

Position-dependent effects on stability in tricyclo-DNA modified oligonucleotide duplexes

Damian Ittig, Anna-Barbara Gerber and Christian J. Leumann*

Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012 Bern, Switzerland

Received June 30, 2010; Revised and Accepted August 3, 2010

ABSTRACT

A series of oligodeoxyribonucleotides and oligoribonucleotides containing single and multiple tricyclo(tc)-nucleosides in various arrangements were prepared and the thermal and thermodynamic transition profiles of duplexes with complementary DNA and RNA evaluated. Tc-residues aligned in a non-continuous fashion in an RNA strand significantly decrease affinity to complementary RNA and DNA, mostly as a consequence of a loss of pairing enthalpy ΔH . Arranging the tc-residues in a continuous fashion rescues T_m and leads to higher DNA and RNA affinity. Substitution of oligodeoxyribonucleotides in the same way causes much less differences in T_m when paired to complementary DNA and leads to substantial increases in T_m when paired to complementary RNA. CD-spectroscopic investigations in combination with molecular dynamics simulations of duplexes with single modifications show that tc-residues in the RNA backbone distinctly influence the conformation of the neighboring nucleotides forcing them into higher energy conformations, while tc-residues in the DNA backbone seem to have negligible influence on the nearest neighbor conformations. These results rationalize the observed affinity differences and are of relevance for the design of tc-DNA containing oligonucleotides for applications in antisense or RNAi therapy.

INTRODUCTION

Chemically modified oligonucleotides are of great current interest as modulators of gene expression in RNA-targeting therapeutic applications (1–3). Their mode of action basically relies on Watson–Crick pairing to a given target RNA, inducing various and complex biological gene-silencing mechanisms that mainly depend on the nature of the chemical modification and the

RNA target. Typically, single-stranded oligonucleotides modulate the biological function of an RNA via antisense mechanisms by either simply binding to it (steric block inhibitors), by concomitantly promoting its degradation with the help of endogenous enzymes such as RNaseH or by interfering with the splice machinery. Double-stranded oligoribonucleotides on the other hand induce silencing of a given mRNA via the RNA interference (RNAi) pathway. In this mode of action one of the two strands of the duplex acts as a guide (antisense) strand, while the other (sense or passenger strand) has no direct function in gene silencing.

It has been shown in the past that chemical modification of oligonucleotides can improve their therapeutic potential (4). The nature of the chemical modification has to be carefully tuned with regard to the envisaged gene-silencing mechanism. For single-stranded antisense oligonucleotides acting either via a steric block or an RNaseH-dependent mechanism, high affinity to the RNA target besides improved biostability and efficient cellular uptake are required. These features are to some extent attainable by sugar/backbone modification. Successful candidates of such chemically modified oligonucleotide analogs include HNA (5), locked nucleic acid (LNA) (6,7), 2'-MOE-RNA and related 2'-O-modified RNA (8), PNA and modifications thereof (9), Morpholino-NAs (10) and tricyclo-DNA (11,12).

For siRNA-mediated mechanisms on the other hand, homogeneously increased stability of chemically modified oligoribonucleotides to their mRNA target may confer adverse thermodynamic effects on guide strand dissociation compared with target RNA binding. In addition, guide strand selection by RNA-induced silencing complex (RISC) exploits a thermodynamic asymmetry of base pairing at the two ends of the siRNA duplex, favoring that strand as a guide strand which shows weaker base pairing at its 5'-end (13,14). This highlights the subtle interplay between target affinity and RNAi activity and poses a challenge for the optimal design of an siRNA duplex. To remedy these drawbacks, several approaches have been evaluated in the recent past. For example, the thermodynamic asymmetry has been exploited to favor

*To whom correspondence should be addressed. Tel: +41(0)31 631-4355; Fax: +41(0)31 631-3422; Email: leumann@ioc.unibe.ch

guide strand selection by the use of siRNAs with judiciously positioned LNA residues (15). Improved antisense strand selection by RISC has also been attained by using small internally segmented siRNAs (sisiRNAs) (16). Meanwhile, a large variety of siRNAs with various chemical modifications have been tested with encouraging results (17,18).

In recent work, we demonstrated that siRNAs containing tricyclo-DNA (tc-DNA, Figure 1) modifications in various positions in the sequence can improve RNAi (19). During thermal melting analyses of tc-modified siRNA duplexes, we became aware of a significant variability in T_m , depending on the position of the modifications in the sequence. In order to rationalize these effects, we set out to systematically determine the structural and energetic effects of single and multiple tricyclo-DNA modifications in a DNA- or RNA backbone on its pairing to complementary DNA and RNA.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification

Oligonucleotides were synthesized on a 1.3 μ mol scale on a Gene Assembler Plus (Pharmacia), using 5-(ethylthio)-1H-tetrazole (0.25 M in CH_3CN) as the activator and following standard phosphoramidite chemistry in the DMT-off mode. The phosphoramidites of the natural DNA-nucleosides were from Vivotide and the 2'-O-triisopropylsilyloxymethyl (TOM)-RNA-amidites as well as DNA- or RNA-solid supports from Glen Research. Tc-DNA nucleotides were attached to a Amino SynBase CPG solid support (Link Technologies) in analogy to published procedures (20). Oligonucleotides **1–11** containing only deoxyribonucleosides were deprotected in 33% aqueous NH_3 for 16 h at 55°C. Oligonucleotides **12–21** containing ribonucleosides were base- and phosphate deprotected with 8 M methylamine in EtOH/ H_2O 1:1 for 12 h at 35°C, followed by TOM deprotection in 1 M tetrabutylammoniumfluoride (TBAF) in tetrahydrofuran (THF) for 10 min at 50°C and then 6 h at 35°C. All oligonucleotides were purified by ion-exchange chromatography on a DNAPac PA200 column (Dionex, 4 \times 250 mm); solvents: A: 25 mM Trizma base in H_2O , pH = 8.0; B: 25 mM Trizma base, 1.25 M NaCl in H_2O , pH = 8.0. Gradient 0–50% B in 30 min. The purity of all

