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Energetically Important C−H···F−C Pseudohydrogen Bonding in Water: Evidence and Application to Rational Design of Oligonucleotides with High Binding Affinity

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Supporting Information

ABSTRACT: It is controversial whether organic fluorine can form energetically important hydrogen bonds in aqueous environments. We previously showed by NMR and molecular modeling that the unexpectedly high binding affinity of 2′F-ANA is largely due to a C−H···F−C pseudohydrogen bond at pyrimidine−purine steps. Comparisons of the melting of duplexes with identical sequence composition but a rearranged sequence confirm that energetically important fluorine-mediated pseudohydrogen bonding is in operation in these sequences. The effect is of particular importance when the H-bond donor (purine H8) is activated by the presence of fluorine at its own 2′-position. These results provide a rational method to increase the binding affinity of antisense oligonucleotides by placement of 2′F-ANA modifications at pyrimidine−purine steps.

Organic fluorine does not generally act as a hydrogen bond acceptor.[1,2] Occasional examples appear: researchers have observed short H−F distances in crystal structures,[2−6] favorable interactions in molecular models,[7,8] H···F scalar couplings in NMR spectra[9−12] and vibrational frequency shifts.[13,14] Yet particularly in aqueous systems, where better hydrogen bond acceptors are abundant, it is rare to find examples of energetically important hydrogen bonds to fluorine.

The field of modified oligonucleotides has experienced a good deal of the controversy over whether fluorine-mediated hydrogen bonds are important. The highest profile example is the Kool group's difluorotoluene base.[15,16] Developed as a non-hydrogen-bonding isostere of thymine,[17] controversy has raged for over a decade after its synthesis about whether it could in fact accept hydrogen bonds after all. A recent crystal structure showed a short N−H···F−C distance in a difluorotoluene-adenine base pair,[5] but the weight of the evidence is that N−H···F−C interactions in difluorotoluene base pairs are of little energetic importance, especially in water where excellent hydrogen bond acceptors are abundant.[15] Evidence for R-H···F-C interactions involving other modified nucleobases has come from crystallographic, computational and spectroscopic methods.[4,8,14,18−20] In this paper we demonstrate modified oligonucleotides containing energetically important pseudohydrogen bonds to fluorine in a non-base-pairing context.

2′-Fluoroarabinonucleic acid (2′F-ANA)[21] is a DNA mimic with high binding affinity to both DNA and RNA.[9] This high binding affinity is surprising because its close cousin arabinonucleic acid (ANA) binds to DNA and RNA with low affinity.[22] We recently determined the NMR structure of a 2′F-ANA•RNA duplex and showed by structural, biophysical and computational methods that its surprisingly high stability was largely due to the presence in the 2′F-ANA strand of a pseudohydrogen bond between 2′F and purine H8 of the 3′-proximal nucleotide.[23] This interaction was optimal at 5′-pyrimidine−purine-3′ steps, where the base stacking geometry can adjust.[6,23]

The sequence dependence of our pseudohydrogen bond suggests a testable hypothesis: if 2′F···H8 interactions at 5′-pyrimidine−purine-3′ steps are of true energetic importance, then an oligonucleotide with more of these steps should have a greater increase in binding affinity upon modification with 2′F-ANA, as compared with a sequence of identical nucleotide composition and degree of 2′F-ANA modification, but fewer pyrimidine−purine steps.

Accordingly, we designed two sequences of identical base composition (Table 1). Sequence **A** contained four pyrimidine−purine steps (all four are 5′-TA-3′), while sequence **B** contained only one (5′-TG-3′). The unmodified sequences have a similar T_m. Upon 2′F-ANA modification of all the T and A nucleotides, the T_m of sequence **A** increased by 12.6 °C while that of sequence **B** increased by less than 6 °C (sequences **A4**, **B4**, Figure 1 and Table 1).

To further explore the nature of this change, we modified only the T or A nucleotides (**A2**, **A3**, **B2** and **B3**, Table 1). Modification of only the adenines in sequence **A3** gave a substantial stabilization (ΔT_m/modification = 1.4 °C, only about 15% lower than the 1.6 °C/modification observed for **A4**). We wondered if this could be related to intraresidue pseudohydrogen bonding in the modified purines.

