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RNA interference with 2′,4′-bridged nucleic acid analogues

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ABSTRACT

In this study, a number of 2′,4′-BNA- and 2′,4′-BNA^{NC}-modified siRNAs were designed and synthesized. Their thermal stability, nuclease resistance and gene silencing properties against cultured mammalian cells were evaluated and compared with those of natural siRNAs. The 2′,4′-BNA- and 2′,4′-BNA^{NC}-modified siRNAs (named siBNA and siBNA^{NC}, respectively) showed very high T_m values, were remarkably stable in serum sample and showed promising RNAi properties equal to those exhibited by natural siRNAs. Thermally stable siBNAs composed of slightly modified sense and antisense strands were capable of suppressing gene expression equal to that of natural siRNA. A number of modifications on the sense strand by 2′,4′-BNA or 2′,4′-BNA^{NC}, either consecutively or separated by natural RNA nucleotides, is tolerable in RNAi machinery. Modifications at the Argonauate (Ago2) cleavage site of the sense strand (9–11th positions from the 5′-end of the sense strand) produced variable results depending on siRNA composition. Mostly, modification at the 10th position diminished siRNA activity. In moderately modified siRNAs, modification at the 11th position displayed usual RNAi activity, while modification at the 9th position showed variable results depending on siRNA composition.

1. Introduction

Since its discovery in 1998,[1] RNA interference (RNAi) has emerged as a very important and powerful molecular biological tool for regulation of gene expression.[2–6] RNAi technology utilizes short ~21 bp double-stranded RNA (dsRNA) with a two nucleotide (nt) 3′-overhang generally termed as small interfering RNA (siRNA), which upon incorporation to RNA-induced silencing complex (RISC) interacts with specific mRNA and ultimately suppresses it.[7,8] In this procedure, the sense strand (passenger strand) of siRNA is typically cleaved at 9 nt from the 5′-end of the sense strand by Argonauate 2 (Ago2) endonuclease,[9,10] and the activated RISC containing the antisense strand (guide strand) binds with target mRNA through Watson–Crick base pairing to cause degradation or translational block of target RNA.

Although natural siRNAs elicit promising RNAi activity in cell culture,[11–15] their in vivo use as a drug remains questionable because of obstacles such as low biostability and undesirable toxicity (off-target effects). To overcome these problems and to improve the pharmacokinetics and delivery of siRNA, efforts to use chemically modified siRNAs are increasing day by day. Various types of chemical modifications applied to siRNA have been nicely presented in some recent reports and reviews.[16–21] Chemical modification in siRNAs in many cases has improved the serum stability of siRNAs, albeit often at the expense of RNAi activity.[22–26] However, careful placement of some specific modified residues has also enhanced siRNA biostability without loss of siRNA potency, even enhancing siRNA potency in some cases.[27–32] Some specific modifications have reduced siRNA side effects, such as induction of recipient immune responses and inherent off-targeting effects.[33–35]

Among the various chemically modified siRNAs investigated, bridged nucleic acid[36] such as 2′,4′-methylene bridged nucleic acid (2′,4′-BNA,[37] also known as locked nucleic acid or LNA, Fig. 1)[38] modified siRNAs (named siLNAs) are some of the most promising

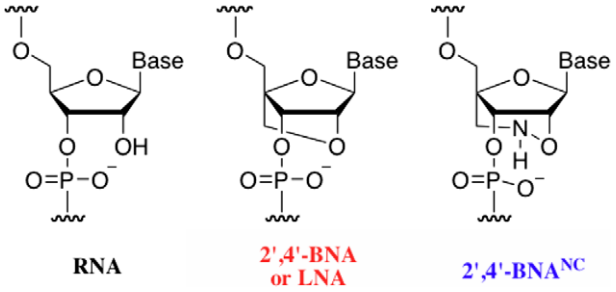

Figure 1. Chemical structures of natural RNA, 2′,4′-BNA, and 2′,4′-BNA^{NC}.

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siRNAs that have been employed in RNAi technology.[22,32,39–42] Corey and co-workers[22] showed that incorporation of LNA (2′,4′-BNA) into siRNA successfully improves the thermal stability of siRNAs without loss of RNAi activity. Another report described LNA-mediated improvement of siRNA stability and functionality, the positional effects of LNA modifications in antisense strands and LNA-mediated reduction of off-target effects.[32] Recently, small internally segmented siRNAs or three-stranded construct siRNAs comprising LNA (also called sisiLNA) have been shown to possess improved silencing properties.[41] Increased serum stability and reduced off-target effect by employing siLNA have also been recently noted in an in vivo study,[40] suggesting that siLNA should be preferred over unmodified siRNA for RNAi technology. SiLNAs have also shown antiviral effects[42] against coxsackie virus B3.

