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Comparison of Duplex Stabilizing Properties of 2′-Fluorinated Nucleic Acid Analogues with Furanose and Non-Furanose Sugar Rings

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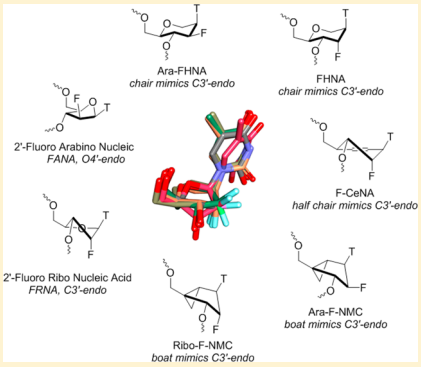
ABSTRACT: We compare the duplex stabilizing properties of 2′-fluorinated nucleic acid analogues with furanose and non-furanose ring systems and dissect the relative contributions of hydration, sugar conformation, and fluorine configuration toward the overall T_m value. We find that the stabilization imparted by fluorine substitution is additive over that obtained by restricting the conformation of the sugar ring itself. Our studies support further evaluation of fluorinated nucleic acid analogues with non-furanose sugar rings as surrogates of 2′-F RNA for therapeutic antisense applications.



2′-Fluoro RNA (FRNA), where the 2′-hydroxyl group of RNA is replaced with a fluorine atom, represents one of the most widely investigated nucleic acid modifications for therapeutic antisense applications. FRNA-modified oligonucleotides (ONs) have found widespread use as nucleic acid aptamers,[1] ribozymes,[2] antisense agents,[3] allele-selective gene silencing,[4] splice-switching,[5] single-stranded siRNA,[6] and anti-microRNA[7] oligomers. FRNA-modified siRNAs possess unique gene silencing properties[8,9] and are currently being evaluated in human trials for the treatment of genetic disorders. However, FRNA modified ONs are still vulnerable to nuclease-mediated metabolism and FRNA nucleosides are substrates for human DNA polymerases,[10] which could complicate development of the modified oligonucleotides as potential human therapeutics. To address these limitations, we recently characterized the antisense properties of both isomers of 2′-fluorohexitol (FHNA)[11,12] and 2′-fluoro-methanocarba (F-NMC) nucleic acids[13] and the "ribo" configured 2′-fluoro analogue of cyclohexenyl nucleic acid (F-CeNA)[14] as potential surrogates of FRNA for gene silencing applications. While the above studies characterized the biophysical, structural, and biological activities of oligonucleotides modified with fluorinated nucleic acid analogues, we remained intrigued by the relative contributions of hydration, conformation of the sugar moiety, and configuration of the fluorine atom on the duplex stabilizing properties of the modified oligonucleotides. To address these questions, we compared the duplex stabilizing properties of 10 nucleic acid analogs at 4 positions within an oligonucleotide sequence to dissect the relative contributions of the above variables and report the results herein.

The furanose ring in DNA (**1**) and RNA (**2**) exists in a conformational equilibrium between the C2′-endo (DNA-like) and C3′-endo (RNA-like) sugar puckers (Figure 1A). As seen with the 2′-hydroxyl group in RNA and the 2′-*O*-methoxyethyl group in MOE (**4**) nucleotides, the 2′-fluorine atom in FRNA (**3**) steers the conformation of the furanose ring into the RNA-like C3′-endo sugar pucker.[15] This structural arrangement positions the electronegative fluorine atom and the 4′-ring oxygen atom in a favorable gauche orientation. However, while the 2′-groups in RNA and MOE participate in an extensive hydration network with water molecules around the sugar−phosphate backbone,[16] the 2′-fluorine atom in FRNA does not form H bonds with water molecules in the minor groove of the modified duplexes.[17,18] Instead, enhanced duplex stabilization is achieved by improved strength of Watson−Crick base pairing, a consequence of increased polarization of the nucleobase by the antiperiplanar fluorine atom.[19] In contrast, the sugar ring of 2′-fluoro arabino nucleic acid (FANA, **5**) exists in the O4′-endo conformation.[20] FANA also has a stabilizing effect upon duplex stability, but these effects are thought to be a result of stabilizing internucleosidic pseudo H···F bonds at the purine−pyrimidine steps.[21]

The furanose ring in FRNA can be replaced with other ring systems which mimic the C3′-endo sugar pucker (Figure 1). The six-membered hexitol ring in HNA (**6**),[22] FHNA (**7**), and Ara-FHNA (**8**)[12] preferentially exists in the chair conformation with the nucleobase in an axial orientation. This chair

