

A structure–activity relationship study of siRNAs with structural variations

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Abstract

Specific knock-down of cellular gene expression using small interfering RNAs (siRNAs) is a powerful gene silencing technique in mammalian cells. Early siRNAs were double stranded, and 19–21 bp in length, but several variations in siRNA structure have been introduced to achieve better silencing efficiency. In addition, siRNA modules have been incorporated into higher-order RNA structures to generate multi-functional RNA molecules. The effects of such structural variations on the activities of siRNAs have not been thoroughly studied. Here, we present a structure–activity relationship study of siRNA structural variants. Specifically, we focus on the effect on silencing efficiency of the attachment of extra, target-unrelated sequences to the conventional short duplex siRNA structure. Interestingly, while some siRNA structural variants efficiently silence target gene expression, others show a reduction in or a complete lack of silencing activity. Off-target effects and innate immune responses triggered by siRNA structural variants were also measured. *In vitro* Dicer cleavage reactions show that all siRNA structural variants are substrates of Dicer, but digestion patterns vary. To our knowledge, this is the first systematic structure–activity relationship analysis of siRNAs bearing structural variations. Our results provide useful guidelines for the design of siRNA structural variants and for the construction of complex RNA molecules bearing functional siRNA modules.

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Small interfering RNAs (siRNAs) are double-stranded RNAs (dsRNAs) 19- to 21-bp in length that are able to target and degrade cellular mRNAs with complementary nucleotide sequences [1]. siRNA, along with its close relative, short hairpin RNA (shRNA), utilizes the naturally occurring RNA interference (RNAi) pathway [2]. Because of high efficiency and specificity, siRNA-based gene silencing has become a powerful molecular tool in target validation and a promising future therapeutic approach [3].

Initially, siRNA was designed as 19-bp double-stranded RNA with 2-nt 3' overhangs (termed the 19+2 structure) to mimic the Dicer-cleaved product of a long dsRNA [1].

Since then, several variations in the siRNA structure have been introduced to achieve better silencing efficiency. The first example of structural variation in the canonical 19+2 siRNA was the development of 27 bp-long siRNAs [4,5] and 29 bp-long shRNAs [6]. These longer RNA duplexes provided better silencing efficiency than did 19+2 siRNAs. In addition, siRNA modules were implemented within a higher-order RNA structure to obtain multi-functional RNA molecules. McNamara et al. [7] recently developed an aptamer–siRNA fusion RNA molecule to achieve aptamer-driven cell type-specific delivery of siRNAs. In another study, pRNAs were developed. These were based on bacteriophage phi29 RNA sequence and included a number of functional RNA modules, including siRNA modules, producing multi-functional RNA nanoparticles [8]. Also, siRNAs with extended sense strands

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have been developed to facilitate quantification of cellular siRNA concentrations [9].

Although many siRNA structural variants exist, to date no systematic structure–activity relationship analyses have been performed on siRNA structures deviating from the canonical 19+2 structure. This is especially true of siRNAs fused to sequences unrelated to target mRNAs. An understanding of the effect of appending extra sequences to siRNAs on silencing efficiency and specificity is important for designing structurally modified siRNAs with optimal gene silencing efficiency. Also, several studies have shown that short dsRNAs such as siRNAs can be recognized by several cellular dsRNA sensors [10–12] to elicit innate immune responses, resulting in the non-specific induction of interferon-regulated genes and inflammatory cytokines. An understanding of the potential of siRNA structural variants to trigger non-specific innate immune responses is important in the design of functional RNA molecules with few side-effects.

In the present study, we designed and synthesized a number of siRNA structural variants and studied their structure–activity relationships, focusing on silencing efficiency, off-target gene silencing, and the potential to trigger innate immune responses. Specifically, we measured the effect of appending extra target-unrelated sequences to the conventional short duplex siRNA structure. We also examined the cleavage patterns of these siRNAs by the recombinant Dicer protein *in vitro*.