These previous studies utilize a number of 2′,4′-BNA-modified siRNAs, most of which are modified in 3′- and/or 5′-termini of sense and antisense strands. At the time of publication by Elmén et al.,[32] we had some similar results to those which they obtained and could not publish; rather, we concentrated on elaborating our investigations using a variety of 2′,4′-BNA-modified siRNAs with modifications at several points either consecutively or separated by natural nucleotides. It is well known that several modifications of the antisense strand of siRNA by 2′,4′-BNA is detrimental to siRNA efficacy. Therefore, we were interested in elucidating the effect of modifications in the sense strand; the effect of modifications at the cleavage site of the sense strand (9–11 nt) was especially investigated. In addition to 2′,4′-BNA, we also examined the efficacy of another highly potential bridged nucleic acid, that is, 2′,4′-BNA^{NC} (2′,4′-aminomethylene bridged nucleic acid)[43–46] in RNAi technology. 2′,4′-BNA^{NC} is a recently developed bridged nucleic acid analogue from our laboratory which possesses superior nuclease resistance and slightly improved target binding affinity compared to 2′,4′-BNA (LNA).[43–46] In this report we analyzed the effect of both 2′,4′-BNA and 2′,4′-BNA^{NC} modifications in gene silencing by RNAi methodology. We observed that 2′,4′-BNA (LNA) and 2′,4′-BNA^{NC} are equally effective in RNAi activity if incorporated appropriately, especially in the sense strand of siRNA.

2. Results and discussion

2.1. Thermal stability of siRNAs modified by 2′,4′-BNA and 2′,4′-BNA^{NC}

Due to inadequate thermal stability, siRNA duplexes may dissociate easily to render the siRNAs ineffective. Therefore, siRNAs possessing high thermal stability may be more effective in gene silencing. To evaluate the thermal stability of synthesized siRNAs, UV melting studies were conducted and UV melting temperature (T_m) was recorded. The results are summarized in Tables 1 and 2. As expected, replacement of natural nucleotides with 2′,4′-BNA or 2′,4′-BNA^{NC} either in the sense or antisense strand improved the thermal stability significantly (calculated ΔT_m/mod. = +2.5 to 4 °C approximately).

2.2. Serum stability

To be effective in vivo, a nucleic acid must be sufficiently stable to digestion by nucleases. Single stranded 2′,4′-BNA and 2′,4′-BNA^{NC} oligonucleotides showed very high nuclease resistance properties, as described in our previous reports.[44,46–50] Therefore, in general, it can be assumed that modification of natural siRNA by 2′,4′-BNA and 2′,4′-BNA^{NC} will increase the functional half-life of siRNA in vivo. To evaluate the stability of these siRNAs, the unmodified and modified siRNAs were incubated in 10% fetal bovine serum (FBS) at 37 °C. As shown in Figures 2A and B, unmodified siRNA (lane 2) was digested completely within 15 min whereas the modified siRNAs (lanes 3–10 in Fig. 2A, and lanes 3–8 in Fig. 2B) showed remarkable resistance in the medium. No or only slight degradation was found for the modified siRNAs (siBNA7, siBNA9, siBNA10, siBNA11, siBNA^{NC}7, siBNA^{NC}9, siBNA^{NC}10, siBNA^{NC}11 in Fig. 2A and siBNA17–19 and siBNA^{NC}17–19 in Fig. 2B) after 15 min of incubation. Most of the modified siRNAs also showed marked resistance even after 45 and 120 min of incubation; in most cases, the bands for the modified siBNAs in Figure 2 probably represent the products of 3′-overhang degradation. The modified siRNAs, siBNA7, and siBNA^{NC}9, having consecutive modifications at the 5′-end of the sense strand, showed striking stability even after 120 min of incubation.