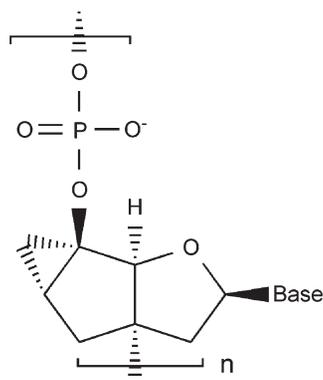


Figure 1. Chemical structure of tricyclo(tc)-DNA.

tc-modified oligonucleotides (HPLC control) was >96%. The constitution of all oligonucleotides was verified by ESI-mass spectrometry (Supplementary Table S1).

UV-melting curves

Solutions were prepared in standard saline buffer (10 μ M NaH_2PO_4 , 150 mM NaCl, pH 7.0) with duplex concentrations of 2 μ M in 1:1 strand ratio. Thermal melting experiments were carried out on a Varian Cary 100-Bio UV/VIS photospectrometer (Varian Inc.), equipped with a Peltier element at 260 nm with a heating/cooling rate of 0.5°C/min. T_m values were obtained from the maxima of the first derivatives of the melting curves using WinUV software.

CD measurements

CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a Jasco PFO-350S temperature controller, using the same buffer conditions and oligonucleotide concentrations as for UV melting curves. All CD spectra were collected at 25°C between 210 to 320 nm, using a wavelength step of 5 nm. Subsequently, the graphs were smoothed with a noise filter (convolution width: 7) and analyzed using the J-700 control driver. The reported spectra correspond to the average of at least three scans.

Thermodynamic parameter

Thermodynamic parameters were determined by fitting a simulated curve to the experimental UV-melting curve. The simulated curves were based on a bimolecular system obeying the two-state model (21) and were obtained using our fitting function 'alpha' which was built in analogy to published procedures (22). The fitting process was performed with the program Origin 8 (OriginLab).

Molecular modeling

Molecular modeling was performed with the Amber force field as incorporated in the software package HyperChemTM Release 8.0.4 for Windows (Hypercube, Inc.). Only original Amber parameters were used and no explicit water or counterions were included. A distance-dependent scale factor of $\epsilon = 1$ was used. One to four non-bonded interactions were scaled by 0.5. No cutoffs were applied. DNA/DNA structures were built on the basis of a B-DNA, all structures containing RNA on the basis of an A-DNA. All sequences were first minimized until the gradient was <0.1 kcal/mol Å. The structures were then heated from 0 to 300 K over 200 ps and submitted to 200 ps of unrestrained molecular dynamics at 300 K. After simulation, the structures were minimized again until the gradient was <0.1 kcal/mol Å.

RESULTS

Tc-modification of DNA

To determine the effects of tc-modifications in the context of the DNA backbone, we synthesized a series of decamers with a randomized, generic base sequence (Table 1). Single

Table 1. Cooperative and anti-cooperative effects on thermal duplex stability with DNA and RNA complements of oligodeoxyribonucleotides containing an increasing number of tc-DNA residues

Sequence ^a	T_m (°C, 260 nm) with DNA complement ^{b,c}	T_m (°C, 260 nm) with RNA complement ^{b,c}
1 d(AACTGTCACG)	46.0	43.6
2 d(AACTGTcACG)	48.3 (+2.3)	48.3 (+4.7)
3 d(AACTGTcCACG)	45.6 (−0.4)	46.0 (+2.4)
4 d(p-aACTGTcACG)	45.6 (−0.4)	44.6 (+1.0)
5 d(AACTGTCACg)	48.2 (+2.2)	48.2 (+4.6)
6 d(AAcTGTcACG)	48.3 (+1.1)	51.0 (+3.7)
7 d(AaCtGtCaCG)	45.0 (−0.2)	49.3 (+1.4)
8 d(AACTGtcACG)	51.0 (+2.5)	51.6 (+4.0)
9 d(AActgtcaCG)	54.3 (+1.4)	59.9 (+2.7)
10 d(AactgtcacG)	56.9 (+1.4)	63.9 (+2.5)
11 tc(p-aactgtcacg)	58.5 (+1.3)	64.3 (+2.1)

^aCapital letters: 2'-deoxynucleotides; small letters bold: tricyclo-nucleotides; p, phosphate.

^bConditions: c, 2 μ M in 10 mM NaH₂PO₄, 150 mM NaCl, pH 7.0. Estimated error in T_m : \pm 0.5°C.

^cValues in parenthesis: ΔT_m /modification.

modifications were introduced at the 5'- and 3'-ends and in the center of the sequence (**2-5**) followed by sequences containing either two or four tc-units spaced by natural nucleotides (**6, 7**) or two, six and eight tc-residues in a continuous fashion (**8-10**). The unmodified (**1**) and the fully modified (**11**) oligonucleotides served as references.

T_m -data. With DNA as a complement, single modifications in the center of the sequence (**2, 3**) revealed deviations in T_m ranging from -0.4 to $+2.3$ °C relative to the unmodified duplex. The differences are likely due to base-specific effects. The situation with RNA as a complement is similar with a distinctly higher tendency to duplex stabilization. The 5'-end modification leads to no major change in T_m with DNA as complement, while 3'-end modification stabilizes both duplexes with DNA or RNA complements by $+2.2$ and $+4.6$ °C, respectively. The T_m -data of the 5'-end-modified duplexes have to be interpreted and compared carefully, as for chemical stability reasons they contain a 5'-phosphate group which is not the case for the reference duplex containing **1**.

