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Effect of incorporation of alkyl linkers into siRNAs on RNA interference

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ABSTRACT

Unintended (off-target) transcript silencing is a critical problem associated with RNA interference (RNAi)-based therapeutic applications. This paper shows that the incorporation of appropriate alkyl linkers at the center of the sense strands can suppress the off-target effects induced by the sense strands without reducing the RNAi-inducing activity of the antisense strands.

RNA interference (RNAi) is a homology-dependent gene-silencing process that is triggered by exogenous or endogenous, long double-stranded RNA molecules (dsRNA). The process is initiated by a processive cleavage of dsRNA into 21- to 23-nucleotide (nt) duplexes containing a 2-nt overhang at the 3′-end of each strand termed short interfering RNAs (siRNAs) by the RNase III-like enzyme Dicer. The siRNAs are incorporated into a protein complex designated as the RNA-induced silencing complex (RISC). Directed by the antisense strand of the siRNA, the RISC recognizes and cleaves the target mRNA.[1–3] siRNAs have considerable potential as new therapeutic drugs for intractable diseases because they can be rationally designed and synthesized if the sequences of disease-causing genes are known.[2,3]

Microarray profiling studies have demonstrated that siRNAs can potentially silence multiple genes in addition to the intended target.[4–10] This unintended (off-target) transcript silencing is a critical problem associated with RNAi-based therapeutic applications. Both the sense and the antisense strands of an siRNA can contribute to the off-target effects. Thus, minimizing the extent of sense-strand incorporation into an activated RISC increases the targeting specificity. Recently, it has been reported that 2′-*O*-methyl[11] and 5′-*O*-methyl[12] modifications of siRNAs can effectively reduce the siRNA off-target effects. Bramsen et al. showed that the segmentation of siRNA with locked nucleic acids (LNAs) at the center of the sense strand can suppress the off-target effects induced by the sense strand.[13] Here, we show that the incorporation of appropriate alkyl linkers at the center of the sense strand (Fig. 1) can reduce the off-target effects caused by the sense strand without reducing the RNAi-inducing activity of the antisense strand.

First, we assessed the silencing activity of the segmented siRNAs by performing a dual-luciferase assay using the psiCHECK-2 vector. The sequences of the siRNAs used are shown in Figure

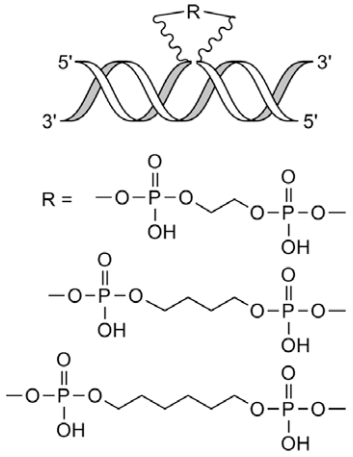

Figure 1. Structures of the siRNAs containing alkyl linkers.

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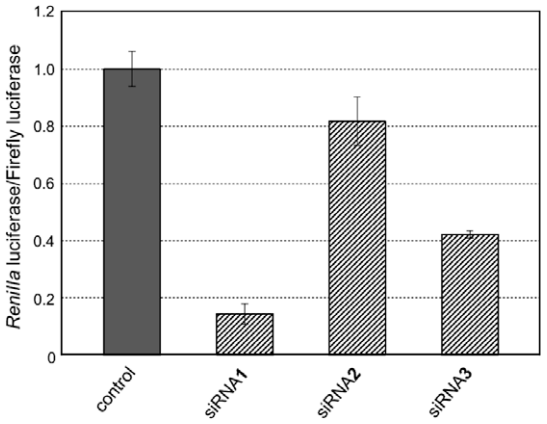

Figure 2. Dual-luciferase assay (1). Final concentration of siRNAs: 10 nM.

2.[14] siRNA **2** comprised 9- and 12-nt sense strands, while siRNA **3** comprised 10- and 11-nt sense strands. HeLa cells were co-transfected with the vector and the siRNAs. The signals of *Renilla* luciferase were normalized to those of the firefly luciferase.[18] As shown in Figure 2, it was found that the segmentation of siRNAs considerably reduced their silencing activity. The RNAi-inducing ability of siRNA **2** was weaker than that of siRNA **3**. Table 2 shows the melting temperatures (T_ms) of the siRNAs. The T_m value of the unsegmented siRNA **1** was 76.1 °C, whereas the T_ms of siRNAs **2** and **3** were 49.5 and 61.5 °C (2 transitions) and 55.1 °C, respectively. Therefore, we considered that the reduced silencing activity of the segmented siRNAs was attributable to the low thermal stability of the complexes.

