LNA units present in \([R_P\text{--}PS\text{--}(DNA\#LNA)]\) chimeras enhance the thermal stability of parallel duplexes and triplexes formed with \((2'\text{--}OMe)\text{--}RNA\) strands\(^{†‡}\)

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The results of CD measurements indicate that \(2\text{--}4\) LNA units distributed along \(12\) nt P-stereodefined phosphoroiothioate \([R_P\text{--}PS\text{--}(DNA\#LNA)]\) chimeras impose a \(C3'\text{--}endo\) conformation on the \(2'\text{--}\)
deoxyribonucleosides. Under neutral and slightly acidic conditions homopurine \([R_P\text{--}PS\text{--}(DNA\#LNA)]\) hybridizes with \(9\text{--}12\) nt Hoogsteen-paired \((2'\text{--}OMe)\text{--}RNA\) strands to form parallel duplexes, which are thermally more stable than the reported earlier analogous complexes containing LNA-free \([R_P\text{--}PS\text{--}DNA\) oligomers \(\Delta T_m = 7 \text{ °C} per\) LNA unit at \(pH 5.4\). Upon addition of the corresponding Watson–Crick-paired \((2'\text{--}OMe)\text{--}RNA\) strands, parallel triplexes are formed with further increased thermal stability.

Introduction

Nucleic acid triple-helical forms have been known for sixty years.\(^3\) Their formation seems to play a role in sequence-specific recognition of a double helix,\(^2\text{--}4\) as well as in chromatin organization, DNA repair, transcriptional regulation and RNA processing.\(^5\text{--}6\) The third strand binds to an antiparallel duplex either in a parallel or an antiparallel orientation with respect to the purine strand, utilizing either a Hoogsteen or reverse Hoogsteen hydrogen bonding scheme, respectively.\(^7\) Hoogsteen \(G\text{--}C^+\) base pairing requires protonation of the \(N3\) nitrogen atoms in cytosines\(^8\) (for \(CMP\) and \(dCMP\), \(pK_a\) of \(4.3\) and \(4.6\) was found at \(25^\circ\text{C}\), respectively). Triplexes may comprise RNA and/or DNA strands, with consequences for thermal stability.\(^10\text{--}11,12\)

Usually, the Hoogsteen paired (Hp) parallel duplexes are thermally much less stable than the Watson–Crick paired (WCp) duplexes, but their stability increases when a Hp chain is linked to a pyrimidine chain (of inverted polarity) by a \(3'\text{--}3'\) or \(5'\text{--}5'\) linker.\(^13\text{--}16\) There are a few reports on parallel stretches in native DNA\(^17\text{--}19\) and in *Escherichia coli* mRNA.\(^20\) Hp-DNA duplexes seem to be involved in regulation of cell processes and evolution of neurodegenerative diseases.\(^21\text{--}23\)

Since natural DNA molecules are easily degraded by phosphodiesterases, several modifications of the sugar-phosphate backbone have been proposed. Among them, phosphoroiothioate analogs of DNA (PS-DNA) were found to be very useful because their electronic and steric properties are remarkably close to those of DNA.\(^24\text{--}26\) However, as short as \(10\text{--}12\) nt PS-DNA oligomers prepared by standard chemical methods (a phosphoramidite or an \(H\)-phosphonate approach) exist as mixtures of hundreds or even thousands of \(P\)-diastereomers.\(^27\) Developed in this laboratory an oxathiaphospholane approach\(^28\text{--}29\) (utilizing the OTP monomers 1, Chart 1) allows for preparation of \(P\)-stereodefined PS-DNA.

It was found that the antiparallel duplexes formed by \([R_P\text{--}PS\text{--}DNA\) (sDNA) or the \(S_P\)-counterparts (sDNA) with DNA or RNA oligomers were thermally less stable than analogous complexes formed by unmodified oligomers.\(^30\) However, we discovered that homopurine sDNA form thermodynamically highly stable parallel duplexes RNA\&sDNA (e.g. 1, “&” indicates the parallel orientation of strands) and even more stable parallel triplexes RNA\&sDNA:RNA (e.g. 11),\(^31\text{--}33\)

![Chart 1](image)

