

Chemical modification of siRNAs to improve serum stability without loss of efficacy

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Abstract

Development of RNA interference as a novel class of therapeutics requires improved pharmacokinetic properties of short interfering RNA (siRNA). To confer enhanced serum stability to Sur10058, a hyperfunctional siRNA which targets survivin mRNA, a systematic modification at the 2'-sugar position and phosphodiester linkage was introduced into Sur10058. End modification of three terminal nucleotides by 2'-OMe and phosphorothioate substitutions resulted in a modest increase in serum stability, with 3' end modification being more effective. Alternating modification by 2'-OMe substitution significantly stabilized Sur10058, whereas phosphorothioate modification was only marginally effective. Through various combinations of 2'-OMe, 2'-F and phosphorothioate modifications that were directed mainly at pyrimidine nucleotides, we have identified several remarkably stable as well as efficient forms of Sur10058. Thus, our results provide an effective means to stabilize siRNA in human serum without compromising the knockdown efficiency. This advancement will prove useful for augmenting the in vivo potency of RNA interference.

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Gene silencing by RNA interference (RNAi) has emerged as a powerful method that is useful for the study of functional genomics, drug target identification and validation, and therapeutic development [1,2]. In the process of RNAi, double stranded RNA is first cleaved into 21–25 nucleotide (nt) RNA duplexes by the RNase III enzyme, named dicer [3–5]. The resulting siRNAs are then associated with a protein complex which is called the RNA-induced silencing complex (RISC) [6]. The siRNA duplex is unwound by the putative helicase in RISC through an ATP-dependent process [7] and the single stranded RNA subsequently guides the activated RISC to homologous mRNA targets. After binding to target mRNA, the Ago2 protein in the RISC acts as a slicer and functions to cleave target mRNA

[8–10]. Degradation of cleaved target RNA is accomplished by ribonuclease activity as a result of the lack of protection by 5' caps or poly(A) tails.

The execution of successful RNAi experiments depends upon multiple factors. Specifically it is important to: (1) design and identify effective and specific siRNA sites, (2) enhance pharmacokinetic properties, including serum stability, and (3) to perform efficient and specific delivery of siRNA to the desired target cell types. As a first step toward developing siRNA therapeutics, we have screened 82 siRNAs which specifically target survivin. This important protein has been previously implicated in both anti-apoptosis and the regulation of cytokinesis [11–15]. Screening of 82 siRNAs resulted in the identification of several highly effective siRNA inhibitors including Sur10058. The identified inhibitors were hyperfunctional and were capable of silencing at the sub-nanomolar level in vitro (IC₅₀ values are below 100 pM). Due to the potential promise of Sur10058 as

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an effective siRNA inhibitor, we were interested to study this particular siRNA in detail. In order to develop Sur10058 as an efficient anti-cancer agent, we subsequently introduced various chemical modifications into Sur10058 and tested their effects on the serum stability of Sur10058. Since unmodified siRNA duplexes are highly unstable especially in serum, it is often desirable to enhance serum and intracellular stability of siRNA duplexes by chemical modification as an effective means to increase in vivo potency.

In addition to modification at phosphodiester linkages by phosphorothioate (PS) substitution, chemical modifications, such as 2'-sugar modification by 2'-OMe, 2'-F, and locked nucleic acid (LNA) substitutions have been widely used to enhance serum and intracellular stability of siRNA [16–23]. These studies have shown that in principle, chemical modification is capable of enhancing the serum stability of siRNA without compromising and affecting the functionality of siRNA. Furthermore, it has been reported that when transfected into cells, modified siRNA was capable of maintaining the knockdown effect for a longer period as compared to unmodified siRNA [17,21]. In the case of in vivo experiments, when a fully modified siRNA targeting hepatitis B virus was introduced into mice by the hydrodynamic injection method, the modified siRNA was significantly more effective in reducing serum HBV DNA and hepatitis B surface antigen than the unmodified siRNA [24]. In addition, when modified siRNA was formulated into a stable nucleic-acid-lipid particle (SNALP) by using a liposome and was injected into mice intravenously, the modified siRNA provided a high improvement of in vivo stability as compared to unmodified siRNA. It should be particularly noted that unmodified siRNA caused an immune stimulation, whereas modified siRNA did not induce interferon α and inflammatory cytokines [25]. Taken together, these results have clearly demonstrated that the use of modified siRNA is capable of greatly improving pharmacokinetic properties.

In this study, we have introduced various combinations of chemical modifications into Sur10058 in order to identify the modified structures which can greatly enhance its serum stability without reducing its knockdown efficiency. In contrast to the unmodified Sur10058 which was rapidly degraded in human serum, we have identified several modified structures of Sur10058 which exhibited remarkably enhanced serum stability. Collectively, our results presented here will serve as a useful guideline for the design of stable siRNA.

Materials and methods

RNA and DNA oligonucleotides. 2'-OMe-, 2'-F- and phosphorothioate-modified siRNA duplexes were synthesized by Prologo (Boulder, CO) and IDT (Coralville, IA). All siRNA duplexes have the sense strand sequence of 5'-AAG GAG AUC AAC AUU UUC A dTdT-3'. The 19 nucleotides represent the target sequence in survivin (NM_001168) and dTdT is a 2-nt 3' overhang. The antisense strand was composed of nucleotides that are complementary to the target sequence and the dTdT

3' overhang sequence. All siRNAs were resuspended in DEPC-treated water to give the final concentration of 30 μ M. DNA oligonucleotides were obtained from Bioneer (Daejeon, Republic of Korea).

Cell culture. HeLa cells were maintained at 37 °C under 5% CO₂ in RPMI 1640 medium (Gibco-BRL/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotics (100 U of penicillin per ml and 100 μ g of streptomycin per ml).

