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Thermal stability of G-rich anti-parallel DNA triplexes upon insertion of LNA and α -L-LNA†

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G-rich anti-parallel DNA triplexes were modified with LNA or α -L-LNA in their Watson–Crick and TFO strands. The triplexes were formed by targeting a pyrimidine strand to a putative hairpin formed by Hoogsteen base pairing in order to use the UV melting method to evaluate the stability of the triplexes. Their thermal stability was reduced when the TFO strand was modified with LNA or α -L-LNA. The same trend was observed when the TFO strand and the purine Watson–Crick strand both were modified with LNA. When all triad components were modified with α -L-LNA and LNA in the middle of the triplex, the thermal melting was increased. When the pyrimidine sequence was modified with a single insertion of LNA or α -L-LNA the ΔT_m increased. Moreover, increasing the number of α -L-LNA in the pyrimidine target sequence to six insertions, leads to a high increase in the thermal stability. The conformational S-type structure of α -L-LNA in anti-parallel triplexes is preferable for triplex stability.

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Introduction

DNA triplexes are formed when a DNA duplex containing a polypurine tract interacts with a third strand by means of specific hydrogen bonds in the major groove of the duplex. The formation of triple helices between DNA duplexes and external DNA single strands was introduced theoretically in 1953 by Pauling and Corey¹ and demonstrated experimentally by Rich and co-workers in 1957.²

Depending on the orientation of the third strand, triplexes are classified into two main categories: parallel and anti-parallel oriented triplexes.³ The parallel-oriented triplexes (also named pyrimidine triplexes) are defined by three types of Hoogsteen base triads: d(T-A.T), protonated d(C-G.C⁺) and d(C-G.G), where the last base refers to the Hoogsteen strand. The dot and hyphen refer to Hoogsteen and Watson–Crick binding, respectively, Fig. 1. Anti-parallel triplexes (also named purine triplexes) are classified by three reverse-Hoogsteen base triads: d(T-A.T), d(T-A.A) and d(C-G.G) as shown in Fig. 1.

Historically, guanine containing triplex forming oligonucleotides (G-rich TFOs) have been far less explored than TFOs based on pyrimidines (CT-TFOs). However, C-triplexes

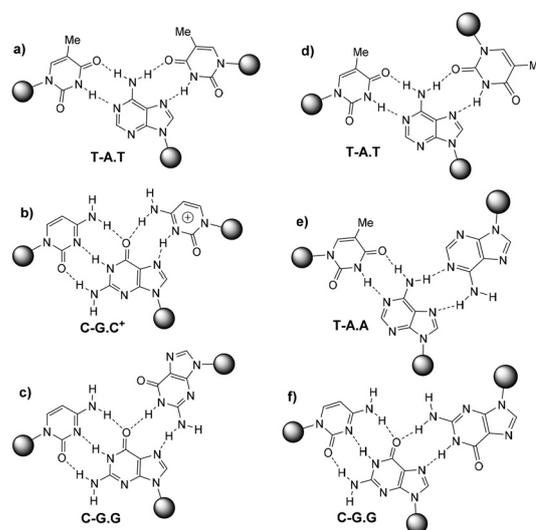


Fig. 1 First column: Hoogsteen pairings found in parallel triplexes (a, b and c), second column: reverse Hoogsteen pairings found in anti-parallel triplexes (d, e and f).

are unstable at neutral pH due to lacking protonation of cytosines which is required to form hydrogen bonds with the guanines in the duplex. Therefore, it has been attempted to focus on pH-independent TFOs containing GA and/or GT nucleotides in order to design TFOs that combine DNA target-specificity with strong binding under physiological conditions.⁴ However this approach is hampered by the tendency of G-rich TFOs to form highly stable aggregates, mostly in the

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form of inter- or intramolecular G-quadruplexes, thus preventing triplex formation.^{5–7}