**Chart 1** Structure of the 2-thio-1,3,2-oxathiaphospholane derivatives of nucleosides of the DNA (1) and LNA (2) type.
check if a few LNA units (here denoted AL or GL) present in energetic toll for the C2 (H–P–O–S–P)–RNAs (mRNA), which are known for adopting more Hp-mRNA strands gave rise to significantly enhanced thermal stability. Our experiments showed that 2-4 pyrimidine LNA units present in 9-12 nt Hp-mRNA strands gave rise to significantly enhanced thermal stability of the (mRNA/LNA) complexes.7 We wanted to check if a few LNA units (here denoted AL or GL) present in homopurine [R-P-PS]-DNA/LNA chimeric oligomers (αDL, 3, Scheme 1) would impose a C3'-endo conformation on the 2'-deoxyribonucleosides in the phosphorothioate strand. If so, an energetic toll for the C2'-endo → C3'-endo transition of the phosphorothioate central strand (Scheme 1) should be smaller giving rise to increased thermal stability of the parallel complexes. For that purpose we used recently developed P-diastereomerically pure OTP,L monomers 2 (Chart 1).8

Results and discussion

Synthesized oligomers

In the codes shown in Table 1 and used throughout the text, the prefixes α/β indicate R-P/β-P analogs, respectively, and the ending digit indicates the number of LNA units in a 12 nt oligomer. Accordingly, a code αA2 indicates an [R-P-PS]-DNA/LNA chimera bearing 10 2'-deoxyribonucleosides (dA, dG) and 2 AL units, whereas sG4 stands for an [α-P-PS]-DNA/LNA analog bearing 4 GL units. Four reference, non-palindromic, homopurine 12 nt oligonucleotides were synthesized (Table 1): (1) B0, a basal phosphate, LNA-free oligomer d(GGAGAAAGAGAG), (2) G4, an analog of B0 containing four GL units, (3) sB0, an R-P-PS analog of B0; and (4) sG4, an S-P-PS analog of G4.

Since only the homopurine [R-P-PS]-DNA oligomers form the complexes I and II, we hypothesized (based on IR measurements and molecular modeling) that the Hp-strand is anchored not only by the hydrogen bonding but also by water bridge(s) (via charge assisted hydrogen bonds54) between the sulfur atoms of the R-P-phosphorothioate moieties and the O2 atoms in pyrimidine nucleobases.5 The overall A-like conformation of the RNA&aDNA and RNA&aDNA:RNA complexes, which is required for the interactions of that type, was imposed by the RNA strand(s), and was confirmed by CD measurements. The conformational factor is important because the complexes I and II are more stable when formed with the participation of (2'-OMe)-RNAs (mRNA), which are known for adopting more profound C3'-endo conformation than RNA molecules. To analyze further this phenomenon we employed LNA units (Chart 1) would impose a C3'-endo conformation on the 2'-deoxygenucleosides in the phosphorothioate strand. If so, an energetic toll for the C2'-endo → C3'-endo transition of the phosphorothioate central strand (Scheme 1) should be smaller giving rise to increased thermal stability of the parallel complexes. For that purpose we used recently developed P-diastereomerically pure OTP,L monomers 2 (Chart 1).8

CD spectra for single-stranded chimeric αDL oligonucleotides

The impact of the LNA units on conformation of the αDL oligomers (single strands) was assessed by CD spectroscopy (Fig. 1). As a reference we used a spectrum recorded for wD12, in which the 2'-deoxyribonucleosides exist in a 2'-endo form. It was found that 2-4 pyrimidine nucleobases.

† The mechanism of stabilization (proposed by C. Hélène and also discussed by others44-46), based on the hydrogen bonding between the 2'-OH group of ribose in the Hp-strand and the pro-Rp oxygen atom (or the Rp sulfur atom in our work) cannot operate because parallel triplexes and parallel duplexes formed with Hp-mRNAs are thermally more stable. Also hydrogen bonding pyrimidine-C2'-O–H–S–P(O)OR,R analogous to Cys-S–H–O–C in proteins,47 is less likely because at neutral pH phosphorothioate diesters are fully ionized.

‡ We assumed high melting temperatures of the investigated complexes, therefore, to avoid degradation of RNA oligomers at elevated temperatures we decided to use (2'-OMe)-RNA oligomers.
that in the spectra recorded for sA4, sG2, and sG4 the isoelectrostatic points shifted from 263 nm to 255–257 nm, so the conformations changed towards that observed for the mRNA oligomer hR12 (the isoelectrostatic point 250 nm, λmax = 273 nm, θ = 5.66 mdeg), in which the nucleosides adopt a 3'-endo form. That change is more developed for sA4 and sG4 as the intensities of the bands around 270 nm are ca. 50% higher than for sG2.