There is some evidence for pseudohydrogen bonding between a top-face 2′-fluorine and its own nucleobase.[7,9,11,24] For a single 2′F-ANA nucleoside, evidence for intramolecular R-H···F−C interactions has come from NMR splittings of purine H8 or

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Table 1. Sequences and T_m Values of Designed Oligonucleotides[a]

name	sequence (5′−3′)	T_m[b]	ΔT_m[c]	ΔT_m/*mod*	ΔG[d]
A1	ctatagtatac	27.5	–	–	−36.8
A2	c**T**a**T**ag**T**a**T**ac	31.0	3.5	0.9	−40.6
A3	ct**A**t**A**gt**A**t**A**c	33.2	5.7	1.4	−43.2
A4	c**TATA**g**TATA**c	40.1	12.6	1.6	−50.2
B1	caattgaattc	27.7	–	–	−36.2
B2	caa**TT**gaa**TT**c	28.4	0.7	0.2	−37.2
B3	c**AA**ttg**AA**ttc	28.2	0.5	0.1	−37.1
B4	c**AATTgAATT**c	33.5	5.8	0.7	−43.1

[a] Legend: 2′F-ANA, bold uppercase; dna, lowercase. [b] In °C, for a duplex of the strand with complementary RNA. [c] Change in T_m relative to **A1** or **B1**, respectively. [d] In kJ/mol at 298 K, derived from the shape of the UV melt curves.

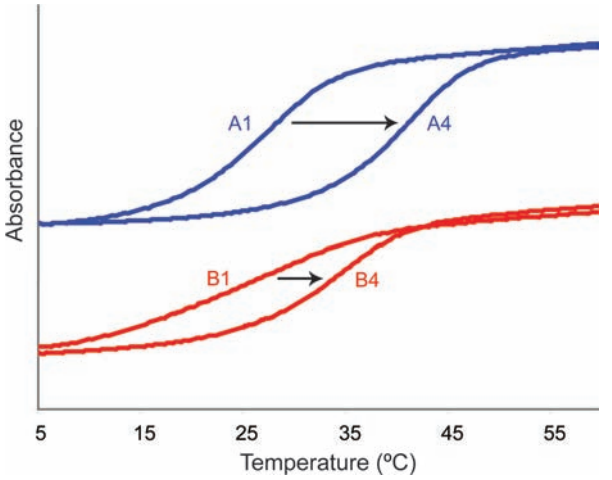

Figure 1. A sequence containing multiple pyrimidine−purine steps is more heavily stabilized upon 2′F-ANA modification.

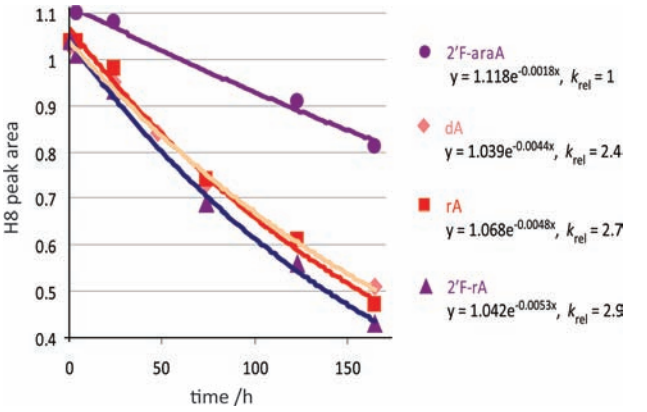

Figure 2. Deuterium exchange rates at H8 of DNA, RNA, 2′F-ANA, and 2′F-RNA adenine nucleosides.

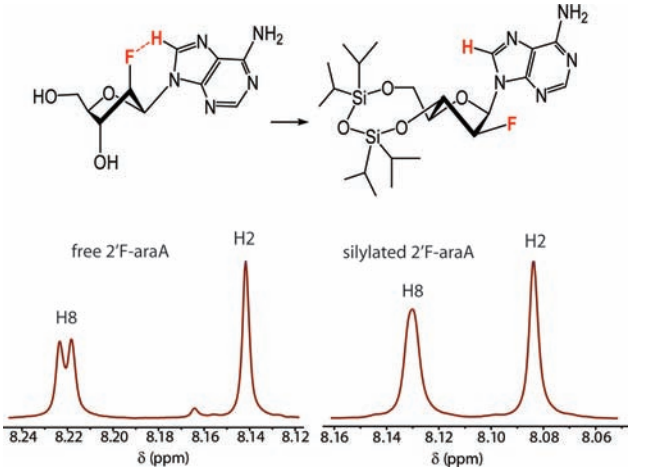

Figure 3. Inducing a northern conformation by connecting the 5′- and 3′-hydroxyl groups of 2′F-araA causes disruption of the otherwise stable H8/F2′ coupling.

