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Improvements in siRNA properties mediated by 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (FANA)

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ABSTRACT

RNA interference (RNAi) has emerged recently as an efficient mechanism for specific gene silencing. Short double-stranded small interfering RNAs (siRNAs) are now widely used for cellular or drug target validation; however, their use for silencing clinically relevant genes in a therapeutic setting remains problematic because of their unfavourable metabolic stability and pharmacokinetic properties. To address some of these concerns, we have investigated the properties of siRNA modified with 2′-deoxy-2′-fluoro-β-D-arabinonucleotide units (araF-N or FANA units). Here we provide evidence that these modified siRNAs are compatible with the intracellular RNAi machinery and can mediate specific degradation of target mRNA. We also show that the incorporation of FANA units into siRNA duplexes increases activity and substantially enhances serum stability of the siRNA. A fully modified sense 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (FANA) strand when hybridized to an antisense RNA (i.e. FANA/RNA hybrid) was shown to be 4-fold more potent and had longer half-life in serum (∼6 h) compared with an unmodified siRNA (<15 min). While incorporation of FANA units is well tolerated throughout the sense strand of the duplex, modifications can also be included at the 5′ or 3′ ends of the antisense strand, in striking contrast to other commonly used chemical modifications. Taken together, these results offer preliminary evidence of the therapeutic potential of FANA modified siRNAs.

INTRODUCTION

Numerous strategies for silencing gene expression with nucleic acid-based molecules are under development (1,2). Of these, the hybridization-driven 'antisense' strategies, using ribozymes, DNAzymes and antisense oligonucleotides (AON) such as chimeric 2′-*O*-alkyl-RNA–DNA (gapmers) or phosphorothioate DNA (PS-DNA) have received the greatest attention and are the subject of numerous reviews [e.g. see Refs. (2,3)]. More recently, RNA interference (RNAi) has emerged as an exciting potential alternative to these more classical approaches (4–10). There are now literally thousands of reports describing the utility of this method for silencing genes in living organisms ranging from yeast to mammals (11–14). The utility of small interfering RNA (siRNA) *in vivo* and its possible applications in pharmacotherapy, as with other oligonucleotide-based therapies, faces some key hurdles including delivery, cellular uptake and biostability. Initial work with AONs and siRNAs was undertaken with unmodified, natural molecules. It soon became clear, however, that native oligonucleotides were subject to relatively rapid degradation, primarily through the action of 3′ exonucleases, but not excluding endonuclease attack as well. Oligoribonucleotides (RNA) are, in fact, generally more susceptible to nuclease degradation than DNA.

AON as well as siRNA molecules are now routinely modified to enhance their stability, affinity with RNA and biodistribution since these attributes are required if the molecules are to function as drugs (15–21). In designing new analogues, it is important to recognize that two key features of siRNA differ from traditional antisense approaches: (i) duplex RNAs are recognized and (ii) gene inhibition involves RNA-induced silencing complex (RISC)—rather than RNase HII—to promote recognition and cleavage of the mRNA target (5–7,9,10). Therefore, the most obvious type of modification to introduce into siRNAs is RNA-mimic nucleotides (North or C3′-*endo*

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sugar pucker), that will improve the siRNA properties without perturbing the A-form helical structure required for activity. Among this class, a promising modification is locked nucleic acid (LNA), in which the key benefits of improved thermal stability and biostability, and reduced off target-effects were achieved with relatively few modifications that did not significantly compromise siRNA activity (22). With a few exceptions (23–25), increasing degree of modification with RNA-mimic nucleotides, e.g. 2′F-RNA, LNA, boranophosphate-RNA, 2′-*O*-alkyl RNA and phosphorothioate RNA, impairs activity (25–27). While this may in principle be compensated by the nuclease stability and/or specificity imparted by certain oligonucleotide chemistries, the prediction of effective siRNA chemistries remains an active focus of continued study.

We reported recently on the antisense activity of PS-FANA–DNA chimeras in cultured cells, and showed that these compounds maintain high intracellular concentrations for prolonged periods of time which appears to promote long-term gene silencing (28). These observations, combined with the nuclease stability benefit provided by 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (FANA) nucleotides, prompted us to evaluate whether FANA modified siRNAs are accepted by the RISC and regulate gene expression via the RNAi pathway. Herein we report studies which directly compare the gene silencing efficiency of FANA modified siRNA and native siRNA molecules. Our results indicate that the FANA-containing siRNAs, including FANA:RNA hybrids, trigger the RNAi pathway and are able to induce sequence-specific and selective RNAi to a level surpassing that observed with native siRNAs. This property, together with the observation that FANA modified siRNAs display significantly enhanced nuclease resistance in serum, suggests that these molecules may find utility in therapeutic applications.

MATERIALS AND METHODS

Oligonucleotides

Oligoribonucleotides were obtained commercially from the University of Calgary DNA Synthesis Laboratory (Calgary, AB). FANA and FANA modified siRNAs were prepared according to the published procedures (29). All oligonucleotides were purified by anion-exchange high-performance liquid chromatography followed by desalting (SepPak cartridges, Millipore, Nepean ON). siRNA duplexes were obtained by annealing equimolar ratios of the sense and antisense strands in siRNA suspension buffer (Qiagen, Mississauga, ON) to a final concentration of 20 μM. Duplexes were heated at 95°C for 5 min and slowly cooled at 4°C O/N. siRNA duplexes used in this study are listed in Table 1.

