA oligonucleotide was most efficient for activation of RNase H, while 2′-*O*-methyl was still superior to DNA and the phosphorothioate induced the slowest cleavage.

Stability of LNA/DNA chimeras in human serum

The use of unmodified antisense oligodeoxynucleotides in cell cultures or animals is limited by their sensitivity to nucleolytic degradation in biological media. Therefore, phosphorothioates, which are stabilized by the substitution of a non-bridging oxygen by sulfur, are usually used for *in vivo* studies. However, they have toxic side-effects. Since LNA/DNA oligomers have been shown to be more resistant to nucleolytic attack compared to unmodified DNA and did not cause detectable

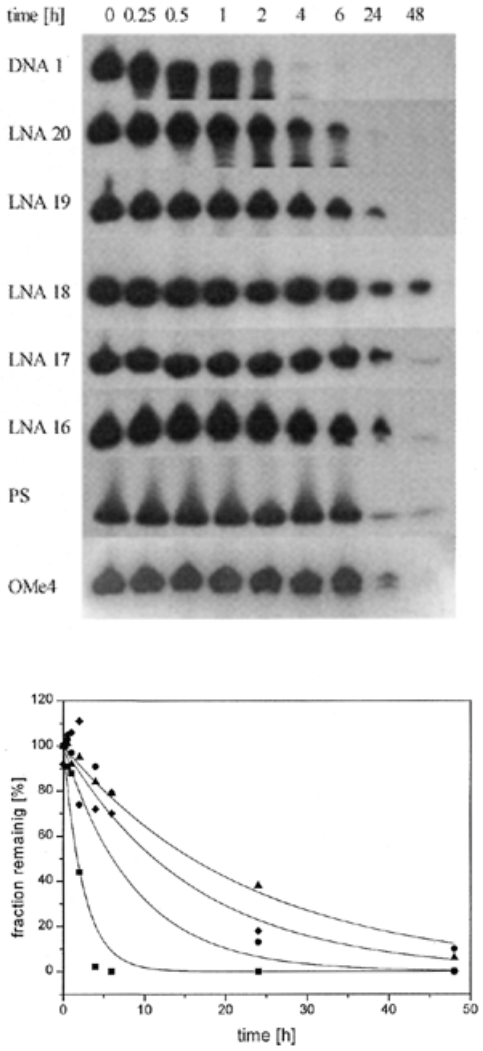

Figure 5. Stability of oligonucleotides in human serum. (**Top**) Autoradiogram of radioactively labeled oligonucleotides (DNA 1, LNA 16–20, phosphorothioate and OMe 4) incubated in human serum at 37°C. Aliquots were taken at the time points indicated and analyzed on a denaturing 20% polyacrylamide gel. (**Bottom**) Quantitative evaluation of the gel for DNA 1 (squares), phosphorothioate (circles), LNA 17 (triangles) and OMe 4 (diamonds) as examples. The fraction of uncleaved oligonucleotides is plotted as a function of time and fitted to a single exponential decay function.

toxic reactions in rat brains (24), the stability of LNA/DNA chimeras of different design in serum was analyzed further.

Radioactively labeled oligonucleotides were incubated in human serum at 37°C for up to 2 days. Aliquots were taken at appropriate time points and frozen in liquid nitrogen. Degradation of oligonucleotides was subsequently analyzed by polyacrylamide gel electrophoresis. Figure 5 (top) shows the results for the all DNA, the end-block, with one to five LNA at the 3′- and 5′-ends (LNA 16–20), the phosphorothioate and a 2′-*O*-methyl gapmer (OMe 4) oligonucleotide. The unmodified oligodeoxynucleotide was almost completely degraded after 4 h. End protection with LNA significantly stabilized the oligonucleotide against nucleolytic attack. Only three LNA monomers at each end were sufficient to increase resistance, so that even after 48 h a significant amount of the oligonucleotide

Table 3. Half-lives of DNA, phosphorothioate and 2′-*O*-methyl and LNA end-block oligonucleotides in human serum

Oligonucleotide	End block (nt)	$t_{1/2}$ (h)
DNA 1	0	1.5 ± 0.3
PS	0	10 ± 2
LNA 20	1	4 ± 2
LNA 19	2	5 ± 2
LNA 18	3	17 ± 1
LNA 17	4	15 ± 1
LNA 16	5	15 ± 2
OMe 4	4	12 ± 1

remained intact. The all phosphorothioate was substantially degraded but still present after 48 h and some uncleaved 2′-*O*-methyl gapmer was detectable after 24 h.

