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ORIGINAL RESEARCH PAPER

Improved serum stability and biophysical properties of siRNAs following chemical modifications

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Abstract Small interfering RNAs (siRNAs) which mediate sequence-specific gene silencing through RNA interference have been harnessed for therapeutic applications. Here, we found combinations of modest 2′-ribose siRNA modifications that permit effective gene silencing and improve siRNA stability in human serum. Introduction of as few as two different or identical 2′-ribose modifications at a specific position of either strand, which has been previously suggested to suppress unintended off-target effects, enhances the thermal and thermodynamic stability of siRNA duplexes, and does not alter the overall A-form helical geometry that is required for silencing activity. Our results provide a useful template for the design for therapeutic siRNAs.

Keywords A-form helix · Melting temperature · 2′-Ribose modification · RNA interference · siRNA · Stability

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Introduction

RNA interference (RNAi) is a biological process in which double-stranded small interfering RNAs (siRNAs) mediate degradation of cognate mRNAs. Effective siRNA duplexes have characteristic 5′-monophosphate ends and 2-nucleotide (nt) 3′-overhangs, which facilitate their entry into RNA-induced silencing complex (RISC) (Ma et al. 2004, 2005). Active RISC contains one of the two siRNA strands, the "guide" strand, which directs RISC-mediated mRNA cleavage and degradation, and the other "passenger" strand which plays no direct role in RNAi is discarded from the RISC (Matranga et al. 2005).

RNAi has been harnessed for gene-specific therapeutics, because it is applicable to any undesirable gene that causes human disease (De Paula et al. 2007). However, several critical obstacles must be resolved before the therapeutic promise of this technology can be completely realized. These include (i) improvement of siRNA stability in physiological fluids; (ii) reduction of off-target effects that arise from partial complementarity between each siRNA strand and mRNAs; and (iii) suppression of innate immune activation.

Various chemical modifications at the 2′-position of the ribose moiety have been evaluated to improve siRNA stability and the prospect of siRNA therapeutics. The most common 2′-ribose modifications include 2′-*O*-methyl (2′-*O*-Me) and 2′-deoxy-

2′-fluoro (2′-F) substitutions, and a locked nucleic acid (LNA) which is a high-affinity RNA analog with a methylene linkage between the 2′ and the 4′ carbons of the ribose. Interestingly, a recent study has suggested that a single 2′-*O*-Me modification introduced at position 2 of the guide strand, as counted from the 5′-end, significantly reduces off-target effects without compromising silencing activity (Jackson et al. 2006). Additionally, introduction of a single LNA modification at the 5′-terminal end (position 1) of the passenger strand was suggested to allow preferential incorporation of the guide strand into RISC, thereby reducing the passenger strand-specific off-targeting activity (Elmen et al. 2005). Because these position- and strand-specific 2′-ribose modifications provide flexibility in siRNA design, we envisaged that their application in a combinatorial fashion within the duplex might lead to superior siRNAs.

In the present study, we examined biological and biophysical properties of siRNA duplexes in which the specific 2′-ribose modification was introduced into one or both strands. Our findings are of potential importance in the development of siRNA therapeutic applications.

Materials and methods

Synthetic siRNA duplexes

Single-stranded siRNAs, including those containing a single 2′-ribose modification, were synthesized by Integrated DNA Technologies and were received in RNase-free HPLC purification formats. All the single-stranded siRNAs contain the 5′-phosphate terminus. The sequence of the EGFP siRNA duplex used in this study was previously reported as a valid target site of the corresponding mRNA (Chiu and Rana 2002). To prepare siRNA duplexes, complementary single-stranded siRNAs each at 20 μM were mixed in annealing buffer (30 mM HEPES/KOH, pH 7.4, 100 mM potassium acetate, and 2 mM magnesium acetate), and annealed at 95°C for 2 min, followed by incubation at 37°C for 1 h. Sequences of all siRNA duplexes and their respective modifications are shown in Table 1.