Several strategies have been developed to avoid the self-aggregation of G-rich TFOs, some of them depend on formation of short duplexes on the 3'- or 5'-ends of G-rich TFOs,⁸ replacement of phosphodiester internucleotide bonds with positively charged phosphoramidites⁹ or modification of the TFO strand with twisted intercalating nucleic acid monomer (TINA) which effectively circumvents guanine-mediated self-association of G-rich anti-parallel TFOs.¹⁰ Also, 8-amino-guanine has a unique profile as a molecule showing simultaneous strong triplex-stabilization and quadruplex-destabilization.¹¹ The introduction of 8-aminoguanine into triplex-forming oligonucleotides seems preferable compared to other modified purines like 6-thioguanine,^{6,12} 9-deazaguanine,¹³ 7-deazaxanthine,¹⁴ 6-thio-7-deazaguanine,¹⁵ or 7-chloro-7-deazaguanine.¹⁶ Although they were found to inhibit quadruplex formation, there was no improvement on triplex stability.

LNA (Locked Nucleic Acid) is a nucleotide modification (Fig. 2) with a 2'-O, 4'-C-methylene linked bicyclic ribonucleoside locked in an 3'-endo conformation. This preorganizes the sugar-phosphate backbone to facilitate a more efficient stacking of the nucleobases.¹⁷ α -L-LNA (α -L-ribo configured LNA, Fig. 2) is a diastereoisomer of LNA. Like LNA it improves stability against nucleases and induces high affinity towards DNA and especially RNA complements when incorporated into oligonucleotides.^{18,19} α -L-LNA can be described as a DNA mimic with respect to helical structure, whereas LNA is an RNA mimic.^{20,21}

It was reported that incorporation of LNA-T or LNA-C in the homo-pyrimidine strand significantly increased the binding affinity of TFOs,^{22–24} but unexpectedly, a continuous stretch of 12–13 LNA-T and LNA-C monomers in the TFO decreased the affinity dramatically so that no triplex formation could be detected.²⁴ There is also a report claiming reverse Hoogsteen

hydrogen bonding with (G,A)- and (G,T)-containing LNA-TFOs to be disfavored, but without reporting the corresponding experimental data.²⁵ In contrast to LNA-TFOs, the fully modified α -L-LNA-TFO forms a stable parallel triplex with a DNA duplex.²⁶

Herein, G-rich triplexes were modified with LNA and α -L-LNA in the TFO and Watson–Crick strands. To the best of our knowledge this is the first report describing the incorporation of α -L-LNA into anti-parallel TFOs.

Results and discussion

In this study, we were dealing with two hairpins that target a pyrimidine strand to form the triplexes **ON1** and **ON2**. These two triplexes were taken from a previous anti-parallel triplex study, where the linking between the Watson–Crick polypurine and the reverse-Hoogsteen strands was done by a tetrathymidine loop,²⁷ Fig. 3.

In our hands the triplexes **ON1** and **ON2** showed melting temperatures at 61.5 °C and 60.5 °C, respectively (Table 1). For **ON1** this is close to the previously reported temperature at 62.7 °C.²⁷ The single strands forming hairpins did not show any melting and melting was observed only when the putative hairpins were mixed with the pyrimidine target sequences. This observation is in agreement with the previous finding that reverse-Hoogsteen hairpins are not fully preorganized before binding to the third strand.²⁷ Interestingly, the control duplexes formed by Watson–Crick 11-mer and corresponding polypurine strand **ON26** and **ON29** (with scrambled anti-parallel strand) melted at lower temperatures (47–48 °C, Table 2).