Increasing the number of tc-residues from two to four in a discontinuous way (at least one natural nucleotide in between) has a T_m -neutral to slightly stabilizing effect with DNA as complement (-0.25 to $+1.1$ °C/modification) and definitely a stabilizing effect with RNA as the complement ($+1.4$ to $+3.7$ °C/modification). More tc-modifications, as in **7**, are clearly less stabilizing on a per-residue basis as compared with fewer modifications in a similar arrangement as in **6**. Extending the number of continuous tc-residues from 2 to 10 (**8-11**) is associated with increasing duplex stability with both DNA and RNA as the complement. The stabilization on a per-residue basis ranges from $+2.5$ to $+1.4$ °C with DNA as complement and from $+4.0$ to $+2.5$ °C with RNA as complement and clearly levels off with increasing number of modifications.

Thermodynamic data of duplex formation. From van't Hoff analysis of representative melting curves of

oligonucleotides **1, 7, 9** and **11** in complex with complementary DNA or RNA, we calculated the transition enthalpies (ΔH , ΔG) as well as the entropy changes ($T\Delta S$) under standard conditions (25°C) (Figure 2) (23,24). Detailed numerical values are available in Supplementary Table S2. In all cases the free enthalpies of duplex formation (ΔG) reflect the order of thermal stabilities as represented by the T_m -values. With DNA as complement, oligonucleotide **7** containing four spaced tc-DNA residues leads to a drop in both the ΔH and $T\Delta S$ term compared with **9** containing six continuous tc-residues. The most favorable pairing enthalpy ΔH was found for the fully modified oligonucleotide **11**. With RNA as a complement a similar trend is observed, however, there is much less variability in the enthalpy and entropy terms. Thus, an increasing number of tc-DNA/DNA junctions in the backbone of the modified strand is associated primarily with a loss of pairing enthalpy ΔH which is compensated to some extent by the entropy term. This is especially significant for duplexes with DNA as complement.

CD spectra of modified duplexes. We determined CD spectra of selected modified oligonucleotides with either complementary DNA or RNA (Figure 3). With DNA as complement, and compared with the unmodified duplex, already the introduction of a single tc-T unit, as in **1**, leads to an increase of the positive cotton effect at around 270 nm and a decrease of the negative cotton effect at ~ 245 nm. The duplex containing tc-units in a discontinuous way reinforces this behavior. The duplex containing six continuous tc-units shows a slight red shift of the positive cotton effect and an overall shape with increased A-conformation character. Thus, increasing the number of tc-units is associated with a gradual transition from a B-type to an A-type duplex and this behavior is more expressed for duplexes containing continuous tc-units. This is in agreement with the structural features of a DNA duplex containing a fully modified tc-DNA strand (25). With RNA as a complement there is almost no structural difference between the unmodified DNA/RNA duplex and that containing various tc-units in different positions. From the decrease of the intensity of the negative band at ~ 240 nm with increasing number of tc-units in the DNA strand it can be anticipated that there is an additional shift from a mixed, A–B-conformation to more A-character. This is in full agreement with earlier observations from CD spectroscopy and molecular modeling which describe tc-DNA structurally as an RNA and not a DNA analog (25). Interestingly, no differences between duplexes containing **7** and **9** are observed, indicating non-detectable structural differences between discontinuous or continuous tc-modification of a DNA strand in complex with RNA.

Tc-DNA modifications in the RNA backbone

T_m -measurements. To complete these structural investigations, we prepared the analogous series of modified RNA oligonucleotides **12-21** with tc-DNA modifications and

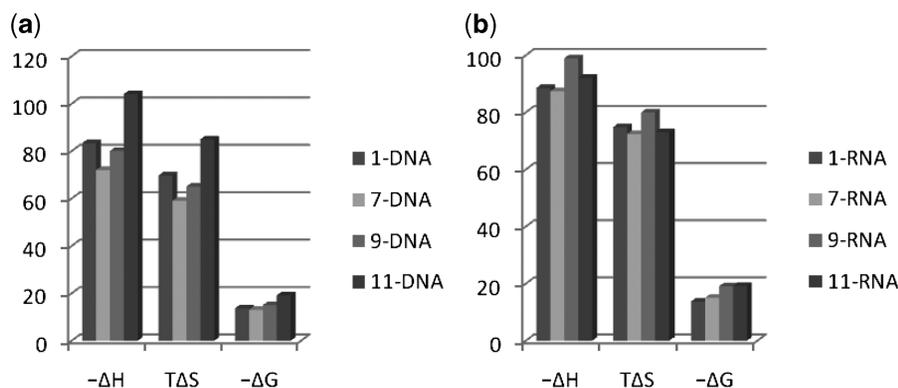


Figure 2. Graphical representation of the thermodynamic data of duplex formation (standard conditions, 25°C, kcal/mol) from van't Hoff analysis of the UV-melting curves of oligonucleotides 1, 7, 9 and 11 in duplex with either complementary DNA (a) or RNA (b).

