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# Evaluation of siRNAs that contain internal variable-length spacer linkages

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ABSTRACT

The most widely accepted mechanism of RNAi-silencing involves the RNA-induced silencing complex (RISC) liberating the active antisense strand from the sense strand of an siRNA duplex to form an active RISC-antisense complex. This involves cleaving the sense strand between positions 9 and 10 from the 5′ end of the strand prior to dissociation. Destabilizing modifications near the center of the duplex in some cases can enhance the efficacy of the resultant construct and may trigger an alternative mechanism through which the sense strand is removed. By introducing alkyl spacers of varying lengths near or within the sense strand's cleavage site, this study illustrates that siRNAs, in most cases, retained potent RNAi-silencing activity. Our results highlight that by substituting the scissile phosphodiester linkage on the sense strand with non-cleavable alkyl chains provides a novel and alternative method to destabilize the central region of siRNAs.



RNA interference (RNAi) is an endogenous gene silencing pathway.[1] The process involves the cleavage of double-stranded RNA into short-interfering RNAs (siRNAs).[2] The siRNAs are comprised of two strands, a sense and an antisense (AS) strand. The antisense strand recognizes the target messenger RNA through Watson-Crick base-pair specificity following sense strand removal. For activation, siRNAs serve as substrates for the RNA-induced silencing complex (RISC) and this complex directs the antisense RNA strand to the template mRNA strand.[3,4]

There has been considerable interest in utilizing siRNAs as biomolecular scaffolds to target genes of interest that may be associated with disease.[5] However, their impact has been delayed due to problems associated with permeability, stability and off-target effects.[6,7] In order to overcome these limitations, there is attention directed at utilizing chemical modifications to overcome some of the inherent problems associated with the native structure of RNA.[8–11]

Over the last number of years, there have been numerous studies aimed at evaluating the mechanism of the RNAi pathway, and elucidating the method through which RISC removes the sense strand.[12–15] This process is important because ultimately the antisense strand must target the desired mRNA. Several studies have confirmed that the catalytic portion of Argonaute 2 (Ago2) cleaves the sense strand between positions 9 and 10, starting from the 5′-end of the strand.[14–16] This cleavage is thought to promote the dissociation of the sense strand from the antisense strand to help initiate an active RISC-antisense RNA complex.

However, there have been a number of studies that suggest that RISC-mediated cleavage of the sense strand is not an essential feature that governs activity.[13,14,17] For example, Zamore and coworkers demonstrated that the substitution of the scissile phosphodiester on the sense strand with a phosphorothioate linkage significantly reduced cleavage by Ago2, yet RNAi activity was retained.[13] This study suggested that an alternative mechanism for sense strand dissociation was possible for RNAi activity. Other studies have illustrated that a pre-cleaved (nicked siRNA) at the sense strand's Ago2 cleavage site resulted in efficient RNAi activity, which indicates that an actual cleavage event per se is not an essential requirement to illicit a potent RNAi response.[14,18,19] Furthermore, Manoharan and coworkers demonstrated that a 2′-*O*-methyl or phosphorothioate modification at the Ago2 cleavage site of the sense strand with destabilizing chemical modifications were well tolerated within functional siRNAs.[17] Among those already mentioned, there are many other examples of chemical modifications performed on, or near the sense strand's cleavage site that are well tolerated by RISC.[20–24]

One of the features that is believed to help promote a bypass mechanism involves destabilization of the central region of a siRNA. Many of the methods to achieve destabilization include the incorporation of chemically modified bases, mismatches and/or abasic sites.[14,24–26] Many of the reported modifications require multiple steps to synthesize and expertise in nucleoside phosphoramidite chemistry. The goal of this study is to retain siRNA efficacy through duplex destabilization using various alkyl linkers as simple alternatives to mismatches or other chemically modified derivatives. Alkyl linker phosphoramidites such as those used in this study are commercially available and thus their preparation does not rely on in-house multi-step phosphoramidite syntheses.

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Based on the aforementioned reports and the well-established principle of thermodynamic asymmetry exhibited within siRNAs,[27–29] a variety of different length alkyl spacer chains was chosen for this study to replace typical destabilizing modifications.

The C3 spacer places three carbons between the oxygens of the phosphodiester linkages, which is exactly the same number of carbons between native RNA. The C4 and C5 spacer naturally have one or two additional carbons, respectively. These were chosen as it had been shown previously that alkyl bulges with increased carbon length can be used for siRNA constructs.[30] Each introduction of these alkyl spacer modifications was designed to replace the distance of a single nucleoside.

The E8 and C9 spacers contain eight or nine atoms between phosphodiester functionalities, respectively. The intention of these spacer derivatives is to span across two nucleotides with each introduction in RNA. Native RNA contains nine atoms between alternating phosphodiesters. Finally, the E17 spacer contains 17 atoms and is a mimic designed to span across three bases. Figure 1 illustrates the structural differences amongst the different alkyl spacer linkers used.

Various siRNAs were synthesized or purchased commercially that contained a combination of the linkers identified in Figure 1. The siRNAs generated target *firefly* luciferase mRNA transcribed from the plasmid pGL3 and expressed within mammalian cells (Table 1). The siRNAs were tested in a dose-dependent manner and their silencing data is illustrated in Figure 2.