Cell culture and transfection

The HeLa X1/5 cell line, expressing the firefly luciferase gene, was maintained in EMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1% MEM vitamins, 500 μl/ml G418, 300 μg/ml Hygromycin as described previously (30). For transfection, 1.0×10^5 cells/well in 24-well plates were seeded the day prior to transfection. The day of transfection, cells were incubated with 60 nM of siRNA complexed with Lipofectamine 2000 (Invitrogen, Burlington, ON) in a 1:2 (i.e. 1 μg of siRNA for 2 μl of Lipofectamine 2000) ratio according to the manufacturer's recommendations. Dose–response studies were performed using a final concentration of 60 nM of siRNA whereby the effective siRNA was serially diluted with a control siRNA thus reducing the effective concentration of active oligonucleotide while keeping the final concentration of siRNA constant. Cell viability was determined by measuring the cell metabolic activity, as an indicator of cellular toxicity resulting from siRNA transfection, using the Alamar Blue™ fluorimetric assay (Medicorp, Montreal, QC) as per the manufacturer's recommendations. This assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. The luciferase RLU values will then be normalized to their respective Alamar blue

Table 1. Oligonucleotides and duplexes synthesized in this study

Name	Sequence[a]	EC_{50} (nM)
siRNA1	5′-GCUUGAAGUCUUUAAUUAAgg-3′ 3′-ggCGAACUUCAGAAAUUAAUU-5′	0.26
siFANA1	5′-GCUUGAAGUCUUUAAUUAA**TT**-3′ 3′-ggCGAACUUCAGAAAUUAAUU-5′	0.25
siFANA2	5′-GC**TT**GAAGUC**TT**UAA**TT**AAtt-3′ 3′-ggCGAACUUCAGAAAUUAAUU-5′	2.0
siFANA3	5′-**GCTTGAAGTCTTTAATTAAGG**-3′ 3′-ggCGAACUUCAGAAAUUAAUU-5′	0.59
siFANA4	5′-GCUUGAAGUCUUUAAUUAAgg-3′ 3′-g**G**CGAACUUCAGAAAUUAAUU-5′	0.16
siFANA5	5′-GCUUGAAGUCUUUAAUUAAgg-3′ 3′-ggCGAACUUCAGAAAUUAAU**T**-5′	0.52
siFANA6	5′-GCUUGAAGUCUUUAAUUAAgg-3′ 3′-ggCGAAC**TT**CAGAAA**TT**AA**TT**-5′	6.3
siFANA7	5′-GCUUGAAGUCUUUAAUUAAgg-3′ 3′-**GG**CGAACUUCAGAAAUUAAUU-5′	0.06
siFANA8	5′-GCUUGAAGUCUUUAAUUAAgg-3′ 3′-**GG**CGAACUUCAGAAAUUAAU**T**-5′	>10
siFANA9	5′-GC**TT**GAAGUC**TT**UAA**TT**AAtt-3′ 3′-g**G**CGAACUUCAGAAAUUAAUU-5′	0.11
siFANA10	5′-GC**TT**GAAGUC**TT**UAA**TT**AAtt-3′ 3′-ggCGAACUUCAGAAAUUAAU**T**-5′	1.7
siFANA11	5′-GC**TT**GAAGUC**TT**UAA**TT**AAtt-3′ 3′-ggCGAAC**TT**CAGAAA**TT**AA**TT**-5′	3.6
siFANA12	5′-GC**TT**GAAGUC**TT**UAA**TT**AAtt-3′ 3′-**GG**CGAACUUCAGAAAUUAAUU-5′	0.06
siFANA13	5′-GC**TT**GAAGUC**TT**UAA**TT**AAtt-3′ 3′-**GG**CGAACUUCAGAAAUUAAU**T**-5′	>10
siFANA14	5′-**GCTTGAAGTCTTTAATTAAGG**-3′ 3′-g**G**CGAACUUCAGAAAUUAAUU-5′	0.24
siFANA15	5′-**GCTTGAAGTCTTTAATTAAGG**-3′ 3′-ggCGAACUUCAGAAAUUAAU**T**-5′	1.5
siFANA16	5′-**GCTTGAAGTCTTTAATTAAGG**-3′ 3′-ggCGAAC**TT**CAGAAA**TT**AA**TT**-5′	>10
siFANA17	5′-**GCTTGAAGTCTTTAATTAAGG**-3′ 3′-**GG**CGAACUUCAGAAAUUAAUU-5′	0.17
siFANA18	5′-**GCTTGAAGTCTTTAATTAAGG**-3′ 3′-**GG**CGAACUUCAGAAAUUAAU**T**-5′	>10
Control siRNAs		
siRNA-CTL	5′-AAGCAAUAUUUAAAUCGUAAAgg-3′ 3′-ggUUCGUUAUAAAUUUAGCAUUU-5′	inactive
siRNA1-Mi	5′-GCUUGAUUCUGAUAAUUAAgg-3′ 3′-ggCGAACUAAGACUAUUAAUU-5′	inactive
siFANA17-Mi	5′-**GCTTGATTCTGATAATTAAGG**-3′ 3′-**GG**CGAACUAAGACUAUUAAUU-5′	inactive

[a]Uppercase letters, RNA; lowercase letters, DNA; bold uppercase letters, FANA; underlined letters, mismatches; siRNA sequences are shown with the sense strand on top and antisense strand below it.

