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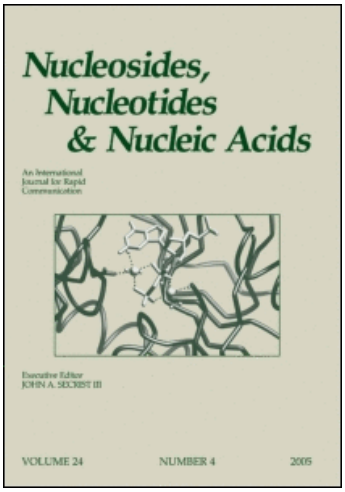

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PROPERTIES OF ARABINONUCLEIC ACIDS (ANA & 20'F-ANA): IMPLICATIONS FOR THE DESIGN OF ANTISENSE THERAPEUTICS THAT INVOKE RNASE H CLEAVAGE OF RNA

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PROPERTIES OF ARABINONUCLEIC ACIDS (ANA & 20′F-ANA): IMPLICATIONS FOR THE DESIGN OF ANTISENSE THERAPEUTICS THAT INVOKE RNASE H CLEAVAGE OF RNA

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ABSTRACT

Inversion of configuration of the C2′ position of RNA leads to a very unique nucleic acid structure: arabinonucleic acid (ANA). ANA, and its 2′-fluoro derivative (2′ F-ANA) form hybrids with RNA that are capable of activating RNase H, resulting in cleavage of the RNA strand. In this paper, we review the properties of duplexes formed between ANA (or 2′F-ANA) and its RNA complement. These studies support the notion that RNase H is sensitive to the minor groove dimensions of the hybrid substrate.

Arabinonucleosides and their analogues represent an important group of biologically active molecules (1). They occur in natural sources, and ara-C and ara-A have been especially suitable for pharmacological purposes (2,3). They are not found in natural polymeric nucleic acids, but their 5′-triphosphate derivatives can act as substrates of DNA polymerases (ref. 4 and 5, and references therein). Because the 2′-OH group is attached to the sugar ring in a β configuration and *cis*-positioned with respect to the heterocyclic base, some steric influence on the

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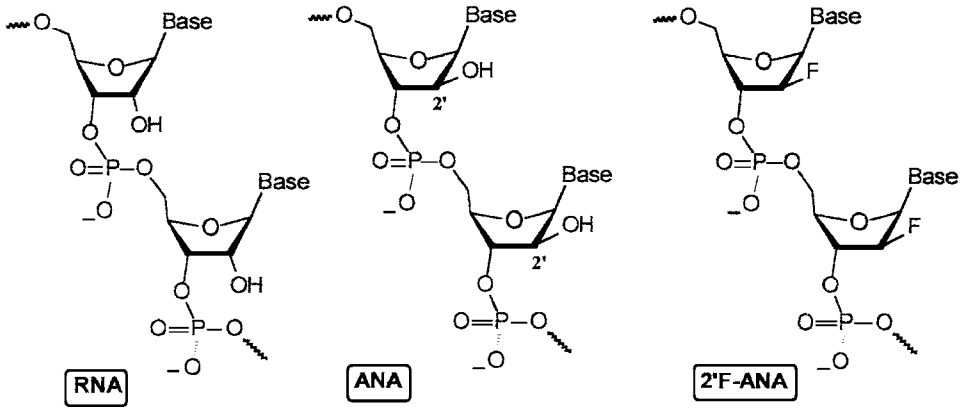

Figure 1. Primary structures of RNA, ANA and 2′F-ANA.

N-glycosidic bond conformation may be anticipated. In fact, this conformational characteristic may explain some of the biochemical properties of these nucleosides (4,5).

