2′-Deoxy-2′-fluoro-β-D-arabinonucleic acid (2′F-ANA) modified oligonucleotides (ON) effect highly efficient, and persistent, gene silencing

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ABSTRACT

To be effective *in vivo*, antisense oligonucleotides (AS ON) should be nuclease resistant, form stable ON/RNA duplexes and support ribonuclease H mediated heteroduplex cleavage, all with negligible non-specific effects on cell function. We report herein that AS ONs containing a 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (2′F-ANA) sugar modification not only meet these criteria, but have the added advantage of maintaining high intracellular concentrations for prolonged periods of time which appears to promote longer term gene silencing. To demonstrate this, we targeted the *c-MYB* protooncogene's mRNA in human leukemia cells with fully phosphorothioated 2′F-ANA–DNA chimeras (PS-2′FANA–DNA) and compared their gene silencing efficiency with AS ON containing unmodified nucleosides (PS-DNA). When delivered by nucleofection, chemically modified ON of both types effected a >90% knockdown of *c-MYB* mRNA and protein expression, but the PS-2′F-ANA–DNA were able to accomplish this at 20% of the dose of the PS-DNA, and in contrast to the PS-AS DNA, their silencing effect was still present after 4 days after a single administration. Therefore, our data demonstrate that PS-2′F-ANA–DNA chimeras are efficient gene silencing molecules, and suggest that they could have significant therapeutic potential.

INTRODUCTION

There has been an explosive resurgence of interest in therapeutically motivated gene silencing since the discovery that RNA interference may be effected in mammalian cells with short interfering RNA (siRNA) molecules (1,2). At the same time, it has become clear that many of the issues that determine the efficiency of more traditional gene silencing molecules such as antisense oligodeoxynucleotides (AS ODN) and ribozymes will also pertain to siRNA molecules. These include appropriate mRNA target selection, effective delivery into cells expressing the target gene, proper selection of nucleotide sequence to target within the mRNA of interest and molecule stability (3–7). It has also become apparent that effectively delivered and rationally targeted AS ODN can silence gene expression as efficiently as the best siRNA molecules (8). These observations, combined with the fact that DNA molecules are generally easier and less expensive to synthesize in bulk than RNA molecules, has sustained the field of therapeutic gene targeting with antisense oligonucleotides (AS ON).

Our group has been particularly interested in the issues of therapeutic target gene selection, mRNA sequence selection for targeting (9) and with chemical modifications of ODN that yield enhanced antisense effects (10,11). Such chemical modifications are ideally thought to enhance ODN hybridization to the selected nucleotide sequence within the targeted mRNA, permit ribonuclease H (RNase H) binding to the ODN/mRNA heteroduplex so that mRNA cleavage can follow, and enhance ODN stability to exo and endonucleases so that intracellular dwell time is increased once the molecules are delivered.

A very large number of chemical modifications have been made to ODN in order to enhance their stability, and raise the melting temperature (T_m) of mRNA hybrids once formed. To a great extent, these modifications have focused on the ribose sugar and the internucleotide linkage group (2,5,7,12,13). With regard to the latter, arguably the most successful, and still widely employed, is the phosphorothioate modification originally synthesized by Eckstein (14,15). Here, one of the non-bridging oxygen atoms in the internucleoside phosphate group is replaced with a sulfur atom creating the phosphorothioate (PS) modification of ODN (PS-DNA). When compared with natural DNA, PS-DNA is moderately nuclease resistant and retains the ability to activate RNase H. However, these desirable properties are offset by the fact that the PS modification lowers the T_m of ODN/mRNA heteroduplexes, and the sulfur atom promotes non-specific binding to many intra- and extracellular proteins (16–18). Unintended, and at times undesirable, biological side effects can be observed as a result (14,19,20).

Many chemical modifications have been developed to enhance the gene silencing properties of PS-DNA molecules. A typical strategy is to flank a central core of PS-DNA with nucleotides modified to promote enhanced nuclease resistance, and to raise the T_m of resulting heteroduplexes. The most promising of these are 2′-*O*-alkyl modifications such as the 2′-*O*-methyl and 2′-*O*-methoxyethyl groups (21,22). These substitutions confer an RNA-like (*north* or C3′-*endo*) conformation to the ONs which has the beneficial effect of raising the T_m of formed heteroduplexes, but unfortunately, this enhanced stability is achieved at the expense of RNase H activation. Since RNase H activation is critical for effective antisense activity in living cells (11), this latter characteristic is highly undesirable.

A recently described 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (2′F-ANA) analog appears to overcome this important disadvantage of the 2′-*O*-alkyl modified gapmer molecules. ANA is a 2′-stereoisomer of RNA based on D-arabinose (23). When the ANA is further modified by substitution of the 2′-OH group with a fluorine atom, the 2′F-ANA is created (24) (Figure 1). The 2′F-ANA has significantly enhanced binding affinity to the target mRNA compared with native or PS modified DNA (25,26). Remarkably, and importantly, 2′F-ANA/RNA duplexes, unlike 2′F-RNA/RNA duplexes retain the ability to activate RNase H (11,26). This is because 2′F-ANA/RNA duplexes are structurally similar to normal DNA/RNA duplexes (27,28). At the same time, because the 2′F-substituent in 2′F-ANA/RNA hybrids projects into the major groove of the helix, it does not significantly interfere with the binding and subsequent catalysis of RNA by RNase H (19,29). Indeed, incorporation of DNA into the 2′F-ANA structure accelerates RNase H mediated RNA cleavage to a level that is superior to that observed with PS-DNA (11). For example, we have reported that PS-2′F-ANA–DNA chimeras consisting of a core of six DNA nucleotides flanked by 2′F-ANA wings resulted in an AS ON with high-affinity RNA binding, excellent RNase H cleavage properties and gene silencing efficacy in the low nanomolar range (<10 nM) (11). Herein we report studies which directly compare the gene silencing efficiency of two different designs of PS-2′F-ANA–DNA chimeric molecules and PS-DNA molecules. Our results indicate that the 2′F-ANA-containing ON with central, or alternating, stretches of PS-DNA had greater potency, and duration of action, than those exhibited by ON composed solely of PS-DNA.

MATERIALS AND METHODS

ODN synthesis

Unmodified phosphorothioate ODNs were synthesized by Integrated DNA Technology (IDT, Coralville). PS-2′F-ANA–DNA chimeras were synthesized in the Department of Chemistry (McGill University, Montreal, QC, Canada) using a previously reported methodology (30). The sequences of ODNs employed in this study are described in Table 1.