Table 1 Sequence, 2′-ribose modification, and RNAi activity of siRNA duplexes

Name[a]	Sequences[b]	RNAi activity[c] (%)
Pp-luc	5′-cguacgcggaauacuucgauu-3′ 3′-gugcaugcgccuuaugaagcu-5′	4.23 ± 6.95
RNA1	5′-gcagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguuc-5′	77.30 ± 2.89
RNA2	5′-gcagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguUc-5′	71.74 ± 4.98
RNA3	5′-gcagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguuc-5′	73.84 ± 3.66
RNA4	5′-***g***cagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguuc-5′	73.23 ± 4.63
RNA5	5′-***g***cagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguUc-5′	68.59 ± 1.84
RNA6	5′-***g***cagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguuc-5′	69.33 ± 1.48
RNA7	5′-gCagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguuc-5′	71.74 ± 3.96
RNA8	5′-gCagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguUc-5′	62.72 ± 7.54
RNA9	5′-gCagcacgacuucuucaaguu-3′ 3′-uucgucgugcugaagaaguuc-5′	77.23 ± 2.13
RNA10	5′-uaguucugugggcuguuuuu3′ 3′-uuaucaagacaccccgacaaa-5′	ND
RNA11	5′-***u***aguucugugggcuguuuuu3′ 3′-uuaucaagacaccccgacaaa-5′	ND
RNA12	5′-uAguucugugggcuguuuuu3′ 3′-uuaucaagacaccccgacaaa-5′	ND

[a] *Pp*-luc siRNA which targets firefly luciferase was used as a specificity control. siRNAs RNA1-RNA9 targeted the EGFP gene, and siRNAs RNA10-RNA12 were directed against the Id-1 gene

[b] Top and bottom strands represent the passenger and guide strands of the siRNA duplex, respectively. LNA, bold and italic lowercase letters; 2′-*O*-Me, uppercase letters; 2′-F, underlined lowercase letters

[c] RNAi activity is presented as the target-gene (EGFP) silencing efficiency of each siRNA duplex at 50 nM when compared with the mock-transfected sample (100%). Reported values are means ± SD from three independent experiments

ND, Not determined

Cell culture and transfection

HeLa cells stably transfected with the pEGFP-N2 vector (Clontech) encoding enhanced green fluorescence protein (EGFP) were maintained at 37°C, in an

atmosphere of 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM, Cambrex) supplemented with 10% fetal bovine serum (Gibco-BRL), 100 units penicillin/ml, and 100 μg streptomycin/ml. EGFP-expressing HeLa cells were transfected with siRNAs, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The final siRNA concentration is indicated in each experiment.

EGFP fluorescence assay

Quantitative analysis of the silencing activity of siRNA duplexes directed against the exogenous EGFP gene in HeLa cells was performed as previously described (Chiu and Rana 2002).

Real-time RT-PCR and Western blotting

Total RNA was isolated from EGFP-expressing HeLa cells transfected with 50 nM siRNA using the RNeasy kit (Qiagen). One μg total RNA from each sample was treated with RNase-free DNase I (Invitrogen) to generate first-strand cDNA using a random hexamer (GE Healthcare) and Superscript III (Invitrogen). The resulting cDNA was subjected to quantitative PCR using iQ SYBR Green SuperMix and an iCycler iQ instrument (Bio-Rad) according to the manufacturer's protocol. The sequences of the gene-specific primers are as follows: *EGFP*: 5′-ACTACCTGAGCACCCAGTCC-3′ (forward) and 5′-CTTGTACAGCTCGTCCATGC-3′ (reverse); and *GAPDH*: 5′-ATCATCCCTGCCTCTACTGG-3′ (forward) and 5′-GTCAGGTCCACCACTGACAC-3′ (reverse). All assays were normalized to *GAPDH* RNA levels that served as an internal control. Western blotting was performed as previously described (Pham et al. 2004). Anti-GFP and anti-GAPDH antibodies were purchased from Roche and Chemicon, respectively. Anti-GAPDH antibody was used to verify equal loading of protein on the gel.