Our research has focused on the synthesis of arabinonucleic acids (Fig. 1) (6–9). Apart from evaluating the suitability of ANA as potential therapeutic agents, the study of their hybridization properties allowed us to address several fundamental questions, for example: *How does sugar structure, particularly the stereochemistry at position 2′ of the furanose ring, affect the formation and stabilization of nucleic acid helices (8,9)? What is the stability of an ANA/ANA duplex compared to that of duplex RNA and DNA (10)? Can ANA form a triplex with target duplexes, and if so, what is its stability compared to triplexes formed by the corresponding RNA strand (11)? Why is ANA (but not RNA) capable of forming C-rich tetrads or "*i*-motif" structures (12)?*

We have also been interested in understanding how an ANA strand is accommodated in a hybrid molecule (8,9). The formation of an ANA/RNA hybrid helix was first demonstrated with homopolymeric and pyrimidine-rich oligoarabinonucleotides (8). Advances in synthetic techniques, particularly in the preparation of ara-G (9,13), have allowed the synthesis of ANA oligomers with mixed base composition (9). These studies not only increased our understanding of the molecular forces that stabilize RNA double and triple helices, but also allowed us to predictably improve the binding properties of arabinonucleic acids. Our studies also led to the exciting discovery that ANA/RNA and 2′F-ANA/RNA hybrids are substrates of ribonuclease H (14), an enzyme implicated in the mechanism of action of antisense drugs (15,16). Since RNA/RNA duplexes are not substrates of RNase H (17), our findings provided an important advancement in understanding the catalytic mechanism and substrate selectivity of RNase H.

In this report, we review the hybridization properties of ANA and 2′F-ANA particularly with respect to binding to an RNA target. We also provide preliminary NMR data on the conformation of ANA/RNA and 2′F-ANA/RNA hybrids, which

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support the notion that RNase H is sensitive to the minor groove dimensions of the hybrid duplex (18,19).

ANA Versus the Epimeric RNA: Is 2′-Stereochemistry Relevant?

There is considerable interest in the contribution made by the 2′-hydroxyl group of ribose residues to the overall stability of helices (20). In addition to stabilizing the C3′-endo conformation of the ribose, the 2′-hydroxyl seems to play a significant part in stabilizing A-helices through interactions with water molecules in the minor groove (ref. 20 and 21, and cited literature). This feature presumably is partly responsible for the very different thermodynamic stability of DNA and RNA duplexes.

We have determined the stability of ANA/RNA hybrids and found that they have a lower value of T_m, compared to the corresponding RNA/RNA duplexes (9). Inversion of configuration at C2′ (*i.e.*, ribose → arabinose) results in a decrease of *ca.* 1–2°C/bp in binding affinity towards an RNA target (Table 1). The relative order of stability of hybrid duplexes with RNA is as follows: RNA > DNA > thioate-DNA > ANA. We also observed that ANA strands of mixed base composition are unable to pair with complementary ssDNA (9). Another striking observation was the inability of the Drew-Dickerson dodecamer ara(CGCGAAUUCGCG) to self-associate, whereas the corresponding RNA and DNA duplexes exhibited T_m values of 64.0 and 60.9°C, respectively (1 M NaCl) (10). These results clearly

Table 1. Properties of Oligonucleotide Analogues[a]

Oligonucleotide Analogue	ΔT_m/mod (°C)	RNase H Activation and Conformation		Ref. #
LNA	>3	no		44
2′F-RNA	+3.0	no		32
MMI-2′OMe-RNA	+2.0	no	A-form	45
2′MOE-RNA	+2.0	no		32
2′OMe-RNA	+1.7	no		32
RNA	+0.7	no		22
CeNA	+2	yes		35
2′F-ANA	+1.2	yes		22
thio-2′F-ANA	+0.5	yes	A/B-form	37
DNA	+0.5	yes		22
thio-DNA	0	yes		22
ANA	−0.5	yes		9,22

[a]Abbreviations: LNA, locked nucleic acid; 2′F-RNA, 2′-deoxy-2′-fluoro-ribonucleic acid; MMI-2′OMe-RNA, methylene(methylimino) 2′OMe-RNA; CeNA, cyclohexene nucleic acid; 2′F-ANA, 2′-deoxy-2′-fluoroarabinonucleic acid; thio-2′F-ANA, phosphorothioate 2′-deoxy-2′-fluoroarabinonucleic acid; DNA, deoxyribonucleic acid; thio-DNA, phosphorothioate DNA; ANA, arabinonucleic acid.