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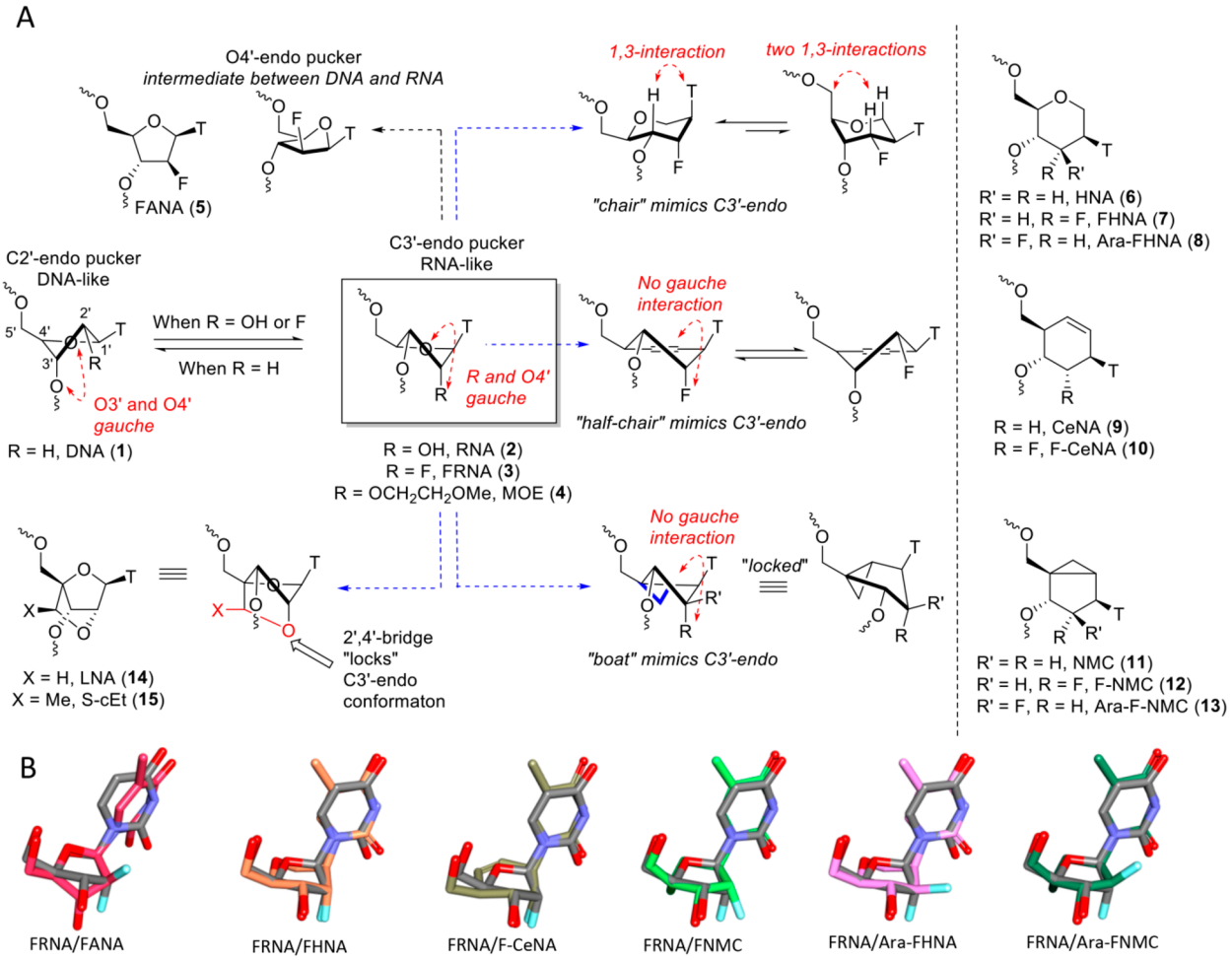

Figure 1. (A) Conformational analysis of fluorinated nucleic acid modifications with furanose ring and non-furanose ring systems. (B) Overlays of FRNA with the different fluorinated modifications: FRNA (gray)/FANA(red) showing different orientations for the 2′-fluoro, the 3′-hydroxyl and the nucleobases; FRNA/FHNA (caramel), FRNA/F-CeNA (olive green), and FRNA/FNMC (light green) showing similar orientations for the 2′-fluoro, 5′- and 3′-hydroxyls, and the nucleobases; FRNA/Ara-FHNA (pink) and FRNA/Ara-FNMC (dark green) showing similar orientations for the 5′- and 3′-hydroxyls and the nucleobases but different orientations for the 2′-fluoro atoms.