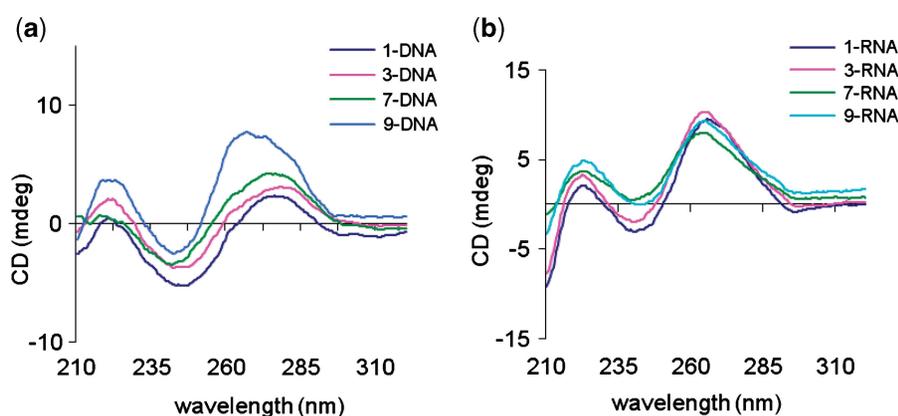


Figure 3. CD spectra of oligonucleotides 1, 3, 7 and 9 in complex with complementary DNA (a) and RNA (b). Experimental conditions as for UV-melting curves, $T = 25^\circ\text{C}$.

investigated their thermal melting properties in duplex with complementary DNA and RNA (Table 2).

When introducing a single modification into the center of RNA strand, as in **13** and **14**, a significant drop in T_m between -3.7 and -5.3°C is observed irrespective of whether the complement is RNA or DNA. The 5'- or 3'-end modifications are slightly stabilizing with DNA as a complement. With complementary RNA, only the 3'-end modification slightly stabilizes the duplex. Again, discontinuous tc-units destabilize duplex formation with complementary DNA and RNA by -3.0 to $-5.1^\circ\text{C}/\text{mod}$. On a per-residue level, the destabilization is more pronounced with a lower number of modifications. Continuously increasing the number of consecutive tc-units from two to eight is associated with changes in $T_m/\text{modification}$ from -2.1 to $+1.7^\circ\text{C}$ with both, the DNA and RNA complement.

Thus, there are considerable differences of tc-modified RNA as compared with tc-modified DNA in thermal stabilities, specifically with RNA as a complement. While modification of a DNA backbone with tc-residues stabilizes in all cases a duplex with RNA, irrespective of the number of tc-DNA/DNA junctions in the backbone, the same modification in the RNA backbone is distinctly destabilizing with increasing number of tc-DNA/RNA junctions in the oligonucleotide. In other words,

Table 2. Cooperative and anti-cooperative effects on thermal duplex stability with DNA and RNA complements of oligoribonucleotides containing an increasing number of tc-DNA residues

	Sequence ^a	T_m ($^\circ\text{C}$, 260 nm) with DNA complement ^{b,c}	T_m ($^\circ\text{C}$, 260 nm) with RNA complement ^{b,c}
12	r(AACUGUCACG)	41.3	54.0
13	r(AACUGUcACG)	37.6 (-3.7)	49.9 (-4.1)
14	r(AACUGtCACG)	36.0 (-5.3)	49.0 (-5.0)
15	r(p-aACUGUCAC)	41.9 ($+0.6$)	53.3 (-0.7)
16	r(AACUGUCACg)	42.9 ($+1.6$)	55.0 ($+1.0$)
17	r(AACtGUcACG)	31.0 (-5.1)	45.2 (-4.4)
18	r(AaCtGtCaCG)	29.3 (-3.0)	41.3 (-3.2)
19	r(AACUGtcACG)	37.0 (-2.1)	49.6 (-2.2)
20	r(AActgtcaCG)	45.7 ($+0.7$)	54.9 ($+0.1$)
21	r(AactgtcacG)	54.8 ($+1.7$)	63.1 ($+1.1$)
11	tc(p-aactgtcaCG)	58.5 ($+1.7$)	64.3 ($+1.0$)

^aCapital letters: ribonucleotides; small letters bold: tricyclo-nucleotides; p, phosphate.

^bConditions: $c = 2\ \mu\text{M}$ in 10 mM NaH_2PO_4 , 150 mM NaCl, pH 7.0. Estimated error in T_m : $\pm 0.5^\circ\text{C}$.

^cValues in parenthesis: $\Delta T_m/\text{modification}$.

discontinuous introduction of tc-residues destabilize duplexes, whereas a continuous substitution pattern increases duplex stability. Again, this does only marginally depend on the nature of the complementary strand

(DNA or RNA) and is thus an intrinsic property of tc-DNA/DNA- versus tc-DNA/RNA-backbone junctions.

Thermodynamic data. We calculated thermodynamic data of duplex formation for oligonucleotides **18** and **20**, containing discontinuous or continuous tc-modifications, respectively, with DNA or RNA as complements and compared them with the duplexes containing the unmodified RNA strand **12** or the fully tc-DNA-modified strand **11** (Figure 4 and Supplementary Table S2). With DNA as complement, the enthalpy of duplex formation is least favorable in the case of discontinuous tc-modification, recovers to some extent in the case of continuous tc-modifications and is most favorable in the case of the fully modified tc-DNA/DNA duplex. As expected this is to some extent counterbalanced by entropy compensation (26,27), leading to ΔG values (25°C) that are in line with the measured T_m -values. A similar tendency occurs with RNA as the complement, however, the relative differences in the enthalpy and entropy terms are smaller as compared with the DNA complement series. This points to the fact that tc-DNA/RNA junctions are associated primarily with a loss of pairing enthalpy ΔH which contributes unfavorably to duplex formation.

CD spectra of tc-modified RNA duplexes. While single incorporations of tc-residues into the RNA backbone change the CD spectra with complementary DNA and RNA only marginally, there exist substantial differences between duplexes containing tc-DNA residues in a discontinuous (**18**) or continuous (**20**) fashion (Figure 5). Both show reduced positive ellipticities around 270 nm compared with the unmodified duplexes. While the structure of duplexes with RNA are more similar and A-like, irrespective of the distribution pattern of the tc-residues, the analogous duplexes with DNA as complement are structurally more diverse showing a distinctly different pattern in the low wavelength region between 210 and 240 nm, and a gradual change of the overall duplex structure from a mixed A,B-like conformation to a more A-like conformation with increasing number of tc-residues. This shows that the number of tc-DNA/RNA junctions

in the backbone leads to significant changes in duplex conformation, especially with DNA as a complement.