pyrimidine H6.[7,9−11,24] Computational work has identified this interaction as a pseudohydrogen bond (i.e., having partial covalent character.)[7] We carried out three types of NMR experiments to characterize this apparent C−H···F−C pseudohydrogen bond in water.

Upon dissolving a purine nucleoside in D_2O, the acidic H8 proton undergoes deuterium exchange and disappears from the NMR spectrum; at neutral pH this exchange takes several days. We dissolved dA, rA, 2′F-rA and 2′F-araA in D_2O and compared their rates of H8 deuterium exchange at pH 7.4. The rate of exchange for 2′F-araA was markedly lower than that of dA, rA, or 2′F-rA ($k_{2'\text{F-rA}}/k_{2'\text{F-araA}} = 2.9$) (Figure 2). Since there is no reason to expect that the 2′F-araA H8 is inherently less acidic than the other three analogues, it is likely stabilized from exchange by its engagement in a hydrogen-bonding interaction.[25]

We then evaluated the thermal stability of this C−H···F−C interaction. The 2 Hz splitting of 2′F-araA H8 was monitored by NMR in D_2O or DMSO at various temperatures, and was stable even up to 100 °C.[26] Most H-bonding interactions of relatively low energy would disappear by this temperature, but this one remained stable—perhaps in part because the nucleobase is constrained in the anti orientation by steric effects from the top-face fluorine.[27]

2′F-ANA nucleosides generally adopt a southeast sugar pucker. To provide further evidence that the H8/F2′ coupling was not a five-bond coupling, we constrained the nucleoside to adopt a northern conformation by connecting the 3′- and 5′-OH groups with the Markiewicz reagent[28] (Figure 3). The disiloxane product showed no H8/F2′ splitting. This result provides strong evidence for a stable intramolecular pseudohydrogen bonding interaction in 2′F-ANA purine nucleosides, particularly when taken together with the previously described NMR experiments, the fact that 2′F-rA does not show splitting at H8, and the computational work mentioned above.[7]

It would not be surprising to find this intraresidual pseudohydrogen bond acting in oligonucleotides as well, if the base pairing geometry can adjust. In our previous structural study,[23] some short intraresidual H···F distances were observed (2.3 Å) but the geometry was more favorable for the interresidual C−H···F bonds, and we focused our analysis on the latter. In a partially modified sequence such as **A3**, however, the base-stacking geometry could readily adjust to optimize intraresidual H···F interactions. Thus the relatively high stabilization observed for sequence **A3** may be related to intraresidue pseudohydrogen bonding within oligonucleotides.

In contrast, for sequence **B3** where the purines are adjacent, the base stacking geometry cannot adjust, and the stabilization is minimal (or if the base stacking does adjust, it imposes a steric penalty with an

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Table 2. Sequences, T_m Values and RNase H Cleavage Velocities of Antisense Oligonucleotides[a]

name	sequence (5′-3′)[b]	T_m[c]	ΔT_m	V[d]
X-DNA	tcatgagtggcagctgcaatt	64.9	–	1.00 ± 0.10
X-gap	**TCAT**gagtggcagctgc**AATT**	68.0	3.1	0.80 ± 0.15
X-1	t**CAT**g**AGT**ggca**GCT**gc**AAT**t	72.1	7.2	0.76 ± 0.03
X-2	tc**ATG**A**gtg**G**C**agCTg**CAAT**t	72.3	7.4	0.83 ± 0.12
X-4	t**CAT**g**AGTG**gCagc**TGCA**att	73.5	8.6	0.85 ± 0.05
X-6	t**CATG**ag**TGgCA**gc**TGCA**att	74.2	9.3	0.59 ± 0.10

[a] Legend as for Table 1. [b] 2′F-ANA-modifed pyrimidine–purine steps are underlined in the altimers. [c] In °C, for a duplex of the strand with complementary RNA. [d] Relative velocity for RNase H cleavage of the hybrid. By comparison, phosphorothioate X-DNA had a relative velocity of 0.27 ± 0.15.

associated loss in binding affinity.) Accordingly, sequence **B3** showed an order of magnitude less stabilization than **A3**.