conformation mimics the C3′-endo conformation of FRNA and has one less 1,3-diaxial interaction in comparison to the chair with the equatorial nucleobase. In contrast, the six-membered cyclohexenyl ring in CeNA (**9**)[23] and F-CeNA (**10**)[14] exists in a conformational equilibrium between two half-chair conformations which mimic the C2′- and C3′-endo sugar puckers of DNA and RNA, respectively. The bicyclo[3.1.0] ring system in NMC (**11**),[24] F-NMC (**12**), and Ara-F-NMC (**13**)[13] nucleotides resembles the "boat" conformation of cyclohexane and is also an effective structural mimic of the C3′-endo sugar pucker.[25] The appended cyclopropyl ring rigidifies the conformation of the cyclopentane ring without the need of a 2′-electron-withdrawing group. Finally, tethering the 2′-*O*-alkyl group back to the 4′-position of the furanose ring gives 2′,4′-bridged nucleic acids (LNA **12** and constrained ethyl BNA cEt **13**) where the [2.2.1] dioxabicycloheptane ring system is a very effective mimic of the C3′-endo sugar pucker.[26]

To systematically compare the effects of hydration, conformation and fluorine substitution on T_m, we measured the duplex stabilizing properties of MOE, *S*-cEt, FRNA, FANA, HNA, FHNA, F-CeNA, NMC, F-NMC and Ara-F-NMC using a single incorporation of the modified nucleotide at four locations within a previously described oligonucleotide sequence (Table 1).[27] The modified nucleotides were inserted at different locations and were flanked on either side by different nucleobases, thus providing a position and sequence context for the evaluation.

A single incorporation of MOE (T) or FRNA (U) at four different locations within the sequence resulted in a net stabilization effect of +0.9 °C/mod and +0.8 °C/mod, respectively. In comparison, FANA (T) provided an overall stabilization of +1.3 °C/mod. It should also be noted that FRNA was evaluated using uracil, while the rest of the panel was evaluated using thymine as the nucleobase. It is known that the 5-Me group in thymine contributes roughly +0.5 °C/mod toward overall duplex thermal stability by increased stacking interactions within the duplex.[28] Thus, FANA and FRNA most likely have very similar duplex stabilization properties which appear to be slightly superior to those of MOE. Our data suggest that polarization of the nucleobase by the 2′-fluorine atom has a greater impact on enhancing duplex stability in comparison to improved hydration around the sugar–phosphate backbone.[29]

A single incorporation of HNA (+2.5 °C/mod) provided a greater magnitude of increase in duplex stability in comparison to MOE, FRNA, and FANA. This increase could be further

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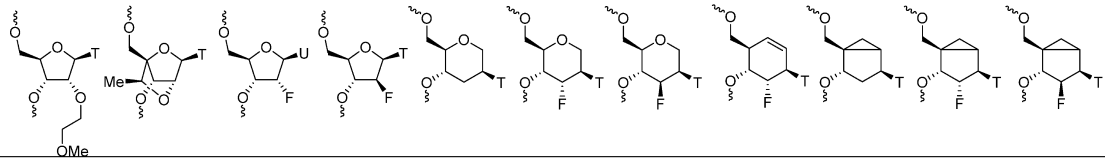
Table 1. Positional Effect of Fluorinated and Other Modifications on Thermal Stability of Modified Duplexes[a]

Sequence (5′ to 3′)	ΔT_m/mod. (°C) vs. RNA										
	MOE (T)	S-cEt (T)	FRNA (U)	FANA (T)	HNA (T)	FHNA (T)	Ara-FHNA (T)	F-CeNA (T)	NMC (T)	F-NMC (T)	Ara-F-NMC (T)
											
d(GGA**T**GTTCTCGA)	+1.7	+6.6	+1.1	+1.2	+2.2	+3.2	--	+0.7	+0.4	+2.2	-2.6
d(GGATG**T**TCTCGA)	+0.4	+5.3	-0.3	+0.6	+2.9	+2.0	--	-0.7	+1.6	+2.7	-2.2
d(GGATGT**T**CTCGA)	+0.9	+4.6	+1.3	+1.3	+2.2	+3.5	--	+1.5	+2.5	+2.0	-4.3
d(GGATGTTC**T**CGA)	0	+6.5	+1.2	+1.8	+2.3	+3.5	--	-0.4	+1.7	+2.6	-2.1
Average ΔT_m/mod. (°C)	+0.9	+5.8	+0.8	+1.3	+2.5	+3.2	-2.0*	+0.3	+1.6	+2.2	-2.8