Cell culture and transfection

K562 cells were cultured in RPMI medium supplemented with 10% of fetal bovine serum (FBS) and 0.5% penicillin/streptomycin at 37°C in a humidified incubator (95% humidity) and 5% CO_2. Culture media was changed every second day or as required by rapidity of cell growth.
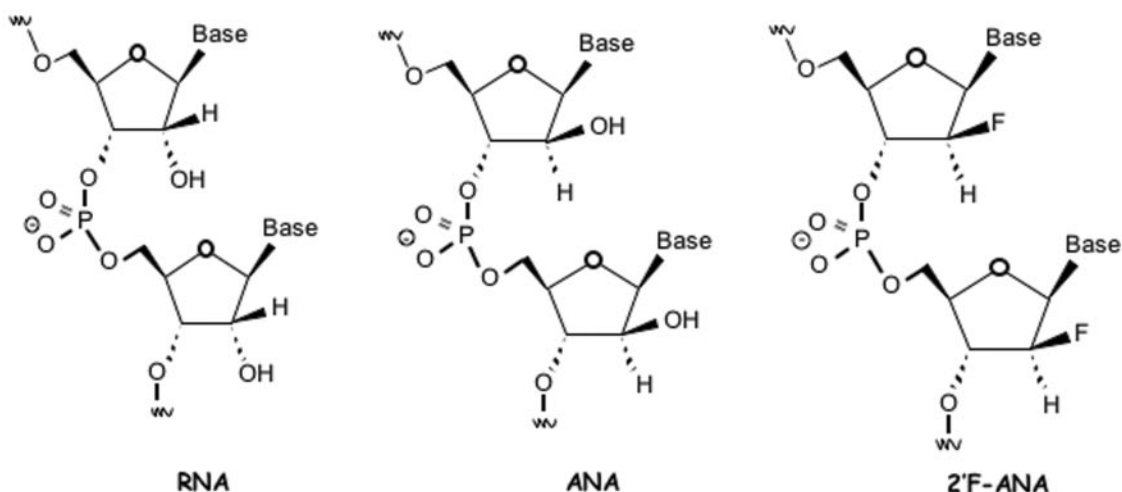

Figure 1. Chemical structure of 2′F-ANA compared with RNA and ANA.

Table 1. Sequences of ODNs employed in this study

Name	Sequence
MOH1	5′-CAAcgtTTCggaCCGtatTTC-3′
MOH2	5′-CAACGTTtcggaccGTATTTC-3′
MOH10	5′-CGTTTCGgaccgtaTTTCTGT-3′
Gapmer control	5′-ATATCCttgtcgTATCCC-3′
Altimer control	5′-ATAtccTTGtcgTATccc-3′
PS-DNA 321 as	dccaacgtttcggaccgtatt
PS-DNA control	dgaatgtgacatttcgacacg

All nucleotides linked by phosphorothioate bridges. Upper case letters, 2′F-ANA modified nucleotides. Lower case letters, unmodified nucleotides.

Human CD34$^+$ cells were cultured in IMDM medium supplemented with 2% FBS and 0.5% penicillin/streptomycin.

Cells were transfected with ODN using an Amaxa nucleofector (Amaxa Inc., Gaithersburg) as reported previously (10). In brief, 3×10^6 cells were washed two times in phosphate-buffered saline (PBS), and then suspended in 100 μl of nucleofection solution supplied by the manufacturer containing 5 μg of ODN (final concentration = 7.9 μM). For dose response studies, concentrations of 1 and 0.5 μg of ODN were used as well. Immediately after nucleofection, the cell suspension was moved into 5 ml of culture medium and maintained in 6-well plates for 24, 72 and 96 h.

Quantitative real-time PCR (QRT-PCR)

Total RNA was isolated from cells harvested after 24, 72 and 96 h using an RNeasy mini Kit (Qiagen Scientifics, MD) according to the manufacturer's instructions. Reverse transcription was carried out with an Iscript reverse transcription kit (BioRad, Hercules, CA). The resulting cDNA was used as a template for QRT-PCR, which was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and iCycler iQ Real-Time PCR Detection System (BioRad).

For *c-MYB* PCR the following primers were used: forward, dGAAGGTCGAACAGGAAGGTTATCT; and reverse, dGTAACGCTACAGGGTATGGAACA.

The *c-MYB* probe was labeled with reporter dye: 6-carboxyfluorescein (FAM) at the 5′ end and Black Hole quencher at 3′ end. The probe sequence was dTCAAAAG-CCAGCCAGCCAGCAGTG.

The *GAPDH* was employed as a reporter gene for QRT-PCR. The product was obtained with the following primers: forward, dGACAGTCAGCCGCATCTTCTT; and reverse, dCCAATACGACCAAATCCGTTGAC.

The *GAPDH* probe was labeled with reporter dye: 6-carboxyfluorescein (FAM) at 5′ end and Black Hole quencher at 3′ end.

The probe sequence was dCGTCGCCAGCCGAGCCA-CATCG.

All reactions were performed in triplicates with 1 μl of cDNA. The volume of reaction mixture was 15 μl. The reaction mixture was pre-incubated at 50°C for 2 min. PCR cycling conditions were as follows: denaturation 95°C for 10 min, followed by 39 cycles 92°C for 15 s, 60°C for 45 s.

Analysis of QRT-PCR data was based on comparison of the target transcript PCR signal in a treatment group to signal measured in an untreated control. Analysis was done using the $2^{-\Delta\Delta CT}$ method as described by Levak and Schmittgen (31).

Western blotting

Controls (untreated cells and Mock nucleofected cells, i.e. in the absence of ODN) and cells nucleofected with ODN were harvested, washed twice in PBS and then pelleted. The pellet was dissolved in triple-lysis buffer composed of 50 mM Tris, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS and 1% Igepal (Sigma, St Louis), and then incubated for 30 min on ice with vortexing every 10 min. The lysate was then spun at maximum speed in a microfuge at 4°C for 15 min. The extracted cell supernatant was used for western blot analysis.