RFU values. Therefore, if any toxicity is induced by the siRNAs in the tested experimental conditions, it will then be corrected by this normalization.

Luciferase activity assay

Luciferase activity assays were performed using the luciferase assay system (BD Bioscience, Mississauga, ON) according to the manufacturer's protocol. Briefly, following transfection with the siRNA, cells were washed with phosphate-buffered saline (Invitrogen, Burlington, ON) and lysed. Cell lysates were centrifuged to remove cellular debris and aliquots were transferred to 96-well lumitrac plates (Ultident; Greiner Bio-one, Longwood FL). Luminescence was measured using a microplate luminometer (Luminoskan Ascent, Thermo LabSystem) immediately following addition of the luciferin substrate solution. Luminescence values were then normalized to the cell viability values.

Luciferase mRNA quantification

For real-time PCR analysis, total RNA was extracted using the RNeasy mini kit (Qiagen, Mississauga, ON) according to the manufacturer's protocol and cDNA prepared from 1 μg total RNA using the SuperScript™ II Reverse Transcriptase and random primers (Invitrogen, Burlington, ON). Quantitative real-time PCR was performed using the SYBR green reagent and gene-specific primers for the luciferase (LUC5013 F1, 5′-acgctgggcgttaatcagag-3′; LUC5013 R1, 5′-gtcgaagatgttggggtgttg-3′; TIB MOLBIOL, Berlin, Germany) and the housekeeping gene GAPDH (huGAPD forward, 5′-ggtggtctcctctgacttc-3′; huGAPD reverse, 5′-ctcttcctcttgtgctcttg-3′; TIB MOLBIOL) according to optimized conditions and using the LightCycler (Roche, Laval, QC).

Stability of oligonucleotides in serum

siRNAs were diluted in 10% FBS (Wisent, St Bruno, QC) in DMEM (Invitrogen, Burlington, ON) (1 μg in 50 μl) and incubated at 37°C. Aliquots of 5 μl were collected after 0, 0.25, 0.5, 0.75, 1, 2, 6 and 24 h and frozen at −20°C in 10 μl of 1× TBE-loading buffer until analysed. Samples were separated on 20% polyacrylamide gel under non-denaturing conditions and stained with SYBR gold (Invitrogen, Burlington, ON). Densitometry analysis was performed using the TotalLab software and half-life of each siRNA was determined. For stability studies in human plasma, 2 μg of each siRNA was resuspended in 10 μl of ice-cold plasma and incubated at 37°C. Aliquots of 1 μl were collected at the different time points and frozen at −20°C in 39 μl of loading buffer (0.5× TBE, 6% Ficoll). Samples were separated as described above.

RESULTS

Compatibility of FANA with the cellular RNAi machinery

To assess the compatibility of FANA with the siRNA machinery, we have evaluated a series of different FANA-containing siRNA (Table 1 and Figure 1) for their ability to specifically inhibit firefly luciferase expression in HeLa X1/5 cells. Cells were transfected with 60 nM siRNA, and both luciferase mRNA and luciferase activity were measured 24 h following transfection and compared with a set of siRNA controls (Table 1), and results are compiled in Figure 2. Dose–response studies were also performed and estimated EC_{50} are presented in Table 1 while best-fit curves of the most promising siRNAs are shown in Figure 3. The native siRNA had potency in the subnanomolar range, consistent with several studies using similar cell/assay systems [e.g. see (22,25,31)].

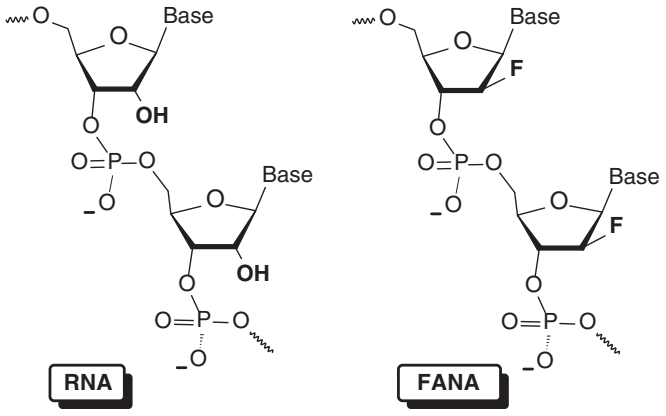

Figure 1. Chemical structures of FANA and RNA.

Sense and/or antisense strand modifications. We first investigated the effect of introducing FANA units in the sense strand of the duplex, as this strand has been shown to better tolerate chemical modifications [e.g. (22,25)]. Our results indicate that FANA units are well tolerated in the sense strand of the siRNA as shown by the selective inhibition of luciferase mRNA and luciferase activity by a series of sense-modified siRNA (Table 1 and Figure 2A). Remarkably, an siRNA having an all-FANA sense strand (siFANA3) showed only minimal loss of activity when compared with the unmodified siRNA (Figure 2A). Activity is 'rescued' by introducing FANA at the 3′-terminus of the antisense RNA strand (dGG-3′ → araF-GG-3′). In fact, the resulting duplex siFANA17 (>50% FANA) was more active than the native siRNA duplex (Table 1 and Figure 3). Interestingly, a duplex with a fully modified sense strand (siFANA3, E_{50} ∼0.6 nM) was more potent than a duplex with a partially modified sense strand (siFANA2, E_{50} 2 nM). None of the siRNA controls, namely siRNA-CTL, siRNA1-Mi and siFANA17-Mi (Table 1), had an effect on luciferase activity confirming the specificity of the FANA-modified siRNAs.