In order to overcome this problem, we then designed and synthesized siRNAs containing alkyl linkers. The segmented sense strands of siRNA **3** were linked with alkyl linkers of various lengths

Table 1
Sequences of the oligonucleotides (ONs) and siRNAs used in this study

No. of siRNA	No. of ON	Sequence
siRNA**1**	ON**12**	5′-CUUCUUCGUCGAGACCAUG*tt*-3′
	ON**13**	3′-*tt*GAAGAAGCAGCUCUGGUAC-5′
siRNA**2**	ON**14**	5′-CUUCUUCGU-3′
	ON**15**	5′-CGAGACCAUG*tt*-3′
	ON**16**	3′-*tt*GAAGAAGCAGCUCUGGUAC-5′
siRNA**3**	ON**17**	5′-CUUCUUCGUC-3′
	ON**18**	5′-GAGACCAUG*tt*-3′
	ON**19**	3′-*tt*GAAGAAGCAGCUCUGGUAC-5′
siRNA**4**	ON**20**	5′-CUUCUUCGUC**pO(CH$_2$)$_2$Op**GAGACCAUG*tt*-3′
	ON**21**	3′-*tt*GAAGAAGCAG—CUCUGGUAC-5′
siRNA**5**	ON**22**	5′-CUUCUUCGUC**pO(CH$_2$)$_4$Op**GAGACCAUG*tt*-3′
	ON**23**	3′-*tt*GAAGAAGCAG—CUCUGGUAC-5′
siRNA**6**	ON**24**	5′-CUUCUUCGUC**pO(CH$_2$)$_6$Op**GAGACCAUG*tt*-3′
	ON**25**	3′-*tt*GAAGAAGCAG—CUCUGGUAC-5′
siRNA**7**	ON**26**	5′-CUUCUUCGU–**pO(CH$_2$)$_4$Op**GAGACCAUG*tt*-3′
	ON**27**	3′-*tt*GAAGAAGCAG—CUCUGGUAC-5′
siRNA**8**	ON**28**	5′-CUUCUUCGU—CGAGACCAUG*tt*-3′
	ON**29**	3′-*tt*GAAGAAGCA**pO(CH$_2$)$_2$Op**GCUCUGGUAC-5′
siRNA**9**	ON**30**	5′-CUUCUUCGU—CGAGACCAUG*tt*-3′
	ON**31**	3′-*tt*GAAGAAGCA**pO(CH$_2$)$_4$Op**GCUCUGGUAC-5′
siRNA**10**	ON**32**	5′-CUUCUUCGU—CGAGACCAUG*tt*-3′
	ON**33**	3′-*tt*GAAGAAGCA**pO(CH$_2$)$_6$Op**GCUCUGGUAC-5′
siRNA**11**	ON**34**	5′-CUUCUUCGU—CGAGACCAUG*tt*-3′
	ON**35**	3′-*tt*GAAGAAGCA**pO(CH$_2$)$_4$Op**–CUCUGGUAC-5′

The capital letters indicate ribonucleosides, while the small italicized letters represent 2′-deoxyribonucleosides.

Table 2
Hybridization data

No. of siRNA	T_m (°C)
siRNA**1**	76.1
siRNA**2**	49.5 and 61.5
siRNA**3**	55.1
siRNA**4**	69.6
siRNA**5**	68.3
siRNA**6**	66.5
siRNA**7**	64.8

The T_ms were measured in a 0.01-M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl.

(Table 1). The sense strands of siRNAs **4**, **5**, and **6** contained ethylene (C_2), tetramethylene (C_4), and hexamethylene (C_6) linkers, respectively. The antisense strands of siRNAs **8**, **9**, and **10** contained the C_2, C_4, and C_6 linkers, respectively. siRNAs **7** and **11** lacked the complementary bases, C and G, opposite sites of G and C, respectively. The T_ms of the siRNAs **4**, **5**, and **6** were 69.6, 68.3, and 66.5 °C, respectively (Table 2). Thus, the thermal stability of the complexes was found to improve with the attachment of alkyl linkers to the segmented strands.

In order to study the global conformation of the siRNAs containing alkyl linkers, we examined the circular dichroism (CD) spectra of the siRNAs. As shown in Figure 3, negative and positive CD bands were observed at ~209 nm and ~263 nm, respectively; the bands were attributable to A-type duplexes. Although the intensity of the positive bands obtained for siRNAs **4**, **5**, and **6** was slightly lower than that of siRNA **1**, the shapes of their spectra were similar. These results imply that the global conformations of siRNAs containing alkyl linkers do not significantly differ from those of siRNAs lacking linkers.

