
Triplex-stabilizing properties of parallel clamps carrying LNA derivatives at the Hoogsteen strand

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DNA parallel clamps with a polypurine strand linked to a polypyrимidine Hoogsteen strand containing locked nucleic acids bind their corresponding polypyrимidine targets with high affinity.

Introduction.- Triplex-forming oligonucleotides (TFOs) constitute interesting DNA sequence-specific binders that have wide potential applications in diagnosis, gene analysis and therapy. Under experimental conditions, triplex formation can be used to extract and isolate specific nucleotide sequences [1], inhibit DNA transcription and replication [2], generate site-directed mutations [3], cleave DNA [4] or induce homologous recombination [5].

In a classical DNA triplex, the triplex-forming oligonucleotide binds to a polypurine-polypyrимidine region of dsDNA in the major groove through specific hydrogen bonds. Depending on the orientation and base composition of this third strand, triplexes are classified as parallel or antiparallel. Homopyrимidine TFOs bind parallel to the duplex purine strand via Hoogsteen hydrogen bonds to form T·AT and C⁺·GC triplets, whereas purine-rich TFOs bind in an antiparallel orientation by reverse Hoogsteen bonding and form G·GC, A·AT and T·AT triplets [6].

Because cytosine bases in a homopyrимidine TFO must be protonated at N3 to form C⁺·GC triplets, the formation of parallel triplexes requires acidic conditions and is thus extremely unstable at pH 7. Therefore, stabilization of the homopyrимidine TFOs
at physiological conditions is of great importance in improving its biological potential and a great effort has been made to modify oligonucleotides to enhance triple helix stability [7].

In 1998, novel conformationally restricted nucleotide analogues known as locked nucleic acid (LNA) were described [8]. 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 flexibility of the ribose and increasing the local organization of the phosphate backbone. This pre-organization is believed to increase the strength of base-stacking interaction [9]. Several studies demonstrate that LNAs have greater affinity for complementary DNA and RNA sequences [10].

The triplex-forming properties of oligonucleotides containing LNA in the Hoogsteen chain have been reported elsewhere [11]. Except Petersen and colleagues [11b], who studied an intramolecular dsDNA:LNA triplex, the remaining authors focused on adding a homopyrimidine LNA-modified TFO to an existing WC duplex.

An alternative approach consists in the triple-helix formation of a pyrimidine RNA/DNA strand with a parallel-strand Hoogsteen hairpin duplex. Such parallel-stranded hairpins were first described by Agrawal and Kandimalla [12] and have been reported to form triplex with greater thermal stability than conventional triplexes. In addition, the use of parallel clamps not only allows the possibility of forming triplexes with mRNA targets [1, 12] but also decreases the susceptibility of oligonucleotides to degradation by cellular nucleases [12, 13].

In our experiments the homopyrimidine LNA-containing Hoogsteen strand and the natural homopurine WC strand are covalently attached by 5’-5’ internucleotide junctions, and the WC pyrimidine strand is added. The sequence was selected from a triplex studied by Xodo et al. [14] which we had also studied in the context of 8-
aminopurines substitutions [15]. We have previously shown that the presence of 2’-O-methyl-RNA residues improves triplex formation [15c]. Here, we show that parallel clamps containing LNA residues at the Hoogsteen polypuridimine strand bind to the corresponding polypuridimine target strand via triple-helix formation with greater affinity than unmodified hairpins.

**Results and Discussion.** *1. Design of oligonucleotides.* Oligonucleotides used in this study are listed in Table 1. Oligonucleotide sequences are based on a triplex characterized previously.\(^{14}\) Here, the polypuridimine Hoogsteen strand was linked to the Watson-Crick (WC) polypuridimine strand (Figure 1). Clamps are formed by two chains of eleven bases connected by 5’-5’ through a tetrathymidin loop. Hairpin B22 is a control sequence that contains only the natural bases without modifications. Hairpin B22-T contains three LNA thymidine nucleotides and B22-C contains three 5’-Me-C-LNA bases. In addition, a hairpin bearing two T-LNA and three 5’-Me-C-LNA was prepared (B22-CT) to test whether the stabilizing properties of the two modifications are additive.

To study the effect of the sugar type of the target on triplex stability, the polypuridimine target strand was synthesized with either DNA or 2’-O-methyl-RNA backbone. A 2’-O-methyl-RNA strand was used as a nuclease-stable model for a natural RNA target.