Modeling of tc-DNA and -RNA junctions. To correlate the thermal and thermodynamic properties with structure on a more detailed level, we performed molecular dynamics calculations of duplexes of the mono-modified oligonucleotides **3** and **14** with complementary DNA and RNA (Figure 6).

It turned out that a tc-T residue in the DNA strand **3**, paired to DNA (Figure 6a), does not significantly alter the overall B-DNA conformation expected for such a duplex. No unusual backbone torsion angles or nucleoside conformation are observed for the two flanking natural nucleotides in the strand. The tc-T residue shows an interchange in the conformation of torsion angle β and γ to *gauche*, *trans* instead of the usual *trans*, *gauche*. This interchange is structurally compensatory in nature and very closely reproduces the local conformation observed in an X-ray structure of a tc-DNA modified DNA duplex (28). Thus, there seems to be no conflicting situation at the DNA/tc-DNA junction, which is well reflected by the mostly invariant ΔT_m /modification of duplexes containing single incorporations in a discontinuous way. A similar situation is encountered when **3** is paired to complementary RNA (Figure 6b). Also in this case, the flanking natural deoxynucleotides are in their normal 2'-endo conformation and for the tc-T residue the same changes apply as before. The complementary RNA strand shows all nucleotides in the 3'-endo conformation, giving rise to a mixed duplex conformation. Also here, no structural violations within and around the modification occurs which is again reflected in the ΔT_m /modification data (Table 1), which are positive for all duplexes containing single or multiple tc-DNA/DNA junctions. If tc-nucleosides are incorporated into RNA, as in **14**, the picture changes significantly. In the duplex of **14** with complementary DNA (Figure 6c), the rC-nucleotide 3' to the tc-T modification occurs in the unusual 2'-endo conformation with an unusual torsion angle γ and phosphodiester conformation. The 5'-rG-residue occurs more or less in a canonical A-RNA

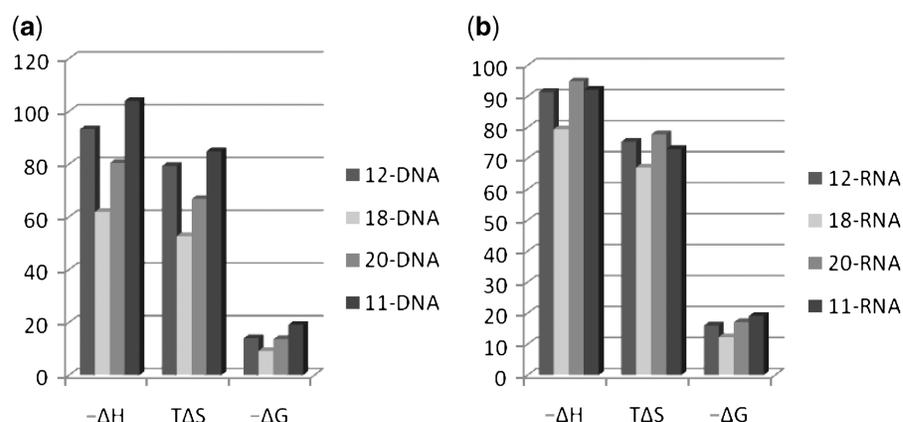


Figure 4. Graphical representation of the thermodynamic data of duplex formation (standard conditions, 25°C, kcal/mol) from van't Hoff analysis of the UV-melting curves of oligonucleotides **12**, **18**, **20** and **11** in duplex with either complementary DNA (a) or RNA (b).

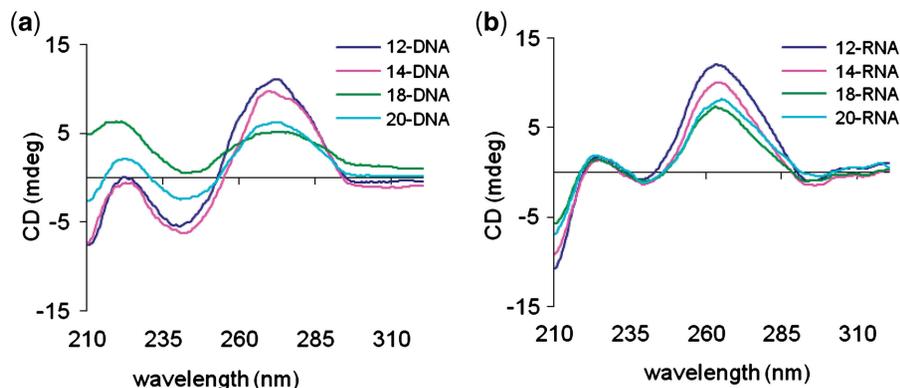


Figure 5. CD spectra of oligoribonucleotides **12**, **14**, **18** and **20** in complex with complementary DNA (a) and RNA (b). Experimental conditions as for UV-melting curves, $T = 25^{\circ}\text{C}$.

backbone conformation. The complementary nucleotides on the DNA complement show less structural variation. The $\Delta T_m/\text{modification}$ data (Table 2) clearly show that an increasing number of tcDNA/RNA backbone junctions have an additive negative effect on the T_m . Modeling thus suggests that this negative effect is the consequence of unfavorable conformational changes of the neighboring ribonucleotides. This is essentially also found for the case of **14** paired to complementary RNA (Figure 6d). Also here, the flanking ribonucleotides switch into the higher energy 2'-endo conformations, which is again in agreement with the $\Delta T_m/\text{modification}$ data (Table 2) which show a negative tendency that is additive with the number of tc-RNA/RNA junctions. In summary, the structural data suggest that the influence of a tc-residue within a DNA backbone leads to less conformational changes to its nearest neighbors, while a tc-residue in a RNA backbone significantly changes its nearest neighbors into higher energy conformations. This is well reflected in the corresponding thermal and thermodynamic data set.