Firstly, we modified the TFO with adenine-LNA (**A^L**), guanine-LNA (**G^L**), thymine-LNA (**T^L**) or thymine- α -L-LNA (**T ^{α}**),

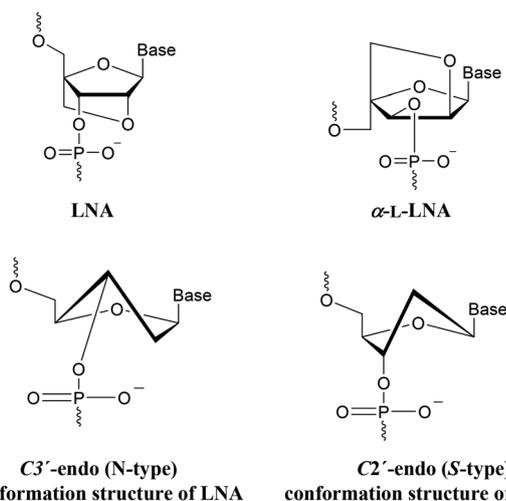


Fig. 2 Structure of LNA (**T^L**, **C^L**, **A^L**, **G^L**) and α -L-LNA (**T ^{α}** , **C ^{α}**) nucleotide monomers.

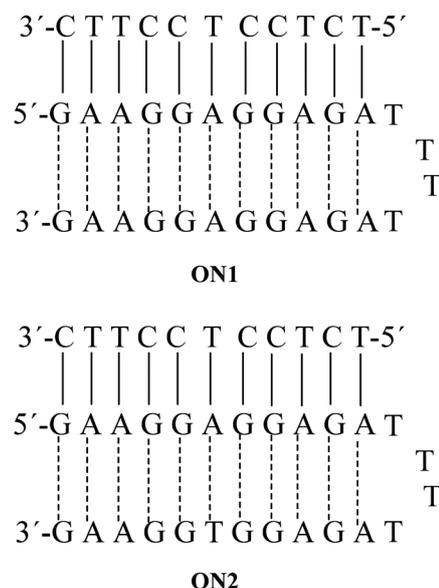


Fig. 3 Anti-parallel triplexes **ON1** and **ON2**.



Table 1 Thermal melting T_m [°C] of triplexes melting ON1–ON25, taken from UV melting curves ($\lambda = 260$ nm) recorded in 10 mM sodium cacodylate, 50 mM $MgCl_2$, and 0.1 mM EDTA buffer solution at pH 7.2^a

ON	Watson–Crick	Hairpin		T_m (°C)	ΔT_m (°C)	
	Target	Watson–Crick	TFO		Ref. ON1	Ref. ON2
1	5'TCTCCTCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGAGGAAG ^{3'}		61.5		
2	5'TCTCCTCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		60.5		
3	5'TCTCCTCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGG ^L AGGAAG ^{3'}		54.5	-7.0	
4	5'TCTCCTCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGA ^L GGAAG ^{3'}		56.5	-5.0	
5	5'TCTCCTCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGT ^L GGAAG ^{3'}		53.5		-7.0
6	5'TCTCCTCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGT ^L GGAAG ^{3'}		55.0		-5.5
7	5'TCTCCTCCTTC ^{3'}	5'GAAGGAG ^L GAGA-TTTT-AGAGGAGGAAG ^{3'}		60.5	-1.0	
8	5'TCTCCTCCTTC ^{3'}	5'GAAGGA ^L GGAGA-TTTT-AGAGGAGGAAG ^{3'}		62.0	0.5	
9	5'TCTCCTCCTTC ^{3'}	5'GAAGGA ^L GGAGA-TTTT-AGAGGA ^L GGAAG ^{3'}		58.5	-3.0	
10	5'TCTCCTCCTTC ^{3'}	5'GAAGGAG ^L GAGA-TTTT-AGAGG ^L AGGAAG ^{3'}		56.5	-5.0	
11	5'TCTCCTCCTTC ^{3'}	5'GAAGGA ^L GGAGA-TTTT-AGAGGT ^L GGAAG ^{3'}		58.5		-2.0
12	5'TCTCCT ^L CCTTC ^{3'}	5'GAAGGA ^L GGAGA-TTTT-AGAGGT ^L GGAAG ^{3'}		64.0		3.5
13	5'TCTCCT ^L CCTTC ^{3'}	5'GAAGGA ^L GGAGA-TTTT-AGAGGT ^L GGAAG ^{3'}		63.5		3.0
14	5'TCTCCT ^L CCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGT ^L GGAAG ^{3'}		60.0		-0.5
15	5'TCTCCT ^L CCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGT ^L GGAAG ^{3'}		57.5		-3.0
16	5'TCTCCT ^L CCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGT ^L GGAAG ^{3'}		59.0		-1.5
17	5'TCTCCT ^L CCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		64.0		3.5
18	5'TCTCCT ^L CCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		64.5		4.0
19	5'TCT ^L CCT ^L CCT ^L TC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		69.5		9.0
20	5'TCT ^L CCT ^L CCT ^L TC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		72.0		11.5
21	5'TCTCC ^L TCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		66.0		5.5
22	5'TCTCC ^L TCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		73.5		13.0
23	5'TCTCC ^L TCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		66.5		6.0
24	5'TC ^L TCC ^L TCC ^L TTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		76.0		15.5
25	5'TC ^L TCC ^L TCC ^L TTC ^{3'}	5'GAAGGAGGAGA-TTTT-AGAGGTGGAAG ^{3'}		82.0		21.5