*2. Thermal stability of the Hoogsteen parallel duplexes.* First melting curves of clamps (B22, B22-T, B22-C and B22-CT) alone, in the absence of the polypuridimine target, were recorded (Table 2). Depending on the pH we observed two different transitions. At pH 5 we observed a transition with 15% hyperchromicity at 260 nm that
decreased in intensity at higher pH. This transition was assigned to the denaturation of the parallel duplex, as described previously [15, 16]. To confirm that this transition corresponds to the denaturation of the Hoogsteen parallel duplex to a random coil, all the melting curves were also recorded at 295 nm. In all cases we observed the presence of a melting curve with hypochromicity as expected for a parallel duplex. It has been established that the change of absorbance at 295 nm indicates the formation of a nucleic-acid structure that requires protonation / deprotonation of cytosines [17]. As 295 nm corresponds to a negative peak of the differential spectrum between protonated and non-protonated cytosines, the melting profile recorded at 295 is expected to be inverted. On the other hand, the absence of hyperchromism or hypochromism at 295 nm seems to be a general phenomenon for many WC duplex transitions.

At pH above 6, a new transition appears at higher temperatures. We suggest that at pH above 6 the structure rearranges and short intermolecular antiparallel duplexes can be formed with the central part of the oligonucleotides (like a 7mer duplex between -AGGAGGA- and -TCCTCCT-). This effect is mainly observed in clamps carrying LNA modifications, probably because LNA can enhance hybridization of complementary sequences. These transitions are only observed at 260 nm. No transitions are observed at 295 nm which indicates the presence of an antiparallel Watson-Crick duplex.

The melting temperatures for clamps carrying the LNA were much higher than those for the unmodified sequence (Table 2). At pH 5.0 the increase in melting temperature ($T_m$) is 24 °C for B22-C and 22 °C for B22-T. The B22-CT hairpin, with the highest LNA content, is the most stable especially at pH 5 (more than 40 °C difference). These results show that LNA modifications in the polypyrimidine strand enhance the stability of parallel duplexes, and therefore the stability of Hoogsteen bonds.
3. Thermal stability of triplexes formed by parallel clamps and their DNA / RNA polypyrrimidine target strands. In the presence of a polypyrrimidine target strand (WC-11mer or WC-11mer/2’-OMe) hairpins B22 and derivatives form stable triplexes with higher stability than the Hoogsteen parallel duplexes alone.

The thermal stability as indicated by the $T_m$ value of all triple helices is given in Tables 3 (for DNA target) and 4 (for RNA target). As in the case of hairpins, the melting curves for the triplexes were recorded at both 260 and 295 nm to ensure that changes corresponded to triplex-to-random-coil transition.

Although the stability of all these triplexes decreased when the pH was raised (caused by a destabilization of Hoogsteen bond due to a lower proportion of C\(^+\)) the transition was observed even at pH 7.

In all cases the resulting triplexes showed single, cooperative melting transitions (sigmoidal curves) with a pH-dependent hyperchromicity (ranging from 15% to 30% when pH decreases from pH 7.0 to pH 5.0 for triplexes with the DNA target, and from 10% to 30% in the case of triplexes with the RNA target. This single transition was observed at both 260 and 295 nm throughout the pH range studied, indicating that the Hoogsteen and WC hydrogen-bonded strands dissociate simultaneously and that the change of absorbance in the melting profile is due to triplex-to-random-coil transition as described for clamps carrying 8-aminopurines [15].

In all cases the triplexes formed by LNA-containing hairpins showed higher $T_m$ values than the unmodified triplexes, especially when the target was an RNA strand. The increase in $T_m$ is presented in parentheses next to the $T_m$ data for each pH value.

When the target was a DNA sequence, B22T and B22C hairpins stabilized the triplex to a similar extent with an increase in $T_m$ of 8-12\(^\circ\)C relative to the unmodified
triplex. For B22-CT, differences in parallel duplex stability relative to B22-C and B22-T disappeared when the triplex was formed, except for pH 5, where B22-CT still showed a significant increase in T\textsubscript{m}.

RNA or DNA backbone composition is reported to have dramatic effects on triple helix stability [18]. To study such effects, a target RNA polypyrimidine strand was synthesized.

Although the T\textsubscript{m} for the triplex formed by the unmodified B22 hairpin and the RNA target is lower than that of the triplex formed with the DNA target, the LNA-containing hairpins form a more stable triplex in the presence of complementary RNA strand. Previous studies showed that combination of RNA pyrimidine and DNA purine strands forms a highly stable triplex [18]. As LNA has a locked C3’-endo-sugar pucker it can be considered as an RNA analogue derivative. So, when a polypyrimidine target is an RNA strand and the Hoogsteen pyrimidine strand of the clamp contains LNA, the resulting triplex will contain this favourable combination. According to this, the B22-CT, with a higher LNA content and the most A-like (RNA) geometry, would form the most stable triplex, which is consistent with our experimental data.

