RNA Interference

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Unique Gene-Silencing and Structural Properties of 2′-Fluoro-Modified siRNAs**

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Dedicated to Professor Fritz Eckstein and Dr. P. Dan Cook

Although fluorine is not a naturally occurring component of genetic material, fluorine-containing drugs have many medical applications.[1] Fluorine is highly electronegative, and 2′-deoxy-2′-fluoro (2′-F) analogues of nucleosides adopt C3′-*endo* conformations characteristic of the sugars in RNA helices. The 2′-F substitution was first introduced in ribozymes[2] and then evaluated in 1993 in the context of antisense oligonucleotides.[3] Macugen (pegaptanib), an oligonucleotide therapeutic agent substituted with 2′-F pyrimidines, has been approved by the U.S. Food and Drug Administration. It is an RNA aptamer that selectively antagonizes vascular endothelial growth factor (VEGF) and is used clinically in the treatment of exudative (wet) age-related macular degeneration.

RNA interference (RNAi) is a mechanism for the regulation of gene expression that operates in organisms ranging from plants to flies to humans. Synthetic duplexes called short interfering RNAs (siRNAs) can enter the RNAi pathway and silence expression of virtually any gene. As part of a protein complex called RISC, siRNA recognizes and cleaves mRNA strands complementary to the "antisense" or "guide" strand of the siRNA. The mRNA is cleaved between the nucleotides paired to bases 10 and 11 of the siRNA guide strand.[4] With molecular weights of around 13 kDa and with about 40 negatively charged phosphates, siRNAs are not typical "druglike" molecules. Native oligonucleotides are rapidly degraded in serum and are not readily taken up by cells. Chemical modification can impart "druglike" properties to siRNA,[5] but these modifications may also inhibit various steps in the process that leads to gene silencing.[6]

In cell culture, siRNAs substituted with 2′-F have activity similar or superior to that of unsubstituted controls independent of the position or strand, which indicates that the modification is well-tolerated by RISC.[7–9] Although siRNAs modified with 2′-F clearly have greater stability toward nucleases and a prolonged half-life in human plasma relative to that of 2′-OH-containing siRNAs,[7,10,11] and although they are less immunostimulatory than unmodified siRNA,[12,13] conflicting results have been obtained when these modified siRNAs were evaluated in vivo. The increase in stability did not translate into an enhanced or prolonged decrease in target-gene expression in mice in two separate studies.[10,14] In contrast, in a study in which 2′-F-modified siRNA and the target hepatitis B virus (HBV) vector were coinjected in vivo, the modified siRNA was significantly more potent than unmodified siRNA.[11] To resolve these discrepancies, we set out to evaluate the properties of the 2′-F-modified siRNA in vitro and in vivo in an established model system that targets the endogenously expressed coagulation factor VII (FVII).

The siRNAs A and B were designed to target factor VII (FVII) mRNA; this siRNA sequence was previously shown to effectively inhibit factor VII expression in rodent and primate animal models.[15,16] Factor VII is a blood-clotting factor and is ideal for evaluating the efficacy of siRNAs, as it is a secreted protein readily measured in serum. Furthermore, the protein has a short half-life, so mRNA silencing can be measured at the protein level with minimal lag. An siRNA duplex with 2′-F at all pyrimidine residues (Table 1, siRNA B) was synthesized by previously reported methods (see the Supporting Information). The 2′-F modification imparted significant thermal stability to the siRNA duplex. The melting temperature (T_m) of siRNA B was almost 15 °C higher than that of

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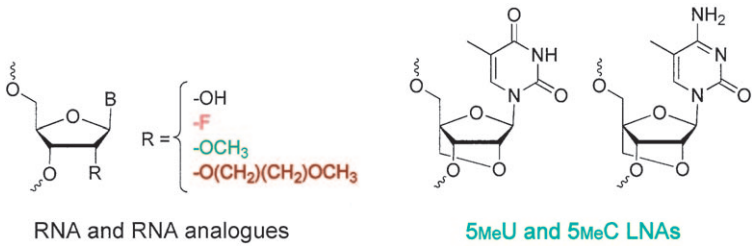
Table 1: SiRNA sequences and chemical modifications used in the in vivo FVII silencing study.[a]