We next examined the effect on siRNA activity of incorporating FANA units at internal and terminal positions of the antisense strand, which has been reported to be more sensitive to chemical modifications compared with sense strand (22,25). This is most likely due to the importance of this strand for incorporation into the RISC (5′ terminus), nucleation of binding with target mRNA (positions 2–5 starting from the 5′ end) and mRNA cleavage (between positions 10 and 11) (22,26,32,33).

Our data show that modifications of the 5′ end (siFANA5) or the 3′-dangling dinucleotides (siFANA4 and siFANA7) were very well tolerated (Table 1 and Figure 2B). For example, the siRNA with two FANA residues at the 3′ end retains activity and is, in fact, 4-fold more potent than the control (siFANA7; EC_{50} ∼0.06 nM). The benefits of 3′ end modifications are

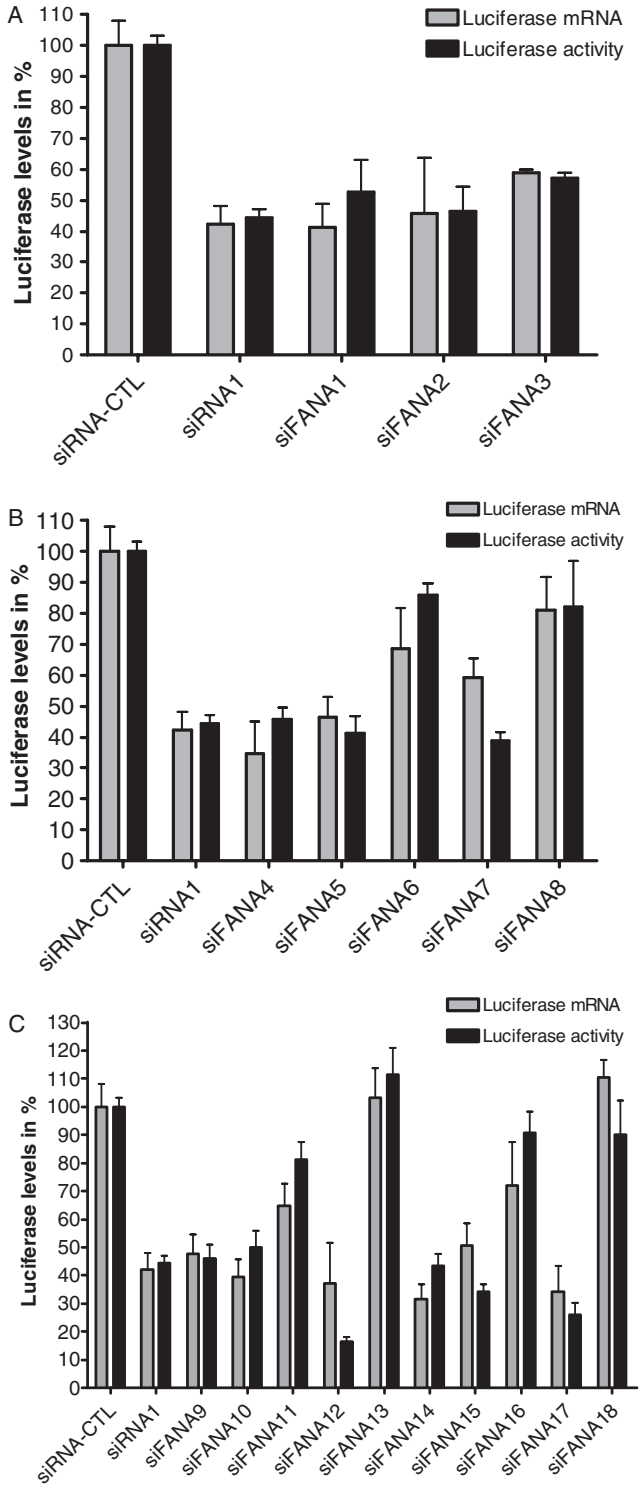

Figure 2. Efficacy of the different siRNAs at inhibiting luciferase in HeLa X1/5 cells. Cells were transfected with 60 nM siRNA with modifications in the sense strand only (**A**), in the antisense strand only (**B**) or in both sense and antisense strands (**C**). Luciferase activity levels were measured 24 h post-transfection and normalized to metabolic activity. The normalized luciferase activity was then determined as a percentage of luciferase activity as compared with an unrelevant control siRNA (siRNA-CTL) set at 100%. Data represent mean normalized luciferase activity ± SEM. Luciferase mRNA levels were quantified by real-time PCR analysis (relative to expression of the house keeping gene GAPDH) 24 h post-transfection. Bars show mean Luciferase/GAPDH ratios ± SEM.