Protein concentration was determined by Bradford protein assay (BioRad). A total amount of 100 μg protein extract was resolved on 10% polyacrylamide gel (150 V, 60 min) then transferred on to PVDF membrane (25 V, 60 min). Condensed milk (5%) was used as blocking solution. The membrane was incubated overnight at 4°C with primary mouse antibody against *c-MYB* clone 1-1 (Upstate, Lake Placid) at 1:1000 dilution. After incubation the membrane was washed three times in TBS-T buffer and probed with secondary, HRP conjugated anti-mouse antibody (Amersham Biosciences, Piscataway) at dilution 1:1000 for 1 h at room temperature.

Blots were developed using enhanced chemiluminescence western blotting detection kit ECL+ (Amersham Biosciences). Membrane was then stripped with Re-blot stripping solution (Chemicon, Temecula) and incubated with antibody against β-actin clone AC –15 (Sigma) in dilution 1:3000.

Slot blotting

Control cells, and cells transfected with 5 μg of PS-DNA, or PS-2′F-ANA–DNA antisense were harvested at 5 min, and 24, 72 and 96 h post nucleofection, washed twice with PBS and pelleted. Cell pellets were then lysed in 200 μl of lysis buffer (50 mM HEPES, pH 7.5; 85 mM KCl and 0.5% IGEPAL) for 20 min on ice. After lysis, cells were homogenized using a 1 ml dounce homogenizer (cells were dounce 50 times), and centrifuged for 10 min at 5000 r.p.m. ($6000 \times g$). The supernatant was removed, and put into a clean tube. The cell pellet was resuspended in 120 μl of DEPC water. Both, supernatant and pellet were used for slot blotting.

Samples were mixed with NaOH (0.4 M final concentration) and EDTA (10 mM final concentration) to denature DNA, and heated for 10 min at 100°C. The samples were then applied to a pre-hydrated Zeta-Probe membrane (BioRad) using a Bio-Dot SF Microfiltration Apparatus (BioRad). After sample filtration, 500 μl of 0.4 M NaOH was added to each sample-well and aspirated through, until the wells were empty. The blotted membrane was then removed from the apparatus and air-dried. The DNA in the filtrate was UV cross-linked to the membrane. The membrane was pre-hybridized by incubation with hybridization solution (1 mM EDTA, 7% SDS, 0.5 M NaHPO$_4$, pH 7.2) for 30 min at 65°C. After the pre-hybridization, radiolabeled probe was added, and the membrane was incubated at 65°C for 12 h. Next, the membrane was washed twice with buffer containing 0.2% SSC and 0.2% SDS. It was then autoradiographed for 6 h at −80°C.

A c-*MYB* sense probe, complementary to the sequence transfected into the cells, was radiolabeled with T4 polynucleotide kinase (New England Bio Labs, Beverly) and P-32 gamma ATP 0.075 mCi (Amersham Biosciences). To purify the probe, Quick Spin (TE) Columns G-25 sephadex (Roche Diagnostics, Indianapolis) were used.

RESULTS

Gene silencing effect of PS-2′F-ANA–DNA chimeras

To evaluate the ability of PS-2′F-ANA–DNA chimeras to silence gene expression we employed a previously described, well-established model system in which the human *c-MYB* gene's mRNA is targeted in K562 leukemia cells (10). Cells of this line express c-Myb protein at high levels, and their proliferation is *c-MYB* dependent. The 2′F-ANA modified sequences were directed to a region of the *c-MYB* mRNA that was identified previously as being accessible for hybridization (9). Efficiency of silencing was compared with a PS-DNA that had been shown previously to silence *c-MYB* expression with high efficiency (10). Three PS-2′F-ANA–DNA chimeras, namely, MOH1, MOH2 and

MOH10 were evaluated. The sequences of these compounds are shown in Table 1. MOH1 and MOH2 were targeted to the identical mRNA sequence, while MOH10 was targeted to the same region but shifted 3 bases downstream. The 2′F-ANA modifications were introduced into the 21mer AS sequences as either triplets alternating with unmodified nucleosides ('altimer,' e.g. MOH1) (19), or as 7mer 2′F-ANA modified sequences flanking a 7mer core of unmodified nucleosides ('gapmers' e.g. MOH2 and MOH10) (11). Control altimer and gapmer sequences were included in each assay.

In our first series of experiments, we directly compared the silencing efficacy of PS-2′F-ANA–DNA chimeras with natural sugar PS DNA. ON (5 μg) were transfected into K562 cells using an Amaxa device as we have reported previously (10). After 24 h, cells were harvested for extraction of mRNA and protein, which were measured using QRT-PCR and western blotting, respectively. Results were compared with untreated (Figure 2) or mock-transfected cells (Figure 3) whose levels of *c-MYB* mRNA and protein were arbitrarily set at 100%. MOH1 and MOH2 inhibited *c-MYB* mRNA expression by 90 and 84%, respectively, compared with the untreated control (Figure 2). Under these conditions, the PS-DNA appeared to be somewhat
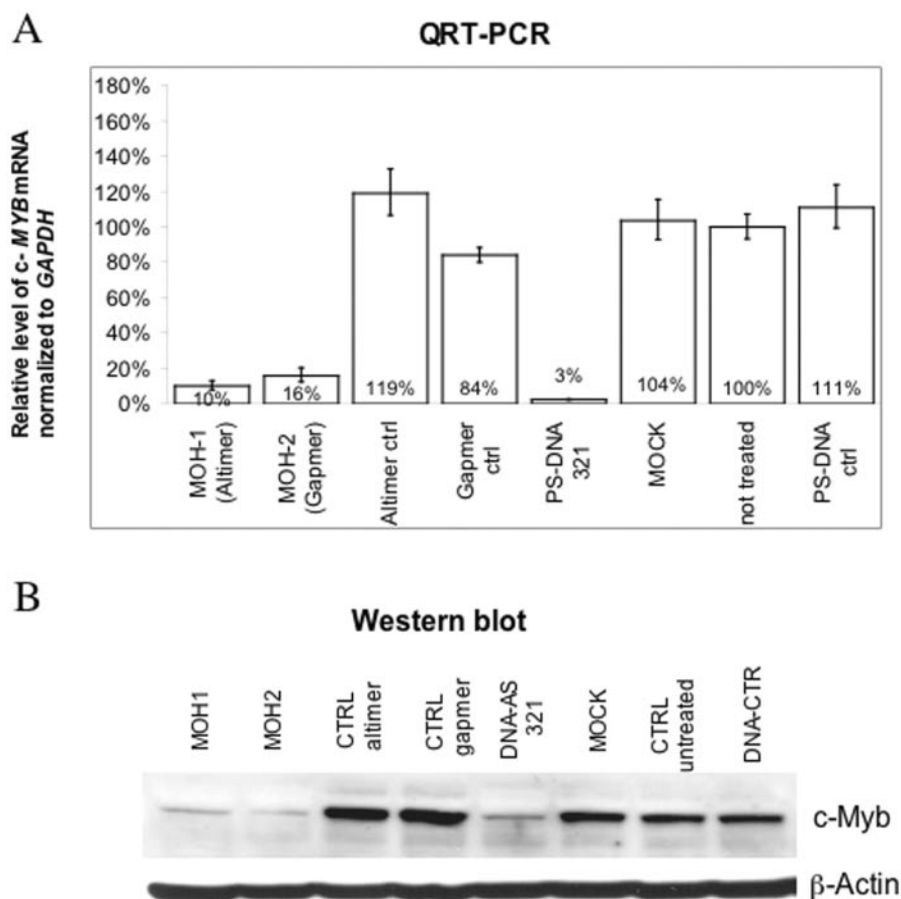