DISCUSSION

Tc-DNA was introduced as a second-generation conformationally constrained oligonucleotide analog of the bicyclo-DNA molecular platform some 13 years ago. While in the past, most biophysical and biological evaluations of tc-DNA were performed on the basis of fully modified oligonucleotides, this is the first systematic study of the thermal, thermodynamic and structural effect of single or multiple incorporations of tc-nucleosides in either the DNA- or the RNA backbone. Given the substantial structural changes in a tc-nucleoside compared with a natural nucleoside, it is not surprising that these influence nucleic acid affinities in a distinct way. In this study, we did not intend to determine base-specific effects on structure or stability, but we focused on sugar/backbone-specific changes. We note that base-specific effects may be operative in addition, but we believe that they will have lower structural or energetic impact.

From earlier work, it is evident that fully modified tc-oligonucleotides stabilize duplexes with complementary

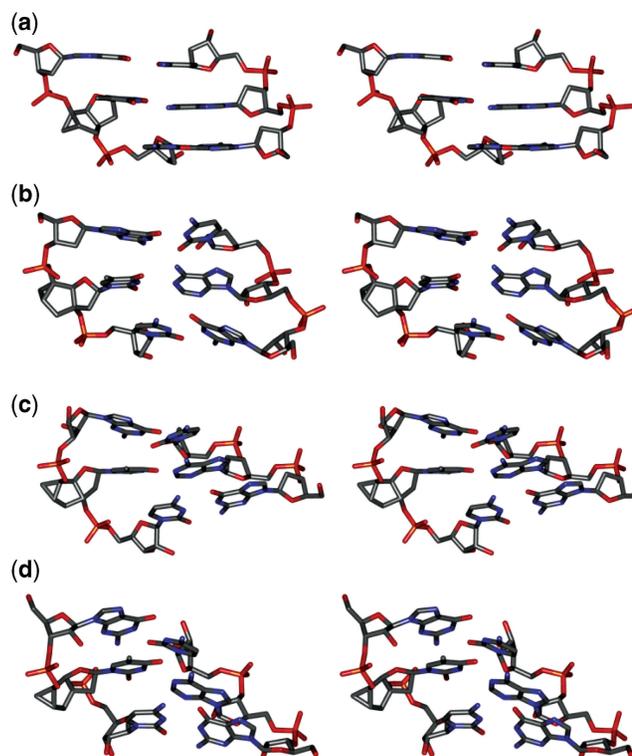


Figure 6. Stereoviews of the central section of the duplexes of (a) **3**/DNA, (b) **3**/RNA, (c) **14**/DNA and (d) **14**/RNA, containing the modified tc-DNA- as well as its 3'- and 5'-flanking natural base pairs after a total of 400 ps dynamic simulation followed by energy minimization using the AMBER force field as implemented in the software package *Hyperchem*TM.

DNA and RNA by 1–3°C per modification, compared with DNA. Structural investigations by CD spectroscopy, molecular modeling and, more recently X-ray analysis (28), revealed that major deviations in the backbone structure occur within torsion angles β and γ , which show more a *trans*, *gauche* than a *gauche*, *trans* arrangement as observed for DNA and RNA. This leads to an overall strand conformation that is nearer to an A-conformation (25). Single or multiple substitutions of

a DNA strand by tc-nucleosides in either a continuous or discontinuous way, as investigated in this work, leads to relatively small variations in T_m /modification with DNA as complement (Table 1). Nevertheless, a slightly negative tendency with increasing number of tc-DNA/DNA heterojunctions in the backbone is evident. With RNA as a complement, only stabilization occurs which is again less pronounced if the number of heterojunctions in the backbone is high. The CD spectra (Figure 3) clearly show that the structural changes are higher in the duplexes with complementary DNA as compared with RNA. The higher the amount of tc-units in the strand, the more the duplex conformation shifts towards an A-like structure, comparable to that of a fully modified tc-DNA/DNA duplex. Interestingly, there is a negligible energetic penalty to this, and molecular modeling (Figure 6a and b) suggests that this shift in conformation is mainly a direct effect of the modification itself and not a consequence of structural changes imposed to nearest neighbor natural nucleosides in the strand.

The picture is different if the RNA backbone is modified with tc-nucleosides. Here, a significant thermal destabilization is observed when tc-units are spaced by natural ribonucleosides and thus the number of tc-DNA/RNA heterojunctions in the backbone is high. The destabilization is mainly of enthalpic origin. The opposite effect occurs if tc-units are introduced in a consecutive manner, where an additive stabilization with increasing number of tc-residues is observed. Structural modeling of a tc-DNA heterojunction reveals that with both, DNA and RNA complements, substantial changes of the nearest neighbor nucleosides occur. The most relevant changes are a shift from 3'- to 2'-endo conformation of the adjacent ribonucleotides and changes of the phosphodiester bonds to higher energy *gauche*, *trans* conformations. From the modeled structures (Figure 6c and d), it seems that at least for the nucleotides 5' to the tc-modification the adjustment of their ribose conformation is driven by minimizing steric conflicts between their 2'-OH function and the furanose ring of the tc-unit. This conflict comes from the dominant *trans*, *gauche* arrangement of torsion angle γ and β , imposed by the tricyclo-scaffold, which is of course absent in natural backbone junctions and is substantially reduced in tc-DNA/DNA junctions (Figure 6a and b) because of the replacement of an OH group by a sterically less-demanding hydrogen atom. This structural rationalization is reminiscent to that invoked in the case of the completely different nucleotidic pairing system glucopyranosyl-NA. The presence of a 2'-OH group in this case substantially decreased duplex formation efficiency compared with homo-DNA in which this 2'-OH function is replaced by a hydrogen atom ('too many atoms') (29,30).