SiRNA	Sense strand (5′–3′) antisense strand (5′–3′)	Modification	T_m [°C]
A	GGAUCAUCUCAAGUCUUACdTdT GUAAGACUUGAGAUGAUCCdTdT	unmodified	71.8
B	GGAUCAUCUCAAGUCUUACdTsdT GUAAGACUUGAGAUGAUCCdTsdT	2′-F pyrimidines	86.2
C	GGAUCAUCUCAAGUCUUACdTsdT GUAAGACUUGAGAUGAUCCdTsdT	2′-*O*-Me pyrimidines	80.0
D	GGAUCAUCUCAAGUCUUACdTsdT GUAAGACUUGAGAUGAUCCdTsdT	2′-*O*-MOE pyrimidines	87.1
E	GGAUCAUCUCAAGUCUUACdTsdT GUAAGACUUGAGAUGAUCCdTsdT	5MeC-LNA 5MeU-LNA	>100.0
F	GGAUCAUCUCAAGUCUUACdTsdT GUAAGACUUGAGAUGAUCCdTsdT	Mixed 2′-*O*-Me/2′-F	83.0
G	GGAUCAUCUCAAGUCUUACdTsdT GUAAGACUUGAGAUGAUCCdTsdT	Mixed 2′-*O*-MOE/2′-F	91.0
H	GGAUCAUCUCAAGUCUUACdTsdT GUAAGACUUGAGAUGAUCCdTsdT	Mixed LNA/2′-F	≈94.0

[a] The modifications in each strand are indicated by color coding corresponding to that in the column "Modification".



the unmodified duplex (Table 1). SiRNA B was significantly more stable when incubated in serum than unmodified siRNA A (see Figure S1 in the Supporting Information). The unmodified siRNA was completely degraded within 4 h, whereas the 2′-F-modified siRNA had a half-life greater than 24 h. The 2′-F-modified siRNA was not immunostimulatory in an assay in human peripheral blood mononuclear cells: the unmodified siRNA stimulated the production of IFNα and TNFα; the 2′-F-substituted siRNA did not (see Figure S2 in the Supporting Information).

When siRNAs were transfected into HeLa cells that stably express mouse FVII by using Lipofectamine 2000, siRNA B proved roughly twice as potent as the unmodified control: the IC_{50} value for siRNA B was 0.50 nM, and that of siRNA A was 0.95 nM (data not shown). To evaluate the activity of the 2′-F-modified siRNA in vivo, siRNAs were formulated with LNP01, a liver-specific liposome formulation previously described.[16] Silencing was evaluated in mice given a single intravenous injection of formulated siRNA. Again, siRNA B was approximately twice as potent as siRNA A (Figure 1a).

Many examples exist of potent siRNAs containing several types of chemical modifications.[13,17] In some cases, the stability toward nuclease degradation or the potency of these siRNAs with several modifications were higher than for siRNAs with the same sequence with individual modifications.[11,18] Individual siRNA strands were synthesized with 2'-F, 2'-*O*-methyl (2'-*O*-Me), 2'-*O*-methoxyethyl (2'-*O*-MOE), or locked nucleic acid (LNA) modifications at all pyrimidine residues, as shown in Table 1. Even with just pyrimidine residues modified in the LNA siRNA, the duplex had a T_m value above 100°C (Table 1; sequence E). Not all pyrimidine residues were modified in the LNA siRNA, as even when just uridine residues were modified, the duplex had a T_m value above 100°C (Table 1). As strand separation is required for RISC activity, exceptionally high duplex stability may be detrimental to siRNA activity. The siRNAs shown in Table 1 were evaluated in the mouse model for silencing of FVII gene. Mice were given a single intravenous injection of LNP01-formulated siRNA (3 mg kg^{-1}) or phosphate-buffered saline (PBS). SiRNA B with 2′-F-modified pyrimidine residues on both the sense and the antisense strand was the most active (Figure 1b). Neither the 2′-*O*-Me nor the LNA modification were tolerated on the antisense strand; the 2′-*O*-MOE modification was not tolerated on either strand. The results of an in vitro dose-response evaluation of the 2′-F- and 2′-*O*-Me-substituted duplexes are shown in Figure 2.