The gels were densitometrically evaluated and the uncleaved fraction was plotted as a function of incubation time (Fig. 5, bottom). Average half-lives including standard deviations from two independent experiments were obtained by fitting these data to a single exponential decay function. The results are summarized in Table 3. The unmodified oligodeoxynucleotide had a half-life of 1.5 h. Introduction of three or four LNA at each end increased the half-life of the oligonucleotide in human serum ~10-fold, to ~15 h. The end-block LNA/DNA/LNA oligonucleotide LNA 23 against the second target sequence had an even higher half-life of 28 ± 1 h (data not shown). This finding is indicative of a sequence dependence of stability. Further experiments are needed to clarify this point. Regardless of this open question, it is obvious that the stability of chimeric LNA/DNA oligonucleotides is even higher than that of the all phosphorothioate and the 2′-*O*-methyl gapmer, which have half-lives of 10 and 12 h, respectively.

DISCUSSION

Despite an increasing number of successful applications of antisense strategies to specifically prevent the expression of a gene, some basic problems remain to be solved. Unmodified antisense oligodeoxynucleotides are susceptible to nucleolytic attack in biological media and are degraded after a few hours. Therefore, modified oligonucleotides have to be used for most antisense experiments. To date, *in vivo* studies have usually been performed with phosphorothioates. These oligonucleotides are protected against degradation by nucleases but they exhibit toxic side-effects (for a review see 9). Therefore, new types of modified nucleotides have been developed (11). LNA are a very promising new type of antisense molecule, since they have been shown to exhibit biological activities without being toxic in rat brains (24).

However, the optimal design of chimeric LNA/DNA antisense oligonucleotides has not yet been investigated. Therefore, in the present study antisense oligonucleotides with a variety of designs were studied systematically. Three main points were addressed: (i) are there restrictions for LNA/DNA chimeras with respect to the activation of RNase H; (ii) in which way does the introduction of LNA influence the binding of complementary RNA and its degradation by RNase H; (iii) is the stability of antisense oligonucleotides containing LNA in human serum increased compared to other types of oligonucleotides? Five types of antisense oligonucleotides were investigated: (i) unmodified oligodeoxynucleotides (DNA); (ii) phosphorothioates; (iii) LNA/DNA mixmers; (iv) LNA/DNA/LNA gapmers, with a stretch of DNA monomers in the center and either isolated LNA residues or LNA end-blocks; (v) gapmers with 2′-*O*-methyl wings, which have been intensively studied (14–16,29).

We first analyzed the activation of RNase H by a set of LNA/DNA mixmers (LNA 1–10). None of these induced significant cleavage of the complementary mRNA by RNase H. This result does not confirm the finding of Wahlestedt *et al.* (24), who reported that LNA/DNA mixmers stimulated RNase H. We therefore checked whether a mixmer with a different sequence (LNA 21) induces RNase H cleavage. Again, no significant degradation of the mRNA was found. Since Wahlestedt *et al.* (24) performed their experiments with a short target RNA, we also investigated cleavage of an 18mer RNA complementary to the LNA/DNA oligonucleotide. Even after 1 h incubation in the presence of RNase H hardly any cleavage product could be observed. We therefore conclude that a contiguous DNA stretch within a chimeric LNA/DNA oligonucleotide is needed to induce RNase H cleavage of the target RNA. However, the possibility remains that mixed LNA/DNA oligonucleotides show antisense activity by steric blocking of translation. This mode of inhibition has recently been shown in HeLa cells for a 12mer mixed 2′-*O*-methyl/LNA oligonucleotide directed against the HIV-1 *trans*-activation responsive element (TAR) RNA (30).

Secondly, the length of the DNA stretch in chimeric LNA/DNA oligonucleotides necessary to recruit RNase H was determined. A stretch of four or five DNA monomers does not lead to activation of RNase H and a DNA stretch of 6 nt only partially recruits RNase H cleavage. Seven or eight DNA monomers are sufficient to induce full cleavage activity of RNase H. In contrast, only six DNA residues were needed for activation of *E.coli* RNase H by gapmers with 2′-*O*-methyl wings, i.e. LNAs induce more severe conformational changes than 2′-*O*-methyl derivatives. For 2′-*O*-methyl DNA stretches of 4 and 5 nt were reported to activate *E.coli* and human RNase H, respectively (14,15). These differences might be due to the different assay conditions as well as to the use of phosphorothioate oligonucleotides and shorter target RNAs in the literature.

Our findings for LNA/DNA/LNA oligonucleotides are supported by an NMR and CD spectroscopy study of the structure of DNA:RNA and LNA:RNA hybrids (28). DNA:RNA duplexes display CD features of intermediates between A- and B-type structures. A minor groove width that is intermediate between that of A-type RNA:RNA duplexes and B-type DNA:DNA duplexes was proposed as a recognition element for RNase H. Introduction of LNA into the DNA oligonucleotide induces a conformational change towards the A-type helix. Even the replacement of every third nucleotide with a LNA nucleotide shifts the conformation of the duplex completely to the A-form. This observation supports our finding that LNA/DNA mixmers with short stretches of up to five DNA monomers do not induce RNase H cleavage. However, since the structural

effects of LNA are local and affect only the nucleotides flanking the modification, it is not surprising that LNA/DNA chimeras with longer DNA stretches activate RNase H.