The RNAi-inducing ability of the siRNAs containing alkyl linkers was assessed by performing a dual-luciferase assay, the results of which are shown in Figure 4. The silencing activity of siRNAs **4**, **5**, and **6** was found to be comparable to that of siRNA **1** at each concentration of the siRNA. siRNA **6**, which contained the C6 linker, seemed to be most effective relative to others. Intriguingly, the silencing activity of siRNA **7**, which lacked the complementary base opposite site of G, was equivalent to that of siRNA **5**, which contained the complementary base. On the other hand, the introduction of the alkyl linkers into the antisense strands was found to significantly reduce the silencing activity of the siRNAs. Among these siRNAs, siRNA **10**, which contained the C6 linker, showed the lowest silencing activity.

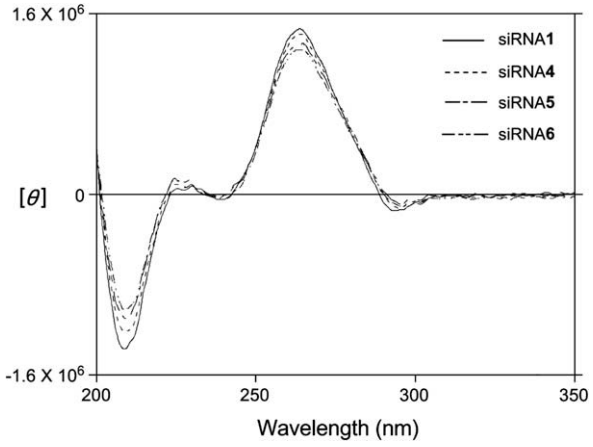

Figure 3. Circular dichroism (CD) spectra. CD spectra were measured at 20 °C in a 0.01-M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl.

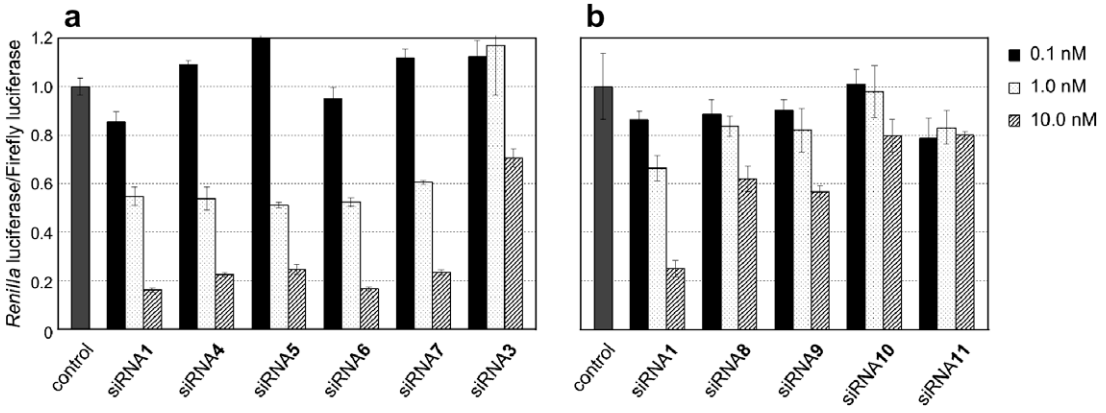

Figure 4. Dual-luciferase assay (2).

In conclusion, we synthesized siRNAs containing simple alkyl linkers at the center of the sense and antisense strands. We found that the incorporation of alkyl linkers at the center of the sense strands could reduce the off-target effects caused by these strands without reducing the RNAi-inducing activity of the antisense strands. Thus, the siRNAs containing the alkyl linkers, especially those containing the C6 linker, are novel siRNAs that can reduce the off-target effect induced by the sense strand.