Materials and methods

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were plated 24 h before transfection at 50% confluence in complete medium without antibiotics, in 12-well plates. All transfection assays were performed using oligofectamine, following the manufacturer's protocol (Invitrogen) at the indicated concentrations, and cells were harvested 24 h after transfection.

siRNAs. Chemically synthesized RNAs were purchased from Bioneer and annealed according to the manufacturer's protocol. The sense and antisense strands of siRNAs were:

19+0	5'-UGUUCUUCUGGAAGUCCAG-3' (antisense) 3'-ACAAGAAGACCUUCAGGUC-5' (sense)
19+2	5'-UGUUCUUCUGGAAGUCCAGdTdT-3' (antisense) 3'-dTdTACAAGAAGACCUUCAGGUC-5' (sense)
27+0	5'-UGUUCUUCUGGAAGUCCAGUCCUCCU-3' (antisense) 3'-ACAAGAAGACCUUCAGGUCAAGGAGGA-5' (sense)
3'LAS	5'-UGUUCUUCUGGAAGUCCAGCGCUCGCCCCGG-3' (antisense) 3'-ACAAGAAGACCUUCAGGUC-5' (sense)
5'LAS	5'-CCGGGCGAGCGUGUUCUUCUGGAAGUCCAG-3' (antisense) 3'-ACAAGAAGACCUUCAGGUC-5' (sense)

3'LS	5'-UGUUCUUCUGGAAGUCCAG-3' (antisense) 3'-GGCCCGCUCGCACAAGAAGACCUUCAGGUC-5' (sense)
5'LS	5'-UGUUCUUCUGGAAGUCCAG-3' (antisense) 3'-ACAAGAAGACCUUCAGGUCGCGAGCGGGCC-5' (sense)
3'LS3'LAS	5'-UGUUCUUCUGGAAGUCCAGCGCUCGCCCCGG-3' (antisense) 3'-GGCCCGCUCGCACAAGAAGACCUUCAGGUC-5' (sense)
5'LS5'LAS	5'-CCGGGCGAGCGUGUUCUUCUGGAAGUCCAG-3' (antisense) 3'-ACAAGAAGACCUUCAGGUCGCGAGCGGGCC-5' (sense)
3'LS5'LAS	5'-CCGGGCGAGCGUGUUCUUCUGGAAGUCCAG-3' (antisense) 3'-GGCCCGCUCGCACAAGAAGACCUUCAGGUC-5' (sense)
5'LS3'LAS	5'-UGUUCUUCUGGAAGUCCAGCGCUCGCCCCGG-3' (antisense) 3'-ACAAGAAGACCUUCAGGUCGCGAGCGGGCC-5' (sense)

Quantitative real-time PCR. Total RNA was extracted from cell lysates using the Tri-reagent kit (Ambion). One microgram of total RNA was used as a template for cDNA synthesis performed with the ImProm-II™ Reverse Transcription System (Promega), according to the manufacturer's protocol. Aliquots (1/20) of the cDNA reactions were analyzed by quantitative real-time PCR on a Rotor-Gene 3000 PCR machine (Corbett Research) according to the manufacturer's protocol. Gene-specific primers for GAPDH, lamin, and OAS2 genes were mixed separately with Power SYBR Green PCR Master Mix (Applied Biosystems) containing cDNAs to be analyzed. Samples were run in duplicate and data obtained were analyzed with Rotor-Gene6 software (Corbett Research). Primer sequences for each gene were:

Lamin—forward	5'-CCGAGTCTGAAGAGGTGGTC-3'
Lamin—reverse	5'-AGGTCACCTCTCTTCTTGGT-3'
GAPDH—forward	5'-GAGTCAACGGATTTGGTCGT-3'
GAPDH—reverse	5'-GACAAGCTTCCCGTTCTCAG-3'
OAS2—forward	5'-TCAGAAGAGAAGCCAACGTGA-3'
OAS2—reverse	5'-CGGAGACAGCGAGGGTAAAT-3'

***In vitro* Dicer cleavage assay.** One hundred picomoles each siRNA duplex was cleaved using the Turbo Dicer siRNA Generation Kit (Genlantis) for 8 h, according to the manufacturer's protocol. The concentrations of Dicer used varied from 0.1 to 1 U, as indicated. A 2-µl aliquot of each reaction (20 pmol) was separated in a 15% (w/v) non-denaturing polyacrylamide gel, stained with EtBr and visualized by UV transillumination.