Transfection. A single day prior to transfection, HeLa cells were plated in 6-well culture dishes at a density of 2.5×10^5 cells per well. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) with methods as recommended by the manufacturer. Briefly, cells were washed once with Opti-MEM (Gibco-BRL/Invitrogen, Carlsbad, CA) and 500 μ l of Opti-MEM was added to each well. For each transfection, 3.5 μ l of lipofectamine was mixed with 250 μ l of Opti-MEM and incubated for 5 min at room temperature. In a separate tube, siRNA was added to 250 μ l of Opti-MEM and the solution was added to the lipofectamine mixture. In order to allow complex formation, the siRNA mixture was incubated for an additional 20 min at room temperature. The solution was subsequently added to the cells in the 6-well plate, giving an end volume of 1 ml. Cells were incubated at 37 °C in the presence of the transfection solution for 6 h. The Opti-MEM medium containing the complexes was then replaced with 2 ml of standard growth media and cultured at 37 °C for 18 h. Twenty-four hours after transfection, total RNA was isolated from transfected cells by using the RNeasy mini kit (Qiagen, Hilden, Germany).

Northern blot analysis. Northern blot analysis was performed as previously described [26]. Briefly, total RNA (8 μ g per lane) was fractionated on a 1% agarose gel containing formaldehyde. Resolved RNA was blotted onto Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ) via capillary transfer with 20 \times SSC. After UV cross-linking, blots were maintained at 65 °C in a prehybridization buffer (1% bovine serum albumin, 2 mM EDTA, 0.5 M sodium phosphate, pH 7.2, and 7% NaDodSO₄) for 1 h [27]. Blots were subsequently hybridized with a ³²P-labeled cDNA probe for 18 h. The blot was washed twice at room temperature for 10 min in 1 \times SSC, 0.1% NaDodSO₄, and subsequently, at 65 °C for 1 h in 0.5 \times SSC, 0.1% NaDodSO₄. During the initial hybridization, the membranes were first probed with survivin cDNA and were subsequently rehybridized with positive control β -actin cDNA which effectively monitored the loading of total RNA in each lane. The probes were synthesized from 832 bp within the survivin cDNA (Accession No. NM_001168; nucleotide 756–1587) and 788 bp of the β -actin cDNA (Accession No. NM_001101; nucleotide 433–1220) using a random primer labeling kit (Stratagene, La Jolla, CA), and [α -³²P]dCTP (Amersham Biosciences, Piscataway, NJ) [28].

Real-time quantitative PCR analysis. Triplicate real-time PCR were performed in 384-well plates using an Applied Biosystems Prism 7900 Sequence Detection System (Applied Biosystems). Each reaction mixture (20 μ l) consisted of 10 μ l of SYBR Green Master Mix (Applied Biosystems) and 0.4 μ l of forward and reverse primers (10 pmol/ μ l) that were specific for survivin and GAPDH. PCR conditions were as follows; initial incubation at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The sequences of the primers specific for survivin were: forward primer, 5'-GCACCACTTC CAGGGTTTAT-3'; reverse primer, 5'-CTCTGGTGCCACTTTCAA GA-3'. The sequence for the GAPDH primers were 5'-TGCACCACTCA ACTGCTTAGC-3' (forward primer), and 5'-GGCATGGACTGTGGTC ATGAG-3' (reverse primer). Serial dilutions (1, 0.5, and 0.25) of control cDNA were included in order to generate relative standard curves for survivin and GAPDH. The amount of survivin transcript was divided by that of GAPDH as a means to obtain normalized expression levels.

Serum stability. siRNA duplex (9 μ g) was incubated at 37 °C in 90 μ l of 10% human serum. Aliquots of 12 μ l were taken at various time points and immediately frozen at –70 °C. Samples (2.5 μ l) were analyzed on 15% non-denaturing polyacrylamide gels in TBE buffer and were visualized on a UV-transilluminator subsequent to EtBr staining.

Nuclear staining. HeLa cells (100,000 cells/well) were plated in 6-well culture dishes and the next day, cells were transfected with unmodified and modified Sur10058 siRNAs (2 nM). Four days after transfection, cells

were stained with DAPI (Roche Applied Science, Indianapolis, IN) at a concentration of 1 $\mu\text{g}/\text{ml}$ dissolved in methanol for 15 min at 37 °C. Afterward, cells were washed once with methanol, 1 ml of PBS was added to each well, and DAPI-stained nuclei were observed by using a Nikon Eclipse TS100 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a ProgRes C10 digital camera (Jenoptik laser, Echting/Munich, Germany).

Western blot analysis. HeLa cells (100,000 cells/well) were plated in 6-well culture dishes and the next day, cells were transfected with unmodified and modified Sur10058 siRNAs (2 nM). Seventy-two hours after transfection, cells were harvested and total protein extracts were prepared using PRO-PREP protein extraction solution (Intron Biotechnology, Seongnam, Republic of Korea) following the manufacturer's instruction. Total protein extracts (40 μg) were electrophoresed on 4–20% precast protein gel (Pierce, Rockford, IL) and transferred to Protran Nitrocellulose membrane (S&S, Dassel, Germany). Immunoblotting was carried out with an anti-survivin monoclonal antibody D-8 (Santa Cruz, CA) using the rabbit anti-mouse IgG/HRP (Pierce, Rockford, IL) as a secondary antibody. Peroxidase activity was detected by using the ECL plus Western blotting reagent (Amersham Biosciences, Piscataway, NJ).

Results and discussion

Effect on stability and RNAi activity of 5' and 3' end modification

We were interested to identify specific chemical modifications which affect the structure of Sur10058 and function to greatly enhance its serum stability. In our first line of investigation, we examined the effect of nucleases on the stability of siRNA in human serum by introducing 2'-OMe or PS modification into three terminal nucleotides at 5' and 3' ends of Sur10058 (Fig. 1).

The unmodified Sur10058 was very unstable in 10% human serum. As a result, unmodified Sur10058 was mostly degraded after 1 h incubation at 37 °C (Fig. 2B). When three terminal nucleotides at the 5' end were modified with 2'-OMe or PS substitution, the stability of modified siRNA was not substantially increased (Fig. 2B, Sur10058-PS-5, and -Me-5). On the other hand, 3' modifications to the

end of the siRNA resulted in significant changes in stability. Specifically, the intact siRNA was retained at significant levels subsequent to 3 h incubation at 37 °C. Furthermore, intact siRNA was also detected even after 6 h incubation in serum (Fig. 2B, Sur10058-Me-3, and -Me-53). These results suggested that the 3' terminal modification is more efficient for enhancing the serum stability of Sur10058 and supports the supposition that 3'-specific exonuclease will act on the degradation of Sur10058 in human serum.