It is not uncommon that chemically modified residues can influence their nearest neighbors in a cooperative fashion in nucleic acid duplexes and has, for example, been found in the case of LNA. It has been shown by high-resolution NMR experiments that nearest neighbor deoxynucleotides next to a LNA unit show a higher

population of the 3'-endo conformation (31–33). This conformational steering induced by the LNA nucleotides has been rationalized by a change in electronic density at the rim of the minor groove, causing an alteration of the pseudorotational profile of the 3'-flanking nucleotide toward N-type conformation. A direct comparison of LNA- and tc-DNA units as strand modifiers highlights substantial differences between these two members of the class of conformationally stabilized analogs. While LNA units drive adjacent deoxynucleotides into an N-type conformation, this seems not to be the case for tc-DNA. While tc-DNA/DNA heterojunctions are associated with a slight loss in duplex formation enthalpies, LNA/DNA heterojunctions are in most cases enthalpy stabilized (34). While tc-DNA residues in an RNA backbone drive ribonucleotides more into an S-type conformation, this seems not to be the case for LNA, and also no energetic penalties arise from LNA/RNA backbone junctions. This can be inferred from a steric block antisense experiment in which tcDNA/2'-OMe-RNA mixmers were compared with LNA/2'-OMe-RNA mixmers (35). It clearly emerged that fully modified tc-DNA sequences were more active compared with tc-DNA/2'-OMe mixmers, while the corresponding LNA/2'-OMe mixmers did not show any signs of compromised activity. Thus, LNA mixes well with RNA or 2'-OMe-RNA, while tc-DNA does not.

CONCLUSIONS

In this systematic study, we found that there are significant positional effects on T_m upon incorporation of tc-DNA residues into DNA or RNA homo- or heteroduplexes. In particular, introducing tc-residues into an oligoribonucleotide in a discontinuous fashion significantly decreases the T_m , while continuous tracts of tc-residues or fully modified tc-oligonucleotides stabilize duplexes. These findings are of relevance for the design of tc-DNA containing oligonucleotides for therapeutic purposes. For antisense oligonucleotides acting by a steric block mechanism, where high RNA affinity is required, the use of fully modified tc-oligonucleotides seems to be the appropriate choice. For applications in RNAi, the positional stabilization/destabilization of tc-residues can be used to design thermodynamic asymmetry into a siRNA duplex. For example, it seems advisable to substitute a passenger strand of a siRNA duplex at the 3'-end in a discontinuous way with tc-nucleotides to thermodynamically destabilize the duplex at the 5'-end of the guide strand. Similarly the 5'-end of the passenger strand should be modified in a continuous way to thermodynamically stabilize the duplex at the 3'-end of the guide strand, thus increasing the thermodynamic asymmetry and directing guide strand selection by RISC.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online

FUNDING

Swiss National Science Foundation (grant number 200020-115913); Association Monégasque Contre les Myopathies. Funding for open access charge: University of Bern.

Conflict of interest statement. None declared.