To shed light on the increased siRNA activity in vitro and in vivo of 2′-F-modified RNA relative to unmodified RNA and other 2′ modifications, we first carried out detailed calorimetric and UV melting experiments with the octamers r(CGAAUUCG) and f(CGAAUUCG) (Table 2). These experiments revealed, surprisingly, that the higher stability of 2′-F-modified RNA was primarily due to favorable enthalpy. Therefore, the common assumption that conformational preorganization for interaction with the target strand and a resultant entropic benefit is the main cause of the superior RNA affinity of the 2′-F modification may not be correct. Given the reputation of fluorine as a poor hydrogen-bond acceptor,[19] it was particularly unexpected that the entropic contributions to the pairing stability of RNA and 2′-F-RNA were virtually the same.

The 2′-hydroxy groups on RNA stabilize an intricate water structure in the minor groove, as visualized first in the crystal structure of the duplex $[r(CCCCGGGG)]_2$ (Figure 3a).[20] In principle, one would expect this feature to be

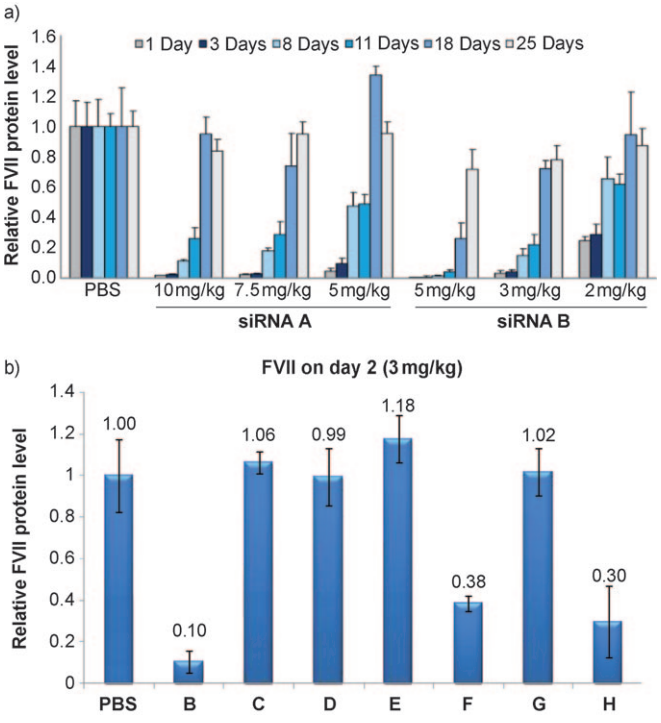


Figure 1. In vivo activity of 2′-F-modified siRNA and comparison with native RNA and other modifications. a) 2′-F-modified siRNA was approximately twice as potent as the unmodified siRNA in vivo. Mice ($n=5$) received a single intravenous dose of LNP01-formulated siRNA A or siRNA B or PBS. FVII protein levels were measured from serum collected at the indicated time points postadministration by using a chromogenic assay (Coaset Factor VII, DiaPharma Group or Biophen FVII, Aniara Corp.). b) In vivo FVII gene silencing as a function of the siRNA sequences and chemical modifications described in Table 1.

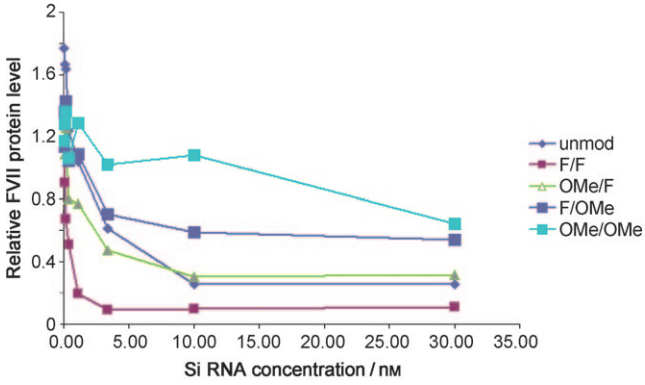


Figure 2. In vitro activity of 2′-F- and 2′-OMe-modified and unmodified siRNAs in the FVII assay.