Alternating modification significantly stabilizes siRNA

Because the modification of three terminal nucleotides showed only some increase in the serum stability, additional nucleotides along the length of siRNA were modified with 2'-OMe or PS substitution. In the previous study, it has been reported that alternating modification with 2'-OMe could increase the serum stability of siRNA without reducing their knockdown efficiency [17]. Accordingly, we have examined the effect of alternate modification with 2'-OMe or PS substitutions on the serum stability of Sur10058.

As shown in Fig. 3B (Sur10058-Me-33), when a stretch of three nucleotides was modified by 2'-OMe alternately with two or three unmodified nucleotides, intact siRNA was present at a significant amount even after a 24 hr incubation period in 10% human serum. The modified Sur10058-Me-33 has the structure wherein 2'-OMe-modified nucleotides of sense and antisense strands face one another. In the case of Sur10058-Me-23, the antisense strand is identical to that of Sur10058-Me-33 but 2'-OMe-modified nucleotides of the sense strand are present alternately with those of the antisense strand. As demonstrated in Fig. 3B, Sur10058-Me-23 was less stable than Sur10058-Me-33. This reduction in stability was most likely due to the small number of 2'-OMe-modified nucleotides of the sense strand in Sur10058-Me-23 (the number of 2'-OMe-modified nucleotides was reduced from 12 in Sur10058-Me-33 to 7 in Sur10058-Me-23). Alternatively, this decrease in stability could have resulted from a difference in RNA structures between them.

Because Sur10058-Me-23 and Sur10058-Me-33 showed a significantly reduced knockdown efficiency as compared to unmodified Sur10058 (Figs. 3C and D), we next examined the structure of siRNAs wherein modified nucleotides are alternately present at every other position along the length of siRNA. Sur10058-Me-AL1 to AL4 have structures which differ in the position of nucleotide to initiate 2'-OMe modification and Sur10058-PS-AL1 contains alternating modification with PS substitution (Fig. 3A). As it can be seen in Fig. 3B, all four of the siRNAs that were alternately modified with 2'-OMe showed a great improvement in stability. In contrast to these data, modifications with PS substitution to Sur10058-PS-AL1 did not exhibit the substantial enhancement in serum stability (Fig. 3B). Since it was reported that the extensive modification with

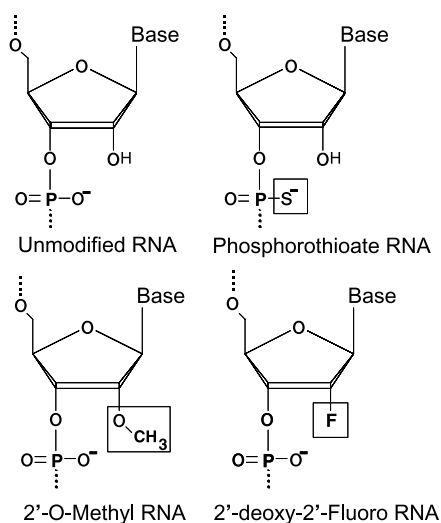


Fig. 1. Structure of modified RNAs used in this study. Modified moieties are boxed.

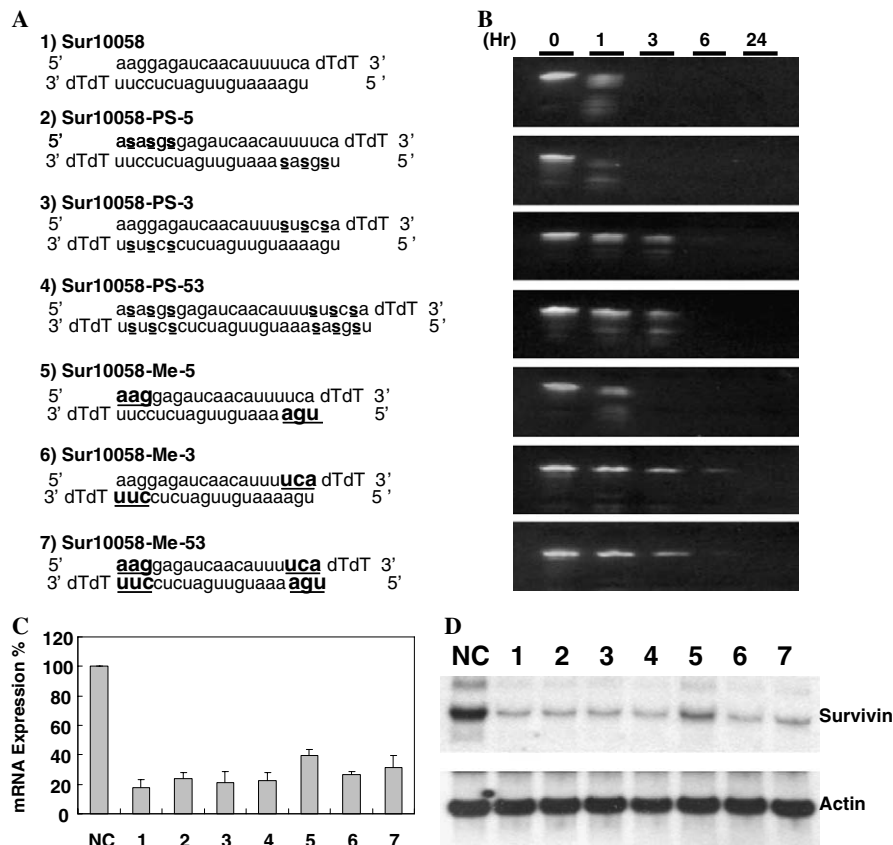


Fig. 2. Stability and knockdown efficiency of siRNAs modified at 5' and 3' terminal three nucleotides by 2'-OMe and PS substitutions. (A) Name, sequence, and chemical modifications of unmodified and modified Sur10058. Lowercase letters = unmodified RNA, underlined bold letters = 2'-OMe, small underlined s between nucleotides = phosphorothioate. (B) Serum stability of siRNAs. Unmodified and modified siRNAs were incubated in 10% human serum at 37 °C for 0, 1, 3, 6, or 24 h and aliquots were analyzed on 15% native polyacrylamide gels. (C). Inhibition of survivin mRNA expression in HeLa cells transfected with modified Sur10058 siRNAs (2 nM). Knockdown efficiency was quantitatively measured by real-time PCR analysis. Survivin mRNA expression levels were obtained subsequent to normalization with constitutively expressed GAPDH mRNA. (D) Northern blot analysis of total RNA extracts. Non-silencing siRNA (NC) and unmodified Sur10058 were transfected along with modified siRNAs to serve as negative and positive controls, respectively. Blots were first probed with survivin cDNA and were subsequently reprobed with β -actin cDNA for visualization of lane-to-lane variation.