Conclusions.
We have shown that LNA-modified parallel clamps bind to their polypyrimidine target strands via triple-helix formation with greater affinity than hairpins containing only natural bases. In addition, the presence of LNA residues stabilizes the parallel duplex, as can be deduced from the Tm values of hairpins alone at pH 5.0. As LNA nucleotides have a C3’-endo sugar pucker, the backbone conformation seems to have a strong positive effect on triplex and parallel duplex structures. Triplex stabilization by LNA
residues is even stronger when the polypyrimidine target strand is an RNA sequence. These results are relevant for the design of clamps for potential applications based on triplex formation such as triplex affinity capture [1] and antisense inhibition of gene expression [12].

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Experimental Part

General. Phosphoramidites and ancillary reagents used during oligonucleotide synthesis were from Applied Biosystems (PE Biosystems Hispania S.A., Spain). The rest of the chemicals were purchased from Aldrich, Sigma or Fluka (Sigma-Aldrich Química S.A., Spain).

Oligonucleotide synthesis. Oligonucleotides were prepared on an automatic Applied Biosystems 3400 synthesizer using phosphoramidite chemistry on polystyrene solid support (LV200). 5’-5’-Parallel clamps were prepared in three steps. First, the pyrimidine part was assembled using the appropriate C, T, LNA-T and LNA-5’-methyl-C phosphoramidites. Longer oxidizing and coupling times were used with LNA monomers, as indicated by the manufacturer. The phosphoramidite of 5’-methyl-C-LNA was dissolved in a acetonitrile / tetrahydrofuran (3:1) mixture. Second, four additional thymidine phosphoramidites were added. This tetranucleotide loop was used
to connect the two strands. From there onward, synthesis was continued using reversed G and A phosphoramidite monomers generating a 5’-5’ phosphate bond. After the assembly of the desired sequence, oligonucleotides were deprotected and cleaved from the support with concentrated aqueous ammonia at 55 °C overnight. Ammonia solutions were concentrated to dryness and the products were purified by reverse-phase HPLC. Oligonucleotides were synthesized on a 0.2 µmol scale without performing final detritylation to facilitate reverse-phase purification. All purified products (DMT on protocol) presented a major peak, which was collected. HPLC solutions were as follows: solvent A, 5% ACN in 0.1 M triethylammonium acetate pH 6.5; solvent B, 70% ACN in 0.1 M triethylammonium acetate pH 6.5. Column Nucleosil C18, 250 x 8 mm. A 20 min linear gradient from 15 to 80% B (DMT on) with a flow rate of 3 ml/min was used. Finally oligonucleotide clamps were detritylated, desalted by NAP-10 and quantified by measuring the absorbance at 260 nm. Their molar absorptivities were calculated assuming identical absorptivities for LNA and DNA monomeric nucleotides. Masses of the oligonucleotides were measured by MALDI-TOF-MS in negative mode recorded on a Perseptive Voyager DETMRP mass spectrometer, equipped with nitrogen laser at 337 nm using a 3ns pulse. The matrix used contained 2,4,6-trihydroxyacetophenone (THAP, 10 mg/ml in acetonitrile/ water 1:1) and ammonium citrate (50 mg/ ml in water). Measured masses of the oligonucleotides (calculated masses for M): WC-11mer: 3194 (3194); WC-11mer/ 2’-O-Me: 3453 (3454); B22: 7950 (7952); B22-T: 8028 (8036); B22-C: 8072 (8078); B22-CT: 8122 (8134).

*Thermal denaturation studies.* Melting experiments were performed as follows. Solutions of equimolar amounts of hairpins with or without the target Watson–Crick pyrimidine strand were mixed in 0.1 M sodium phosphate/citric acid buffer (pH ranging
from 5.0 to 7.0) containing 1 M NaCl, with a final duplex concentration of 2 µM. The solutions were heated to 90°C, allowed to cool slowly to room temperature, and stored at 4°C until UV was measured. Thermal melting curves were recorded at 260 and 295 nm at a heating rate of 1°C/min with a Jasco V-650 Elmer spectrophotometer. The melting temperatures (Tm) were determined at the maximum of the first derivative of the melting curve. Melting temperatures are an average of at least two independent measurements within ±1.0 °C.

REFERENCES


LEGENDS

Fig. 1. A) Structure of the triplex studied, B) T.A.T and C+G.C triads C) Structure of LNA-T and LNA-Me-C phosphoramidites used.
Table 1. *Oligonucleotide sequences used in this study.*

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>WC-11mer</td>
<td>5’ TCTCCTCCTTC 3’</td>
</tr>
<tr>
<td>WC-11mer/2’-OMe</td>
<td>5’ UCUCUCUUC 3’</td>
</tr>
<tr>
<td>B22</td>
<td>3’ AGAGGAGGAAG 5’ - 5’ TTTT - CTTCCCTCTCT 3’</td>
</tr>
<tr>
<td>B22-T</td>
<td>3’ AGAGGAGGAAG 5’ - 5’ TTTT - CTtCCtCCtCT 3’</td>
</tr>
<tr>
<td>B22-C</td>
<td>3’ AGAGGAGGAAG 5’ - 5’ TTTT - CTTeCTeCTeT 3’</td>
</tr>
<tr>
<td>B22-CT</td>
<td>3’ AGAGGAGGAAG 5’ - 5’ TTTT - CTTeCTeCTeT 3’</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lower bold case letters t and c are LNA thymidine and LNA 5’-methylcytosine respectively. Underlined upper case letters U and C are 2’-O-methyl-RNA derivatives.
Table 2. *Melting temperatures (°C) of transitions observed with B22 and derivatives alone, without target.* Two transitions are observed. The first transition was observed at 260 nm (hyperchromic) and 295 nm (hypochromic). The second transition was only observed at 260 nm (hyperchromic).