^a C = cytosine, C^L = 5-methylcytosine LNA, C^L = 5-methylcytosine α -L-LNA.

Table 2 Thermal melting T_m [°C] data of duplexes melting ON26–ON36, taken from UV melting curves ($\lambda = 260$ nm) recording in 10 mM sodium cacodylate, 50 mM $MgCl_2$, and 0.1 mM EDTA buffer solution at pH 7.2^a

ON	Pyrimidine sequence	Purine sequence	T_m (°C)	ΔT_m (°C)	
				Ref. ON26	Ref. ON29
26	5'TCTCCTCCTTC ^{3'}	5'GAAGGAGGAGA ^{3'}	48.0		
27	5'TCTCCTCCTTC ^{3'}	5'GAAGGA ^L GGAGA ^{3'}	53.5	5.5	
28	5'TCTCCTCCTTC ^{3'}	5'GAAGGAG ^L GAGA ^{3'}	53.5	5.5	
29	5'TCTCCTCCTTC ^{3'}	5'GAAGGAGGAGA-TTTT-GAGAGGAAAGA ^{3'}	47.0		
30	5'TCTCCTCCTTC ^{3'}	5'GAAGGA ^L GGAGA-TTTT-GAGAGGAAAGA ^{3'}	51.0		3.0
31	5'TCTCCTCCTTC ^{3'}	5'GAAGGAG ^L GAGA-TTTT-GAGAGGAAAGA ^{3'}	51.0		3.0
32	5'TCTCCT ^L CCTTC ^{3'}	5'GAAGGAGGAGA ^{3'}	51.0	3.0	
33	5'TCT ^L CCT ^L CCT ^L TC ^{3'}	5'GAAGGAGGAGA ^{3'}	56.0	8.0	
34	5'TCTCC ^L TCCTTC ^{3'}	5'GAAGGAGGAGA ^{3'}	54.0	6.0	
35	5'TC ^L TCC ^L TCC ^L TTC ^{3'}	5'GAAGGAGGAGA ^{3'}	62.0	14.0	
36	5'TC ^L TCC ^L TCC ^L TTC ^{3'}	5'GAAGGAGGAGA ^{3'}	68.0	20.0	

^a C = cytosine, C^L = 5-methylcytosine α -L-LNA.

but unfortunately, the T_m was decreased in all cases. The ΔT_m was -7 °C in case of G^L (ON3) and T^L (ON5). In case of A^L (ON4) and T^L (ON6) it was -5 °C and -5.5 °C, respectively. This confirms the previously published statement that LNA in the TFO destabilize the anti-parallel triplex.²⁵ Also, we tried to modify the Watson–Crick part in the middle with LNA. However, the T_m increased only 0.5 °C with A^L (ON8) and decreased 1 °C with G^L (ON7), Table 1.