PS caused a cytotoxicity, it appears that PS modification should only be used with careful attention and the appropriate precautionary measures [16,19].

Interestingly, AL2 and AL4 showed an approximate 2-fold decrease in their knockdown efficiency, whereas the silencing efficiency of AL1 and AL3 was comparable to unmodified Sur10058. Consistent with a previous report, these data showed that the alternate modifications which initiate from the 5' terminal nucleotide of antisense strand RNA provide better knockdown efficiency than the alternate modifications which initiate from the second nucleotide at the 5' end of antisense strand [17].

Enhanced stability of siRNAs containing fully modified sense strand

Since it has been reported that modification to the sense strand is better tolerated than the modification of the antisense strand, we next examined the stability and efficiency of siRNAs containing the sense strand fully modified with

2'-OMe. As can be seen in Fig. 4B (Sur10058-Me-S), when only two 3' terminal nucleotides of the antisense strand were modified, the stability was not greatly increased in comparison to the alternating modification. Even in the case of Sur10058-Me-S-AS, wherein the antisense strand was modified with nine 2'-OMe pyrimidine nucleotides, the stability in human serum was not greatly increased when compared to Sur10058-Me-S. In Sur10058-Me-S-AS, 9th and 10th uridine nucleotides from the 5' end of antisense strand were not modified since the cleavage of target mRNA occurs in the center of the antisense strand [29,30]. As a result, there is a possibility that 2'-OMe modification in that region may inhibit the target RNA cleavage reaction.

In addition to 2'-OMe modification, 2'-F and PS modifications were introduced into the antisense strand to give rise to Sur10058-Me-F and Sur10058-Me-SP. As it can be seen in Fig. 4B, the stability of both siRNA duplexes was greatly increased and significant fractions of intact siRNA remained even after 48 h incubation in human serum.

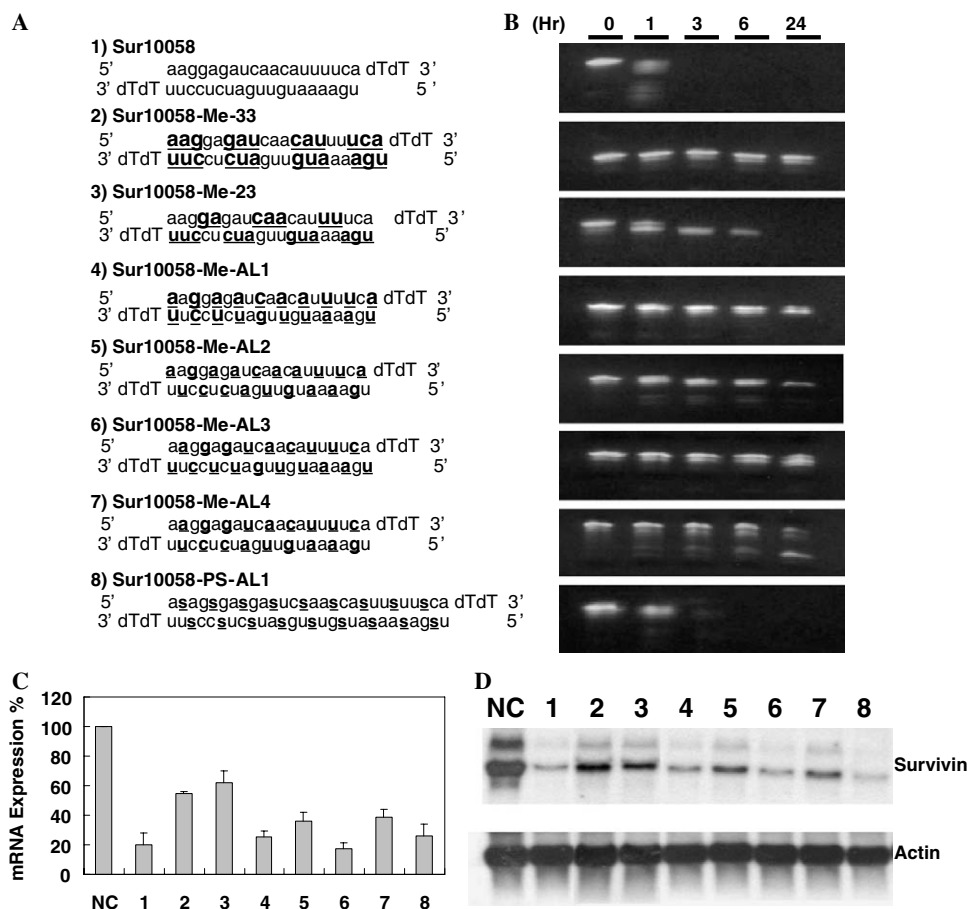


Fig. 3. Enhanced stability of siRNAs modified by 2'-OMe and PS substitutions at alternating nucleotide positions. (A) Name, sequence, and chemical modifications of unmodified and modified siRNAs. Lowercase letters = unmodified RNA, underlined bold letters = 2'-OMe, small underlined s between nucleotides = phosphorothioate. (B) Serum stability of siRNAs. Unmodified and modified siRNAs were incubated in 10% human serum at 37 °C for 0, 1, 3, 6, or 24 h. Aliquots were withdrawn at indicated time points and were analyzed by electrophoresis on 15% native polyacrylamide gels. (C) Knockdown efficiency was quantitatively measured by Real-time PCR analysis and survivin expression levels were normalized to those of GAPDH. (D) Potency of modified siRNAs targeting survivin. HeLa cells were transfected with modified siRNAs (2 nM), total RNA was extracted, and subjected to Northern blot analysis. Non-silencing siRNA (NC) and unmodified Sur10058 were included as negative and positive controls, respectively. A probe specific for β -actin was used to monitor equal loading between lanes.