<table>
<thead>
<tr>
<th>hairpin</th>
<th>pH 5</th>
<th>pH 5.5</th>
<th>pH 6</th>
<th>pH 6.5</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>B22</td>
<td>52 /--</td>
<td>-- /--</td>
<td>-- /55</td>
<td>-- /55</td>
<td>-- /56</td>
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<tr>
<td>B22-T</td>
<td>74 /--</td>
<td>52 /--</td>
<td>40 /--</td>
<td>-- /75</td>
<td>-- /70</td>
</tr>
<tr>
<td>B22-C</td>
<td>76 /--</td>
<td>55 /--</td>
<td>45 /--</td>
<td>-- /77</td>
<td>-- /75</td>
</tr>
<tr>
<td>B22-CT</td>
<td>&gt;90 /--</td>
<td>64 /--</td>
<td>51 /--</td>
<td>-- /80</td>
<td>-- /80</td>
</tr>
</tbody>
</table>
Table 3. *Melting temperatures (°C) for the triplexes formed by B22 and derivatives with the DNA target WC-11mer.* One single transition is observed at 260 nm (hyperchromic) and 295 nm (hypochromic).

<table>
<thead>
<tr>
<th>hairpin</th>
<th>target</th>
<th>pH 5</th>
<th>pH 5.5</th>
<th>pH 6</th>
<th>pH 6.5</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>B22</td>
<td>WC-11mer</td>
<td>62</td>
<td>55</td>
<td>45</td>
<td>31</td>
<td>18</td>
</tr>
<tr>
<td>B22-T</td>
<td>WC-11mer</td>
<td>74 (+12)</td>
<td>65 (+10)</td>
<td>53 (+8)</td>
<td>39 (+8)</td>
<td>28 (+10)</td>
</tr>
<tr>
<td>B22-C</td>
<td>WC-11mer</td>
<td>73 (+11)</td>
<td>63 (+8)</td>
<td>52 (+7)</td>
<td>40 (+9)</td>
<td>29 (+11)</td>
</tr>
<tr>
<td>B22-CT</td>
<td>WC-11mer</td>
<td>79 (+17)</td>
<td>64 (+9)</td>
<td>52 (+7)</td>
<td>39 (+8)</td>
<td>28 (+10)</td>
</tr>
</tbody>
</table>

* Values in parenthesis indicates the ΔTm relative to unmodified triplexes. All Tm values are calculated with the curves recorded at 260 nm.
Table 4. *Melting temperatures (°C) for the triplexes formed by B22 and derivatives with the RNA target WC-11mer/2’-OMe.* One single transition is observed at 260 nm (hyperchromic) and 295 nm (hypochromic).

<table>
<thead>
<tr>
<th>hairpin</th>
<th>target</th>
<th>pH 5</th>
<th>pH 5.5</th>
<th>pH 6</th>
<th>pH 6.5</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>B22</td>
<td>WC-11mer/2’-OMe</td>
<td>62</td>
<td>53</td>
<td>41</td>
<td>24</td>
<td>n.d.</td>
</tr>
<tr>
<td>B22-T</td>
<td>WC-11mer/2’-OMe</td>
<td>77 (+15)</td>
<td>68 (+15)</td>
<td>56 (+15)</td>
<td>39 (+15)</td>
<td>25</td>
</tr>
<tr>
<td>B22-C</td>
<td>WC-11mer/2’-OMe</td>
<td>77 (+15)</td>
<td>67 (+14)</td>
<td>53 (+12)</td>
<td>40 (+16)</td>
<td>27</td>
</tr>
<tr>
<td>B22-CT</td>
<td>WC-11mer/2’-OMe</td>
<td>84 (+22)</td>
<td>73 (+20)</td>
<td>60 (+19)</td>
<td>44 (+20)</td>
<td>30</td>
</tr>
</tbody>
</table>

*Values in parenthesis indicates the ΔTm relative to unmodified triplexes. All Tm values are calculated with the curves recorded at 260 nm. n. d. not detected.*
A) Parallel hairpin B22 and derivatives

WC polypyrimidine target

B)

C)
Graphical illustration for the table of contents