Results and discussion

Structure–activity relationships of siRNAs with single strand extensions on one strand

We first synthesized an siRNA targeting lamin A/C mRNA as described by Elbashir et al. [1] (Fig. 1A and

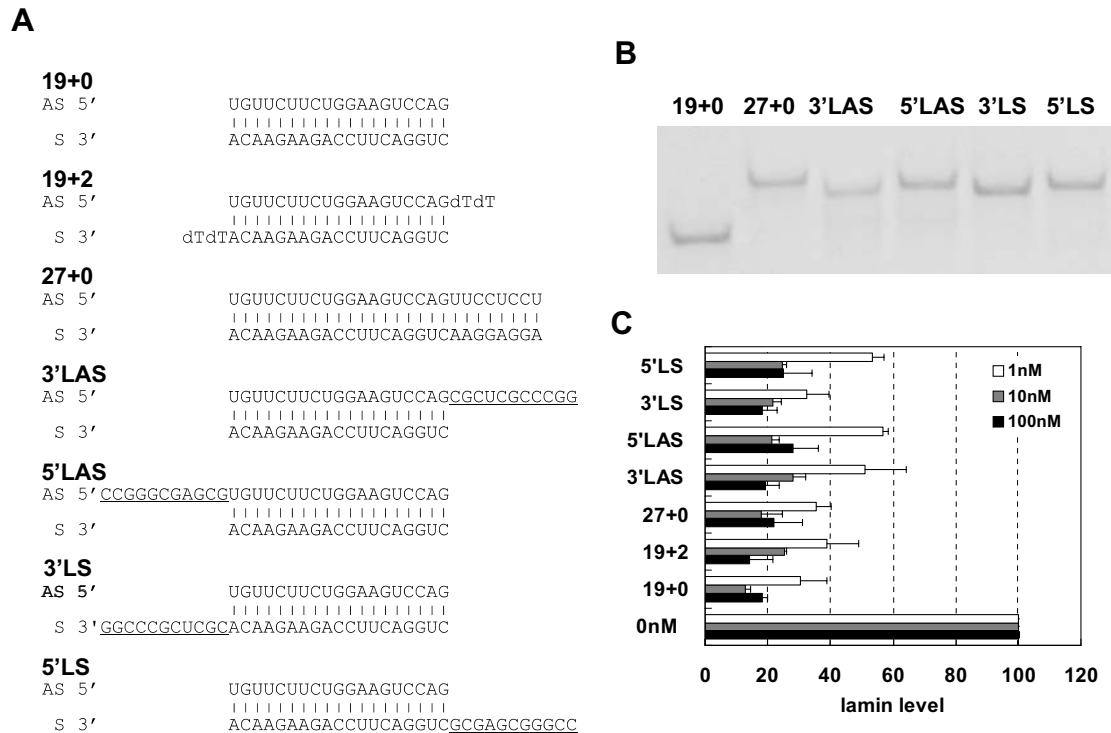


Fig. 1. Structures and activities of siRNAs with single strand extensions on one strand. (A) Structure of control siRNAs and siRNAs with single strand extensions on one strand. Extended sequences are underlined. (B) Non-denaturing PAGE analysis of the siRNAs in (A). (C) Lamin gene silencing activity of siRNAs in (A). Lamin mRNA level was measured 24 h after transfection. Data were calculated as ratios of lamin mRNA level/GAPDH mRNA level. Transfection with oligofectamine alone (0 nM) was the control and the lamin/GAPDH ratio was set at 100%. All data in the graph show means \pm SD values from three independent experiments.

B). The conventional 19+2 siRNA was transfected into HeLa cells using oligofectamine. As expected, good silencing efficiency was observed at all concentrations tested (Fig. 1C). siRNAs with a 19-bp duplex with blunt ends (19+0), and a 27-bp duplex with blunt ends (27+0), also showed effective gene silencing (Fig. 1A–C). We used these siRNAs as control siRNAs in comparisons with siRNA structural variants.