Furthermore, the knockdown efficiency was comparable to that of unmodified Sur10058 in both cases. On the other hand, the modification of 9 pyrimidine nucleotides of antisense strand with 2'-OMe in Sur10058-Me-S-AS resulted in significant decrease in the gene-silencing efficiency (Figs. 4C and D). These results contrast to the previous data which showed that the modification of either sense or antisense strand fully with 2'-OMe substitution made siRNA non-functional [17,29]. Even in case where the sense strand was fully modified with 2'-OMe, the knockdown efficiency was not reduced if the antisense strand was modified with 2'-F or PS substitutions. However, when both the sense strand and the antisense strand were extensively modified with 2'-OMe, as in Sur10058-Me-S-AS, the knockdown efficiency was greatly reduced. Consistent with our results, it was recently reported that one out of four siRNAs targeting PTEN mRNA was as potent as unmodified siRNA duplex even when the sense strand was fully modified with 2'-OMe [31]. Interestingly, full modification of sense strand with 2'-OMe was better tolerated in 20-bp blunt-end

siRNA duplexes targeting the same four sites and in some cases, complete modification with PS in either or both strand was tolerated. Taken together, these results demonstrated that the extensive modification of the sense strand with 2'-OMe is a viable option and is dependent upon the siRNA structure and the types of chemical modification that are directed towards the complementary antisense strand.

Identification of the stable as well as efficient modification through various combinations of pyrimidine modifications

Since RNA can be sufficiently stabilized by only modifying pyrimidine residues [20,21], we examined the stability and knockdown efficiency of siRNAs modified with 2'-F-pyrimidine and 2'-OMe-pyrimidine nucleotides at various positions along the entire length of siRNA duplexes.

As shown in Fig. 5B, in the case where all pyrimidine nucleotides of the sense and antisense strands were substituted with 2'-OMe-pyrimidine, the serum stability of

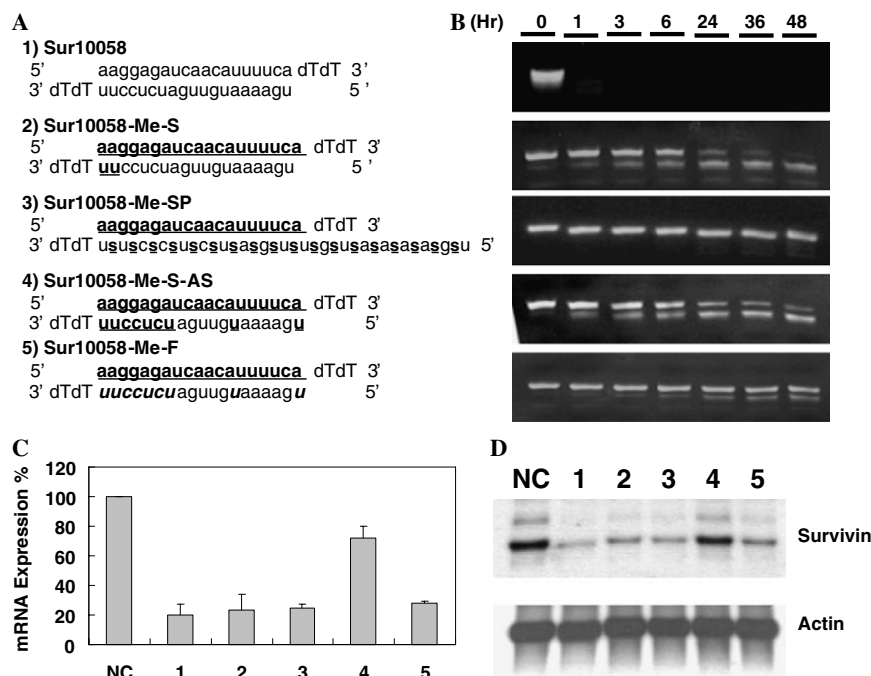


Fig. 4. Enhanced stability of modified siRNAs containing a fully modified sense strand. Sense strand RNA, which was fully modified with 2'-OMe substitution, was duplexed with 2'-OMe-, 2'-F-, and PS-modified antisense strand RNA. (A) Name, sequence, and chemical modifications of unmodified and modified siRNAs. Lower case letters = unmodified RNA, underlined bold letters = 2'-OMe, italic letters = 2'-F, small underlined s between nucleotides = phosphorothioates. (B) Serum stability. Unmodified and modified siRNAs were incubated in 10% human serum at 37 °C for 0, 1, 3, 6, 24, 36, and 48 h. Aliquots were analyzed by electrophoresis on 15% native polyacrylamide gels. (C) Potency of modified siRNAs targeting survivin was analyzed by real-time PCR analysis. Survivin expression levels were normalized to those of GAPDH. (D) HeLa cells were transfected with siRNAs (2 nM). Twenty-four hours post-transfection, total RNA was isolated and subjected to Northern blot analysis. Non-silencing siRNA (NC) and unmodified Sur10058 served as negative and positive controls, respectively. A β -actin probe was used as a control to monitor equal loading of samples between lanes.

Sur10058-Me-Py1 was greatly increased. It is important to note that even after a 48 h incubation in human serum, most siRNA duplexes remained intact. However, concomitant to this remarkable increase in stability, a great reduction in the knockdown efficiency was observed (Figs. 5C and D). These data were consistent to the case of Sur10058-Me-S-AS wherein the efficiency was greatly reduced when both the sense and antisense strands were extensively modified with 2'-OMe.

Since the modification of the antisense strand has a greater effect on the reduction of knockdown efficiency, the number of 2'-OMe pyrimidine in the antisense strand was reduced from a total of 11 in Sur10058-Me-Py1 to 5 in Sur10058-Me-Py2. In this particular case, the silencing efficiency was vastly improved, but at the same time, the stability of Sur10058-Me-Py2 was reduced. In this case, intact siRNA was not substantially evident subsequent to a 24 h incubation in serum (Fig. 5B). With the exception of 2'-OMe pyrimidines, even when the remaining nucleotides were modified with PS substitution, there was no evident increase in stability (Fig. 5B; Sur10058-Me-Py3). Since the cleavage of target mRNA occurs in the center portion of antisense strand, every pyrimidine residue except the 9th and 10th uridine of the antisense strand was modified with 2'-OMe in Sur10058-Me-Py4. In this case, the stability was enhanced but the knockdown efficiency was

greatly reduced; bearing similarity to the other cases where both sense and antisense strands were extensively modified with 2'-OMe.