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emphasize the role of the "down" 2′-OH as a binding determinant of ribonucleic acid helices.

The loss of stability on inverting the configuration at C2′ is significant. This destabilization might well arise from the fact that, in arabinonucleosides, the 2′-hydroxyl group is "*cis*" oriented to the heterocyclic base, leading to changes about the N-glycosidic bond conformation which, in turn, would result in local deformation (unstacking) of the base pairs. In principle, this can be probed by replacing the 2′-OH group with smaller substituents. We therefore synthesized 2′F-ANA and found that, indeed, the 2′-F substitution results in significantly more stabilization than observed with the ANA (2′-OH) analogue (*see below*) (14,22).

Of course, other possible explanations must be considered. First, inversion of stereochemistry at C2′ is most likely accompanied by a conformational change in the ribose sugar from the northern (C3′-endo) to some other pucker affecting the stability of the duplex. For example, B-DNA duplexes that contain arabinonucleotide residues show the Southern conformational range (C2′-endo pucker) (23,24). Further, computational simulation of ANA/RNA hybrids show that ANA sugars prefer the C2′endo pucker due to an intranucleoside O2′-H....O5′ hydrogen bond (25). Crystal structures of Z-DNA duplexes that contain ANA residues also revealed the C2′-endo pucker form (26,27). This feature is expected to pre-organize ANA strands to a more B-like conformation, leading to less favorable ANA-RNA interactions in an A/B hybrid geometry. It should be noted, however, that the solution NMR structure of an ANA/RNA hybrid (see below) show no evidence for such a C2′-OH/O5′ interaction. Further, it was observed that the hybrid ANA strand adopts the East or O4′-endo conformation, with no evidence of steric interactions between the flanking bases and ara 2′OH groups. Slight steric contacts, however, have been noted between ara C2′-OMe groups and C6/C8 carbons of the flanking bases (25).

Another contributing factor to the low stability observed for duplexes containing ANA strands, *e.g.* T_m of ANA/ANA $\ll$ ANA/RNA $<$ RNA/RNA, may be attributed to the differential hydration pattern of these helices. The 2′-OH groups in RNA duplexes are prominent in the *minor groove* of the helix, where they propagate stable and conserved networks of water molecules (ref. 21, and cited literature). In addition, the 2′-OH groups lock the ribose-phosphate backbone in a conformation (C3′-endo) that allows water molecules in the *major groove* to bridge adjacent phosphates, while mediating hydrogen bonding to adjacent sugars (O4′). These interactions would be disturbed in ANA-containing helices (e.g. ANA/ANA, ANA/RNA) since the sugars adopt different conformations and expose their 2′-hydroxyls into the major groove.

Improving the Binding Properties of Arabinonucleic Acids: 2′F-ANA

The ability of fluorine to serve as a hydroxyl mimic and participate in hydrogen bonding (H-bond acceptor) has been widely discussed (ref. 28, and cited

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references). Pioneering studies originating from the laboratories of Chattopadhyaya (29) and Barchi (30) have established that the furanose ring pucker of a nucleoside/oligonucleotide is influenced by a variety of factors, particularly the electronegativity of the ring substituent, steric bulk, and stereoelectronic effects (gauche and anomeric effects). Fluorine has a van der Waals radius (1.47 Å) that is intermediate between that of hydrogen (1.2 Å) and oxygen (1.6 Å). From an electronic point of view, both hydroxyl (OH) and fluorine groups have similar electronegativities, the latter with a value of 4.0 (versus 3.5 for oxygen atoms) based on the Pauling scale (31). Thus, it is not surprising that replacing one of the 2′-H atoms of 2′-deoxyribose with fluorine alters significantly the conformation of the furanose ring.