Table 1
Sequence and T_m value (°C) of natural, 2′,4′-BNA- and 2′,4′-BNA^{NC} -modified siRNAs used in this study[a]

Name	Sequence	T_m (°C)
siRNA1	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	70
siBNA1	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	71
siBNA2	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	70
siBNA3	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	84
siBNA4	5′-CUUACGCUGAGTACTUCGATT-3′ 3′-TTGAAUGCGACTCATGAAGCU-5′	88
siBNA5	5′-CUTACGCUGAGUACTUCGATT-3′ 3′-TTGAAUGCGACTCAUGAAGCU-5′	>95
siBNA6	5′-CUUACGCUGAGTACTUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	84
siBNA^{NC}6	5′-CUUACGCUGAGTACTUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	86
siBNA7	5′-CTTACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	83
siBNA^{NC}7	5′-CTTACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	83
siBNA8	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACTCAUGAAGCU-5′	86
siBNA^{NC}8	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACTCAUGAAGCU-5′	86
siBNA9	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCT-5′	83
siBNA^{NC}9	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCT-5′	83
siBNA10	5′-CTUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	87
siBNA^{NC}10	5′-CTUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	88
siBNA11	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACTCAUGAAGCU-5′	88
siBNA^{NC}11	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACTCAUGAAGCU-5′	89
siBNA12	5′-CUTACGCUGAGUACTUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	86
siBNA^{NC}12	5′-CUTACGCUGAGUACTUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	87
siBNA13	5′-CTUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	83
siBNA14	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	73
siBNA15	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	86
siBNA16	5′-CUUACGCUGAGUACUUCGATT-3′ 3′-TTGAAUGCGACUCAUGAAGCU-5′	74

[a] Top and bottom strands depict the sense strand in the 5′···3′ direction (same as the target sequence) and the antisense strand in the 3′···5′ direction (complementary to the target), respectively. 2′,4′-BNA residues are shown as bold red and 2′,4′-BNA^{NC} residues are shown as bold blue.

Table 2
Sequence and T_m value (°C) of natural, 2′,4′-BNA- and 2′,4′-BNA^{NC} -modified siRNAs used in this study[a]

Name	Sequence	T_m (°C)
siRNA2	5′-GCUGAGUACUUCGAAAUGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	69
siBNA17	5′-GCUGAGUACUUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	84
siBNA^{NC}17	5′-GCUGAGUACUUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	84
siBNA18	5′-GCUGAGUACTUCGAAAUGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	85
siBNA^{NC}18	5′-GCUGAGUACTUCGAAAUGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	85
siBNA19	5′-GCTGAGTACUTCGAAAUGTTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	85
siBNA^{NC}19	5′-GCTGAGTACUTCGAAAUGTTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	86
siBNA20	5′-GCTGAGTACTUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	90
siBNA^{NC}20	5′-GCTGAGTACTUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	92
siBNA21	5′-GCTGAGTACUUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	88
siBNA^{NC}21	5′-GCTGAGTACUUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	89
siBNA22	5′-GCTGAGTACTUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	88
siBNA^{NC}22	5′-GCTGAGTACTUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	89
siBNA23	5′-GCTGAGTACUUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	84
siBNA^{NC}23	5′-GCTGAGTACUUCGAAATGUTT-3′ 3′-TTCGACUCAUGAAGCUUUACA-5′	86
siBNA24	5′-GCUGAGUACUUCGAAAUGUTT-3′ 3′-TTCGACTCATGAAGCUUTACA-5′	85
siBNA^{NC}24	5′-GCUGAGUACUUCGAAAUGUTT-3′ 3′-TTCGACTCATGAAGCUUTACA-5′	85
siBNA25	5′-GCUGAGUACUUCGAAAUGUTT-3′ 3′-TTCGACTCATGAAGCUUTACA-5′	89
siBNA^{NC}25	5′-GCUGAGUACUUCGAAAUGUTT-3′ 3′-TTCGACTCATGAAGCUUTACA-5′	91
siBNA26	5′-GCUGAGUACUUCGAAAUGUTT-3′ 3′-TTCGACUCAUGAAGCTTTACA-5′	84
siBNA^{NC}26	5′-GCUGAGUACUUCGAAAUGUTT-3′ 3′-TTCGACUCAUGAAGCTTTACA-5′	85

[a] Top and bottom strands depict the sense strand in the 5′···3′ direction (same as the target sequence) and the antisense strand in the 3′···5′ direction (complementary to the target), respectively. 2′,4′-BNA residues are shown as bold red and 2′,4′-BNA^{NC} residues are shown as bold blue.