Figure 2. (**A**) Quantitative real-time PCR (QRT-PCR) assay performed on RNA isolated from K562 cells transfected with PS-2′F-ANA and PS-DNA molecules. Data are presented as a function of *c-MYB* mRNA copies relative to *GAPDH* mRNA. Mock (control) cells were subjected to nucleoporation, but in the absence of DNA. (**B**) c-Myb protein western blot analysis performed on cell lysate obtained from K562 cells transfected with PS-2′F-ANA and PS-DNA molecules. Real-time PCR and western blot were performed 24 h post nucleofection.
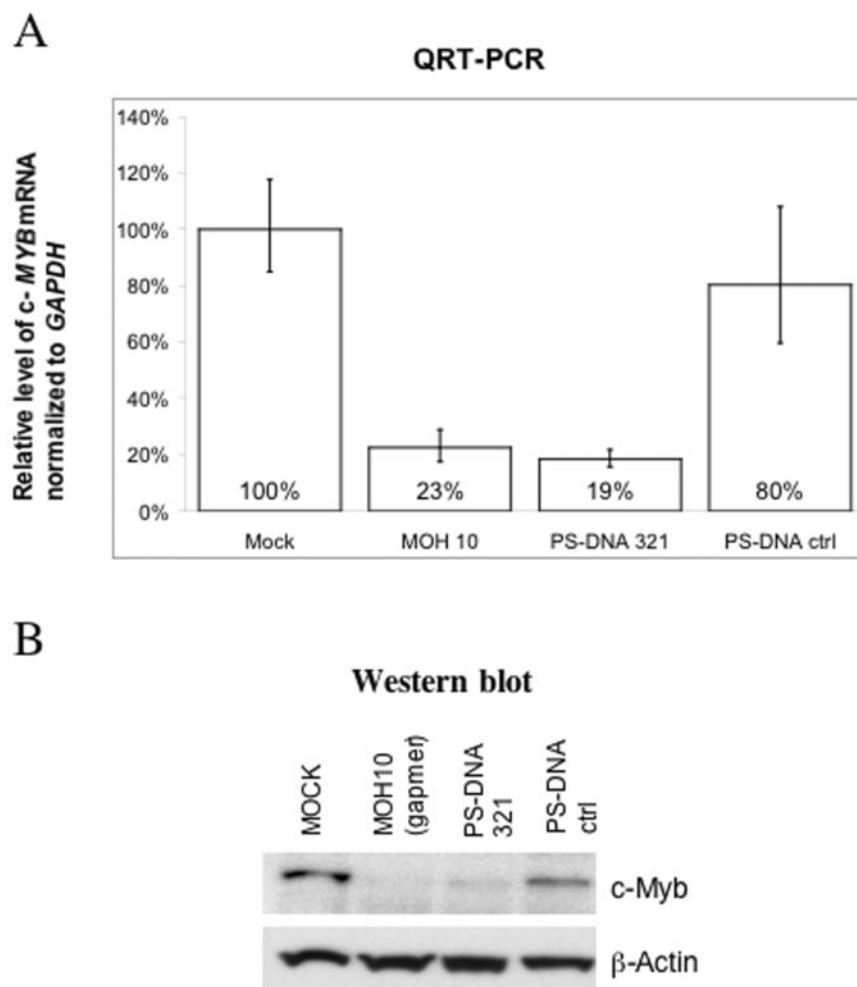
A

QRT-PCR

B

Western blot

Figure 3. (**A**) QRT-PCR assay performed on RNA isolated from K562 cells transfected with 2′F-ANA and PS-DNA molecules. Data are presented as a function of *c-MYB* mRNA copies relative to *GAPDH* RNA. Mock (control) cells were subjected to nucleoporation, but in the absence of ODN. MOH10 antisense is designed to target *c-MYB* mRNA region slightly shifted toward the 3′ end compared with the MOH1 and MOH2 sequences. (**B**) c-Myb protein western blot analysis performed on cell lysate obtained from K562 cells transfected with 2′F-ANA and PS-DNA molecules. Real-time PCR and western blot were performed 24 h post nucleofection.

more efficient inhibiting the mRNA 97% compared with the control. Neither the mock transfection procedure, nor treatment with any of the control ON resulted in inhibition >16% (Gapmer Control sequence) and all others actually had no effect at all or slightly stimulated the expression level of the target mRNA. The mRNA results were accurately reflected in the western blots (Figure 2). The MOH10 sequence also performed well in comparison with the PS DNA. Each of these compounds suppressed *c-MYB* mRNA by 77 and 81%, respectively, in this series of experiments (Figure 3). Therefore, the 2′F-ANA modification was compatible with efficient gene silencing but at the conclusion of these experiments it was unclear if these molecules would be of greater utility than unmodified PS-DNA for this purpose.

Pharmacodynamic comparison of PS-2′F-ANA chimera with PS-DNA

To address the issue of practical advantage in employing 2′F-ANA modified ON, we investigated the kinetics of knockdown and dose response of 2′F-ANA versus unmodified ON. The kinetics of silencing was investigated by analysing *c-MYB* mRNA and protein levels at 72, 96 and 120 h post transfection of 5 μg of ON (Figure 4A–C, respectively). At 72 h, MOH1, MOH2 and MOH10 treated cells had *c-MYB* levels 22, 35 and 7%, respectively, of mock-transfected cells. In marked contrast, cells treated with the AS PS-DNA expressed levels of *c-MYB* mRNA and protein equivalent to or greater than control ON treated cells. MOH1 and MOH10 continued to be highly effective at 96 h as well with ~85% 'knock down' compared with mock controls. For unclear reasons, activity of the MOH2 gapmer was lost at 96 h. However, by 120 h, all the 2′F-ANA modified ON had also lost activity.