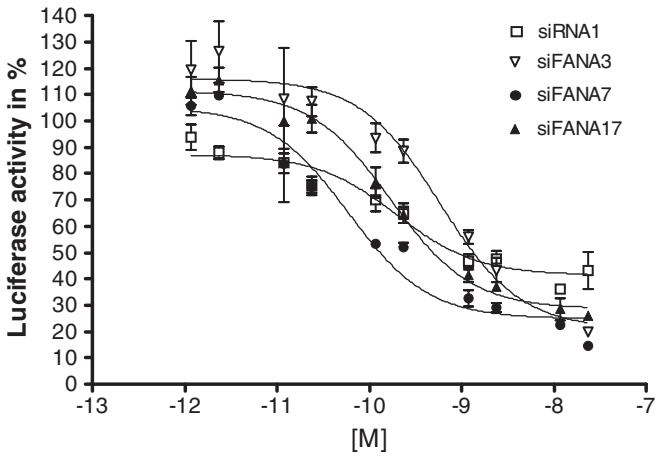

Figure 3. Potency of FANA-containing siRNA at inhibiting the luciferase activity. Dose-responses were obtained for each siRNA by transfecting cells with different amounts of active siRNA for 24 h. Luciferase activity was measured and values normalized to the metabolic activity and compared with a control siRNA (siRNA-CTL) set at 100%. Data represent mean normalized luciferase activity ± SEM.

clearly seen for siFANA12 (EC_{50} ~0.06 nM) and siFANA17 (EC_{50} ~0.17 nM) which are significantly more potent than the corresponding 3′-unmodified duplexes (siFANA2, EC_{50} ~2.0 nM; siFANA3, EC_{50} ~0.59 nM; Table 1). In addition, no significant effect on siRNA potency was observed when a FANA residue is placed at the 5′ end of the antisense strand (siFANA5; EC_{50} ~0.52 nM). When FANA units were placed at both termini of the antisense strand, a different picture emerged. For example, a large negative effect on siRNA potency was observed with siFANA13 (Table 1; EC_{50} >10 nM). The same trend was observed for the series siFANA15, siFANA17 and siFANA18 (Table 1).

Our results also show that FANA substitutions at position 1–2, 5–6 and 13–14 of the antisense strand (siFANA6) reduced siRNA activity suggesting that (i) these positions may play a critical role for the siRNA function (Figure 2B), and/or (ii) that some property of the FANA T nucleoside is responsible for the observed lowered activity. Interestingly, this loss of activity is compensated by introducing six other FANA units at juxtaposed positions within the sense strand, providing a duplex that now has excellent activity (siFANA11, EC_{50} 3.6 nM). This shows that the effects observed depend on not only the number of modifications but also the precise location and arrangement of the FANA sugar with the siRNA duplex.

Persistence of activity of FANA-containing siRNA

To further characterize the inhibitory properties of the FANA siRNAs in our cells stably expressing the luciferase mRNA target, we carried out a time-course experiment, observing luciferase expression on 4, 8, 24, 48, 72 and 96 h after transfection (Figure 4). Luciferase suppression lasted over the 4 day period, consistent with previous observations (31,34). Our results also indicate that the higher potency of FANA-modified siRNA over native siRNA was also maintained over this time period. For example, at the 96 h time point, siFANA7, siFANA3 and siFANA 17 were more potent than the unmodified siRNA duplex (Figure 4). The data given below suggest that increased nuclease stability of FANA modified siRNAs

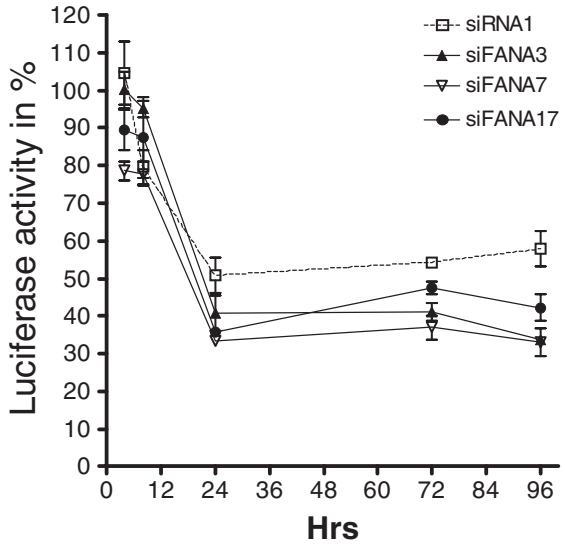

Figure 4. Duration of activity of modified siRNA. Cells were transfected with 60 nM siRNA. Luciferase activity was measured 4, 8, 24, 48, 72 and 96 h post-transfection. Data represent mean normalized luciferase activity ± SEM compared with a control siRNA (siRNA-CTL) set at 100%.

may be responsible, at least in part, for the persistent and potent activity of FANA-containing siRNAs.