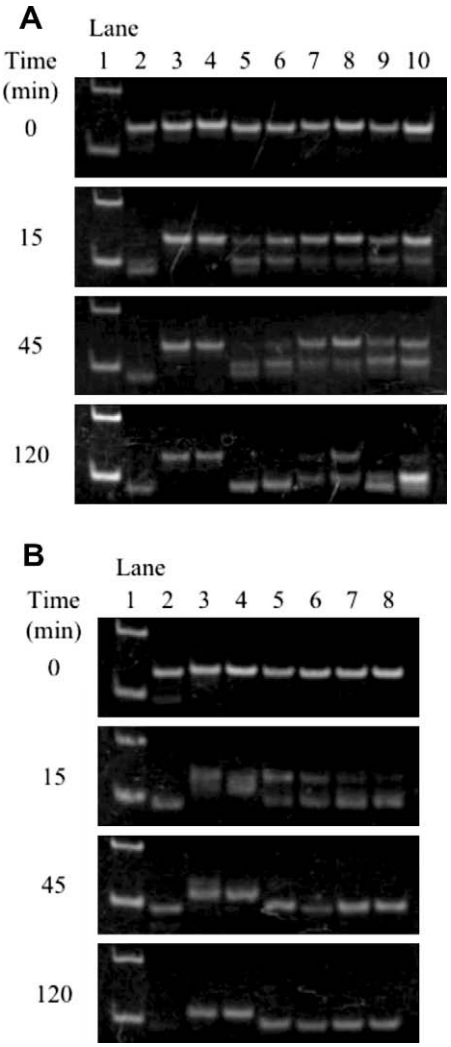

Figure 2. Serum stability of siRNA, siBNA, and siBNA^{NC} in 10% FBS after incubation time of 0, 15, 45 and 120 min. (A) Lanes 1–10 represent marker, siRNA1, siBNA1, siBNA7, siBNA^{NC}7, siBNA9, siBNA^{NC}9, siBNA10, siBNA^{NC}10, siBNA11, and siBNA^{NC}11 respectively. (B) Lanes 1–8 represent marker, siBNA2, siBNA17, siBNA^{NC}17, siBNA18, siBNA^{NC}18, siBNA19, and siBNA^{NC}19, respectively.

2.3. Inhibition of gene expression by 2′,4′-BNA- and 2′,4′-BNA^{NC}-modified siRNAs

To examine the effect of modifications by 2′,4′-BNA and 2′,4′-BNA^{NC} on siRNA activity, a range of modified siRNAs (Tables 1 and 2) were analyzed for their ability to inhibit firefly luciferase expression in CHO-luc cells; the results are presented in Figures 3–6. Table 1 shows the sequences of 2′,4′-BNA- and 2′,4′-BNA^{NC} (T, C residues)/2′,4′-BNA (A, G residues)[51]-modified siRNAs used in this study. Similar to that observed by Elmén et al.,[32] we found that introduction of 2′,4′-BNA modifications at 3′-overhangs in the sense and antisense strands of the siRNA (siBNA1 and 2) completely retained the usual RNAi property of natural RNA (Fig. 3). Modifications either on the sense or antisense strand alone are also effective (data not shown). Of course, these observations contrasted results found for modification by ethylene bridged nucleic acid (ENA) by Taira and co-workers where replacement of 3′-overhang nucleotides either of the sense or antisense strands resulted in loss of siRNA functionality.[52] Modification in the middle of both the sense and antisense strand including the 10th position from the 5′-end of the antisense strand (siBNA3 and 4) displayed usual

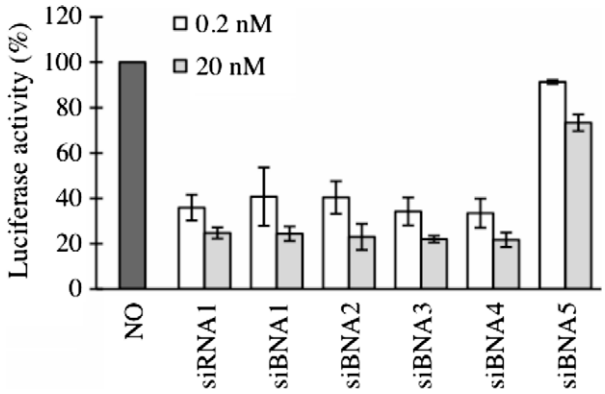

Figure 3. Effect of 2′,4′-BNA-modified siRNAs (siBNA1–5) on luciferase activity of CHO-luc cells. The cells were transfected with several siRNAs and the luciferase activity was assessed at 24 h post-transfection. Two different concentrations of the natural and modified siRNAs (0.2 nM and 20 nM) were used. Data are shown in mean and SD which were derived from three or more experiments. 'NO' means the luciferase expression in the absence of siRNA.