However, when the antisense strand was modified with 2'-F-pyrimidine in Sur10058-Me-Py5 and Sur10058-Me-Py6, the stability was greatly increased and these modifications did not reduce knockdown efficiency. It is important to note that these modifications to the antisense strand did not have deleterious effects on knockdown ability like the case of Sur10058-Me-F which contained a completely modified sense strand. Taken together, these results clearly indicated that 2'-F and PS modifications were better tolerated in antisense strand than 2'-OMe modification, especially when the sense strand was extensively modified with 2'-OMe substitutions.

Stabilized Sur10058 siRNAs are as potent as unmodified Sur10058

To compare knockdown efficiencies more accurately, transfection was carried out with much lower concentrations of siRNA duplexes. Considering both increased stability and maintenance of knockdown efficiency, we have chosen Sur10058-Me-SP, Sur10058-Me-F, Sur10058-Me-Py5, and Sur10058-Me-Py6 for titration experiments. Although Sur10058-Me-Py1 was the most stable structure,

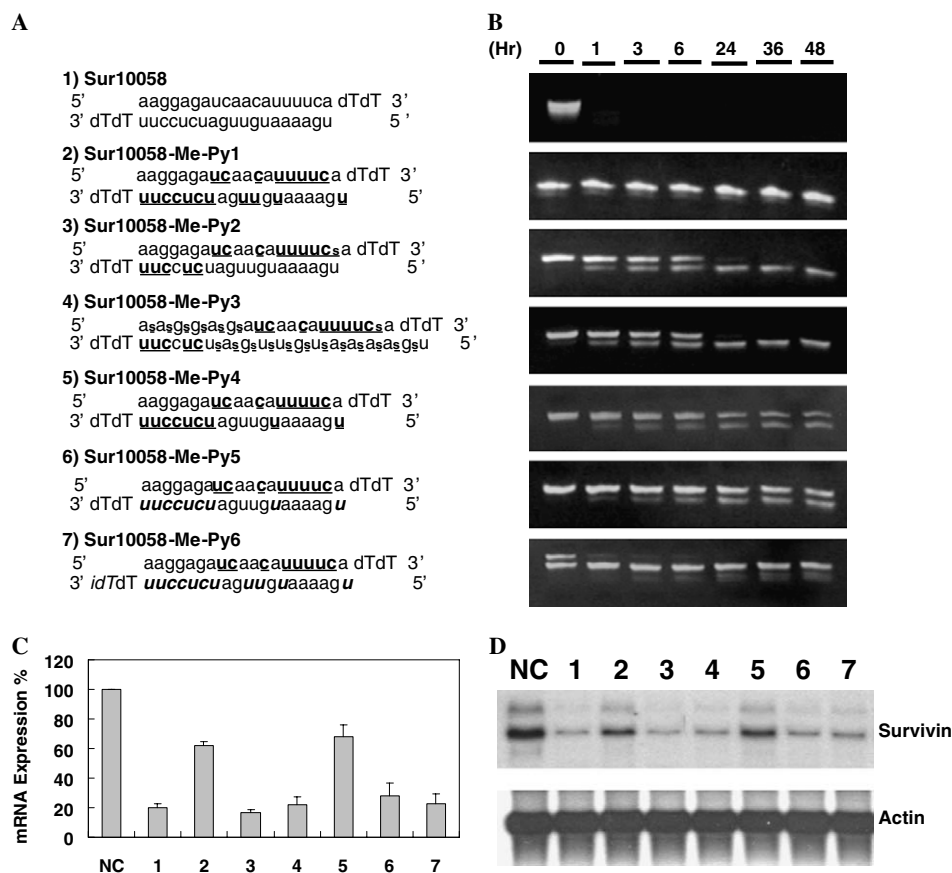


Fig. 5. Systematic analysis of pyrimidine-modified siRNAs. Various combinations of modifications including 2'-OMe, 2'-F, and PS substitutions at pyrimidine nucleotides were used to identify stable as well as efficient modifications of Sur10058. (A) Name, sequence, and chemical modifications of Sur10058. Lowercase letters = unmodified RNA, underlined bold letters = 2'-OMe, italic letters = 2'-F, small underlined s between nucleotides = phosphorothioates, *idT* = inverted deoxythymidine in Sur10058-Me-Py6. (B) Serum stability. Unmodified and modified Sur10058 siRNAs were incubated in 10% human serum at 37 °C for 0, 1, 3, 6, 24, 36, and 48 h. Aliquots were analyzed by electrophoresis on 15% native polyacrylamide gels. Lower bands appearing in Sur10058-Me-Py2, -Py3, -Py4, and -Py5 after hours of incubation in serum seem to be a degradation product. It was not a denatured RNA molecule because single stranded RNA moved much faster than the lower band under our condition. In the case of Sur10058-Me-Py6 containing inverted deoxythymidine on 3'-end of antisense strand, a lower band appeared immediately after incubation in serum, indicating that it is not a degradation product nor a denatured single stranded RNA. It is likely that an inverted deoxythymidine residue may have something to do with altered mobility of Sur10058-Me-Py6 in the presence of serum since another modified Sur10058 siRNA containing inverted deoxythymidine residues on 3'-end of both sense and antisense strands also exhibited faster migration patterns under same conditions (data not shown). (C) Knockdown efficiency of modified siRNAs targeting survivin was analyzed by Real-time PCR. HeLa cells were transfected with modified siRNAs (2 nM). Expression levels obtained for GAPDH were used for normalization of survivin expression. (D) Total RNA was isolated from HeLa cells transfected with siRNAs (2 nM). Eight µg of total RNA was then subjected to Northern blot analysis. Non-silencing siRNA (NC) and unmodified Sur10058 were negative and positive controls, respectively. A β-actin probe was used as a means to assess loading variations between lanes.

it did not maintain knockdown efficiency as well as the aforementioned examples. As can be seen in Fig. 6, all four stabilized siRNAs were as potent as unmodified Sur10058 with IC₅₀ values around 50 pM. These results demonstrate that the dramatic increase in serum stability of siRNAs can be achieved without loss of knockdown efficiency depending on the structure of modified RNA.