In 2′F-RNA, the two dominant operational effects are the O4′-C1′-C2′-F *gauche* effect, and an additive *anomeric* effect which locks the sugar pucker in the northern (N) or C3′-endo conformation (Fig. 2). Such a pre-organization favors an A-form geometry and greatly improves the binding affinity to the target RNA (+2.5°C/nucleotide linkage relative to ssDNA; see Table 1) (32,33).

In 2′F-ANA, with an "up" F atom, the [O4′-C1′-C2′-F] *gauche* effect now opposes the weaker anomeric effect, causing the equilibrium to favor the east/south (E/S) conformation (Fig. 2) (32). This modification offers *ca.* +0.7°C increase in binding affinity/modification compared to DNA (Table 1). Pre-organization of the sugar or increased base stacking on the part of 2′F-ANA, or both, could lead to the observed stability differences between 2′F-ANA/RNA and DNA/RNA (22). While 2′F-ANA has a weaker RNA affinity compared to 2′F-RNA (34), 2′F-ANA is significantly more resistant towards nuclease hydrolysis (9). The relative order

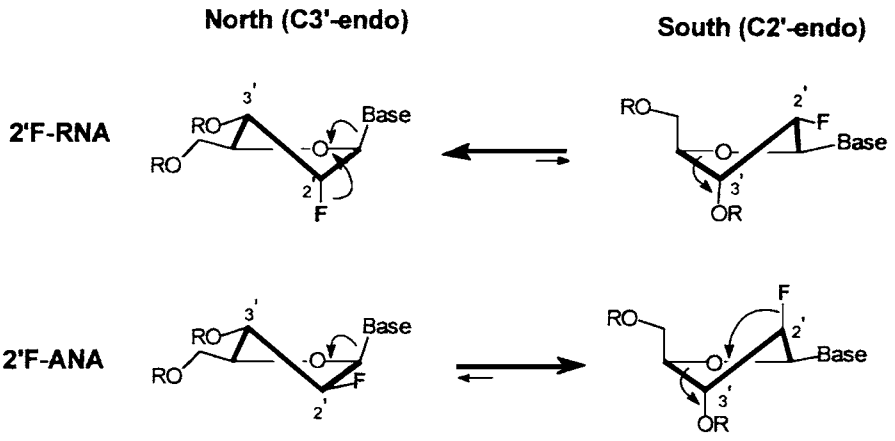

Figure 2. Sugar puckering conformational equilibrium for 2′F-RNA and 2′F-ANA. In the most stable conformation of 2′F-RNA (C3′-endo) and 2′F-ANA (C2′/O4′-endo), the 2′F-atom is pseudoaxial and gauche with respect to ring oxygen O4′ ("gauche effect").

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of stability against hydrolysis by snake venom phosphodiesterase is as follows (9,37),

thioate-DNA ≈ thioate-2′F-ANA > ANA > 2′F-ANA > RNA > 2′F-RNA > DNA,

whereas the relative order of stability of hybrid duplexes with RNA is (22,37):

2′F-RNA > 2′F-ANA > RNA > thioate-2′F-ANA > DNA > thioate-DNA > ANA.

Conformational Features of ANA/RNA and 2′F-ANA/RNA Hybrids: Implications for RNase H Specificity Toward DNA/RNA Hybrids

The formation of a duplex between an oligodeoxynucleotide (ODN) and cellular RNA prevents the translation of such RNA, either by "translation arrest" and, most importantly, by activation of ribonuclease H (RNase H), an endogeneous enzyme that specifically degrades the RNA strand of the ODN/RNA hybrid (15). Oligonucleotide analogues that modulate gene expression by more than one mechanism of action are highly desirable as this increases the potential efficacy of these compounds *in vivo*.