When only the thymines of sequence **A2** were modified, substantially smaller stabilization was observed (0.9 °C/2′F-ANA, Table 1). Thus, the inter-residual pseudohydrogen bonding at pyrimidine–purine steps is most favorable when both residues are modified.

Two possible explanations emerge. Hydrogen bonding interactions are optimal when the donor is an acidic hydrogen. The presence of electronegative fluorine at a nucleoside's 2′-position may render the corresponding purine H8 more acidic. For example, during the D_2O exchange experiment described above, 2′F-rA had a rate of exchange about 20% faster than that of dA at pH 7.4 (Figure 2). Besides the proton acidity, the exchange rate depends on conformation and other factors, so the relative acidity may in fact be smaller or larger.

Alternatively, the pyrimidine–purine dimer may adopt a more favorable conformation for interresidual pseudohydrogen bonding when both nucleotides are 2′F-ANA-modified. It is not yet clear whether both intra- and inter-residual interactions can occur simultaneously at the same residues, since the base pairing geometry may need to adjust to optimize each independently.

Thus while we provide strong evidence that pseudohydrogen bonding exists, it does appear to require cooperativity between several favorable circumstances. This too is consistent with previous work.[18] Corey et al. coined the term "induced" or "cooperative" C–H···F hydrogen bonding for a related phenomenon.[29] Likewise, Koller et al. showed that fluorine exerts both direct and indirect effects (through pseudohydrogen bonding and electronegativity) in stabilizing modified base pairs.[20] Accordingly, to obtain the highest affinity oligonucleotide, both nucleotides of each pyrimidine–purine step should be modified with 2′F-ANA – one fluorine serves as a H-bond acceptor and the other activates a suitable C–H donor.

Can pseudohydrogen bonding at pyrimidine–purine steps be used to increase binding affinity in a rational way and thus aid in sequence design for therapeutically relevant oligonucleotides? To find out, we synthesized five versions of a clinically relevant antisense oligonucleotide.[30] In comparison with a traditional gapmer design (2′F-ANA wings and a central DNA section), we designed altimer oligonucleotides containing 1, 2, 4 or 6 2′F-ANA-modified Py-Pu steps (Table 2). For the four altimers, an equal number of total nucleotides and an equal number of purines were modified with 2′F-ANA. Furthermore, all four strands consisted of nine alternating segments – four regions of 2′F-ANA and five regions of DNA. The termini were native DNA in all cases. These parameters were kept constant to help avoid confounding influences on the binding affinity.

As predicted, the T_m of the four altimers increased with the number of modified Py-Pu steps (Table 2). Thus the same number of 2′F-ANA nucleotides, including the same number of purines, can give higher binding affinity when pyrimidine–purine steps are selectively modified. This provides a rational means to increase the binding affinity of an antisense oligonucleotide without increasing the degree of modification.

To ensure the therapeutic relevance of this strategy, we carried out an RNase H assay on all of the modified oligonucleotides. The four altimers had excellent RNase H activity; all four were superior to phosphorothioate DNA (Table 2 and legend). Altimers **X-1**, **X-2**, and **X-4** had comparable rates to the gapmer; while altimer **X-6** was slightly slower (Supporting Information Figure S1). More pseudohydrogen bonding may lead to higher duplex rigidity as well as higher binding affinity. This could explain the slower rate for **X-6** since the RNase H enzyme reacts more quickly with duplexes containing flexible antisense strands.[31] Flexible strands may enable the enzyme to deform the duplex more easily as required during binding.[31,32]

In conclusion, these data provide compelling evidence for C–H···F–C pseudohydrogen bonding in water. We previously showed using NMR structural studies and computational methods that short H···F distances exist and are predicted to be favorable.[23] Now we have shown that this rare interaction is in fact energetically important in real systems and can be used to tune the binding affinity of oligonucleotides in a rational way.

The principles described here may also be relevant to other fluorinated oligonucleotides, although it appears that an array of favorable circumstances are required for pseudohydrogen bonding to have such a large effect.

■ ASSOCIATED CONTENT

Supporting Information. RNase H cleavage data and all experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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