Chemically modified Sur10058 siRNAs induce dramatic morphological changes including the increase in cell size and multinucleation

To determine whether chemically modified Sur10058 siRNAs can cause phenotypic changes, we first examined the effects of siRNA treatments on the reduction of sur-

vivin protein by Western blot. As shown in Fig. 7A, unmodified and all four chemically modified Sur10058 siRNAs effectively downregulated survivin protein levels 72 h after transfection. More importantly, the treatment with all four chemically modified siRNAs resulted in pronounced morphological changes; 4 days post-transfection, most transfected cells became abnormally gigantic and multinucleated, indicating that their cell division was severely inhibited but their nuclei continued to divide (Fig. 7B). It is also important to note that the number of cells is much less in survivin siRNA-treated cells compared to non-silencing siRNA-treated cells, suggesting that the treatment of survivin-specific siRNAs caused growth inhibition and apoptosis (11–15). These results demonstrated that like unmodified Sur10058

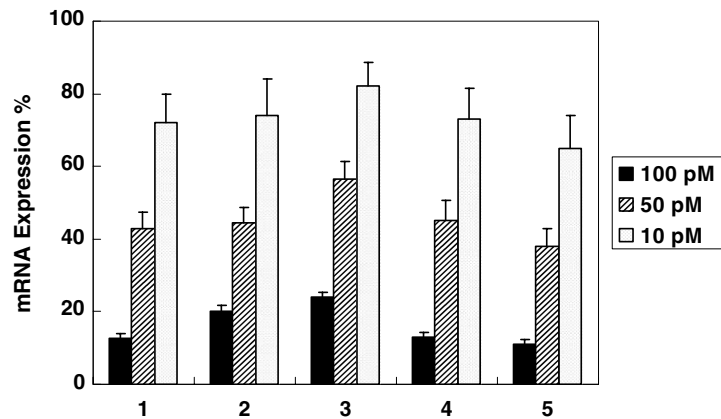


Fig. 6. Potency of four stabilized Sur10058 siRNAs. HeLa cells were transfected with siRNAs at 10, 50, and 100 pM. Knockdown efficiency was analyzed by real-time PCR. 1, Sur10058; 2, Sur10058-Me-SP; 3, Sur10058-Me-F; 4, Sur10058-Py5; and 5, Sur10058-Py6.

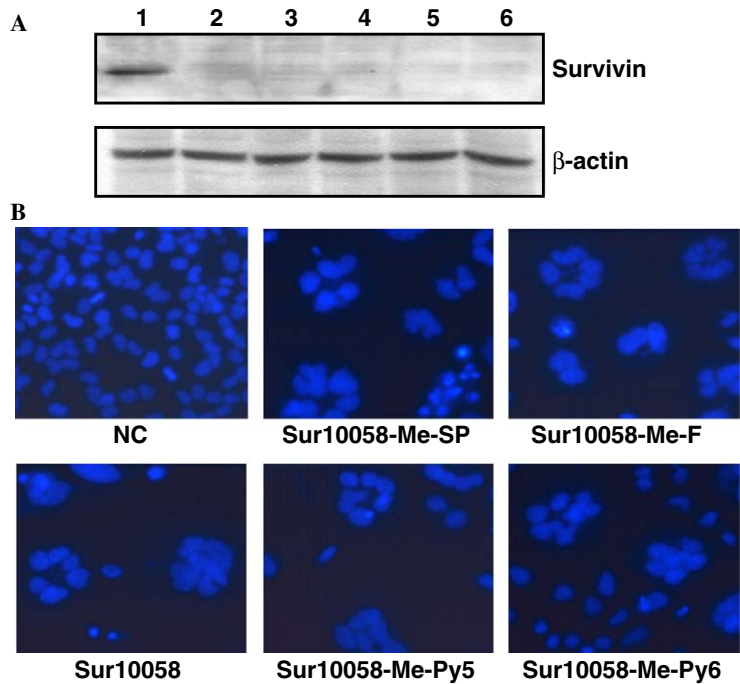


Fig. 7. Chemically modified Sur10058 siRNAs can induce morphological changes. (A) Knockdown of survivin protein expression. Total cellular proteins were isolated from HeLa cells transfected with siRNAs (2 nM) and 40 µg of protein was subjected to Western blot analysis. Lane 1, non-silencing siRNA; lane 2, Sur10058; lane 3, Sur10058-Me-SP; lane 4, Sur10058-Me-F; lane 5, Sur10058-Me-Py5; and lane 6, Sur10058-Me-Py6. (B) Nuclei morphology as analyzed by DAPI staining 4 days after transfection with unmodified and chemically modified Sur10058 siRNAs (2 nM).

siRNA, chemically modified siRNAs can downregulate survivin mRNA and protein which can be translated into dramatic phenotypic changes. In summary, we systematically analyzed approaches that were directed towards achieving an optimized balance between stability and knockdown efficiency of Sur10058. We accomplished this goal by incorporating various combinations of chemical modifications into Sur10058 via various substitutions that were designed to greatly increase the stability without reducing the knockdown efficiency. In the future, in vivo experiments that are designed to explore

pharmacokinetic properties and immune responses will further prove the potential utility of the stabilized siRNA as an effective therapeutic agent.