Melting and CD experiments in pH 7.2 buffer

For the annealing/melting and CD experiments, equimolar amounts of the homopurine oligomers (sDL, sG4, or the reference oligomers) and the 12 nt mRNAs (wR12 and/or hR12) or the reference wD12 were mixed. The temperatures of association (Tassoc) determined during annealing and melting (Tm) were determined using the first order derivative method. The hysteresis values (Tm–Tassoc) for the two component mixtures did not exceed 3 °C, unless otherwise stated (Table 2).

The complex formed by the phosphate oligomer G4 with hR12 was less stable than G4:wR12 (Tm = 61 °C and 64 °C, respectively; Fig. S6, ESI†) and this indicates the formation of an imperfect duplex (VI).

Fig. 1 CD spectra for the chimeric sDL and the reference hR12 and wD12 oligomers, recorded at pH 5.4 at room temperature.

Table 2 Melting temperatures [°C] for the complexes formed with the WCP and/or Hp 12 nt mRNA matrices. The samples were dissolved in 10 mM Tris–HCl, 100 mM NaCl, 10 mM MgCl2 buffer (pH 7.2)

| Homopurine oligomer | wR12 | hR12 | hR12 + wR12 |
|---------------------|------|------|-------------|
| B0                  | 51   | 47   | 53          |
| G4                  | 64   | 61   | 69          |
| sB0                 | 62 (I)| 67 (II)|<80 (II)    |
| sA2                 | 79   | 80   |<80 (II) |
| sG4                 | 75 (I)| 80 (II) |

a Roman numerals in parentheses refer to the proposed structures of complexes. b Numbers in brackets indicate the temperature of association found during the annealing in the cases where the hysteresis value exceeded 3 °C.

The WCP duplex sG4:wR12 was more stable than B0:wR12 (ΔTm = 11 °C) thus, the earlier identified stabilizing effect of the LNA units overrode the commonly observed destabilizing effect of the phosphorothioate modification (vide supra). The Tm values found for the intended hR12:sG4 duplex (52 °C) and the hR12:sG4:wR12 triplex (60 °C) were close to that for sG4:wR12 (57 °C), indicating that the parallel complexes were not formed.

As mentioned earlier, sDNA or sRNA and WCP DNA or RNA matrices form the corresponding antiparallel duplexes of significantly lower thermal stability than natural DNA. Thus, taking into account Tm = 50 °C noted for the putative sB0:wR12 complex, which was higher than for B0:wR12 (Tm = 46 °C), and Tm = 62 °C noted for the mismatch-free hR12:sB0, one can conclude that the imperfect (im)wR12:sB0 complexes (VII, 3 mismatches) was formed, rather than the sB0:wR12. This phenomenon is supported by the relevant CD spectrum, in
which an intense negative signal around 210 nm (similar to that for hR12&RB0) was noted (Fig. S7, ESI†) and indicates an enormous stabilizing effect of the [R7-PS]-modification.

As expected, the parallel duplexes hR12&RA2 and hR12&RG4 were thermally substantially more stable (Tm > 75 °C, Fig. 2) than hR12&RB0 (ΔTm = 17 °C and ΔTm = 13 °C, respectively). The latter effect, formally generated by 4 G4 units, was assumed to be more marked, but neutral pH apparently renders the C&GL interactions less effective.

Interestingly, whereas (im)wR12&RB0 (VII) was less stable than hR12&RB0 (ΔTm = −12 °C, vide supra), a Tm value for sG4:wR12 was higher than for hR12&sG4 (77 °C vs. 75 °C). This phenomenon might be explained by strong stabilizing effect of four G4 units at the WC interface, analogously to sG4:wR12. However, the CD spectrum for sG4:wR12 (Fig. 3, a green line) contained an intense negative signal around 210 nm and the isoelectric point at 249 nm, and was similar to that for hR12&sG4:wR12 (an orange line). Therefore, we suggest an additional favorable factor, where the G4 units effectively promote the C2′-endo → C3′-endo conformational change of the central strand and allow formation of imperfect VIII.

This process seems to occur for sA2 mixed with wR12 or hR12 because the observed Tm = 73 °C or 79 °C, respectively, were close to those for sG4:wR12 and hR12&sG4 (Tm = 77 °C and 75 °C, respectively). This suggestion is supported by an 8 °C hysteresis, which for a bimolecular association should be very low (typically <3 °C), whereas it exceeds 13–15 °C for the trimolecular systems hR12&hR12&wR12 and hR12&sA2:wR12. Unlike commonly known triplexes containing a PO-DNA central strand, which dissociate in two discrete steps, those formed by sDNA undergo a single-step melting transition, followed by return of sDNA to the C2′-endo conformation. Thus, the reverse process is “more trimolecular” and in terms of entropy it is less favored than a bimolecular one, giving rise to the hysteresis. For wR12&sG4:wR12 and hR12&sG4:hR12 the observed hysteresis values were low because 4 G4 units substantially shift the conformational of the central PS-strand in the C3′-endo direction and make it “ready” for rapid association.