Moreover, when the TFO and Watson–Crick parts both were modified with LNA in the middle of the sequence, the T_m also decreased, but we noticed that the decrease in the T_m is much lower than in case of LNA in the TFO only. For example, when we compared the T_m values of ON3 and ON10, we observed that the T_m increased about 2 °C (from 54.5 °C to 56.5 °C, Table 1). The same trend in T_m values was observed when comparing ON4 with ON9 and ON6 with ON11.



One insertion of T^α in the pyrimidine target sequence and in the TFO part decreased the thermal stability of the anti-parallel triplex by only 0.5 °C (ON14). In contrast, using T^L in the same two positions the decrease in T_m was 3 °C (ON15).

Interestingly, the stability of anti-parallel triplex increased, when all the three strands were modified with LNA. The T_m data revealed that, the difference in T_m values between triplexes ON11 with LNA in TFO and Watson–Crick parts and ON12 with LNA in all three parts is 5.5 °C (ΔT_m changed from -2 °C to 3.5 °C, Table 1). We assume that, this dramatic increase in the T_m when the pyrimidine target sequence was modified with LNA, is due to increased stacking between the nucleobases in the duplex part (see molecular modeling section).

It has been reported that 2–4 insertions of twisted intercalating nucleic acid (TINA) in the TFO strand increase the stability of G-rich TFOs and prevent G-quadruplexes formation. The pyrene moiety is positioned inside the DNA duplex between base pairs during the triplex formation which increases the stacking in the duplex part.¹⁰ This means that, the stability of anti-parallel TFO depends on the stacking in Watson–Crick duplex. Based on this observation, it was found interesting to modify only the pyrimidine target sequence with LNA and therefore the triplexes ON17–ON25 were investigated. As expected, one insertion of LNA in the pyrimidine target sequence increases the T_m by 4 °C in case of ON18 with T^α and by 3.5 °C in case of ON17 with T^L . When 5-methylcytosine (C) was used as base in LNA and α -L-LNA, it was found that one insertion of α -L-LNA-C (C^α) in ON23 and LNA-C (C^L) in ON21 increase the melting temperature by 6 °C and 5.5 °C, respectively.

The effect seems additive since three insertions of T^α in ON20 or T^L in ON19 gave higher thermal stabilities with $\Delta T_m = 11.5$ °C and 9 °C, respectively, compared to the wild type ON2 (Table 1). The same trend was observed for three modifications C^α (ON24) and C^L (ON22) in the pyrimidine target sequence, $\Delta T_m = 15.5$ °C and 13 °C, respectively (Table 1), reflecting higher melting temperatures for LNAs-C than for LNAs-T.

From the results, we noticed that α -L-LNA gave higher thermal stability in all cases compared to LNA. Concerning the conformational structure of anti-parallel triplexes, it has been reported that only S-type (south-type) sugars are favorable for the anti-parallel triplex.²⁸ On the other hand, It has been found that the conformation structure of the furanose ring in α -L-LNA is the S-type,²⁰ and this explain that α -L-LNA is more suitable for anti-parallel TFOs. This encouraged us to increase the number of α -L-LNA in the pyrimidine target sequence to six α -L-LNA nucleobases using three C^α and three T^α (ON25) which leads to a significant increase in the thermal stability with $\Delta T_m = 21.5$ °C (Table 1).

In order to be sure that the TFO strand is contributing to the stability of the target complex, the corresponding duplexes ON26–ON36 were considered. In all cases the T_m was lower than for the corresponding triplexes. This was also the case for ON29–ON31 with a mismatched dangling TFO part (Table 2).

CD measurements

All the triplexes with or without modification of T in the pyrimidine target sequence showed a positive band at 270 nm and a negative band at 242 nm in their CD spectra. This means that the spectra of ON12–ON20 with T^L and T^α are in agreement with the CD spectra of (G,A) anti-parallel triplexes,²⁹ Fig. 4a.