Serum stability assay

The guide strand of siRNAs was 5′-radiolabeled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (6,000 Ci/mmol; Perkin Elmer). To prepare labeled siRNA duplexes, the 5′-labeled guide strand was annealed to an equimolar amount of the corresponding unlabeled passenger strand in 1× annealing buffer, and gel-purified. The labeled siRNA duplex was added to 10% (v/v) human serum (Sigma) to give 46 nM. Each sample was incubated at 37°C. Aliquots were withdrawn periodically and quenched with a stop mix (100 mM Tris/HCl, pH 7.4, 12.5 mM EDTA, 150 mM NaCl, 1% SDS, and 1.11 mg proteinase K/ml) at 25°C for 15 min. Following phenol extraction and ethanol precipitation, RNA samples were resolved on 8% polyacrylamide gels containing 7 M urea and visualized on a BAS-2500 Phosphorimager (Fujifilm).

Circular dichroism (CD) and thermal denaturation analysis

Both CD and thermal denaturation studies were performed using a Jasco spectropolarimeter (model 715) equipped with a temperature controller. For the CD spectra, the measurements were carried out at ambient temperature with 2 μM siRNA duplex in 1 ml buffer containing 5 mM sodium phosphate (pH 7.2), 140 mM KCl, and 1 mM $MgCl_2$. Spectra were baseline-corrected with respect to a blank containing the buffer without siRNA duplex. For thermal denaturation profiles, melting curves were obtained with siRNA duplexes prepared under the same conditions as for the CD analysis. Samples were equilibrated at 30°C for 10 min, and the change in hyperchromicity at 265 nm was then monitored upon heating to 95°C at a rate of 1°C/min. Melting temperatures (T_m) were calculated by the first-order derivative method. Thermodynamic parameters ΔH^o, ΔS^o, and ΔG^o were calculated as previously described (Breslauer 1995; SantaLucia and Turner 1997).

Results and discussion

RNAi activity of siRNAs with selective 2′-ribose modifications

In an effort to combine the benefits of the position- and strand-specific 2′-ribose modifications and improve properties of siRNAs, we introduced a 2′-*O*-Me modification at position 2 of either strand, a 2′-F modification at position 2 of the guide strand, or a LNA modification at position 1 of the passenger strand. Next, these selective 2′-ribose modifications were introduced in a combinatorial fashion into the

canonical siRNA duplex in which one or both strands contained the specific modification. We first examined the effect of our selective 2′-ribose modifications on the activity of a previously validated siRNA duplex (siEGFP) which targets the exogenous EGFP gene in HeLa cells. Unmodified siEGFP and its 2′-modified derivatives are listed in Table 1. As expected, the unmodified siEGFP effectively inhibited expression of the target gene, whereas an unrelated *Pp*-luc siRNA directed against firefly luciferase did not affect the EGFP gene expression (Fig. 1a). As shown in Fig. 1b, transfection of each 2′-modified siEGFPs resulted in a dose-dependent reduction in EGFP fluorescence levels comparable to the unmodified siEGFP (RNA1), although we observed less inhibition by RNA5 (differential modifications of passenger and guide strands with LNA and 2′-*O*-Me, respectively) at 1 and 10 nM, and RNA8 (symmetric 2′-*O*-Me modifications of both strands) at 50 nM. Modified siEGFPs reduced EGFP mRNA and protein levels with similar efficacy compared with unmodified siEGFP (Fig. 1c, d),