Normally, an increase in affinity for complementary mRNAs has been sought by the use of either conformationally biased (*e.g.*, 2′F-RNA) or conformationally locked (*e.g.*, bicyclo and tricyclo-DNA) oligonucleotide analogues (AON) (ref. 32 and cited references). All of these antisense oligonucleotide analogues (AON) favor the C3′-endo or North conformation. However, pre-organization of the AON into a pure north conformation (A-DNA like) produces an AON/RNA duplex not generally recognized by RNase H, a critical step in the mechanism of action of AONs. The lack of recognition of 'RNA-like'/RNA duplexes by RNase H has been partly solved by the use the so-called "gapmer technology", where conformationally South-biased deoxynucleotide gaps (B-DNA like) are flanked at either end of the DNA gap with North-biased nucleotide units (*e.g.* 2′-OMeRNA-DNA gap-2′OMeRNA) (16,32). The DNA gap, when bound to RNA, forms a hybrid that has an 'intermediate' A/B-DNA conformation, which is then efficiently recognized by the enzyme RNase H. This supports the notion that discrimination between RNA/RNA and DNA/RNA hybrids by RNase H is based on their different minor groove widths, which in turn is dependent on the sugar puckers of the constituent strands (18,19). Duplex RNA has a minor groove width of 11 Å and is not degraded at all, whereas the DNA/RNA substrate has a width of 9 Å.

Recently, we discovered that ANA and 2′F-ANA invokes RNase H activity when hybridized with complementary RNA (14). As far as we know, these compounds together with the cyclohexene nucleic acids (CeNA) (35), represent the first examples of sugar-modified oligonucleotides that activate this enzyme. CD studies demonstrated that ANA/RNA and DNA/RNA hybrids share the same A/B-helical ("A-like") conformation (9,14). This feature, combined with the expected B-like

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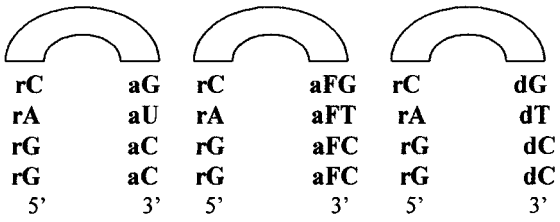

Figure 3. Secondary structure of hairpins studied by NMR (38). Residues aN, aFN, rN, and dN residues are arabinonucleotides, 2′-deoxy-2′-fluoroarabinonucleotides, ribonucleotides and 2′-deoxyribonucleotides, respectively. The loop sequence (not shown) is dTTCG. Helical parameters of these hairpins are given in Table 2.

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conformation of ANA residues (11,23–27), led us to propose the structural basis of cleavage by RNase H: (a) ANA/RNA mimics DNA/RNA in structure, and (b) the 2′OH and 2′F substitutents of the arabinose sugar ring projects into the major groove of the helix, at a site where it should not interfere with the binding and catalytic processes of RNase H (14).

Once the properties of ANA and 2′F-ANA were determined, we began to use them to study the mechanism by which RNase H recognizes and cleaves DNA/RNA hybrids. One obvious study was to determine the structure of these compounds when complexed to RNA (36,37). As model duplexes, we chose the hairpin structures shown in Figure 3. The loop consisted of a tetradeoxynucleotide (dTTCG) which is relatively flexible and presumably has little influence on the stem configuration. As the hairpin stem, we studied the following three combinations: 2′F-ANA/RNA (36), ANA/RNA (37), and DNA/RNA (37,38). Scalar coupling constants and NOE intensities were measured and used as constraints in simulated annealing and energy minimization of the 3D structure. A detailed structural analysis will be published elsewhere (38).