From Fig. 4b it can be seen that there is an indication of shoulders on both sides of the 270 nm band in the CD spectra of ON21 with one C^L insertion in the target sequence and of ON23 with one C^α insertion in the target sequence. When the number of C^L or C^α was increased, the CD spectra changed more for ON22, ON24 and ON25 and shoulders appeared clearly at 255 nm and 285 nm.

The increasing number of C^L and C^α in the target sequence may change the conformation of the duplex part of the triplex. When inserted into a duplex, LNA is known to change the flanking nucleotides into an N-type sugar conformation.³⁰ In fact, the shoulders in the CD spectra of ON21–ON25 corresponds to the main bands observed by Shibata *et al.* in an

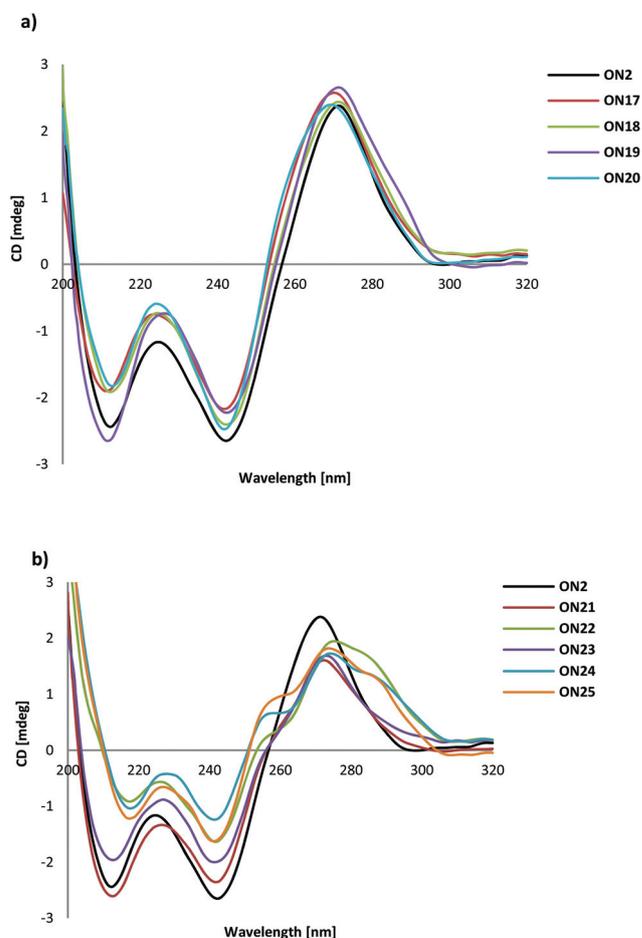


Fig. 4 (a) Typical examples of CD spectra of ON2 and ON17–ON19 with T^L and T^α . (b) CD spectra of ON2 and ON21–25 with C^L and C^α .



anti-parallel triplex with a higher G/A ratio and with 5-amino-methyl modification for one of the thymidines.³¹

Molecular modeling

In order to understand the binding and stacking properties of the anti-parallel triplex modified with LNA and α -L-LNA, we decided to get insight into their structures *via* molecular modeling studies. An AMBER* force field in Macro Model 9.1, molecular modeling was used to generate representative low energy structures of the modified structures with LNA and α -L-LNA at different positions in the Watson-Crick and TFO parts of the triplexes. Patel's structure of the anti-parallel triplex d(TGGTGGT) which contains d(G-GC) and (T-AT) triads (PDB entry pdb134d)³² was used and modified with LNA and α -L-LNA.

The results showed that, multi insertion of α -L-LNA (T^α and C^α) in the pyrimidine target sequence increase the stacking between the nucleobases in the three strands of the anti-parallel triplex which reflects the high thermal stability of ON25 (Fig. 5a).

In contrast, a larger distortion of the triplex structure was observed when the TFO strand was modified with T^L (Fig. 5d), the LNA moiety and some other TFO's nucleobases were forced to twist out of plane of Watson-Crick base pair which is weakening the stacking interactions with the TFO nucleobases and the binding with the duplex part. The same trend was

observed in case of T^α in the TFO strand (Fig. 5c). This explains the lower thermal stability of ON5 and ON6.