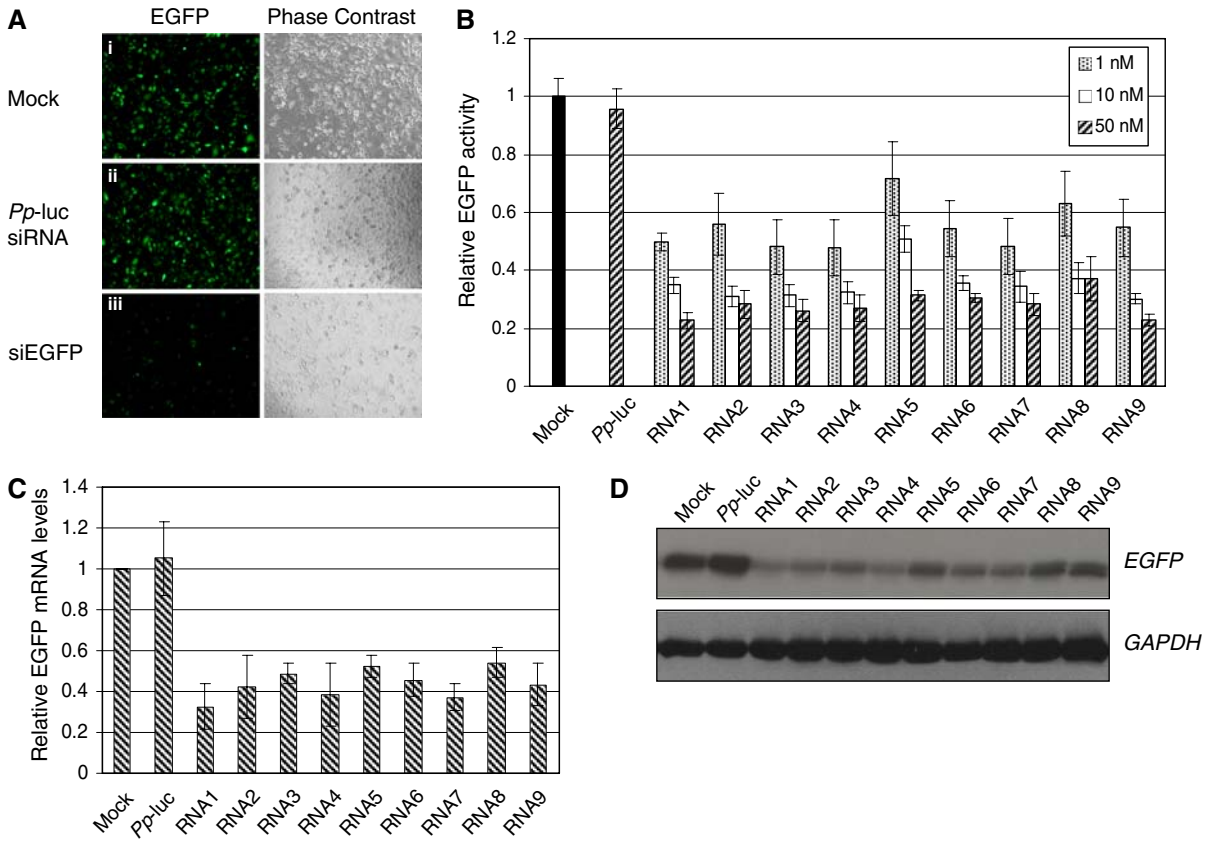

Fig. 1 Potency of unmodified and 2′-modified siRNAs (siEGFPs) targeting the exogenous EGFP gene in HeLa cells. EGFP-expressing HeLa cells were transfected with siRNAs at 50 nM, otherwise indicated. (**a**) Representative fluorescence (left panels) and phase contrast (right panels) images of EGFP-expressing HeLa cells at 65 h following transfection with siRNAs. (i) Mock-transfected cells; (ii) *Pp*-luc siRNA-transfected cells; (iii) unmodified siEGFP-transfected cells. *Pp*-luc siRNA which targets firefly luciferase served as a specificity control. (**b**) Quantitative analysis of EGFP silencing. EGFP-expressing HeLa cells were transfected with siRNAs at the indicated concentrations and harvested 65 h later, followed by EGFP fluorescence assay. The EGFP fluorescence measured in lysates prepared from siRNA-transfected cells was compared with that of mock-transfected cell lysate to determine the relative inhibition of EGFP gene expression. The EGFP activity of mock-transfected cells was set to 1. The results are means ± SD from three independent experiments. (**c**) Inhibition of EGFP mRNA expression. EGFP mRNA levels were measured at 65 h post-transfection by real-time RT-PCR, and normalized to GAPDH mRNA level. The normalized EGFP mRNA level of mock-transfected cells was set to 1. The results are means ± SD from two independent experiments. (**d**) Inhibition of EGFP protein expression. EGFP protein levels were monitored at 65 h post-transfection by Western blotting. The amount of GAPDH was used as a loading control

implying that they were acting through the RNAi pathway. Our data indicate that our selective 2′-modifications in one or both strands of the siRNA duplex permit effective RNAi-mediated gene silencing in HeLa cells.