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References

- [1] Y. Dorsett, T. Tuschl, siRNAs: applications in functional genomics and potential as therapeutics, *Nat. Rev. Drug Discov.* 3 (2004) 318–329.
- [2] G.J. Hannon, J.J. Rossi, Unlocking the potential of the human genome with RNA interference, *Nature* 431 (2004) 371–378.
- [3] E. Bernstein, A.A. Caudy, S.M. Hammond, G.J. Hannon, Role for a bidentate ribonuclease in the initiation step of RNA interference, *Nature* 409 (2001) 363–366.
- [4] P. Provost, D. Dishart, J. Doucet, D. Frendewey, B. Samuelsson, O. Radmark, Ribonuclease activity and RNA binding of recombinant human Dicer, *EMBO J.* 21 (2002) 5864–5874.
- [5] H. Zhang, F.A. Kolb, V. Brondani, E. Billy, W. Filipowicz, Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP, *EMBO J.* 21 (2002) 5875–5885.
- [6] S.M. Hammond, S. Boettcher, A.A. Caudy, R. Kobayashi, G.J. Hannon, Argonaute2, a link between genetic and biochemical analyses of RNAi, *Science* 293 (2001) 1146–1150.
- [7] A. Nykanen, B. Haley, P.D. Zamore, ATP requirements and small interfering RNA structure in the RNA interference pathway, *Cell* 107 (2001) 309–321.
- [8] G. Meister, M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, T. Tuschl, Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs, *Mol. Cell* 15 (2004) 185–197.
- [9] J. Liu, M.A. Carmell, F.V. Rivas, C.G. Marsden, J.M. Thomson, J.J. Song, S.M. Hammond, L. Joshua-Tor, G.J. Hannon, Argonaute2 is the catalytic engine of mammalian RNAi, *Science* 305 (2004) 1437–1441.
- [10] J.J. Song, S.K. Smith, G.J. Hannon, L. Joshua-Tor, Crystal structure of Argonaute and its implications for RISC slicer activity, *Science* 305 (2004) 1434–1437.
- [11] D.C. Altieri, Validating survivin as a cancer therapeutic target, *Nat. Rev. Cancer* 3 (2003) 46–54.
- [12] F. Li, G. Ambrosini, E.Y. Chu, J. Plescia, S. Tognin, P.C. Marchisio, D.C. Altieri, Control of apoptosis and mitotic spindle checkpoint by survivin, *Nature* 396 (1998) 580–584.
- [13] G. Ambrosini, C. Adida, G. Sirugo, D.C. Altieri, Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting, *J. Biol. Chem.* 273 (1998) 11177–11182.
- [14] A.G. Uren, L. Wong, M. Pakusch, K.J. Fowler, F.J. Burrows, D.L. Vaux, K.H. Choo, Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype, *Curr. Biol.* 10 (2000) 1319–1328.
- [15] H. Uchida, T. Tanaka, K. Sasaki, K. Kato, H. Dehari, Y. Ito, M. Kobune, M. Miyagishi, K. Taira, H. Tahara, H. Hamada, Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth in vitro and in vivo, *Mol. Ther.* 10 (2004) 162–171.
- [16] M. Amarzguoui, T. Holen, E. Babaie, H. Prydz, Tolerance for mutations and chemical modifications in a siRNA, *Nucleic Acids Res.* 31 (2003) 589–595.
- [17] F. Czauderna, M. Fechtner, S. Dames, H. Aygun, A. Klippel, G.J. Pronk, K. Giese, J. Kaufmann, Structural variations and stabilizing modifications of synthetic siRNAs in mammalian cells, *Nucleic Acids Res.* 31 (2003) 2705–2716.
- [18] D.A. Braasch, S. Jensen, Y. Liu, K. Kaur, K. Arar, M.A. White, D.R. Corey, RNA interference in mammalian cells by chemically-modified RNA, *Biochemistry* 42 (2003) 7967–7975.
- [19] J. Harborth, S.M. Elbashir, K. Vandenburgh, H. Manninga, S.A. Scaringe, K. Weber, T. Tuschl, Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing, *Antisense Nucleic Acid Drug Dev.* 13 (2003) 83–105.
- [20] J.M. Layzer, A.P. McCaffrey, A.K. Tanner, Z. Huang, M.A. Kay, B.A. Sullenger, In vivo activity of nuclease-resistant siRNAs, *RNA* 10 (2004) 766–771.
- [21] Y.L. Chiu, T.M. Rana, siRNA function in RNAi: a chemical modification analysis, *RNA* 9 (2003) 1034–1048.
- [22] C.R. Allerson, N. Sioufi, R. Jarres, T.P. Prakash, N. Naik, A. Berdeja, L. Wanders, R.H. Grifley, E.E. Swayze, B. Bhat, Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA, *J. Med. Chem.* 48 (2005) 901–904.
- [23] J. Elmen, H. Thonberg, K. Ljungberg, M. Frieden, M. Westergaard, Y. Xu, B. Wahren, Z. Liang, T. Orum, T. Koch, C. Wahlestedt, Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality, *Nucleic Acids Res.* 33 (2005) 439–447.
- [24] D.V. Morrissey, K. Blanchard, L. Shaw, K. Jensen, J.A. Lockridge, B. Dickinson, J.A. McSwiggen, C. Vargeese, K. Bowman, C.S. Shaffer, B.A. Polisky, S. Zinnen, Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication, *Hepatology* 41 (2005) 1349–1356.
- [25] D.V. Morrissey, J.A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C.S. Shaffer, L.B. Jeffs, A. Judge, I. MacLachlan, B. Polisky, Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs, *Nat. Biotechnol.* 23 (2005) 1002–1007.
- [26] Y.-C. Choi, W. Gu, N.B. Hecht, A.P. Feinberg, C.-B. Chae, Molecular cloning of mouse somatic and testis-specific H2B Histone genes containing a methylated CpG island, *DNA Cell Biol.* 15 (1996) 495–504.
- [27] G.M. Church, W. Gilbert, Genomic sequencing, *Proc. Natl. Acad. Sci. USA* 81 (1984) 1991–1995.
- [28] A.P. Feinberg, B. Vogelstein, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, *Anal. Biochem.* 137 (1984) 266–267.
- [29] S.M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate, *EMBO J.* 20 (2001) 6877–6888.
- [30] J. Martinez, A. Patkaniowska, H. Urlaub, R. Luhrmann, T. Tuschl, Single-stranded antisense siRNAs guide target RNA cleavage in RNAi, *Cell* 110 (2002) 563–574.
- [31] B.A. Kravynack, B.F. Baker, Small interfering RNAs containing full 2'-O-methylribonucleotide-modified sense strands display Argonaute2/eIF2C2-dependent activity, *RNA* 12 (2006) 163–176.