To study the structure–activity relationship of siRNA structural variants, we first designed and synthesized lamin siRNA variants with 11-nt extensions of target-unrelated sequences at either the 3' or the 5' ends of the antisense or sense strands, to generate the 3'LAS, 5'LAS, 3'LS, and 5'LS structures (Fig. 1A and B). These siRNA structural variants were transfected into HeLa cells, and the lamin gene silencing efficiency was measured at three concentrations of the siRNAs (100, 10, and 1 nM) and compared with the gene silencing efficiency of control siRNAs (19+0, 19+2, and 27+0). At 100 and 10 nM concentrations, all four variant siRNAs showed good silencing activity (Fig. 1C). However, at the lowest siRNA concentration (1 nM), the silencing activities of the 5'LS and 5'LAS, and the 3'LAS siRNAs, which contained sequence extensions at the 5' end of the sense strand, the 5' end of the antisense strand or the 3' end of the antisense strand, respectively (Fig. 1A), were somewhat reduced (40–50% silencing) compared with control siRNAs (60–70%

silencing) (Fig. 1C). However, 3'LS, which has a sequence extension at the 3' end of the sense strand (Fig. 1A), showed gene silencing efficiency (\sim 70% silencing) similar to that of the control siRNAs (Fig. 1C). Single strand extension of extra sequences unrelated to the target mRNA thus has different effects on the gene silencing activity of siRNAs, depending upon the position of extension.

Structure–activity relationships of siRNAs with single strand extensions on both strands

Next, we synthesized siRNAs with both strands extended in opposite directions, resulting in bidirectional single-strand extension structures (Fig. 2A and B). The 3'LS3'LAS siRNA had extensions at the 3' end of both strands, while the 5'LS5'LAS siRNA had sequence extensions at the 5' end of both strands (Fig. 2A). We transfected these variant siRNAs and measured their gene silencing efficiencies at different siRNA concentrations (Fig. 2C). Interestingly, the gene silencing efficiencies of the two variant siRNAs were dramatically different. We found that even at the highest concentration tested (100 nM), the silencing efficiency of 5'LS5'LAS was reduced (\sim 50% silencing), compared with control siRNAs. On the other hand, 3'LS3'LAS siRNA effectively silenced lamin gene expression at this concentration. At 1 nM concentration, the 5'LS5'LAS siRNA completely lost gene silencing