Serum stability of unmodified versus selectively 2′-modified siRNAs

Unmodified siRNAs are unstable in serum due to degradation by nucleases (De Paula et al. 2007). Chemical modifications of siRNAs might therefore be necessary to achieve prolonged stability required for systemic siRNA delivery in vivo. Having verified the compatibility of our selective 2′-ribose modifications with the RNAi machinery, we next examined whether these modifications increased the stability of siRNAs in serum. The unmodified and 2′-modified siEGFPs containing the 5′-labeled guide strand were incubated in 10% human serum, and stability to serum-derived nuclease digestion was measured following various incubation times (Fig. 2). As expected, the unmodified siEGFP (RNA1) was rapidly degraded during the incubation in serum (Fig. 2a). siEGFPs RNA4 and RNA7, which have LNA and 2′-*O*-Me modifications, respectively, in the passenger strand, exhibited a slightly increased stability relative to the unmodified siEGFP (Fig. 2a). The other siEGFPs with the 2′-ribose modification in either the guide strand or both strands were degraded more gradually than siEGFPs RNA4 and RNA7 (Fig. 2a). These results indicate that the selective 2′-modifications are capable of protecting siRNAs against nuclease digestion in serum. Similarly, 2′-*O*-Me modifications at positions 1 and 2 from the 5′-ends of a siRNA duplex conferred resistance to nucleolytic degradation in serum (Hoerter and Walter 2007). However, those modifications of siRNA substantially reduced in vitro RISC-mediated target RNA cleavage, presumably due to impairment of RISC assembly and/or target RNA cleavage by the modified guide strand-loaded RISC.

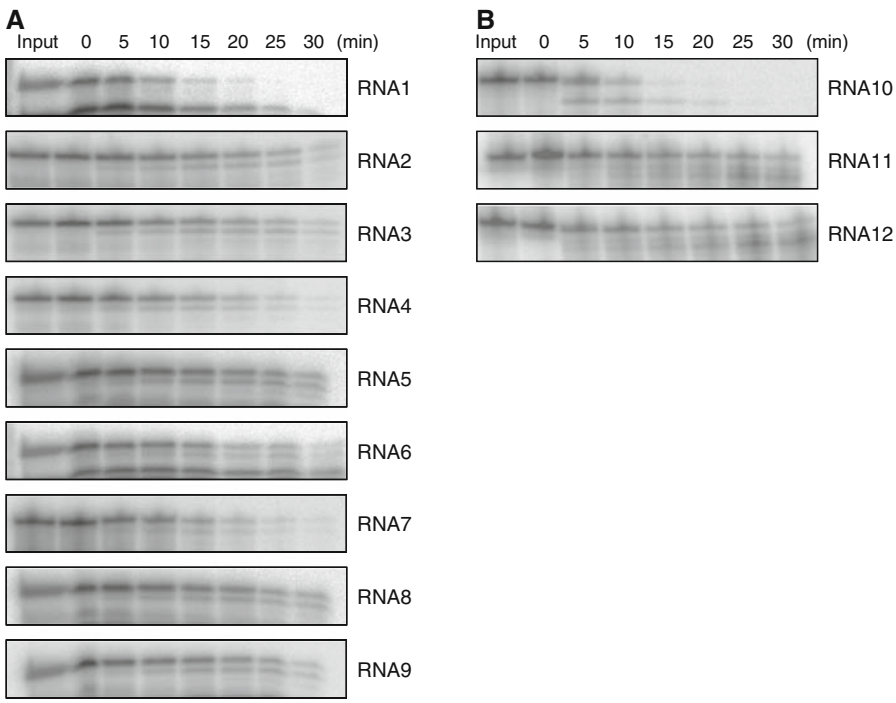

Fig. 2 Stability of unmodified and 2′-modified siRNAs in human serum. siRNAs containing the 5′-labeled guide strand were incubated in 10% human serum for 0, 5, 10, 15, 20, 25, and 30 min at 37°C, and then analyzed by electrophoresis on 8% denaturing polyacrylamide gels, followed by phosphorimage analysis. Equal amount of the labeled siRNA was loaded as an input control. The zero time point samples were treated with the stop mix prior to the addition of the labeled siRNA. (**a**) Serum stability of unmodified (RNA1) and 2′-modified siEGFPs (RNA2–RNA9). (**b**) Serum stability of unmodified (RNA10) and 2′-modified siRNAs (RNA11 and RNA12) directed against the Id-1 gene