We also examined the potency of the 2′F-ANA chimeras compared with the unmodified PS-DNA. In dose finding experiments of the type just described, we found that 1 μg of PS-2′F-ANA chimeras, or just 20% of the dose employed in the initial electroporation experiments, was still able to induce very effective silencing of the *c-MYB* mRNA target
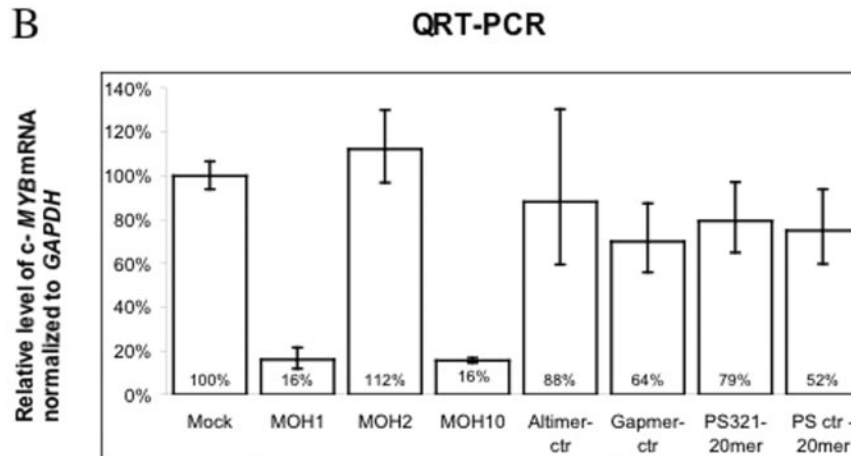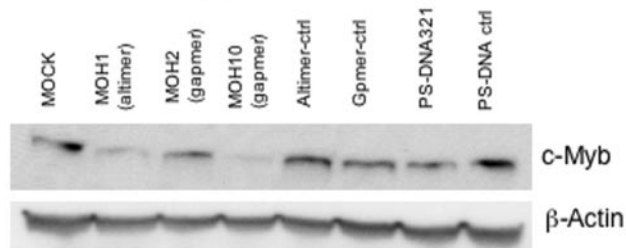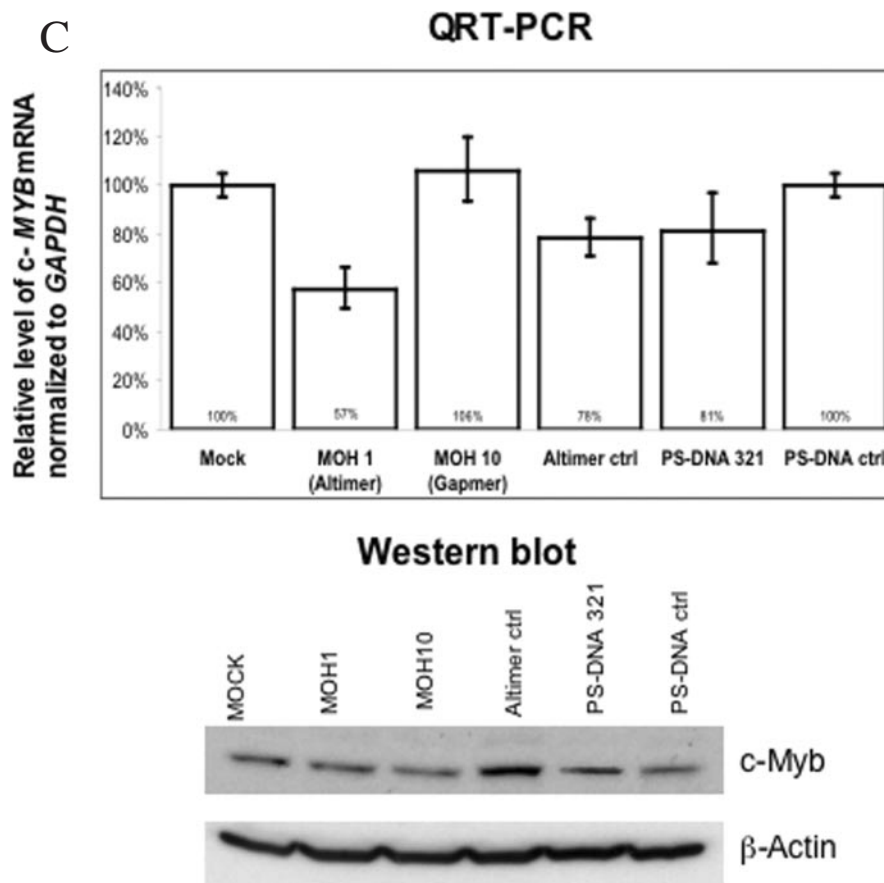
A

QRT-PCR

Western blot

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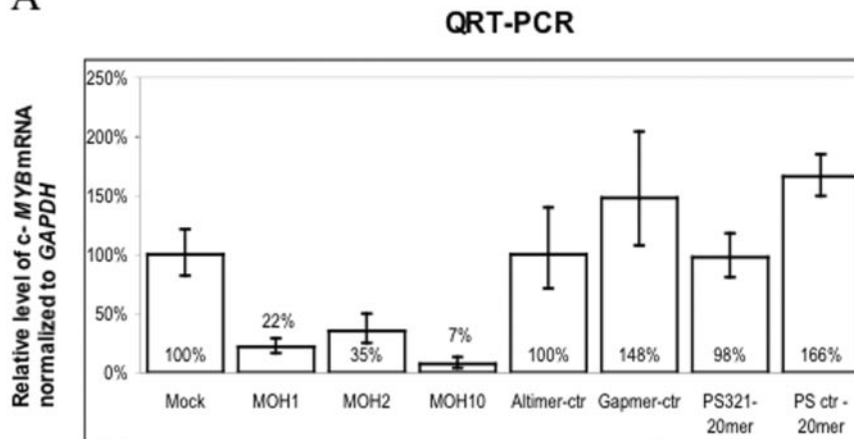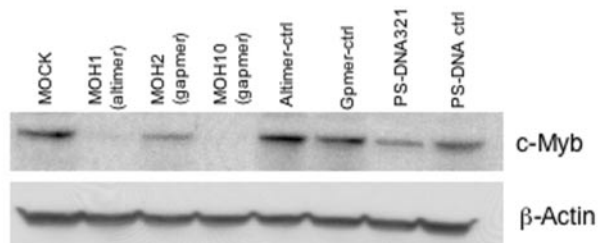
QRT-PCR