Serum stability

FANA AONs are more resistant to endo and exonucleases, and this resistance is further enhanced if the FANA-modified nucleosides are connected by phosphorothioate linkages. PS-FANA AONs, for example, are >20-fold more stable than PS-DNA towards 3′-exonuclease hydrolysis [(30) and M. J. Damha and K.-L. Min, unpublished data). To investigate whether phosphodiester (PO)-linked FANA would provide similar enhancement of nuclease resistance to siRNA duplexes, we assessed the stability of unmodified and modified siRNA in fetal bovine (Figure 5) and human plasma (data not shown). As expected, the unmodified siRNA was rapidly degraded within 15 min (Figure 5A). A similar degradation profile was observed with siFANA7 (two araF-G residues at the 3′ end of the antisense strand) whereas siFANA3 with a fully modified sense strand has a half-life of ∼5 h (Figure 5B). When siFANA3 is further modified to give siFANA17, the serum half-life increases to ∼6 h (Figure 5B). Comparable stability profiles where observed when the same duplexes were exposed to human plasma (data not shown).

DISCUSSION

A growing number of oligonucleotide-based approaches exist for the regulation of gene expression in mammalian cells (2,3). Selective gene knockdown is now widely used for target validation and therapeutic purposes. The recent discovery of RNAi (4,5) has renewed interest in antisense-based approaches (AON, siRNA). However, their use for *in vivo* applications faces key limitations relating to delivery, cellular uptake and biostability.

We have demonstrated previously that incorporation of FANA residues into PS-DNA AONs leads to significant

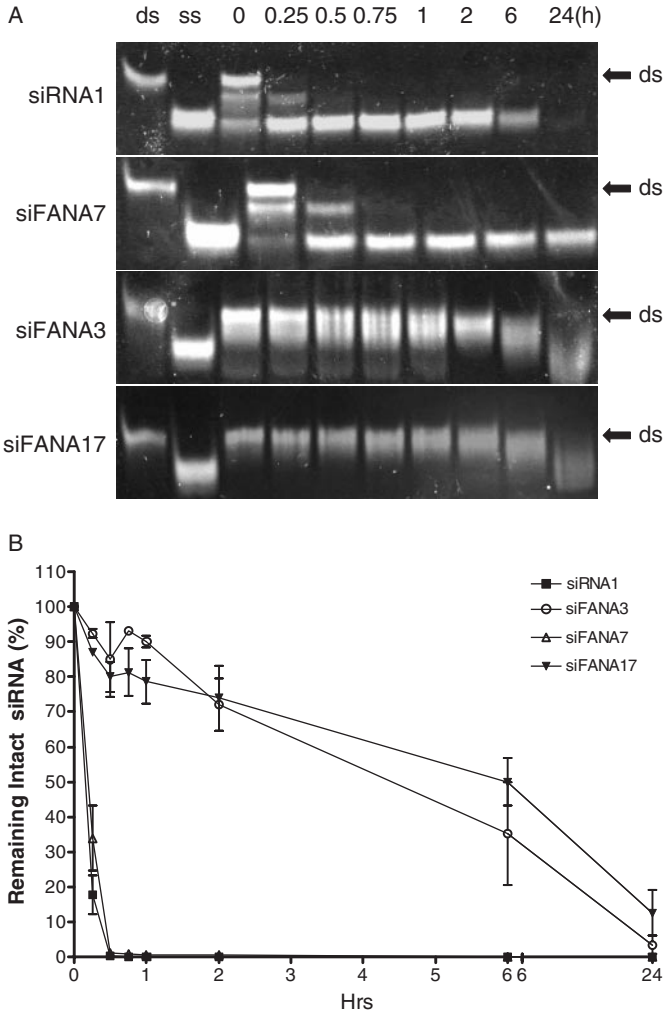

Figure 5. Serum stability of FANA-containing siRNA. The different siRNAs were incubated in 10% FBS at 37°C and aliquots were taken at the time points indicated. (**A**) The siRNA were separated by PAGE and visualized with SYBR gold. 'ds' depicts double-stranded siRNA marker and 'ss' single-stranded. (**B**) Bands were quantified by densitometry and the percentage of intact siRNA from initial amount set at 100%. Data represent mean values from three independent experiments ± SEM.

improvement in terms of potency and stability of the AON (28,30). We have also shown that PS-FANA–DNA chimeras trigger RNase H-mediated mRNA degradation more efficiently than PS-DNA or PS-2′OMe-RNA–DNA chimeras of equal designs, in part because the arabinose sugar does not alter the overall helical structure of the corresponding AON/mRNA hybrid when compared with the native DNA/RNA substrate (35,36). Given that FANA is globally similar in structure to DNA, substitutions of RNA with FANA units is expected to result in structural perturbation of RNA duplexes, and hence influence the ability of siRNA duplexes to serve as substrates for RISC enzymes involved in the RNAi pathway. Remarkably, here we find that FANA is particularly well suited for the RNAi machinery whilst imparting improvements key to the development of siRNA as prospective drugs. Based on the data obtained (Table 1), FANA is one of the very few modifications (24,25) that can drastically enhance serum stability of the duplex (Figure 5) while increasing potency over