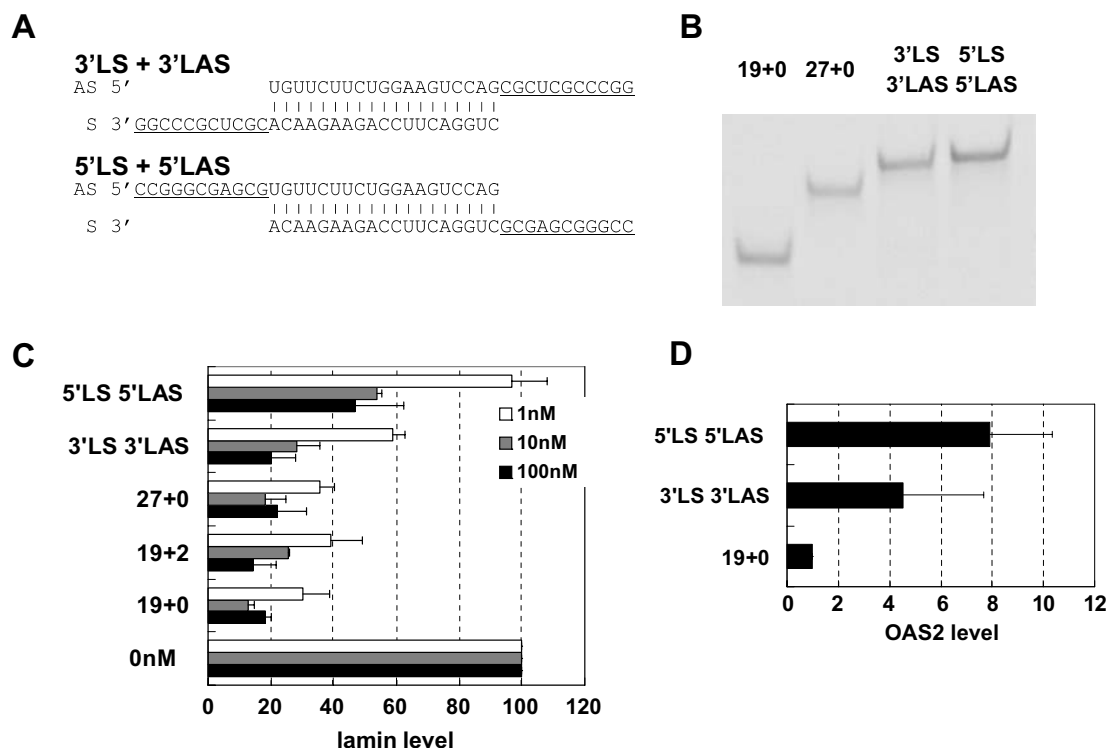


Fig. 2. Structures and activities of, and antiviral responses to, siRNAs with single strand extensions on both strands. (A) Structures of siRNAs with single strand extensions on both strands. Extended sequences are underlined. (B) Non-denaturing PAGE analysis of the siRNAs in (A). (C) Lamin gene silencing activity of the siRNAs in (A). See the legend of Fig. 1C for details. (D) Fold-induction of OAS2 mRNA levels by siRNAs (100 nM) with single strand extensions on both strands. Data were calculated as ratios of OAS2 mRNA level/GAPDH mRNA level. OAS2 induction by 19+0 siRNA was set at unity. All data in the graphs show means \pm SD values from two independent experiments.

activity (Fig. 2C) but 3'LS3'LAS siRNA still maintained some silencing activity, although silencing was somewhat lowered ($\sim 40\%$ silencing). The low silencing activity of the 5'LS5'LAS siRNA was not due to a non-specific general decrease in the mRNA level, which might have been triggered by a dsRNA-mediated antiviral response, because the GAPDH mRNA level was similar in different RNA samples (data not shown). These results again demonstrate that extensions of siRNA strands affect gene silencing differently, depending on the end to which the extra sequence is attached.

Structure–activity relationships of siRNAs with double strand extensions

We also tested the silencing efficiency of long duplex siRNAs generated by adding 11-bp duplexes of target-unrelated sequences to either end of the 19+0 lamin siRNA (Fig. 3A and B). The 5'LS3'LAS siRNA, in which the extra duplex sequence was appended to the 3' end of the antisense strand, showed silencing efficiency comparable to that of the control siRNAs at all concentrations tested (Fig. 3C). However, the 3'LS5'LAS structure, in which the extra duplex sequence was attached to the 5' antisense end, showed a different gene silencing profile. While the silencing efficiency of this siRNA at 100 nM concentration

was similar to that of the control siRNAs, silencing efficiency was reduced at 10 nM, and was further reduced ($\sim 20\%$ silencing) at 1 nM (Fig. 3C). As with other siRNA structural variants studied, we thus observed strong position effects when extra double-strand sequences were appended to gene silencing siRNAs.

Off-target gene silencing triggered by siRNA structural variants

To test whether the attachment of extra, target-unrelated sequence generates off-target gene silencing, that is, to trigger silencing of genes other than lamin A/C [13], we performed DNA microarray experiments using microarrays containing about 8000 human cDNAs [10]. We transfected HeLa cells individually with either 19+0 blunt siRNA as a control or siRNA structural variants such as 3'LS, 3'LS3'LAS, and 5'LS3'LAS siRNAs. Microarray analysis showed that when HeLa cells were transfected with 19+0 siRNA, only a few genes in addition to lamin A/C gene were down-regulated more than twofold (Supplementary Fig. 1). Similarly, when cells were transfected with each siRNA structural variants, only one or two genes other than lamin A/C gene were down-regulated more than twofold (Supplementary Fig. 1). Moreover, the fold reduction of these down-regulated genes was only slightly more