To substantiate the generality of our finding, we designed a siRNA with the sequence corresponding to the Id-1 gene which encodes an inhibitor of basic helix-loop-helix transcription factors. Recently, an RNase A-like activity has been shown to be primarily responsible for initial degradation of siRNAs in mammalian serum by cleaving preferentially after pyrimidine residues in A/U-rich sequences close to the end of siRNAs (Haupenthal et al. 2006; Hoerter and Walter 2007; Turner et al. 2007). It is worth noting that the Id-1 siRNA duplex contains consecutive A/U residues at the ends of the molecule, rendering the regions vulnerable to the RNase A-like enzyme (Table 1). We modified differentially both strands of the Id-1 siRNA duplex with 2′-ribose modifications: a 2′-F modification at position 2 of the guide strand, and either a LNA at position 1 or a 2′-*O*-Me modification at position 2 of the passenger strand (Table 1). The unmodified and modified Id-1 siRNAs were assessed for their resistance to degradation in human serum as described in the above experiment. The unmodified siRNA (RNA10) was rapidly degraded within 20 min, whereas doubly 2′-modified siRNAs (RNA11 and RNA 12) were much more stable than the unmodified siRNA (Fig. 2b). Taken together, it is remarkable that as few as two 2′-ribose modifications in both strands of siRNAs, individually introduced at a particular position known to suppress off-target effects in each strand, can enhance the serum stability of siRNAs without a loss of activity.

Structural and thermodynamic properties of selectively 2′-modified siRNAs

To determine whether the selective 2′-modifications in this study alter the overall A-form helical geometry of siRNA duplex required for activity (Chiu and Rana 2003), circular dichroism (CD) spectra were measured for unmodified (RNA1) and four doubly modified siEGFPs (RNA5, RNA6, RNA8, and RNA9). The CD spectrum of the unmodified siEGFP showed the characteristic A-form pattern, with a strong positive peak at ~265 nm and a negative peak at ~210 nm (Fig. 3a). The CD intensities of siEGFPs RNA6 and RNA9 with a 2′-F-modified guide strand and a passenger strand containing either a LNA or 2′-*O*-Me, respectively, were increased (Fig. 3a), suggesting that these combinations of

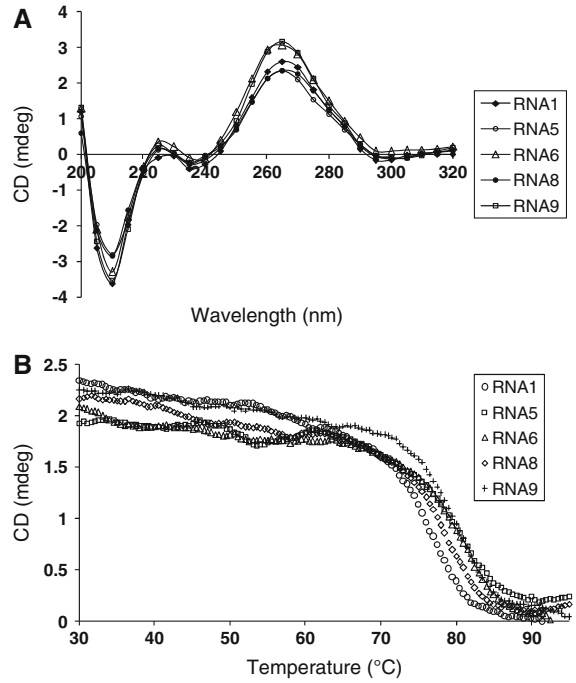

Fig. 3 Circular dichroism and thermal denaturation analysis of unmodified and doubly 2′-modified siEGFPs. (**a**) Circular dichroism (CD) spectra of siRNAs (2 μM) were obtained in duplicate in a buffer of 5 mM sodium phosphate (pH 7.2), 140 mM KCl, and 1 mM $MgCl_2$. (**b**) Thermal melting profiles of siRNAs (2 μM) were monitored by the change in hyperchromicity at 265 nm. The same buffer was used as for CD analysis. Melting temperatures (T_m) were calculated from two independent experiments by the first-order derivative method and are summarized in Table 2