However, when the TFO, Watson-Crick and the target strands were modified with T^α , A^L and T^α , respectively (Fig. 5b), the triplex structure was undisturbed and the T^α -nucleobase in the TFO strand hybridized nicely to the nucleobase A^L in the Watson-Crick duplex, thereby explaining the increase in stability when the three strands were modified with α -L-LNA and LNA (ON12).

Conclusion

The stability of anti-parallel triplexes was increased when the pyrimidine sequence was modified with LNA (C^L and T^L) and α -L-LNA (C^α and T^α). Molecular model building is supportive of base stacking between the nucleobases in the duplex part. However, α -L-LNA gave higher thermal stability in all cases when compared to LNA, because the conformational S-type structure of α -L-LNA corresponding to the sugar conformation in anti-parallel triplexes is preferable for triplex stability.²⁸ A substantial increase in thermal stability was also observed when a base triad was fully modified with LNA and α -L-LNA (T^α, A^L, T^α or T^L, A^L, T^α) in contrast to modification in the purine strands only. When only the purine TFO strand was modified, the triplex stability was reduced. According to molecular modeling, this is due to reduced stacking in the TFO part. We think that our findings about antiparallel triplex stability could be important for developing a strategy for strand invasion by clamping a G-rich purine strand in a duplex. This corresponds to a reported strand invasion method based on formation of a parallel triplex on an A-rich sequence.³³

Experimental section

General

All locked nucleoside amidites were purchased from Exiqon. Unmodified oligonucleotides were purchased from Sigma on a 0.2 mmol scale; the purity was checked by ion-exchange chromatography and MALDI-TOF mass spectrometry.

Oligonucleotide synthesis

Modified oligonucleotides were synthesized in 0.2 mmol scale on 500 Å CPG supports using Expedite™ Nucleic Acid Synthesis System model 8909 from Applied Biosystems. Standard procedures were used for the coupling of commercial phosphoramidites, whereas modified phosphoramidites, LNA-A (A^L), LNA-G (G^L), LNA-T (T^L), α -L-LNA-T (T^α), 5-methylcytosine types LNA-C (C^L) and α -L-LNA-C (C^α), were coupled with 1*H*-tetrazole in CH_3CN as an activator and an extended coupling time (15 min), the coupling efficiency was higher than 95% in all cases. ONs were de-protected and cleaved from the CPG support with 32% aq. NH_3 (1 mL) and left at 55 °C for 20 h. ONs were purified as DMT-on ONs by Reversed phase-HPLC using: (1) Column: XBridge OST C18, 19 × 1000 mm, 5 μ m +

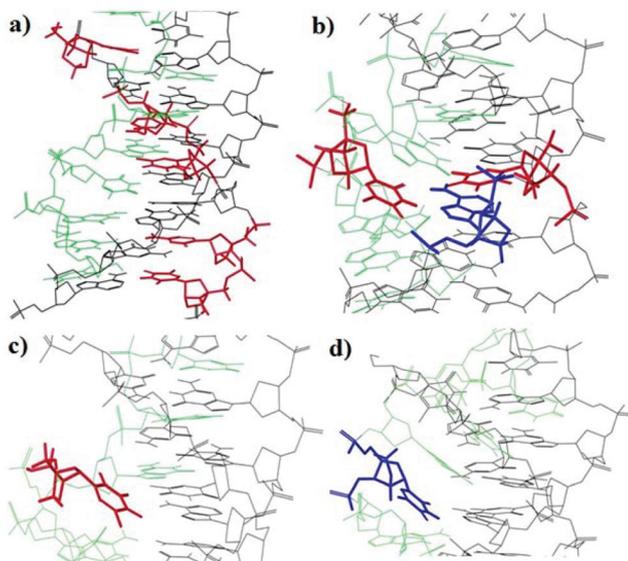


Fig. 5 Representative low-energy conformations of anti-parallel triplex (TFO the green color and Watson-Crick duplex the black color) containing insertion of LNA (blue color) and α -L-LNA (red color) produced by AMBER* calculations (a) α -L-LNA-T and α -L-LNA-C insertions in the target sequence, (b) α -L-LNA-T, LNA-A and α -L-LNA-T insertions as a base triplet in the TFO and duplex parts of the triplex. (c) α -L-LNA-T insertion in the TFO. (d) LNA-T insertion in the TFO.