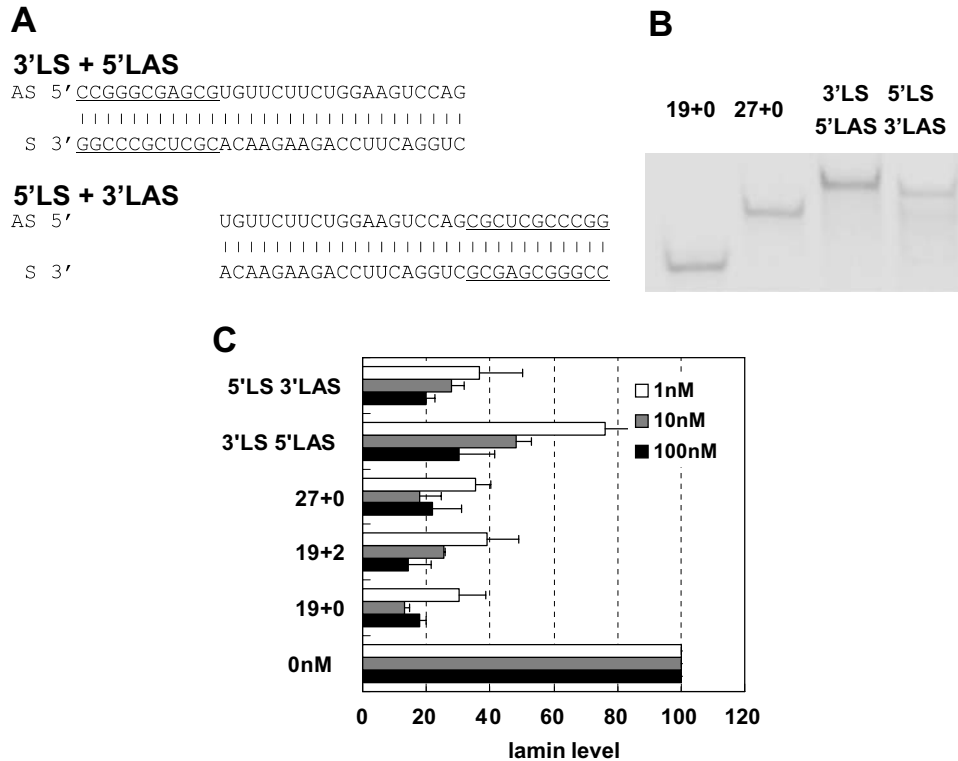


Fig. 3. Structures and activities of long duplex siRNAs. (A) Structures of long duplex siRNAs. Extended sequences are underlined. (B) Non-denaturing PAGE analysis of the siRNAs in (A). (C) Laminin gene silencing activity of siRNAs in (A). See the legend of Fig. 1C for details.

than twofold. From these results, we conclude that the structural variation of siRNA by the attachment of extra target-unrelated sequences does not trigger off-target gene silencing.

Innate immune responses triggered by siRNA structural variants

Several dsRNA sensors exist in cells to detect foreign dsRNAs and to then elicit innate immune responses [14]. OAS2 is one of the key genes located downstream of dsRNA-induced innate immune responses. The gene can be induced by long dsRNAs such as poly(I:C) [15]. We examined if any of the siRNA structural variants used in this work might significantly induce OAS2 gene expression. While the absolute level of OAS2 mRNA was somewhat variable from experiment to experiment, consistent with a previous report [15], we found that most of the siRNA variants we tested did not increase the OAS2 mRNA level when compared with the OAS2 mRNA level induced by 19+0 control siRNA, as measured by quantitative real-time PCR (data not shown). Intriguingly, even 30-bp duplex siRNAs, such as 5'LS3'LAS and 3'LS5'LAS, did not induce OAS2 gene expression above the level induced by the control 19+0 siRNAs. On the other hand, at 100 nM concentrations, siRNAs with single strand extensions on both strands, the 5'LS5'LAS and 3'LS3'LAS siRNAs, induced OAS2 mRNA levels severalfold higher than that induced by 19+0 siRNA (Fig. 2D). The level of

induction was much lower, however, than the OAS2 induction seen with poly(I:C), and while poly(I:C) triggered non-specific reduction of the global mRNA level and cell death, the 5'LS5'LAS or 3'LS3'LAS siRNAs did not show any of these effects (data not shown). Also, at lower concentrations (10 or 1 nM), even 5'LS5'LAS and 3'LS3'LAS siRNAs did not trigger the induction of OAS2 mRNA level. These results suggest that the siRNA structural variants used in this study do not trigger a strong dsRNA-mediated antiviral innate immune response.