2′-ribose modifications might slightly affect the base pair stacking interaction. However, the overall CD traces indicated an A-form helical geometry. On the other hand, no remarkable changes were observed in the CD spectra of the other siEGFPs (RNA5 and RNA8) compared with that of the unmodified siEGFP (Fig. 3a). These results indicate that the selective 2′-modifications introduced into both strands do not perturb the canonical A-form helical structure of the siRNA duplex, which correlates well with the ability of these modified siEGFPs to inhibit target-gene expression as efficiently as the unmodified siEGFP.

To examine whether our selective 2′-modifications stabilize siRNA duplexes, thermal melting profiles were obtained for unmodified and doubly modified siEGFPs (RNA5, RNA6, RNA8, and RNA9). Melting curves for the modified siEGFPs displayed only one transition, resembling that of the unmodified

Table 2 Thermodynamic parameters extracted from equilibrium melting curves[a]

Name	$-\Delta H^{o}$ (kcal/mol)	$-\Delta S^{o}$ (cal/mol * K)	$-\Delta G^{o}{}_{75^{\circ}C}$ (kcal/mol)[b]	T_m (°C)
RNA1	137.5 ± 11.4	368.8 ± 32.0	9.2 ± 0.2	76.25 ± 0.5
RNA5	120.0 ± 10.1	315.0 ± 27.6	10.5 ± 0.5	79.95 ± 0.9
RNA6	115.5 ± 12.8	302.8 ± 37.0	10.5 ± 0.2	79.75 ± 0.7
RNA8	139.0 ± 2.8	369.7 ± 7.2	10.4 ± 0.3	79.15 ± 0.8
RNA9	141.3 ± 10.5	377.0 ± 30.1	10.4 ± 0.0	78.65 ± 0.5

[a] Data are means ± SD of values calculated from two independent measurements

[b] ΔG^{o} was calculated at 75°C, because ΔG^{o} values are most accurate near the T_m of the duplex

siEGFP (RNA1) (Fig. 3b). All the modified siEGFPs exhibited improved thermal stability relative to the unmodified siEGFP, as measured by T_m values (Table 2). In addition, there was a slight increase in thermodynamic stability (ΔG^{o}) of the modified siEGFPs compared with the unmodified siEGFP (Table 2). Considering that the RNase A-like enzyme in mammalian serum recognizes and cleaves preferentially double-stranded RNA rather than single-stranded RNA (Libonati and Gotte 2004), it is conceivable that because of relatively low thermal or thermodynamic stability, the A/U-rich end of siRNA duplexes is transiently separated into single strands susceptible for degradation by the RNase A-like enzyme. Given the differences in the melting temperatures and free energies of the unmodified and 2′-modified siEGFPs, we suggest that thermal or thermodynamic stabilization of the ends particularly vulnerable to an RNase A-like activity by selective 2′-ribose modifications contributes to the observed prolonged stability of the doubly modified siRNAs in serum.

In summary, our present study focused on specific sites in either strand of the siRNA duplex that have been suggested to control strand-specific off-targeting activity (position 1 of the passenger strand) and alleviate off-target effects potentially induced by both strands (position 2 of either strand). Duplex siRNAs with the specific 2′-ribose modifications in both strands exhibited not only effective gene silencing activity, but also enhanced stability in serum. Additionally, these selective 2′-modifications increased the thermal and thermodynamic stability of siRNA duplexes, and did not alter the overall A-form helical geometry required for activity. Future in vivo experiments that aim to investigate pharmacokinetic properties, specificity, and immune responses will further prove the potential utility of our selective 2′-ribose modifications for therapeutic applications.

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