native siRNA (up to 4-fold). For example, our data indicate that the modest substitution of two ribose with arabinose (FANA) at the 3′ end of the antisense strand (siFANA7), was sufficient to increase the potency over the native siRNAs, with gene silencing observed to persist over a 4 day period. Moreover, we showed that an all-FANA:RNA hybrids (e.g. siFANA3, siFANA14) enter the RNAi pathway, and that their activity could also be increased beyond that of the native siRNA (siFANA17) by introducing further modifications at the 3′ end of the antisense strand. Since the plasma stability of siFANA7 was comparable with that of native siRNA, we suggest that the increased potency of siFANA7 (compared with native siRNA) and siFANA17 (compared with siFANA3) is due to a change in relative thermal stabilities of the duplex termini and/or an improved recognition of the FANA 3′-overhang by the Argonaute PAZ domain. Indeed, O'Toole *et al*. (32) have shown that the nature and base stacking of a 3′ double nucleotide overhang can make a significant contribution to the thermodynamic stability of RNA duplexes. As RISC incorporates the siRNA strand whose 5′ end is least stable, this would explain an increase in RISC loading efficacy for siFANA7 (compared with unmodified siRNA and siFANA5) and thus, increased activity (37,38). Furthermore, recent crystallographic studies by Lingel *et al*. (39) showed that the Argonaute PAZ domain forces the 3′ overhanging nucleotides to adopt a DNA-like (South/East) sugar pucker and an *anti* base orientation, both of which are favoured and maintained by FANA nucleotides (35,36). Given the conformational bias of the 2′-deoxy-2′-fluoro-arabinose sugars (35,36), the entropic penalty for PAZ recognition of an FANA overhang is expected to be reduced relative to the more flexible DNA or the C3′-*endo* RNA overhangs. This would favour FANA over DNA or RNA as the dangling nucleotides in siRNAs.

In conclusion, FANA substitutions, particularly when made in the sense strand together with modification of the 3′-overhanging end of the antisense strand, can lead to increased activity of the siRNA. In addition FANA substitution enhances the resistance of the siRNA duplex to degradation in serum-containing medium, a property that may impact the future therapeutic development of chemically modified siRNAs.

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Conflict of interest statement. None declared.

REFERENCES

1. Stephenson,M.L. and Zamecnik,P.C. (1977) Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxynucleotide. *Proc. Natl Acad. Sci. USA*, **74**, 4370–4373.
2. Opalinska,J.B. and Gewirtz,A.M. (2002) Nucleic-acid therapeutics: basic principles and recent applications. *Nature Rev*., **1**, 1–10.
3. Scherer,L.J. and Rossi,J.J. (2003) Approaches for sequence-specific knockdown of mRNA. *Nat. Biotechnol*., **21**, 1457–1465.
4. Fire,A., Xu,S., Montgomery,M.K., Kostas,S.A., Driver,S.E. and Mello,C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806–811.
5. Elbashir,S.M., Lendeckel,W. and Tuschl,T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*., **15**, 188–200.
6. Caplen,N.J., Parrish,S., Imani,F., Fire,A. and Morgan,R.A. (2001) Specific inhibition of gene expression by small dsRNAs in invertebrate and vertebrate systems. *Proc. Natl Acad. Sci. USA*, **98**, 9742–9747.
7. Nishikura,K. (2001) A short primer on RNAi: RNA-directed RNA polymerase acts as a key catalyst. *Cell*, **107**, 415–418.
8. Nykanen,A., Haley,B. and Zamore,P.D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell*, **107**, 309–321.
9. Tuschl,T. (2002) Expanding small RNA interference. *Nat. Biotechnol*., **20**, 446–448.
10. Mittal,V. (2004) Improving the efficiency of RNA interference in mammals. *Nature Rev*., **5**, 355–365.
11. Yu,J.Y., DeRuiter,S.L. and Turner,D.L. (2002) RNA interference by expression of short-interfering RNAs andhairpin RNAs in mammalian cells. *Proc. Natl Acad. Sci. USA*, **99**, 6047–6052.
12. Donze,O. and Picard,D. (2002) RNA interference in mammalian cells using siRNAs synthesized with T7 RNApolymerase. *Nucleic Acids Res*., **30**, e46.
13. Sui,G., Soohoo,C., Affar el,B., Gay,F., Shi,Y., Forrester,W.C. and Shi,Y. (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl Acad. Sci. USA*, **99**, 5515–5520.
14. Paddison,P.J., Caudy,A.A., Bernstein,E., Hannon,G.J. and Conklin,D.S. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev*., **16**, 948–958.
15. Agrawal,S. and Zhao,Q. (1998) Mixed backbone oligonucleotides: improvement in oligonucleotide-induced toxicity *in vivo*. *Antisense Nucleic Acid Drug Dev*., **8**, 135–139.
16. Crooke,S.T. (1999) Molecular mechanisms of action of antisense drugs. *Biochim. Biophys. Acta*, **1489**, 31–44.
17. Nielsen,P.E. (2000) Antisense peptide nucleic acids. *Curr. Opin. Mol. Ther*., **2**, 282–287.
18. Micklefield,J. (2001) Backbone modification of nucleic acids: synthesis, structure and therapeutic applications. *Curr. Med. Chem*., **8**, 1157–1179.
19. Mangos,M.M. and Damha,M.J. (2002) Flexible and frozen sugar-modified nucleic acids—modulation of biological activity through furanose ring dynamics in the antisense strand. *Curr. Top. Med. Chem*., **2**, 1145–1169.
20. Braasch,D.A., Jensen,S., Liu,Y., Kaur,K., Arar,K., White,M.A. and Corey,D.R. (2003) RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry*, **42**, 7967–7995.
21. Opalinska,J.B., Kalota,A., Gifford,L.K., Lu,P., Jen,K.Y., Pradeepkumar,P.I., Barman,J., Kim,T.K., Swider,C.R., Chattopadhyaya,J. *et al*. (2004) Oxetane modified, conformationally constrained, antisense oligodeoxyribonucleotides function efficiently as gene silencing molecules. *Nucleic Acids Res*., **32**, 5791–5799.
22. Elmen,J., Thonberg,H., Ljungberg,K., Frieden,M., Westergaard,M., Xu,Y., Wahren,B., Liang,Z., Orum,H., Koch,T. *et al*. (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res*., **33**, 439–447.
23. Allerson,C.R., Sioufi,N., Jarres,R., Prakash,T.P., Naik,N., Berdeja,A., Wanders,L., Griffey,R.H., Swayze,E.E. and Bhat,B. (2005) Fully 2′-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *J. Med. Chem*., **48**, 901–904.
24. Dande,P., Prakash,T.P., Sioufi,N., Gaus,H., Jarres,R., Berdeja,A., Swayze,E.E., Griffey,R.H. and Bhat,B. (2006) Improving RNA interference in mammalian cells by 4′-thio-modified small interfering RNA (siRNA): effect on siRNA activity and nuclease stability when used in combination with 2′-*O*-alkyl modifications. *J. Med. Chem*., **49**, 1624–1634.
25. Hoshika,S., Minakawa,N., Kamiya,H., Harashima,H. and Matsuda,A. (2005) RNA interference induced by siRNAs modified with