precolumn: XBridge 10 × 10 mm, 5 μm, the temperature of column oven was 50 °C. (2) Buffers: [Buffer A: 0.05 M TEAA (triethyl ammonium acetate) pH 7.4; Buffer B: 75% MeCN/25% Buffer A], flow: 5 mL min⁻¹.

ONS were submitted to DMT deprotection with 80% aq. AcOH (100 μL) for 30 min, followed by addition of doubly filtered H₂O (100 μL), aq. AcONa (5 M, 15 μL), and aq. NaClO₄ (5 M, 15 μL) before precipitation from pure acetone (1 mL). Molecular masses of all modified ONS were confirmed by MALDI-TOF analysis on a Ultraflex II TOF/TOF system from Bruker (a MALDI-LIFT system) with HPA matrix (10 mg 3-hydroxypicolinic acid, in 50 mM ammonium citrate/70% MeCN). The purity of the final ONS were found to be ~99% when checked by ion-exchange chromatography using *La-Chrom* system from Merck Hitachi on *Dionex DNA Pac Pa-100*, 4 × 250 mm anal. column. Buffers: [Buffer A: H₂O Milli-Q; Buffer B: 1 M NaClO₄; Buffer C: 25 mM Tris-Cl, pH 8.0; Buffer D: MeCN], Flow: 1 mL min⁻¹.

Thermal denaturation studies

Thermal melting measurements were performed on a Perkin-Elmer UV/VIS spectrometer Lambda 35 fitted with a PTP-6 temperature programmer. Melting experiments were performed on equimolar amounts of the appropriate oligonucleotides (target sequence and hairpin) (3 μM) in a buffer solution consisting of sodium cacodylate (10 mM), EDTA (0.1 mM), and MgCl₂ (50 mM) at pH 7.2. The solutions were heated to 90 °C then cooled down slowly to room temperature, and were then kept at this temperature for 2 h. The absorbance of the formed triplexes was measured at 260 nm with a heating rate of 0.5 °C min⁻¹. The melting temperature (*T*_m) was determined as the maximum of the first derivative plots of the melting curves.

Circular dichroism spectra (CD)

Circular dichroism spectra were recorded on a Jasco J-600A spectropolarimeter using 1 mL quartz cuvettes with 5 mm path length. Oligonucleotides (3 μM) were dissolved in a buffer solution consisting of sodium cacodylate (10 mM), EDTA (0.1 mM), and MgCl₂ (50 mM) at pH 7.2. All samples were heated for 2 minutes at 90 °C and slowly cooled to room temperature before data collection. Measurements were performed at 20 °C in the 200–350 nm wavelength range with a continuous scanning mode, 50 nm min⁻¹ as a scanning speed, 4 s for a response and accumulation 5 times. The spectrum of the buffer solution was subtracted from the sample spectra.

Molecular modeling

Molecular modeling was performed with Macro Model v9.1 from Schrödinger. All calculations were conducted with AMBER* force field and the GB/SA water model. The dynamic simulations were performed with stochastic dynamics, a SHAKE algorithm to constrain bonds to hydrogen, time step of 1.5 fs and simulation temperature of 300 K. Simulation for 0.5 ns with an equilibration time of 150 ps generated 250 structures, which all were minimized using the PRCG method with convergence threshold of 0.05 kJ mol⁻¹. Patel's structure of the

anti-parallel triplex³² was downloaded from the protein data bank (PDB entry pdb134d), followed by incorporation of the nucleobases T^L, T^α, C^α and A^L.

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