At pH 7.4, the triple-stranded complex hR12&sB1:wr12 and its congeners containing sA2 or sG4 were thermally so stable that the inflection points could not be determined (the melting profiles not shown).

The above presented data indicate, that at neutral pH, the DNA units present in the sDL oligomers make the Hoogsteen interactions with mRNAs significantly stronger compared to the analogous sDNA. However, concomitantly, the rigid LNA nucleotides enhance hybridization at the WC interface strongly enough to promote the formation of the imperfect triplex VIII. This hybridization may result in the binding of non-target RNAs. This loss of specificity may be avoided using sDL oligomers with Watson–Crick base pairings being hampered due to the presence of N6–Me deoxyadenosine (m6dA) units. We reported recently that the m6dA units present in sDNA stabilize (by up to 4.5 °C per modified unit) parallel duplexes formed by with Hp mRNAs compared to the analogous reference duplex containing only unmodified nucleobases, and prevent the formation of the corresponding parallel triplexes.30

Melting and CD experiments performed using pH 5.4 buffer

Because the Tm values for complexes of the sDL oligomers with wr12 or hR12 formed in pH 5.4 buffer could not be determined, next melting experiments were done for the complexes with wr3 and/or hR3.

Compared to the reference sBo:wr3, the Tm values for complexes formed by sDL and sG4 with wr3 increased by 2–10 °C (Table 3 and Fig. 4) and this increase may be attributed to the stabilizing effect of the LNA nucleosides.

More interesting results were obtained for the complexes formed with hR3. The complex formed with sG4 (hR3&sG4(3) or (im)sG4:hR3) was 25 °C less stable than hR3&sG4(3). Also, a Tm
indicating the formation of the imperfect WCp duplexes (IX). Roman numerals in parentheses refer to representative complexes hR9&G4{3} (no PS units) and hR9&SG4{3} (Fig. 5) mixed with hR9 at a 1 : 1 molar ratio in pH 5.4 buffer. The samples were prepared in 10 mM Tris–HCl, 100 mM NaCl, 10 mM MgCl2 buffer (pH 5.4).

The profiles shown in Fig. 5 indicate that hR9&g2, hR9&g4{3}, and hR9&g4{3} were remarkably more stable than hR9&b0 (ΔT_m = 13 °C, 21 °C, and 21 °C respectively) with an average increase ΔT_m = 7 °C per the “active” LNA unit. But one cannot exclude partial formation of imperfect triplexes X, where a 7 nt part of hR9 acts as a WCp strand.

Of course, complexes analogous to X may be formed with participation of any fully or partially WCp RNA. This non-specific binding may be avoided using already mentioned sDL oligomers with the hampered Watson–Crick base pairings.

Despite very short 9 nt RNAs used, the T_m values for the investigated triplexes hR9&sDL:wR9 exceeded 80 °C and could not be more precisely determined (data not shown). This phenomenon indicates that the C2’-endo → C3’-endo conformational shift caused by the LNA units is of great importance for the thermal stability of investigated parallel duplexes and triplexes, which may be beneficial in in vitro experiments with precisely selected RNA oligomers, but in cellular experiments may lead to unspecific hybridization with non-target oligomers present in the RNA pool.

Conclusions

The CD spectra recorded for single-stranded P-stereodefined phosphorothioate [R_p-PS]-(DNA#LNA) chimeras (sDL) indicate that 2-4 LNA units distributed along 12 nt oligomer changed the C2’-endo → C3’-endo transition of the sDL strand giving rise to an increase in thermal stability of the parallel complexes with (2’-OMe) RNAs compared to those formed by LNA-free [R_p-PS]-DNA oligomers (ΔT_m = 7 °C per the LNA unit at pH 5.4). Thermally more stable complexes are formed upon addition of the corresponding Watson–Crick-paired (2’-OMe)-RNA strands due to the formation of highly stable parallel triplexes. The LNA nucleotides enhance hybridization at the WC interface and may promote the formation of imperfect triplexes VIII. This hybridization may result in the binding of non-target RNAs. This loss of specificity may be avoided using sDL oligomers with the Watson–Crick base pairing hampered due to the presence of N6-Me-2’-deoxyadenosine (m6dA) units. Work on such modified oligonucleotides is in progress.