The RNA strand in these hybrids adopts an A-DNA like conformation, with North or C3′-endo sugars. However, the geometry of the 2′F-ANA and ANA strands share features that are neither A- or B-form, as previously proposed for DNA strands of hybrid duplexes (18,19). Specifically, very strong NOEs ($D_{H1'\text{-}H4'} = 2.35$ Å) and coupling constants analysis (PSEUROT) revealed that ANA and 2′F-ANA sugars are rigid and adopt similar Eastern-type O4′-endo puckers. This conformation is consistent with previous X-ray crystallographic data of duplex B-DNA containing 2′F-ANA residues (39). As can be seen from Figure 4B, the O4′-endo pucker ($P = 90-100^\circ$) lies halfway between the C2′-endo (South, B-DNA) and C3′-endo (North, A-DNA), which is more northern than one can predict for the isolated 2′F-ANA nucleosides ($P = 131^\circ$) (30). Presumably, the O4′-endo conformations of 2′F-ANA and ANA strands in the duplex results from steric consequences that predominate over stereoelectronic effects (gauche effect) caused by the local environment of the 2′-substituent (39).

The structure of the ANA/RNA hybrid is shown in Figure 5, and its helical parameters are given in Table 2, along with those of 2′F-ANA/RNA and DNA/RNA. We have calculated the helical twist, axial rise, base-pair inclination, displacement

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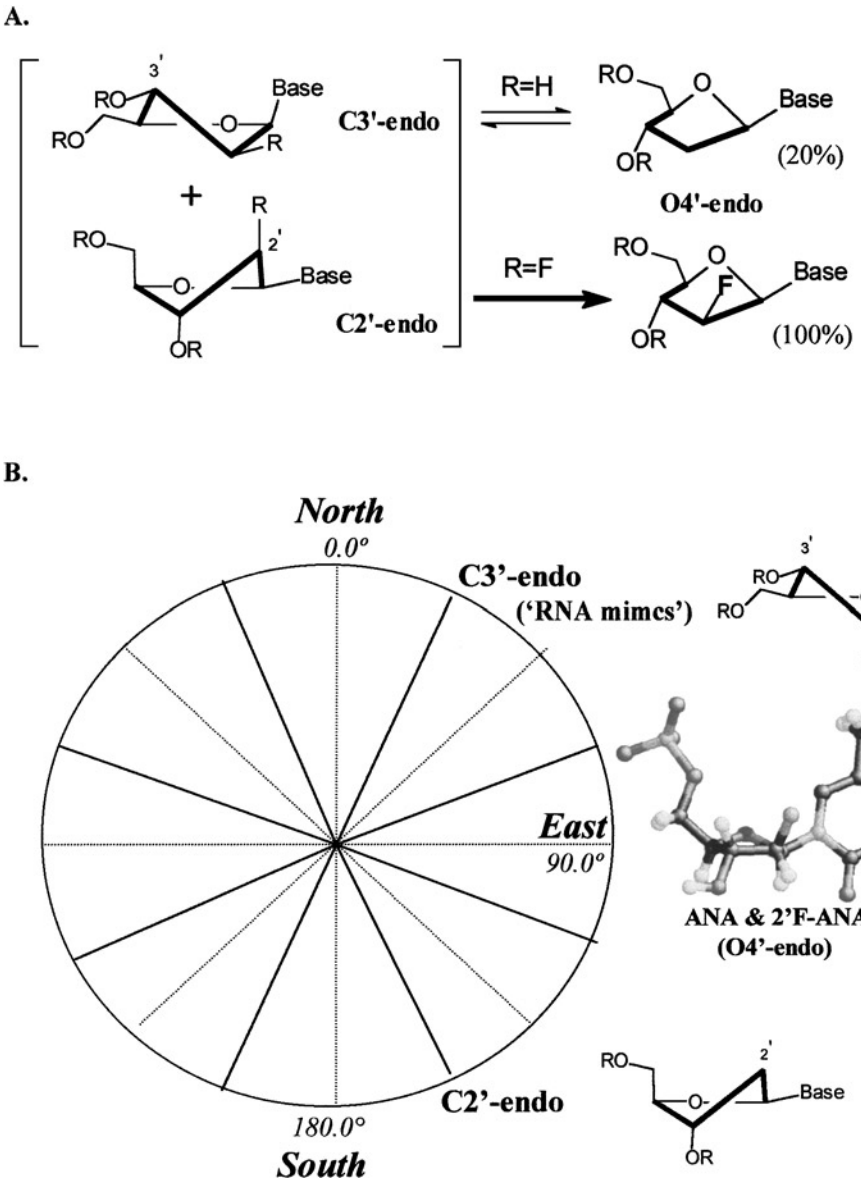