In addition to the correct size of the minor groove, enough flexibility of the antisense oligonucleotide is necessary to support the conformational changes required for RNase H cleavage (12). Since LNAs are locked in the 3′-*endo* conformation they obviously do not possess flexibility. This might be another reason why mixmers do not recruit RNase H.

The introduction of LNA into an oligonucleotide raises the T_m of the hybrid with a complementary DNA or RNA (19). An increase of up to 9.6°C per LNA monomer was reported (28). For longer oligonucleotides less pronounced effects are expected. We found that T_m is raised by 1.5–4°C per LNA monomer introduced into an 18mer oligonucleotide. Isolated LNA residues had greater effects (2.4–4°C per LNA) on T_m than segments of LNA in end-block nucleotides (1.5–2.7°C per LNA). In agreement with the literature (14,29), oligonucleotides with 2′-*O*-methyl modifications increased the T_m only slightly, by <1°C per modified nucleotide. In contrast, the T_m of the all phosphorothioate was reduced by 9°C compared to the isosequential unmodified ODN.

A comparative study of unmodified oligodeoxynucleotides, phosphorothioates and MBOs revealed a correlation between T_m and kinetics of RNase H cleavage (10). The T_m of a 25mer phosphorothioate was 8.4°C lower that that of the isosequential DNA oligonucleotide. As a consequence, its half-life for RNase H cleavage of the target RNA was increased from 8.8 to 22.4 min. An increase in T_m by the introduction of modified nucleotides raised the rate of cleavage.

We therefore compared RNase H cleavage kinetics induced by all DNA, phosphorothioate, 2′-*O*-methyl/DNA and LNA/DNA oligonucleotides. In agreement with the study mentioned above, phosphorothioates decelerated RNase H degradation of the target RNA >2-fold. In contrast, cleavage was accelerated by the chimeric LNA/DNA and 2′-*O*-methyl/DNA oligonucleotides due to their tighter binding to the target RNA. The observed rates correlate with the T_m. The LNA gapmer had the highest efficiency to induce RNase H cleavage (8-fold higher rate than DNA); the rate for the 2′-*O*-methyl gapmer was 4-fold higher than for the DNA oligonucleotide.

Increased oligonucleotide target affinity is known to be correlated with increased antisense activity in cells (15). The higher affinity of LNA-containing oligonucleotides for their complementary target RNA and the acceleration of the RNase H cleavage reaction might be the reason for the observed higher biological potency of chimeric LNA/DNA oligonucleotides compared to the isosequential phosphodiester (24). This improved antisense activity is another important advantage of LNA/DNA/LNA end-blocked oligonucleotides. 2′-*O*-methyl residues have a much smaller effect on the T_m than LNA. Oligonucleotides with isolated LNA residues distributed over the length will have the greatest increase in T_m per modification and can be used if a steric block oligonucleotide is designed and RNase H cleavage is not required.

One of the most desired requirements for antisense oligonucleotides is their stability in biological media. We therefore analyzed the degradation of chimeric LNA/DNA oligonucleotides in human serum. The stability of end-blocked oligomers with between one and five LNA monomers at each end was investigated. Only three LNAs at the 3′- and 5′-ends were sufficient to increase the half-life of the oligonucleotide in human serum from 1.5 (unmodified ODN) to ~15 h. Oligonucleotides with more LNA at the ends were not further stabilized. Obviously, three LNAs are sufficient for protection against exonucleases, whereas endonucleases can still slowly degrade the oligonucleotide. For the phosphorothioate we determined a half-life of 10 ± 2 h in human serum, which is in good agreement with that of 9 h reported by Campbell *et al.* (31) and Phillips and Zhang (3). This means that chimeric LNA/DNA/LNA end-blocked oligonucleotides are even more stable in serum than all phosphorothioates. End-blocked oligonucleotides with three or four phosphorothioate bonds at the 3′- and 5′-ends designed to minimize non-specific side-effects were found to be less stable than all phosphorothioates (32). The 2′-*O*-methyl gapmer had a half-life of 12 h, which is also less than $t_{1/2}$ for the LNA oligonucleotides.

From our study we conclude that chimeric LNA/DNA antisense oligonucleotides should contain a stretch of seven or eight DNA monomers, which is necessary for activation of RNase H cleavage of the target mRNA. One major advantage of LNAs is their high affinity for the complementary target RNA, which results in an acceleration of target RNA cleavage by RNase H. In addition, LNA/DNA/LNA end-block oligonucleotides had the highest stability of all oligonucleotides investigated in the present study. Only three LNA monomers at each end are sufficient to increase the half-life of the oligonucleotide in human serum 10-fold, from 1.5 (unmodified AS ODN) to ~15 h. Together with the finding that LNA have high *in vivo* efficacy in the absence of any toxicity (24), these data demonstrate that chimeric LNA/DNA oligonucleotides are promising new antisense agents. Further experiments to stabilize aptamers, ribozymes and DNA enzymes with LNA are in progress.

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