[a]Bold and underlined letters indicate the modified nucleotides which were inserted at different locations within a phosphodiester DNA sequence. Base code: U = uracil, T = thymine, C = 5′-Me-cytosine, A = adenine, G = guanine. T_m values were measured in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 0.1 mM EDTA using the RNA complement 5′-r(UCGAGAACAUCC)-3′. ΔT_m values were calculated by subtracting the T_m value of the modified duplex from the T_m value of the unmodified DNA/RNA duplex (49.4 °C). The average ΔT_m/mod value was calculated by averaging the individual ΔT_m/mod values for each position. The asterisk denotes that Ara-FHNA phosphoramidites were not available for the comparative analysis. The ΔT_m value for Ara-FHNA was calculated by averaging T_m values from previous studies using two different DNA sequences.[12]

augmented by introducing a fluorine atom antiperiplanar to the nucleobase, at the 2′-position as observed with FHNA (+3.2 °C/mod) modified ONs. The 2′-epimeric analogue of FHNA with an equatorial fluorine (Ara-FHNA) was previously shown to be destabilizing.[12] The improved duplex stabilization observed with HNA- and FHNA-modified ONs can be rationalized by the increased rigidity of the chair conformation of the six-membered ring with the axial nucleobase in HNA and FHNA. In contrast, five-membered furanose rings are conformationally more flexible because of similar thermodynamic stabilities of the intermediate conformers. This hypothesis is further supported by the excellent duplex stabilization observed with the *S*-cEt (+5.8 °C/mod) modification, where the furanose ring is locked in the C3′-endo conformation.[26]

In comparison to the hexitol-modified analogue FHNA, the cyclohexenyl analogue F-CeNA had a smaller overall positive impact on duplex stability (+0.3 °C/mod). However, F-CeNA had a prominent position-specific stabilizing effect on duplex stability, whose origins remain unclear at this time. Previous studies using X-ray crystallography had shown that the cyclohexenyl ring is more flexible and both half-chair conformations were observed in the duplex structure.[14] Unfortunately, CeNA-modified ONs were not available for direct comparison to assess the effect of fluorine incorporation on this scaffold on duplex stability using the sequence employed in this study. However, our previous analysis using two additional sequences had confirmed that fluorination can have a small beneficial effect for CeNA.[14]

For F-NMC, which is also "locked" in the C3′-endo conformation, a single incorporation produced an average stabilizing effect of +2.2 °C/mod, while its nonfluorinated parent NMC was slightly less stabilizing (+1.6 °C/mod) reflecting the positive contribution of fluorine incorporation on duplex stability. The "boat-like" bicyclo[3.1.0] ring system in F-NMC is more rigid as compared to the "half-chair" conformers in F-CeNA, but this conformation is probably not as good a mimic of the C3′-endo conformation in comparison to the "chair conformation" of the hexitol ring in FHNA. Interestingly, the RNA version of NMC was recently shown to be slightly destabilizing, suggesting that the fluorinated analogue F-NMC is less perturbing within the duplex structure.[30]

The Ara-F-NMC analogue had a strong destabilizing effect on duplex stability. This result is similar to that for Ara-FHNA, which also had a destabilizing effect on duplex stability in contrast to FANA, which is stabilizing. We had previously shown that the geometrical constraints imposed by the larger hexitol ring positions the equatorial fluorine atom in Ara-FHNA in close proximity to the 4′-oxygen atom of the 3′-adjacent nucleotide in an A-form DNA duplex. This resulted in a repulsive electrostatic interaction which pushes the 3′-adjacent nucleotide away, causing partial unstacking of the nucleobases leading to duplex instability.[12]

The conformational overlay of FRNA and FANA monomers extracted from structures of the modified duplexes (Figure 1) showed a slightly different orientation around the glycosidic bond (χ) and the 3′-hydroxyl in FANA versus FRNA and the other fluorinated analogues with C3′-endo sugar ring mimics. This suggests that the more flexible five-membered ring of FANA is able to adapt its conformation to the O4′-endo sugar pucker, which fortuitously picks up a stabilizing CH···F interaction with C8 of the 3′-adjacent nucleotide.[31] Interestingly, while the initial report had suggested that this stabilizing interaction is specific for purine/pyrimidine steps,[21] we found