Figure 4. **A**. Conformational equilibrium of DNA and 2′F-ANA strands in DNA/RNA and 2′F-ANA/RNA hybrids. DNA sugars are 'flexible' existing as mixtures of various pucker conformations, whereas 2′F-ANA residues are rigid and adopt almost exclusively the O4′-endo pucker form. **B**. Schematic of the pseudorotation phase angle (P) cycle with the positions of selected pucker types indicated. The P angles of 2′F-ANA and ANA (O4′-endo) are compared with the P angles of C2′-endo and C3′-endo pucker conformations. In the O4′-endo conformation, the β 2′-F (or OH) of arabinose is pseudoaxial and gauche to the ring oxygen O4′ (gauche effect).

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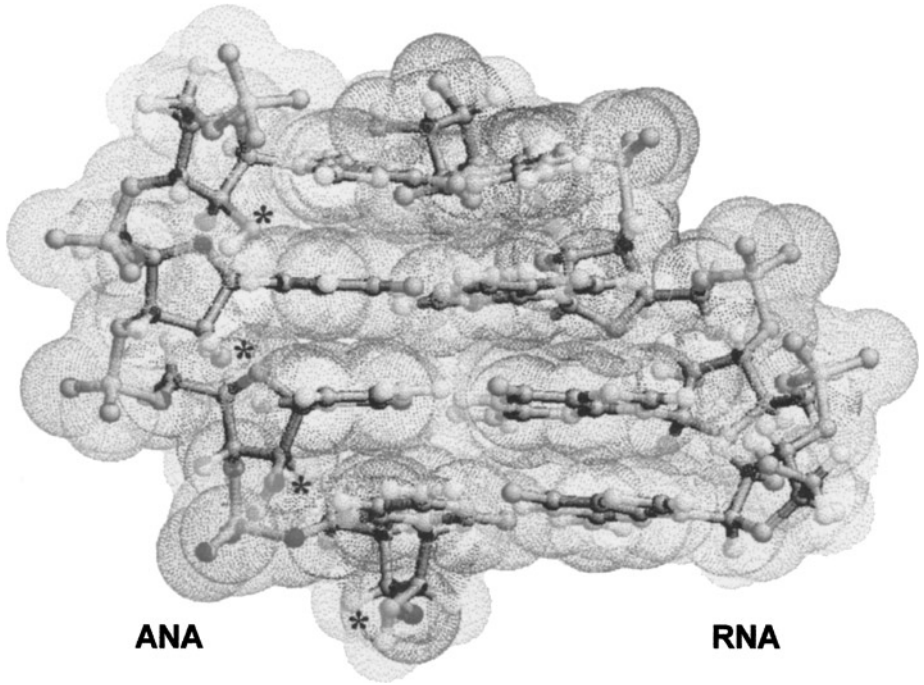

Figure 5. Solution structure of the oligonucleotide hairpin r(GGAC)d(TTCG)a(GUCC) determined by NMR and restrained molecular dynamic calculations. For clarity, the loop residues are not shown, and the positions of the ara 2′-OH groups are indicated by an asterisk.

of the helix axis, and the twist. The axis displacement and the helical twist angles are between those observed for A- and B-DNA. The base-pair inclination also show intermediate values. In the ANA/RNA and 2′F-ANA/RNA hybrids, the width of the minor groove is ∼9 Å, which is narrower than the standard A-DNA (or A-RNA), and more similar to that of the native DNA/RNA hybrid (8–10 Å range) (19,41). We also note that this is much wider than in the B-DNA helix, where the minor groove is ∼6 Å (Table 2).