Reynolds et al. [16] have shown that long (27 bp) siRNAs could trigger induction of a number of genes involved in the antiviral innate immune response, in a cell type-specific manner. It is to note that the HeLa cell line we have used in this study was identified as one of the cell lines which is insensitive to the treatment of long siRNAs [16]. Therefore, the relative lack of innate immune response triggered by siRNA structural variants in our study is consistent with their observation. In fact, in our microarray data, none of the genes induced by the long siRNA in the study of Reynolds et al. [16] was significantly induced by siRNA variants tested, except a moderate increase (~2.5-fold) in the IL-8 mRNA level by the 3'LS siRNA (Supplementary Figs. 2 and 3).

Dicer-mediated cleavage of siRNA structural variants

Some of the siRNA structural variants we designed showed reduced or negligible silencing efficiencies. We

wondered if defective gene silencing might be due to the inefficient recognition and cleavage of such siRNA structural variants by Dicer. To test this, we performed *in vitro* Dicer-mediated cleavage reactions. First, each siRNA variant was digested for 8 h with 1 U recombinant Dicer enzyme, and the cleavage products were analyzed by running a non-denaturing PAGE gel (Fig. 4A). Dicer cleaves dsRNA substrates to generate 19- to 21-bp duplexes [17]. As expected, the 27+0 duplex was cleaved by Dicer to generate products of a size similar to that of the 19+0 siRNA duplex (Fig. 4A). To our surprise, Dicer-mediated cleavage of the siRNA structural variants generated products of various electrophoretic mobilities (Fig. 4A), and most of the cleavage products migrated differently from the 19+0 duplex siRNA (Fig. 4A). While further characterization of each cleavage product is required, this result strongly suggests that Dicer-mediated cleavage of siRNA structural variants generates products whose structures are different from that of the 19-bp duplex.

Each siRNA with a single strand extension on one strand showed a unique cleavage pattern (Fig. 4A). The cleavage product of 3'LAS was similar in size to the 19+0 siRNA and to the cleavage product of 27+0 siRNA. However, 3'LS and 5'LS yielded Dicer-mediated cleavage products of more rapid electrophoretic mobility than 19+0 siRNA and 5'LAS yielded the cleavage product of highest mobility (Fig. 4A and B). On the other hand, siRNAs with single strand extensions at both strands, the 3'LS3'LAS and 5'LS5'LAS siRNAs, produced cleavage products with slower electrophoretic mobilities than that of 19+0 siRNA (Fig. 4A and Supplementary Fig. 4A), and the cleavage patterns of the two siRNAs were different. The cleavage pattern of the long duplex siRNAs, 3'LS5'LAS and 5'LS3'LAS, were also intriguing. While

these two siRNAs share an identical structure (duplexes of 30 bp), and differ in only the site of appended extensions, they yielded Dicer-cleaved products of different sizes (Fig. 4A and Supplementary Fig. 4B). 3'LS5'LAS gave cleavage products migrating faster than 19+0, whereas 5'LS3'LAS yielded species migrating slower than 19+0 (Fig. 4A and Supplementary Fig. 4B). This result suggests that Dicer-mediated cleavage patterns may depend not only on the structures but also on the sequences of siRNAs.

We then tested whether the efficiency of gene silencing by each siRNA variant correlated with the efficiency of cleavage by Dicer, by using varying concentrations of the Dicer enzyme (Fig. 4B and Supplementary Fig. 4). Within the range of Dicer concentrations used, we did not observe significant differences in cleavage efficiencies between siRNA variants (Fig. 4B and Supplementary Fig. 4). From these results, we hypothesize that different gene silencing efficiencies shown by different siRNA variants may not be because of differences in efficiencies of Dicer-mediated cleavage, but rather because of unique patterns and/or sequences of cleaved products generated from each siRNA variant.

Although there have been a number of studies regarding the effect of chemical modification on the activity of siRNAs [3], there have been no reports to date on the effect of siRNA structural modification on silencing activity, off-target gene silencing, and cellular innate immune responses. To our knowledge, this study is the first systematic analysis of structure–activity relationships of siRNAs with structural variations.