Acknowledgments

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References and notes

1. Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391*, 806.
2. Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494.
3. Bumcrot, D.; Manoharan, M.; Koteliansky, V.; Sah, D. W. Y. *Nat. Chem. Biol.* **2006**, *2*, 711.
4. Jackson, A. L.; Bartz, S. R.; Schelter, J.; Kobayashi, S. V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P. S. *Nat. Biotechnol.* **2003**, *21*, 635.
5. Semizarov, D.; Frost, L.; Sarthy, A.; Kroeger, P.; Halbert, D. N.; Fesik, S. W. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6347.
6. Scacheri, P. C.; Rozenblatt-Rosen, O.; Caplen, N. J.; Wolfsberg, T. G.; Umayam, L. U.; Lee, J. C.; Hughes, C. M.; Shanmugam, K. S.; Bhattacharjee, A.; Meyerson, M.; Collins, F. S. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 1892.
7. Qiu, S.; Adema, C. M.; Lane, T. *Nucleic Acids Res.* **2005**, *33*, 1834.
8. Lin, X.; Ruan, X.; Anderson, M. G.; McDowell, J. A.; Kroeger, P. E.; Fesik, S. W.; Shen, Y. *Nucleic Acids Res.* **2005**, *33*, 4527.
9. Birmingham, A.; Anderson, E. M.; Reynolds, A.; Ilsley-Tyree, D.; Leake, D.; Fedorov, Y.; Baskerville, S.; Maksimova, E.; Robinson, K.; Karpilow, J.; Marshall, W. S.; Khvorova, A. *Nat. Methods* **2006**, *3*, 199.
10. Jackson, A.; Burchard, J.; Schelter, J.; Chau, B. N.; Cleary, M.; Lim, L.; Linsley, P. S. *RNA* **2006**, *12*, 1179.
11. Jackson, A.; Burchard, J.; Leake, D.; Reynolds, A.; Schelter, J.; Guo, J.; Johnson, J. M.; Lim, L.; Karpilow, J.; Nichols, K.; Marshall, W.; Khvorova, A.; Linsley, P. S. *RNA* **2006**, *12*, 1197.
12. Chen, P. Y.; Weinmann, L.; Gaidatzis, D.; Pei, Y.; Zavolan, M.; Tuschl, T.; Meister, G. *RNA* **2008**, *14*, 263.
13. Bramsen, J. B.; Laursen, M. B.; Damgaard, C. K.; Lena, S. W.; Babu, B. R.; Wengel, J.; Kjems, J. *Nucleic Acids Res.* **2007**, *35*, 5886.
14. *RNA synthesis.* Synthesis was carried out with a DNA/RNA synthesizer by phosphoramidite method. 1-*O*-(4,4′-Dimethoxytrityl)-2-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphinyl]ethanediol, 1-*O*-(4,4′-Dimethoxytrityl)-4-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphinyl]butanediol, and 1-*O*-(4,4′-dimethoxytrityl)-6-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphinyl] hexanediol were used to introduce the linkers into RNAs.[15] Deprotection of bases and phosphates was performed in concentrated NH_4OH/EtOH (3:1, v/v) at room temperature for 12 h. 2′-TBDMS groups were removed by 1.0 M tetrabutylammonium fluoride (TBAF, Aldrich) in THF at room temperature for 12 h. The reaction was quenched with 0.1 M TEAA buffer (pH 7.0) and desalted on a Sep-Pak C18 cartridge. Deprotected ONs were purified by 20% PAGE containing 7 M urea to give the highly purified ON**20** (9), ON**22** (10), ON**24** (9), ON**26** (8), ON**29** (15), ON**31** (15), ON**33** (5), and ON**35** (11). The yields are indicated in parentheses as OD units at 260 nm starting from 1.0 μmol scale.[16] These ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and observed molecular weights were in agreement with their structures.[17]
15. Ueno, Y.; Kato, Y.; Okatani, S.; Ishida, N.; Nakanishi, M.; Kitade, Y. *Bioconj. Chem.* **2003**, *14*, 690.
16. Puglisi, J. D.; Tinoco, I., Jr.. In *Methods Enzymol.*; Dahlberg, J. E., Abelson, J. N., Eds.; Academic Press: San Diego, 1989; Vol. 180, pp 304–325.
17. *MALDI-TOF/MS analyses of RNAs.* Spectra were obtained with a time-of-flight mass spectrometer. ON**20**: calculated mass, 6703.9; observed mass, 6707.2. ON**22**: calculated mass, 6731.9; observed mass, 6733.4. ON**24**: calculated mass, 6759.9; observed mass, 6762.4. ON**26**: calculated mass, 6426.9; observed mass, 6428.0. ON**29**: calculated mass, 6853.0; observed mass, 6850.9. ON**31**: calculated mass, 6881.0; observed mass, 6878.5. ON**33**: calculated mass, 6909.0; observed mass, 6913.1. ON**35**: calculated mass, 6536.0; observed mass, 6540.1.
18. *Dual-luciferase assay.* HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours before transfection, HeLa cells (4×10^4/mL) were transferred to 96-well plates (100 μL per well). They were transfected, using TransFast (Promega), according to instructions for transfection of adherent cell lines. Cells in each well were transfected with a solution (35 μL) of 20 ng of psiCHECK-2 vector (Promega), the indicated amounts of siRNAs, and 0.3 μg of TransFast in Opti-MEM I Reduced-Serum Medium (Invitrogen), and incubated at 37 °C. After 1 h, MEM (100 μL) containing 10% FBS and antibiotics was added to each well, and the whole was further incubated at 37 °C. After 24 h, cell extracts were prepared in Passive Lysis Buffer (Promega). Activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (Promega) according to a manufacturer's protocol. The results were confirmed by at least three independent transfection experiments with two cultures each and are expressed as the average from four experiments as mean ± SD.