Western blot

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Figure 4. QRT-PCR and corresponding western blot analysis of *c-MYB* expression performed (**A**) 72 h (**B**) 96 h and (**C**) 120 h post nucleofection.

at both the mRNA and protein levels (∼80–90%). In distinct contrast, the PS-DNA sequence had no effect when used at this dose (Figure 5A). Additional dose finding experiments (Figure 5B) revealed a rather tight dose response relationship such that none of the molecules were effective for gene silencing when electroporated at a concentration of 0.5 µg. At a concentration of 1.5 µg MOH1 and MOH10 still gave a >70% knockdown in mRNA expression, in contrast to the PS-DNA 321 which remained ineffective. When exposed to the various modifications at a concentration of 2 µg MOH1 silenced c-*MYB* expression by 80% compared with the mock control, MOH10 by 70%. PS-DNA 321 began to finally show activity at this concentration, decreasing c-*MYB* expression by ∼60%. At 3 µg, MOH1 gave further knockdown of c-*MYB* (∼90%), while MOH10 and PS-DNA 321 effected a 70% decrease in mRNA expression. Specificity of these effects was demonstrated by the fact that knockdown observed with any of the control molecules never exceeded 18%. It is also important to stress that the observed knockdowns with the 2′F-ANA molecules could not be attributed to the 2′F-ANA monomers since the control 2′F-ANA molecules had little or no effect on c-*MYB* gene, or MYB protein expression. The duration of the silencing effect of the 1 µg dose of PS-2′F-ANA–DNA was also examined. At 72 h, the last time point examined, silencing was as robust as with the 5 µg dose (data not shown).

In aggregate, all experimental data described above strongly suggest that the 2′F-ANA modification significantly improves the effectiveness of antisense molecules when compared with

PS-DNA ONs. Nonetheless, to be certain that our findings were not cell type specific, we examined the effectiveness of the 2′F-ANA ON modification in other cell types as well. When tested in Jurkat cells, another human leukemic cell line of lymphoid, as opposed to myeloid origin, we were able to induce inhibition of *c-MYB* mRNA and protein expression with an efficacy essentially identical to that observed in K562 cells (∼80–90%; data not shown). When transfected into normal human CD34^{+} hematopoietic progenitor cells, 2′F-ANA modified ON suppressed *c-MYB* expression ∼60% compared with controls (Figure 5C). While this degree of silencing might appear to be substantially less than that observed in the cell lines, it is important to note that (i) the transfection efficiency in normal human CD34^{+} cells is only ∼60–70% compared with ∼100% observed in the cell lines, and (ii) *c-MYB* mRNA and protein are being measured on a population basis, not at the single cell level. Accordingly, it is highly likely that the results observed are 'diluted' by the mRNA and protein contribution of non-transfected cells and that the decreased efficiency of silencing is more apparent than real.

Intracellular concentration of 2′F-ANA and DNA oligonucleotides

To gain insight into the mechanism for the enhanced dose and kinetic response to PS-2′F-ANA modified ON in comparison with PS-DNA, we examined intracellular concentrations of