Table 2. Helical Parameters for Hybrid Duplexes[a] and Comparison to Parameters Calculated for Canonical Forms

Duplex	Minor Groove Width (Å)	X-Displacement (Å)	Inclination (°)	Rise (Å)	Twist (°)
DNA/RNA	9.2 ± 0.2	−3.3 ± 0.5	10.5 ± 1.5	2.7 ± 0.2	35.0 ± 4.2
F-ANA/RNA	9.3 ± 0.2	−3.0 ± 0.3	6.1 ± 3.5	2.7 ± 0.2	35.5 ± 2.6
ANA/RNA	9.0 ± 0.2	−2.5 ± 0.4	5.5 ± 2.5	2.8 ± 0.2	33.7 ± 4.5
A-form DNA	11.1	−5.4	19.3	2.6	32.7
B-form DNA	5.9	−0.7	−6.0	3.4	36.0

[a]Derived from NMR structures of stem residues in hairpin sequences given in Figure 3. All parameters were calculated using the program CURVES.

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The precise conformation of the DNA strand in DNA/RNA hybrids is somewhat controversial. An eastern sugar geometry (O4′-endo) (18,19) as well as a dynamic equilibrium between C2′-endo and C3′-endo forms have been proposed (40–42). In our case for hybrid DNA/RNA (Fig. 3), the combination of the measured coupling constants and NOE information was consistent with a mixture of C2′/C3′-endo (80%) and O4′-endo (20%) conformations (38). The calculated distance H1′−H4′ for deoxyriboses based on NOE data is 2.8 Å, which represents an average value that results from the exchange of these various sugar conformations. Despite differences in interpretations, all of the above studies yielded DNA/RNA hybrid structures with very similar helical parameters. Notably, the minor groove width is slightly narrower in hybrid duplexes than in duplex RNA, a feature that is shared with ANA/RNA and 2′F-ANA/RNA duplexes (Table 2).

It is worth noting the conformational changes imposed by the "up" fluorine atom. In its absence, 2′-deoxyribose is 'flexible' and exists in a dynamic equilibrium between various conformers, *e.g.* C2′ + C3′ + O4′ endo puckers (38). Upon incorporation of the "up" fluorine atom, the sugar becomes 'rigid' as a result of a strong gauche effect between the 2′F atom and the ring oxygen, and the equilibrium now lies towards the O4′-endo conformation (∼100%) (see Fig. 4A). Because ANA/RNA and 2′F-ANA/RNA hybrids are substrates of RNase H, and arabinonucleic acids do not seem to adopt either the C2′- or C3′-endo geometries when complexed to RNA, Egli (43) and our groups (36) concluded that the sugar pucker of the 'flexible' deoxyribose in DNA/RNA hybrids that is processed by RNase H fall within the O4′-endo range (Fig. 4).

A provocative result from our studies regards the potential use of a fluorine atom (at various positions of the sugar ring) as a "probe" to shift the conformation of North- or South-puckered AON towards the intermediate Eastern sugar geometry (P 75–115°) (e.g., see Fig. 4A). Such fluorinated AON, when paired with the A-type RNA strand, would in principle yield hybrids that adopt the A/B-geometry required for recognition and cleavage of RNase H.

In conclusion, the unique properties of ANA (and analogues) described here are an attribute that highlight their potential for the study of nucleic acid structure and biology. Because of the critical role of the 2′-OH group in RNA, arabinonucleic acids can be used to provide insight into RNA structure and function. Arabinose can also be used to endow nucleic acids with tailored properties such as to increase the stability of nucleic-acid based therapeutics, and importantly, to promote degradation of cellular RNA via RNase H activation. Applications of arabinonucleic acid analogues against cellular mRNA targets are in progress.

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