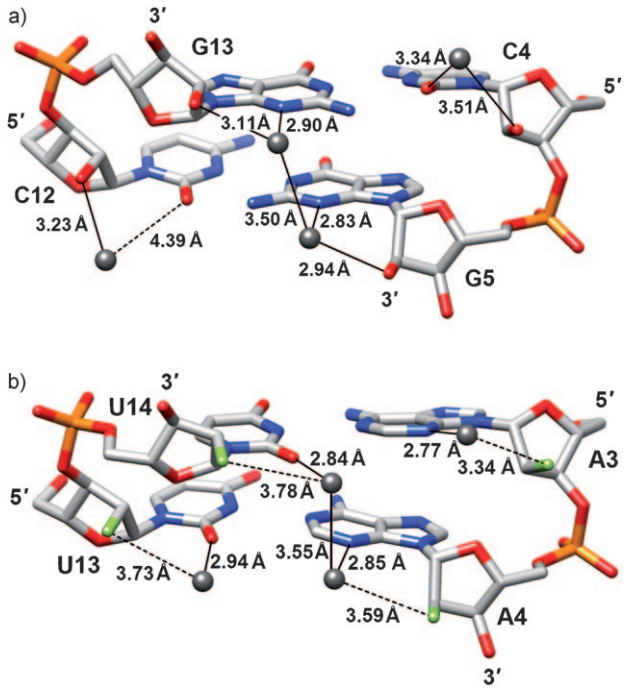


Figure 3. Differences in the minor-groove hydration of a) RNA and b) 2′-F-RNA illustrated by the water structure at individual base-pair steps in the crystal structures of r(CCCCGGGG)]$_2$[20] and [f(CGAAUUCG)]$_2$ (this study). The 2′-F atoms are colored green, water molecules are gray spheres, and hydrogen bonds are thin solid lines.

associated with an entropic penalty. The 2′-*O*-Me,[21] 2′-*O*-MOE,[22] and LNA[23] modifications also affect hydration favorably. To analyze the effects of the 2′-F modification on the minor-groove water structure, we determined the crystal structure of [f(CGAAUUCG)]$_2$, the first for any 2′-F-modified nucleic acid, at a resolution of 1.2 Å (PDB code: 3P4A). The structure revealed that the fluorine atom at the 2′-position of the sugar moiety does not participate in hydrogen bonding to water molecules associated with either the minor-groove base edges or phosphates (Figure 3b). Thus, fluorine was unable to act as a bridgehead in the stabilization of water bridges across the minor groove.

The structural data were consistent with the results of osmotic stressing experiments,[24] which indicated poor hydration of the 2′-F-RNA duplex (Table 2). However, considerable amounts of water were released upon melting of the duplex [r(CGAAUUCG)]$_2$, in line with the wet minor groove

Table 2: Thermodynamic stability and osmotic-stress[a] analysis (Δn_w) for RNA and 2′-F-RNA octamers.

Sequence (r=RNA, f=2′-F-RNA)	T_m [°C] (UV melting)	ΔH [kcal mol^{-1}] (DSC)	ΔH [kcal mol^{-1}] (UV conc.-depend.)	ΔS [eu]	Δn_w (ethylene glycol)	Δn_w (glycerol)	Δn_w (acetamide)
5′-r(CGAAUUCG)-3′	34.1 ± 0.6	39.2 ± 1.4	58.0 ± 9.4	189.7 ± 30.6	18.8 ± 4.9	22.5 ± 6.1	37.9 ± 5.8
5′-f(CGAAUUCG)-3′	53.3 ± 0.3	53.5 ± 0.7	62.3 ± 9.8	192.1 ± 30.2	1.2 ± 3.8	3.0 ± 6.9	14.8 ± 3.3

[a] Δn_w = number of water molecules released upon melting of the duplex (for a discussion of osmotic stress, see Ref. [24]). DSC = differential scanning calorimetry.

in the crystal structure.[20] Changes in hydration are probably not the only consequence of the 2′-F modification. It is possible that the fluorine substituent polarizes the nucleobase and thereby strengthens Watson–Crick hydrogen bonds and enhances base stacking. Such effects would reconcile the structural and osmotic-stressing data with those derived from the thermodynamic studies, and could explain the chiefly enthalpic origin of the stability gains afforded by 2′-F-RNA relative to RNA. The data presented herein provide evidence that the unique activity displayed by 2′-F-modified siRNAs is mirrored in unique physical attributes, such as an unusually "dry" minor groove and a highly favorable enthalpic contribution to duplex formation. A more in-depth analysis of the role of the 2′ substituents in nucleic acid stability will be required to fully explain the in vivo data.