Jiang et al. [9] designed an siRNA structural variant that had a single strand DNA extension at the 3' end of the sense strand. This variant siRNA structure was efficient in target gene silencing, and was utilized as a PCR primer to quantitatively analyze siRNA concentrations inside

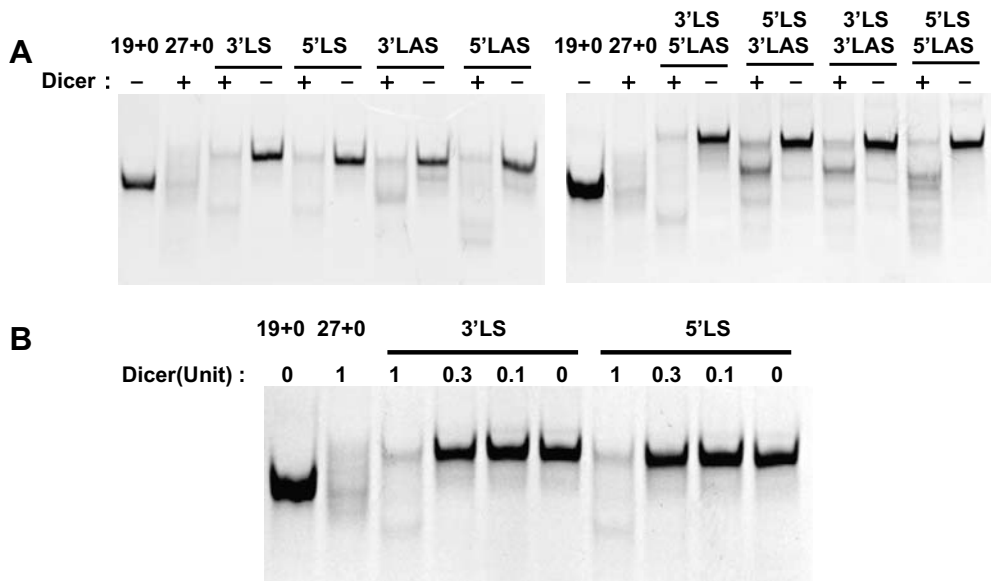


Fig. 4. *In vitro* cleavage of siRNAs by recombinant Dicer. siRNAs before and after treatment with Dicer were analyzed by 15% (w/v) non-denaturing PAGE. (A) Cleavage of siRNAs by 1 U Dicer enzyme for 8 h. (B) Cleavage of 3'LS and 5'LS siRNAs with various concentrations of Dicer enzyme.

cells. We point out that 3' sense strand extension was the only single strand extension modification in our study that did not affect siRNA gene silencing efficiency (Fig. 1C). In addition, McNamara et al. [7] attached aptamer sequences to the 5' end of siRNA sense strands, and observed gene silencing. While the site of single strand extension used in their experiment was not optimal, based on our data, they used very high concentrations of siRNA (400 nM) to observe silencing. Indeed, at 100 nM concentration, all four siRNAs with single strand extensions showed good silencing efficiency (Fig. 1C). At the high concentration of aptamer–siRNA complex, efficient gene silencing should therefore have been observed. We predict that aptamer fusion to the 3' end of the sense strand siRNA might work better in gene silencing, at lower concentrations of the RNA complex, provided that the site of aptamer attachment does not affect aptamer structure.

In summary, our results provide three important rules for the design of modified siRNA structures. First, the 3' end of the sense strand of an siRNA is the site of choice for a strand extension containing extra sequences to maintain maximal gene silencing. Second, extensions of both strands of an siRNA should be 3', not 5', to maintain gene silencing activity. Finally, when duplex extension is designed, addition of extra duplex sequences to the 3' end of the antisense strand of an siRNA is preferred over extension on the 5' end of the antisense strand, to minimize the loss of gene silencing efficiency. We believe our findings will provide useful guidelines in the design of siRNAs with novel structures or in the synthesis of complex RNA molecules which harbor siRNA as one of several functional modules. Toward the comprehensive understanding of the action mechanism of siRNA structural variants, future studies will focus on the characterization of the Dicer cleavage products generated from each siRNA variants, and the cleavage efficiency of each siRNA by Dicer–TRBP complex [18].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2007.06.004](https://doi.org/10.1016/j.bbrc.2007.06.004).

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