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that FANA had a net stabilizing effect at all positions evaluated in the sequence used in our studies. This suggests that the stabilizing CH···F interaction in FANA is generally operative for all sequences or points to a different origin for the improved duplex stabilizing properties of FANA. It is conceivable that fluorine is able to polarize the nucleobase when oriented in either a *trans* (FRNA) or *syn* (FANA) diaxial orientation with the nucleobase and that these interactions further translate to enhanced Watson−Crick base-pairing and nucleobase stacking interactions. Interestingly, a nonbonding interaction between H6 of the pyrimidine nucleobase and fluorine at the 5′-position of bicyclo-DNA enhanced duplex stability by restricting rotation (required for Watson−Crick base pairing) around the glycosidic bond.[27] This observation provides yet another example of the positive impact of fluorination on thermal stability of oligonucleotide duplexes.

In conclusion, we report the comparative duplex stabilizing properties of several nucleic acid analogues and dissect the relative contributions of hydration, sugar conformation, and fluorine configuration toward the overall T_m value. Our comparative analysis shows that the [2.2.1]dioxabicyclo ring system is the best mimic of the C3′-endo conformation, as evidenced by the large increases in duplex stability observed with the family of 2′,4′-bridged nucleic acids (LNA/*S*-cEt). Our data further reinforce the importance of the *trans* diaxial orientation of the 2′-fluorine and the nucleobase (FHNA, F-CeNA and F-NMC) for improving duplex thermal stability[32] and show that the stabilization imparted by fluorine substitution is additive to that obtained by restricting the conformation of the sugar ring itself. Our results also conclusively show that the combination of the "arabino" configuration of fluorine and the C3′-endo sugar pucker (Ara-FHNA and Ara-F-NMC) has a negative impact on duplex thermal stability. Collectively, our studies suggest that fluorinated nucleic acid analogues such as FHNA, F-CeNA, and F-NMC can act as structural and possibly functional mimics of FRNA, and this warrants their further evaluation for therapeutic antisense applications.

EXPERIMENTAL SECTION

Structural Models. Structural models were created by extracting structures of the mononucleotides from published structures of modified duplexes versus complementary RNA: FRNA,[8] FANA,[31] FHNA and Ara-FHNA,[12] F-CeNA,[14] NMC.[25]

Oligonucleotide Synthesis. The syntheses of oligonucleotides (ONs) were performed on a 1.0 μmol scale on a DNA synthesizer using polystyrene support (100 μmol/g). Standard conditions were used for incorporation of DNA phosphoramidites: i.e., 3% dichloroacetic acid in DCM for DMTr deblocking, 1 M 4,5-dicyanoimidazole 0.1 M *N*-methylimidazole in acetonitrile as activator, acetic anhydride in THF as Cap A, 10% methylimidazole in THF/pyridine as Cap B, and 10% *tert*-butyl hydroperoxide as the oxidizing agent. DNA and modified phosphoramidites were dissolved in acetonitrile (0.1 M) and coupled twice with 3 and 6 min contact times, respectively. Phosphoramidites of F-NMC and ara-F-NMC were available in limited quantities and were coupled manually: i.e., amidite (7 mg, 11 μmol) was dissolved in dichloromethane (0.4 mL) and activator (0.6 mL) was added and coupled manually for 30 min via syringes. The synthesis column was returned to the DNA synthesizer, and synthesis of the modified oligonucleotide was completed in the automated mode, as described above. After the synthesis was complete, the support-bound oligonucleotides were treated with a solution of Et_3N/CH_3CN (1/1, v/v) for 25 min and then deprotected and detached from solid support with 33% aqueous NH_3 for 48 h at room temperature. The coupling efficiency of all modified nucleosides was typically >95%, as judged by failure analysis of the crude oligonucleotides after the ammonia treatment. The crude material was purified by ion-exchange HPLC with a linear gradient (0−19%) of buffer B (0.05 M $NaHCO_3$, H_2O/CH_3CN 7/3, 1.5 M NaBr) in buffer A (0.1 M $NaHCO_3$, H_2O/CH_3CN 7/3) as eluent. Oligonucleotides were desalted using a reverse-phase cartridge and lyophilized. The purity and observed and theoretical masses of ONs are given in Table 2.