A

B

C
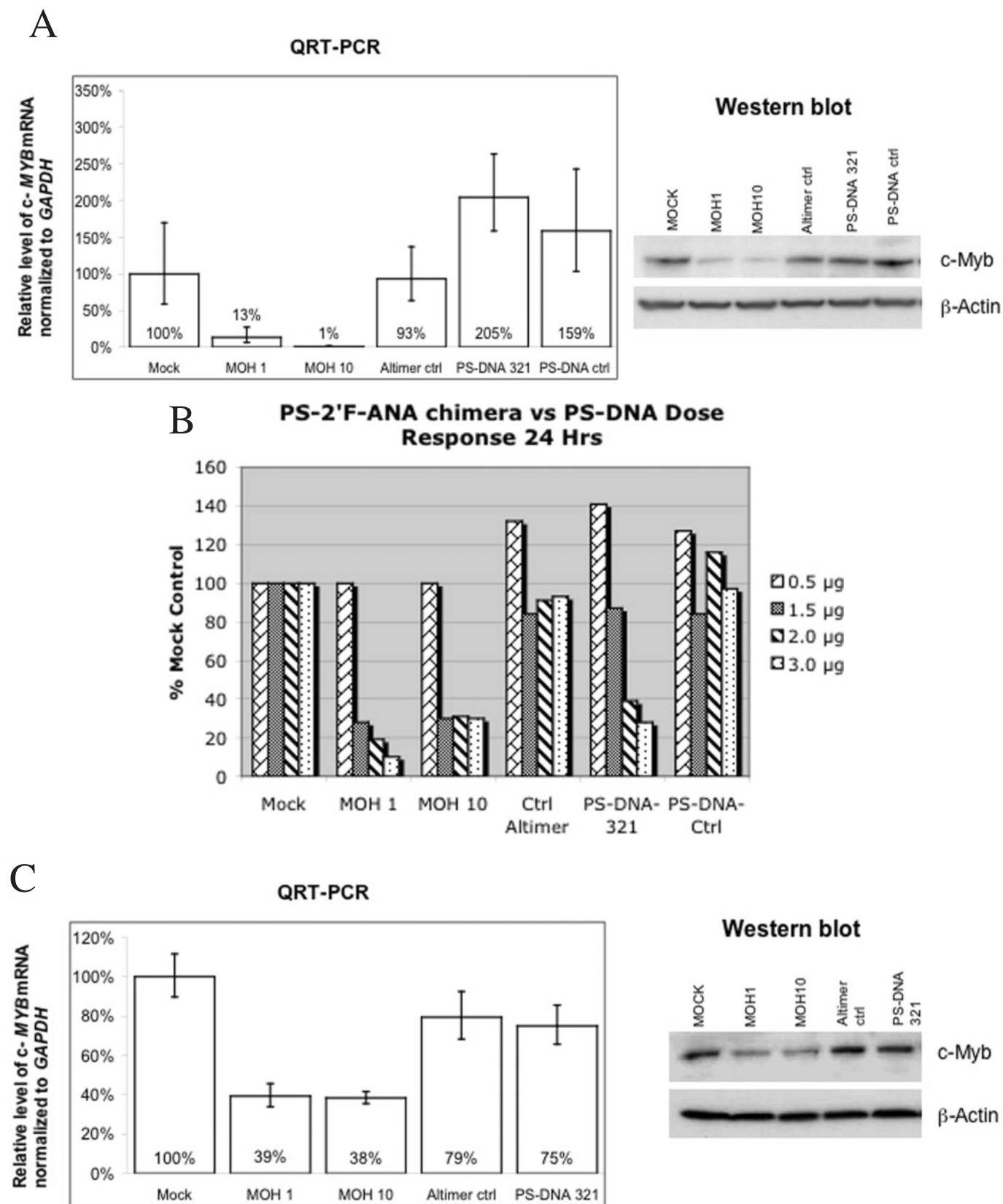
Figure 5. (**A**) QRT-PCR and western blot analysis performed 24 h post nucleofection of cells with 1 µg of indicated ON in K562 cells. (**B**) QRT-PCR of c-MYB expression in K562 cells 24 h after nucleofection of ON in amounts indicated. (**C**) *c*-MYB expression in normal human CD34^{+} marrow cells 24 h post nucleofection with 1 µg of ON. Owing to the difficulty in obtaining CD34^{+} cells for this particular experiment, scrambled control for PS-DNA not tested.
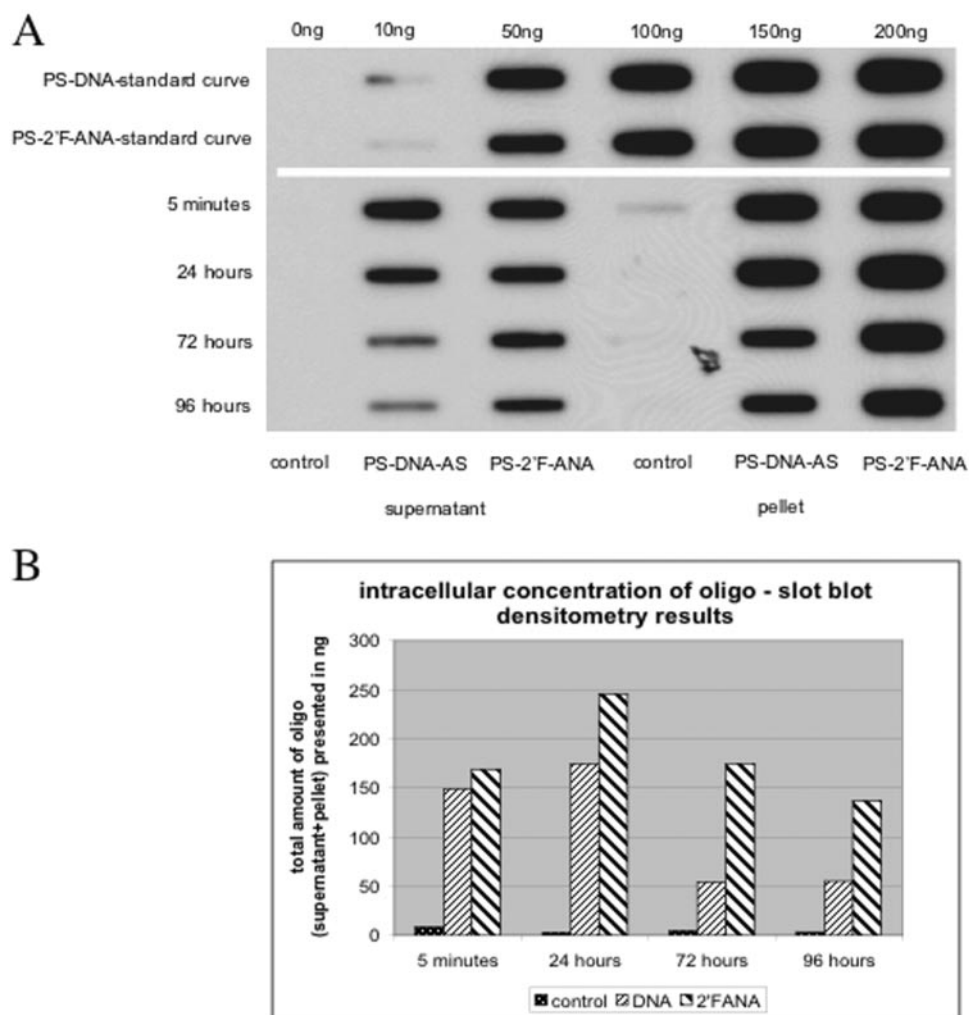
Figure 6. Quantification of ODN delivered to cells by nucleoporation. (**A**) Slot blot of ON obtained from K562 cell lysate supernatants, and residual cell pellet 5 min, as well as 24, 72 and 96 h post nucleoporation. Upper rows are PS-DNA and PS-2′F-ANA–DNA (MOH10) antisense standards applied to membrane for quantification purposes. After transfer to the membrane, the blots were probed with a radiolabeled complementary sequence. Columns show material from untreated control cells (control), and cells nucleoporated with PS-DNA and PS-2′F-ANA–DNA chimera (MOH10). (**B**) Densitometry analysis of slot blot containing ON present in cell lysates, and remaining pellets, 5 min and 24, 72 and 96 h post nucleoporation of material. Control cells were nucleoporated in the absence of exogenous ON.

both compounds 5 min after, and then at 24, 72 and 96 h post nucleofection (Figure 6). At 5 min after transfection, the intracellular concentrations of PS-DNA and PS-2′F-ANA modified oligos were almost identical ($\sim$150–170 ng). However, at the later time points, the intracellular concentration of the PS-2′F-ANA chimera was considerably higher than the corresponding PS-DNA. Even at 96 h, $\sim$70% of the starting concentration of MOH was still detectable in the cells. No intracellular PS-DNA was detectable in the cells at this time point suggesting that the PS-2′F-ANA chimera was either more stable, or less subject to elimination than the corresponding PS-DNA.