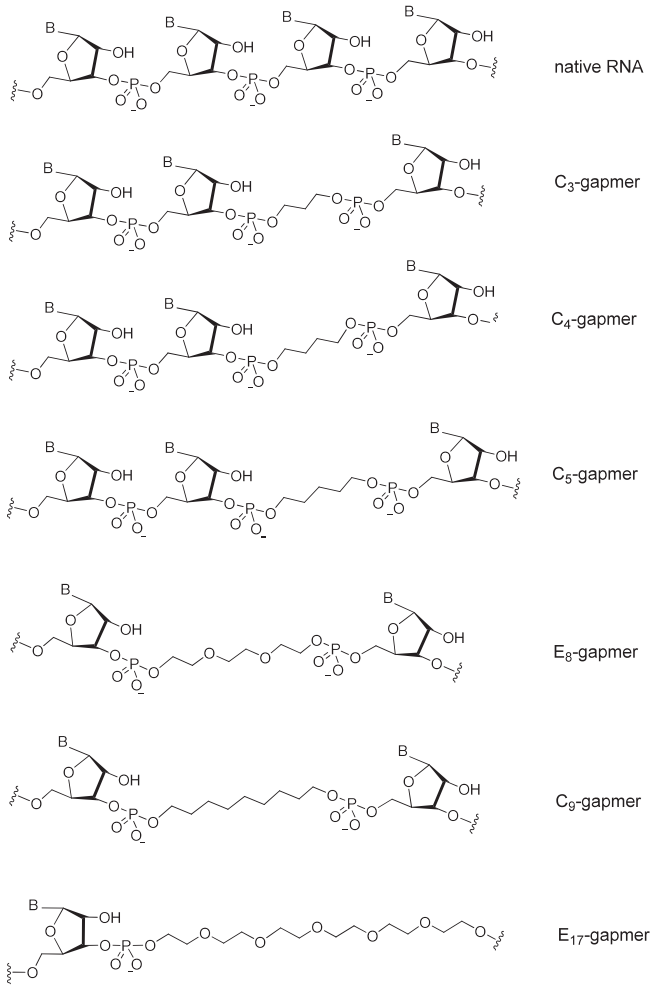


**Figure 1.** Structure of various alkyl spacer linkers used within siRNAs.

**Table 1**
Sequences of anti-luciferase siRNAs and $T_m$ data[31]

| RNA | siRNA duplex | $T_m$ (°C) | $\Delta T_m$ (°C) |
|---|---|---|---|
| wt | 5'- CUUACGCUGAGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 71.4 | -- |
| 1 | 5'- CUU$C_3$CGCUGAGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | -- | -- |
| 2 | 5'- CUUACG$C_4$UGAGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 55.0 | -16.4 |
| 3 | 5'- CUU$C_4$CG$C_4$UGAGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 55.3 | -16.1 |
| 4 | 5'- CUUACGCUGAGU$C_4$CUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 56.8 | -14.6 |
| 5 | 5'- CUUACGCUGAGUACU$C_4$CGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | -- | -- |
| 6 | 5'- CUUACGCUGAGU$C_3$CU$C_3$CGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 53.5 | -17.9 |
| 7 | 5'- CUUACGCUGAGU$C_4$CU$C_4$CGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 56.7 | -14.7 |
| 8 | 5'- CUUACGCUGAGU$C_5$CU$C_5$CGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 50.9 | -20.5 |
| 9 | 5'- CUUACGCU$C_3$AGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 52.3 | -19.1 |
| 10 | 5'- CUUACGCU$C_4$AGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 50.8 | -20.6 |
| 11 | 5'- CUUACGCU$C_5$AGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 50.6 | -20.8 |
| 12 | 5'- CUUACGCUG$C_3$GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 55.3 | -16.1 |
| 13 | 5'- CUUACGCUG$C_4$GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 52.4 | -19.0 |
| 14 | 5'- CUUACGCUG$C_5$GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 52.9 | -18.5 |
| 15 | 5'- CUUACGCU$C_3C_3$GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 49.7 | -21.7 |
| 16 | 5'- CUUACGCU$C_4C_4$GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 51.0 | -20.4 |
| 17 | 5'- CUUACGCU$C_5C_5$GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 50.5 | -20.9 |
| 18 | 5'- CUUACGCUGA$C_4$UACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 51.6 | -19.8 |
| 19 | 5'- CUUACGCU$C_4$A$C_4$UACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 48.8 | -22.6 |
| 20 | 5'- CUUACGCUG$C_4C_4$UACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 50.9 | -20.5 |
| 21 | 5'- CUUACGC-$E_8$-AGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 54.7 | -16.7 |
| 22 | 5'- CUUACGCU-$E_8$-GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 52.2 | -19.2 |
| 23 | 5'- CUUACGCUG-$E_8$-UACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 50.0 | -21.4 |
| 24 | 5'- CUUACGC-$C_9$-AGUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 50.4 | -21.0 |
| 25 | 5'- CUUACGCU-$C_9$-GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 51.3 | -20.1 |
| 26 | 5'- CUUACGCUG-$C_9$-UACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 49.3 | -22.1 |
| 27 | 5'- CUUACGC-$E_{17}$-GUACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 54.4 | -17.0 |
| 28 | 5'- CUUACGCU-$E_{17}$-UACUUCGAtt -3'<br>3'- ttGAAUGCGACUCAUGAAGCU -5' | 50.9 | -20.5 |