Experimental section

MALDI-TOF MS analyses of oligonucleotides were performed with the detection of negative ions, using a Voyager-Elite

Table 3 Melting temperatures [°C] for the complexes formed with WCp and/or Hp 9-nt RNAs. Roman numerals in parentheses refer to the proposed structures of complexes. The samples were prepared in 10 mM Tris–HCl, 100 mM NaCl, 10 mM MgCl2 buffer (pH 5.4).

| Homopurine oligomer | wr9 | hR9 | hR9 + wr9 |
|---------------------|-----|-----|---------|
| 1                   | G4  | 65  | 63 (IX) | 68    |
| 2                   | g0  | 47  | 54 (III) | 77 (V) |
| 3                   | g2  | 54  | 67 (III or X) | >80 (V) |
| 4                   | g4{3} | 50  | 75 (III or X) | >80 (V) |
| 5                   | 49  | 75 (III or X) | >80 (V) |
| 6                   | 57  | 50 (IX) | 57    |

Fig. 4 Normalized melting profiles recorded for nA4, nG2, nG4, or sG4 mixed with wr9 at a 1 : 1 molar ratio in pH 5.4 buffer. A profile for nB0 ([R_p-PS]-DNA, no LNA units) is given as a reference.

Fig. 5 Normalized melting profiles recorded for nA4, nG2, nG4, or sG4 mixed with hR9 at a 1 : 1 molar ratio in pH 5.4 buffer. A profile for nB0 ([R_p-PS]-DNA, no LNA units) is given as a reference.

G4 or sG4

| 5’-d(GGAGAAAGAGAG)-3’ |
|------------------------|
| hR9                    |
| 3’-m(CUCCUUUC)-5’ |

(X)

| 5’-d(GGAGAAAGAGAG)-3’ |
|------------------------|
| hR9                    |
| 3’-m(CUCCUUUC)-5’ |

(IX)

Experimental section

MALDI-TOF MS analyses of oligonucleotides were performed with the detection of negative ions, using a Voyager-Elite
instrument (PerSeptive Biosystems Inc., Framingham, MA) operating in the reflector mode, or a Shimadzu Biotech Axima Performance instrument operating in the linear mode.

Routine UV spectra were recorded on a CINTRA 10e spectrophotometer (GBC, Dandenong, Australia), using a quartz cuvette of 1 cm path length. UV monitored melting experiments were carried out in 1 cm path length cells, using a spectrophotometer CINTRA 4040 (GBC), equipped with a 6 × 1 Peltier thermocell.

For the UV-monitored (at 260 nm) thermal dissociation experiments the oligonucleotide samples were dissolved in pH 7.2 or pH 5.4 buffer containing 10 mM Tris–HCl, 100 mM NaCl, and 10 mM MgCl₂. The annealing was done from 85 °C to 15 °C with a temperature gradient of 1 °C min⁻¹. The melting profiles were recorded over a 15 → 85 °C range (0.5 °C min⁻¹). The temperatures of association (Tₐ) and melting (Tₘ) were calculated using the first order derivative method.

CD measurements were done on a Jasco J-815 dichrograph at room temperature, using a 0.5 cm path-length quartz cell (Hellma). The spectra were recorded over a 210–320 nm range with a 1.0 nm bandwidth, a scanning speed 50 nm min⁻¹, and a data pitch of 1 nm. After 3 spectra were accumulated, the baseline was subtracted and the resultant spectrum was smoothed with a Savitzky–Golay algorithm (the convolution width 7).

P-stereo defined oligonucleotides of the PS-DNA and PS-(DNA/LNA) series were synthesized manually at a 1 μmol scale, according to the previously published protocols. The first nucleoside unit was anchored to the solid support by a sarcosinyl linker. Routine coupling steps were performed using 20 mg of the OTP monomers. In the cycles where LNA-OTP₁ monomers 2 were incorporated, double coupling was executed (20 mg + 20 mg) and in both steps the coupling time was extended to 20 minutes. All synthesized oligomers were routinely purified by two-step reverse phase HPLC (DMT-on and DMT-off). Relevant chromatograms for sDL and sG₄ are shown in Fig. S8–S12, ESF†, and their identity was assessed by MALDI-TOF MS and purity by polycrylamide gel electrophoresis (PAGE).

Unmodified DNA and RNA oligonucleotides were synthesized on a Gene-World synthesizer (K&A Laborgeraete GbR, Schaaheim, Germany).

Conflicts of interest

There are no conflicts to declare.

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