4′-thioribonucleosides in cultured mammalian cells. *FEBS Lett*., **579**, 3115–3118.
26. Hall,A.S., Wan,J., Shaughnessy,E.E., Ramsay Shaw,B. and Alexander,K.A. (2004) RNA interference using boranophosphate siRNAs: structure–activity relationships. *Nucleic Acids Res*., **32**, 5991–6000.
27. Amarzguioui,M., Holen,T., Babaie,E. and Prydz,H. (2003) Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res*., **31**, 589–595.
28. Kalota,A., Karabon,L., Swider,C.R., Viazovkina,E., Elzagheid,M., Damha,M.J. and Gewirtz,A.M. (2006) 2′-Deoxy-2′-fluoro-β-D-arabinonucleic acid (2′F-ANA) modified oligonucleotides (ON) effect highly efficient, and persistent, gene silencing. *Nucleic Acids Res*., **34**, 451–461.
29. Viazovkina,E., Mangos,M.M., Elzagheid,M.I. and Damha,M.J. (2002) Synthesis of 2′-fluoroarabinonucleoside phosphoramidites and their use in the synthesis of 2′F-ANA. In *Current Protocols in Nucleic Acid Chemistry*, Vol. 4.15. John Wiley & Sons, pp. 1–22.
30. Lok,C.N., Viazovkina,E., Min,K.-L., Nagy,E., Wilds,C.J., Damha,M.J. and Parniak,M.A. (2002) Potent gene-specific inhibitory properties of mixed-backbone antisense oligonucleotides comprised of 2′-deoxy-2′-fluoro-D-arabinose and 2′-deoxy nucleotides. *Biochemistry*, **41**, 3457–3467.
31. Kim,D.-H., Behlke,M.A., Rose,S.D., Chang,M.-S., Choi,S. and Rossi,J.J. (2004) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat. Biotechnol*., **23**, 222–226.
32. O'Toole,A.S., Miller,S. and Serra,M.J. (2005) Stability of 2′ double nucleotide overhangs that model the 3′ ends of siRNA. *RNA*, **11**, 512–516.
33. Filipowicz,W. (2005) RNAi: the nuts and bolts of the RISC machine. *Cell*, **122**, 17–20.
34. Reynolds,A., Leake,D., Boese,Q., Scaringe,S., Marshall,W.S. and Khvorova,A. (2004) Rational siRNA design for RNA interference. *Nat. Biotechnol*., **22**, 326-330.
35. Denisov,A.-Y., Noronha,A.M., Wilds,C.J., Trempe,J.F., Pon,R.T., Gehring,K. and Damha,M.J. (2001) Solution structure of an arabinonucleic acid (ANA)/RNA duplex in a chimeric hairpin: comparison with 2′F-ANA/RNA and DNA/RNA hybrids. *Nucleic Acids Res*., **29**, 4284–4293.
36. Damha,M.J., Noronha,A.M., Wilds,C.J., Trempe,J.F., Denisov,A., Pon,R.T. and Gehring,K. (2001) Properties of arabinonucleic acids (ANA and 2′F-ANA): implications for the design of antisense therapeutics that invoke RNase H cleavage of RNA. *Nucleosides Nucleotides Nucleic Acids*, **20**, 429–440.
37. Filipowicz,W., Jaskiewicz,L., Kolb,F.A. and Pillai,R.S. (2005) Post-transcriptional gene silencing by siRNAs and miRNAs. *Curr. Opin. Struct. Biol*., **15**, 331–341.
38. Schwarz,D.S., Hutvagner,G., Du,T., Xu,Z., Aronin,N. and Zamore,P.D. (2003) Asymetry in the assembly of the RNAi enzyme complex. *Cell*, **115**, 199–208.
39. Lingel,A., Simon,B., Izaurralde,E. and Sattler,M. (2004) Nucleoic acid 3′-end recognition by Argonaute2 PAZ domain. *Nature Struct. Mol. Biol*., **11**, 576–577.