DISCUSSION

Our laboratories have had a longstanding interest in defining the structural properties of synthetic nucleic acids, and their mRNA targets, in order to develop more effective gene silencing agents (9,10). The Damha lab has also been actively engaged in defining the substrate specificities of RNase H enzymes in an effort to evolve newer, more potent generations of AS ONs (24,29,32,33). The result of these efforts has been the development of antisense arabino (ANA) (23,32) and 2′F-ANA (Figure1) (11,19,24,26–28,32). The 2′F-ANA modification displays substantial increases in thermal stability when hybridized with cognate DNA or RNA as a result of the fact that each modification increases the T_{m} up to $\sim$1.5°C/2′F-ANA nt (24,26). While this increase in T_{m} is not as high as that observed with ONs containing 2′-O,4′-C-methylene-β-D-ribofuranoside units ('locked nucleic acids'; $\Delta T_{\mathrm{m}} > +3$°C/nt) (34), our results demonstrate that it is clearly sufficient for effective down-regulation of gene expression.

While strength of hybridization is a factor of critical importance in the process of mRNA targeting and degradation, it is clear that other parameters are also very important. One of these is the ability of the AS ON to support cleavage of the mRNA to which it is bound. This in turn is dependent

on the ability of the AS ON/mRNA hybrid to allow cleavage by endogenous RNase H. During the course of our studies, we have discovered that ANA and 2′F-ANA ONs support human RNase H1 activity (11,23) because the arabinose sugar does not alter the overall helical structure of the corresponding ON/mRNA hybrid when compared with the native DNA/RNA substrate (27,28,32). This finding is of fundamental interest for several reasons. First, these ONs represented the first examples of RNase H-competent ONs that lack 2-deoxy-D-ribofuranose. Typically, chemical changes of the sugar moiety (e.g. RNA, 2′OMeRNA and LNA), alterations in the orientation of the sugar to the base, or 2′F or OH groups, completely abolish RNase H activation (27,35). Second, these findings enhance our understanding of the catalytic mechanism and substrate selectivity of RNase H by illustrating how AS ON sugar stereochemistry impacts on hybrid conformation. In this regard, it is also very important to note that RNaseH directed cleavage of the mRNA strand occurs across both the 2′F-ANA and DNA segments (11). This is because 2′F-ANA is a mimic of DNA (27,28), allowing the enzyme (which recognizes only DNA/RNA hybrids) to cleave at both 2′F-ANA/RNA and DNA/RNA segments (i.e. throughout the ON/RNA duplex). In standard 2′-O-alkyl RNA gapmers, such cleavage can only occur across the DNA gap. Of equal if not greater interest, we have also demonstrated that 2′F-ANA directed cleavage can occur when there is but one central DNA gap, while the 2′-O-alkyl RNA gapmers require a DNA gap of at least 8 nt (11). This could also be an important factor contributing to the potency of 2′F-ANA modified ONs. Third, ANA and 2′F-ANA ONs are resistant to endo and exonucleases, and this resistance is further enhanced if the FANA modified nucleosides are connected by phosphorothioate linkages. PS-2′F-ANA, for example, are >20-fold more stable than PS-DNA towards 3′-exonuclease hydrolysis (M. J. Damha and K. L. Min, unpublished data). The increased stability of hybrids formed by 2′F-ANA and target RNA as well as the ability to induce RNase H degradation appears to come from conformational pre-organization of the fluorinated sugars. X-ray crystallographic data from Fedoroff *et al*. (36) and Berger *et al*. (37) demonstrated adoption of an O4′-*endo* (*east*) sugar pucker, a conformation that lies between the C2′-*endo* (*south*) and C3′-*endo* (*north*) puckers, by 2′F-ANA. Amazingly, Salazar *et al*. (38) demonstrated that the sugar of the DNA strand in a DNA/RNA duplex that is recognized by RNase H, has neither *south* nor *north* conformation, but instead is *east* in type.

For all the reasons noted above then, we hypothesized that 2′F-ANA modified ON could have considerable potential as therapeutic gene silencing agents. Accordingly, we sought to test this hypothesis by examining the efficacy of 2′F-ANA as gene silencing agents in living cells, and to compare their efficacy with the chemical modification that has been most widely used in the clinic, namely phosphorothioated DNA (PS-DNA). Our data clearly demonstrate the superiority of ON bearing the 2′F-ANA modification. Gene silencing effected by ON bearing this modification was virtually complete, lasted at least twice as long as the PS-DNA, and could be achieved at ∼20% of the dose needed by PS-DNA to accomplish a similar degree of 'knock down'. It is important to note again that this effect was not due to the fluorinated monomers, which might have been produced as a result of

enzymatic degradation of the parent compounds, because the control 2′F-ANA molecules never gave a similar effect. The apparent basis for this enhanced efficiency was also identified, and ascribed to the significantly higher intracellular concentrations of PS-2′F-ANA–DNA chimeras over the period of observation. This phenomenon in turn was shown not to be due to differences in delivery of the molecules into cells. Rather, enhanced ability to activate RNase H, enhanced resistance of these molecules to nuclease degradation and possibly reduced export from cells in comparison with PS-DNA were thought to be the most probable explanations. We also speculate that these molecules may have increased bioavailability within cells, since increasing intracellular concentration alone does not always enhance gene silencing ability [A. M. Gewirtz, unpublished data and Ref. (39)].

The attributes described above are clearly important with respect to potential therapeutic utilization of the 2′F-ANA modification. Highly efficient inhibition of gene expression is the goal of all therapeutically motivated gene silencing strategies, and the data presented here strongly suggest that the 2′F-ANA modification will support this objective for several reasons. First, the 2′F-ANA modification promotes highly efficient gene silencing in living cells. Second, and potentially of equal importance, 2′F-ANA modified ON suppress gene expression for a prolonged period of time after a single administration. This attribute suggests that longer dosing intervals might be possible with 'drugs' bearing the 2′F-ANA modification. A prolonged interval between doses is clearly advantageous for practitioner, staff and patient alike in both in-patient and out-patient settings. Efficient silencing at substantially lower concentrations also implies the possibility of significant cost savings as well. Accordingly, we have many good reasons to hypothesize that 2′F-ANA modified ON will make a significant addition to the armamentarium of rationally targeted anticancer drugs, and will make an important contribution to the treatment of other diseases where gene silencing is expected to lead to useful therapeutic consequences.

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