Table 2. Purity and Observed and Theoretical Masses of ONs

sequence	mod	mass (calcd), amu	mass (exptl), amu	UV purity, %
5′-GGATGTTCTCGA	MOE	3750.5	3750.5	99.0
5′-GGATGTTCTCGA	MOE	3750.5	3750.5	98.5
5′-GGATGTTCTCGA	MOE	3750.5	3750.5	99.1
5′-GGATGTTCTCGA	MOE	3750.5	3750.4	99.1
5′-GGATGTTCTCGA	*S*-cEt	3718.5	3718.3	98.7
5′-GGATGTTCTCGA	*S*-cEt	3718.5	3718.3	99.0
5′-GGATGTTCTCGA	*S*-cEt	3718.5	3718.3	99.2
5′-GGATGTTCTCGA	*S*-cEt	3718.5	3718.3	98.9
5′-GGAUGTTCTCGA	FRNA	3680.4	3680.1	98.8
5′-GGATGUTCTCGA	FRNA	3680.4	3680.1	98.8
5′-GGATGTUCTCGA	FRNA	3680.4	3680.1	98.9
5′-GGATGTTCUCGA	FRNA	3680.4	3680.0	98.8
5′-GGATGTTCTCGA	FANA	3694.5	3693.3	97.9
5′-GGATGTTCTCGA	FANA	3694.5	3693.6	99.1
5′-GGATGTTCTCGA	FANA	3694.5	3693.6	97.5
5′-GGATGTTCTCGA	FANA	3694.5	3693.6	98.8
5′-GGATGTTCTCGA	HNA	3690.5	3689.7	98.6
5′-GGATGTTCTCGA	HNA	3690.5	3689.7	99.4
5′-GGATGTTCTCGA	HNA	3690.5	3689.4	97.8
5′-GGATGTTCTCGA	HNA	3690.5	3689.7	99.3
5′-GGATGTTCTCGA	FHNA	3708.5	3707.7	98.6
5′-GGATGTTCTCGA	FHNA	3708.5	3707.7	98.8
5′-GGATGTTCTCGA	FHNA	3708.5	3707.7	97.1
5′-GGATGTTCTCGA	FHNA	3708.5	3707.7	99.0
5′-GGATGTTCTCGA	F-CeNA	3704.5	3703.7	98.0
5′-GGATGTTCTCGA	F-CeNA	3704.5	3703.7	99.0
5′-GGATGTTCTCGA	F-CeNA	3704.5	3703.7	98.6
5′-GGATGTTCTCGA	F-CeNA	3704.5	3703.7	99.3
5′-GGATGTTCTCGA	NMC	3686.5	3685.7	99.1
5′-GGATGTTCTCGA	NMC	3686.5	3685.7	99.2
5′-GGATGTTCTCGA	NMC	3686.5	3685.7	98.4
5′-GGATGTTCTCGA	NMC	3686.5	3685.7	98.6
5′-GGATGTTCTCGA	F-NMC	3704.5	3703.8	98.8
5′-GGATGTTCTCGA	F-NMC	3704.5	3703.8	98.8
5′-GGATGTTCTCGA	F-NMC	3704.5	3703.8	98.4
5′-GGATGTTCTCGA	F-NMC	3704.5	3703.8	99.2
5′-GGATGTTCTCGA	Ara-F-NMC	3704.5	3704.0	97.2
5′-GGATGTTCTCGA	Ara-F-NMC	3704.5	3704.0	97.5
5′-GGATGTTCTCGA	Ara-F-NMC	3704.5	3704.0	97.8
5′-GGATGTTCTCGA	Ara-F-NMC	3704.5	3704.0	97.7

Thermal Denaturation Studies. ON and RNA were mixed in a 1:1 ratio (4 μM duplex) in buffer containing 10 mM phosphate, 100 mM NaCl, and 10 μM EDTA at pH 7.0. The duplex was denatured at 85 °C and slowly cooled to the starting temperature of the experiment (15 °C). Thermal denaturation temperatures (T_m values) were measured in quartz cuvettes (path length 1.0 cm) on a UV/vis spectrophotometer equipped with a temperature controller. The absorbance at 260 nm was measured as a function of temperature using a temperature ramp of 0.5 °C per min. T_m values were

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determined using the hyperchromicity method incorporated into the instrument software.

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Notes

The authors declare no competing financial interest.

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