To silence gene expression, siRNAs must be loaded into RISC, the sense strand must be dissociated from the complex, and finally, the mRNA target must be recognized and cleaved. The modifications that disrupt siRNA activity may inhibit any of these steps. For example, when designing siRNAs, one walks a thermodynamic-affinity tightrope: high affinity is desirable so that target recognition is effective, but if the stability is too high, the siRNA duplex may not be dissociated by RISC. It is clear that large groups are not tolerated on the sense strand, even though duplex affinity is not compromised.[9] The relatively bulky 2′-*O*-MOE modification probably compromises binding by proteins involved in the RNAi pathway because of steric clashes (i.e., with the PIWI domain; Figure 4a). By comparison, certain unique properties of the 2′-F modification, such as the small size (Figure 4b), high electronegativity paired with hydrophobicity, and the resulting dry minor groove that precludes the need for dehydration upon binding, appear to have beneficial effects in regard to interactions with proteins.

Overall, the characteristics of the 2′-F modification make it particularly suitable for the design of highly effective siRNAs. Its small size enables position-independent incorporation into both strands, and its high electronegativity locks the sugar in the RNA-compatible C3′-*endo* conformation. Detailed calorimetric and UV melting experiments revealed that the higher thermal stability of 2′-F-modified duplexes was predominantly due to increased enthalpy rather than entropic effects. We also found that siRNAs modified with 2′-F exhibited increased nuclease stability, significantly decreased immune stimulation in an in vitro model, and, in some cases, improved in vitro and in vivo activity relative to that of the unmodified control RNA. Fully modified RNAs with purine and pyrimidine 2′-F modifications are being evaluated now, and their siRNA activities will be reported in due course.

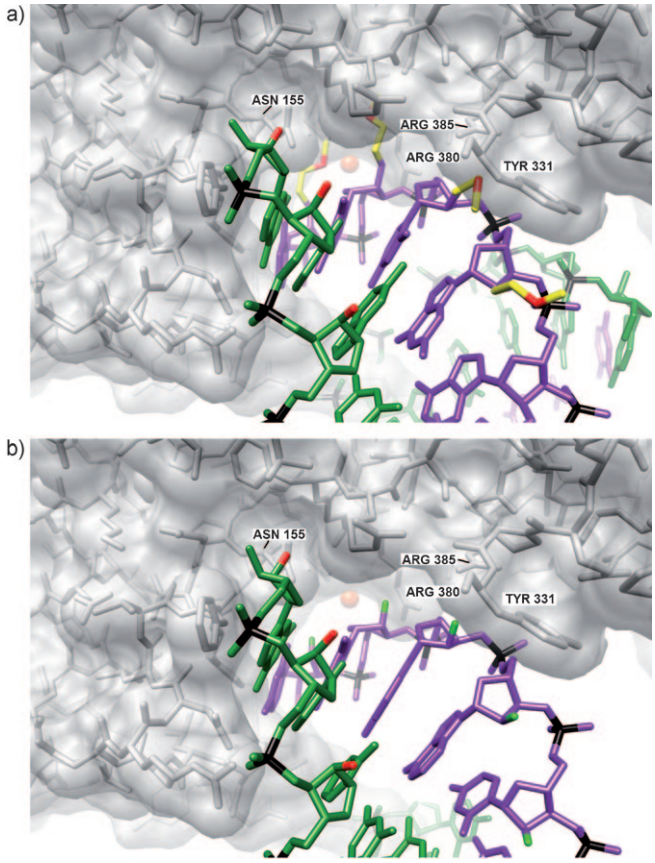


Figure 4. Interactions between antisense RNA (magenta):mRNA (green) duplexes and Piwi protein. The models of the Piwi complexes with a) 2′-*O*-MOE-RNA:RNA and b) 2′-F-RNA:RNA are based on the crystal structure of the protein from *Archaeoglobus fulgidus* (AfPiwi) bound to an siRNA-like duplex that mimics the 5′ end of a guide RNA strand bound to an overhanging target mRNA (PDB ID 2bgg).[25] The models were built by superimposing crystal structures of either 2′-*O*-MOE-RNA[22] or 2′-F-RNA (this study) onto the RNA duplex in the experimental crystal structure. The carbon atoms of 2′-*O*-MOE substituents are yellow, 2′-F atoms are green, 2′-oxygen atoms are red, and selected Piwi residues are labeled. A metal ion that anchors the 5′ nucleotide of the guide RNA is depicted as a red sphere. There are clashes between 2′-*O*-MOE substituents and protein atoms.

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