REFERENCES

- Bell, N.M. and Micklefield, J. (2009) Chemical modification of oligonucleotides for therapeutic, bioanalytical and other applications. *ChemBioChem*, **10**, 2691–2703.
- Tiemann, K. and Rossi, J.J. (2009) RNAi-based therapeutics - current status, challenges and prospects. *EMBO Mol. Med.*, **1**, 142–151.
- Sibley, C.R., Seow, Y. and Wood, M.J.A. (2010) Novel RNA-based strategies for therapeutic gene silencing. *Mol. Ther.*, **18**, 466–476.
- Bennett, C.F. and Swayze, E.E. (2010) RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.*, **50**, 259–293.
- Kang, H., Fisher, M.H., Xu, D., Miyamoto, Y.J., Marchand, A., Van Aerschot, A., Herdewijn, P. and Juliano, R.L. (2004) Inhibition of MDR1 gene expression by chimeric HNA antisense oligonucleotides. *Nucleic Acids Res.*, **32**, 4411–4419.
- Veedu, R.N. and Wengel, J. (2010) Locked nucleic acids: promising nucleic acid analogs for therapeutic applications. *Chem. Biodivers.*, **7**, 536–542.
- Imanishi, T. and Obika, S. (2002) BNAs: novel nucleic acid analogs with a bridged sugar moiety. *Chem. Commun.*, 1653–1659.
- Prakash, T.P., Kawasaki, A.M., Wancewicz, E.V., Shen, L., Monia, B.P., Ross, B.S., Bhat, B. and Manoharan, M. (2008) Comparing in vitro and in vivo activity of 2'-O-[2-(methylamino)-2-oxoethyl]- and 2'-O-methoxyethyl-modified antisense oligonucleotides. *J. Med. Chem.*, **51**, 2766–2776.
- Nielsen, P.E. (2010) Peptide nucleic acids (PNA) in chemical biology and drug discovery. *Chem. Biodivers.*, **7**, 786–804.
- Moulton, J.D. and Jiang, S. (2009) Gene knockdowns in adult animals: PPMOs and vivo-morpholinos. *Molecules*, **14**, 1304–1323.
- Ittig, D., Liu, S., Renneberg, D., Schümperli, D. and Leumann, C.J. (2004) Nuclear antisense effects in cyclophilin A pre-mRNA splicing by oligonucleotides: a comparison of tricyclo-DNA with LNA. *Nucleic Acids Res.*, **32**, 346–353.
- Renneberg, D., Schümperli, D. and Leumann, C.J. (2002) Biological and antisense properties of tricyclo-DNA. *Nucleic Acids Res.*, **30**, 2751–2757.
- Khvorov, A., Reynolds, A. and Jayasena, S.D. (2003) Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell*, **115**, 209–216.
- Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P.D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, **115**, 199–208.
- Elmen, J., Thonberg, H., Ljungberg, K., Frieden, M., Westergaard, M., Xu, Y., Wahren, B., Liang, Z., Orum, H., Koch, T. et al. (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res.*, **33**, 439–447.
- Bramsen, J.B., Laursen, M.B., Damgaard, C.K., Lena, S.W., Ravindra Babu, B., Wengel, J. and Kjems, J. (2007) Improved silencing properties using small internally segmented interfering RNAs. *Nucleic Acids Res.*, **35**, 5886–5897.
- Watts, J.K., Deleavey, G.F. and Damha, M.J. (2008) Chemically modified siRNA: tools and applications. *Drug Discov. Today*, **13**, 842–855.
- Bramsen, J.B., Laursen, M.B., Nielsen, A.F., Hansen, T.B., Bus, C., Langkjaer, N., Babu, B.R., Hojland, T., Abramov, M., Van Aerschot, A. et al. (2009) A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. *Nucleic Acids Res.*, **37**, 2867–2881.
- Ittig, D., Luisier, S., Weiler, J., Schümperli, D. and Leumann, C. (2010) Improving gene silencing of siRNAs via tricyclo-DNA modification. *Artif. DNA PNA XNA*, **1**, 9–16.
- Wagner, T. and Pfeleiderer, W. (2000) Synthesis of 2'-deoxyribonucleoside 5'-phosphoramidites: new building blocks for the inverse (5'-3')-oligonucleotide approach. *Helv. Chim. Acta*, **83**, 2023–2035.
- Marky, L.A. and Breslauer, K.J. (1987) Calculating thermodynamic data for transitions of any molecularity from equilibrium melting curves. *Biopolymers*, **26**, 1601–1620.
- Marky, L.A., Blumenfeld, K.S., Kozlowski, S. and Breslauer, K.J. (1983) Salt-dependent conformational transitions in the self-complementary deoxydodecanucleotide d(CGCGAATTCGCG): Evidence for hairpin formation. *Biopolymers*, **22**, 1247–1257.
- Petersheim, M. and Turner, D.H. (1983) Base-stacking and base-pairing contributions to helix stability: thermodynamics of double helix formation with CCGG, CCGGp, CCGGAp, ACCGGp, CCGGUp and ACCGGUp. *Biochemistry*, **22**, 256–263.
- Epple, C. and Leumann, C. (1998) Bicyclo[3.2.1]-DNA, a new DNA analog with a rigid backbone and flexibly linked bases: pairing properties with complementary DNA. *Chem. Biol.*, **5**, 209–216.
- Renneberg, D. and Leumann, C.J. (2002) Watson-Crick base-pairing properties of tricyclo-DNA. *J. Am. Chem. Soc.*, **124**, 5993–6002.
- Dunitz, J.D. (1995) Win some, lose some: enthalpy-entropy compensation in weak intermolecular interactions. *Chem. Biol.*, **2**, 709–712.
- Marky, L.A., Kupke, D.W., Michael, L. and Johnson, G.K.A. (2000) Enthalpy-entropy compensations in nucleic acids: contribution of electrostriction and structural hydration. In Johnson, M.L. and Ackers, G.K. (eds), *Methods Enzymol.*, Vol. 323. Academic Press, San Diego, pp. 419–441.
- Pallan, P.S., Ittig, D., Heroux, A., Wawrzak, Z., Leumann, C.J. and Egli, M. (2008) Crystal structure of tricyclo-DNA: an unusual compensatory change of two adjacent backbone torsion angles. *Chem. Commun.*, 883–885.
- Beier, M., Reck, F., Wagner, T., Krishnamurthy, R. and Eschenmoser, A. (1999) Chemical etiology of nucleic acid structure: Comparing pentopyranosyl-(2'->4') oligonucleotides with RNA. *Science*, **283**, 699–703.
- Eschenmoser, A. (1994) Chemistry of potentially prebiological natural-products. *Orig. Life Evol. Biosph.*, **24**, 389–423.
- Nielsen, C.B., Singh, S.K., Wengel, J. and Jacobsen, J.P. (1999) The solution structure of a locked nucleic acid (LNA) hybridized to DNA. *J. Biomol. Struct. Dyn.*, **17**, 175–191.
- Petersen, M., Bondensgaard, K., Wengel, J. and Jacobsen, J.P. (2002) Locked nucleic acid (LNA) recognition of RNA: NMR solution structures of LNA : RNA hybrids. *J. Am. Chem. Soc.*, **124**, 5974–5982.
- Bondensgaard, K., Petersen, M., Singh, S.K., Rajwanshi, V.K., Kumar, R., Wengel, J. and Jacobsen, J.P. (2000) Structural studies of LNA:RNA duplexes by NMR: conformation and implications for RNase H activity. *Chem. Eur. J.*, **6**, 2687–2695.
- Kaur, H., Arora, A., Wengel, J. and Maiti, S. (2006) Thermodynamic, counterion, and hydration effects for the incorporation of locked nucleic acid nucleotides into DNA duplexes. *Biochemistry*, **45**, 7347–7355.
- Ivanova, G., Reigadas, S., Ittig, D., Arzumanov, A., Andreola, M.L., Leumann, C., Toulmé, J.J. and Gait, M.J. (2007) Tricyclo-DNA containing oligonucleotides as steric block inhibitors of HIV-1 Tat-dependent trans activation and HIV-1 infectivity. *Oligonucleotides*, **17**, 54–65.