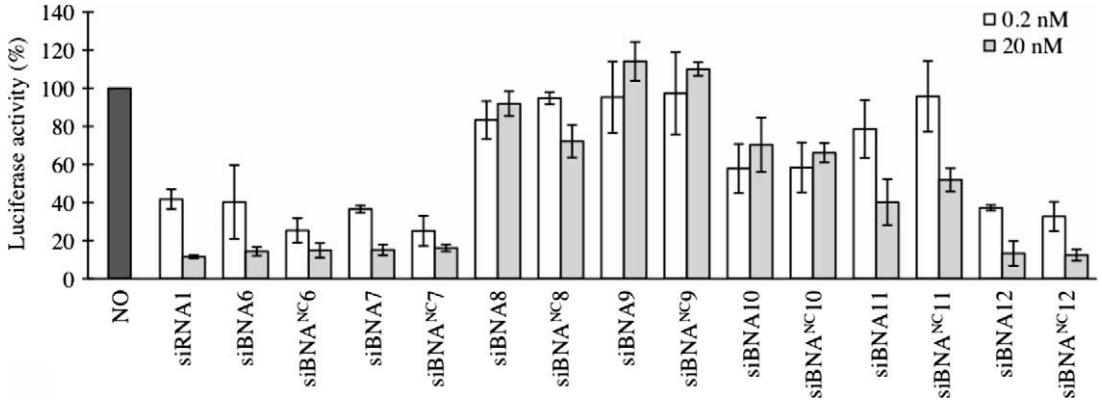

Figure 4. Effect of 2′,4′-BNA- and 2′,4′-BNA^{NC}-modified siRNAs (siBNA6–12 and siBNA^{NC}6–12) on luciferase activity of CHO-luc cells. The cells were transfected with the specific siRNAs and the luciferase activity was assessed at 24 h post-transfection. Two different concentrations of the natural and modified siRNAs (0.2 nM and 20 nM) were used. Data are shown in mean and SD which were derived from three or more experiments. 'NO' means the luciferase expression in the absence of siRNA.

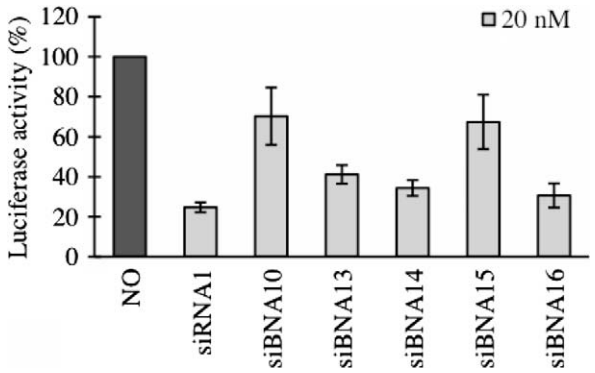

Figure 5. Effect on the modification of the Ago2 cleavage site of sense strand by 2′,4′-BNA nucleotide. Concentration: 20 nM of siRNA was used. Data are shown in mean and SD which were derived from three or more experiments. 'NO' means the luciferase expression in the absence of siRNA.

knockdown property. Moreover, siBNAs composed of one native RNA strand (unmodified strand) and either the sense and antisense strands of siBNA1–4 were found to be equally effective (data not shown). These results contradict the finding by Elmén et al.[32] where the modification at the 10th position from the 5′-end of the antisense strand was found to decrease the silencing property. However, placement of a number of modifications in both the sense and antisense strands (siBNA5) abolished siRNA activity consistent with the previous results from other groups.[22,32]

The effect of several modifications either in the sense or antisense strand was investigated, and results are shown in Figure 4. Incorporation of five consecutive BNA residues either in the middle or at the 5′-end of the sense strand (siBNA6 and 7, and siBNA^{NC}6 and 7, respectively) showed equal gene silencing ability to that of the natural siRNA1. However, similar modifications at the antisense strand (siBNA8 and 9 and siBNA^{NC}8 and 9) diminished the gene silencing ability completely. SiBNAs composed of the sense strands of siBNA6 and 7 with antisense strands of siBNA8 and 9, respectively, were also ineffective (data not shown). These results show that modification of the sense strand consecutively by five BNA residues might be tolerable in RNAi activity, although similar antisense strand modifications are not acceptable. Moreover, the results also show that 2′,4′-BNA^{NC} is equally effective in RNAi activity. Next, modification of the sense strand at several points including near the Ago2 cleavage sites (9–11th positions from the 5′-end of sense strand) were investigated. Modifications including the 10th position from the 5′-end did not show significant inhibitory activity as exhibited by the modified siRNAs: siBNA10 and siBNA^{NC}10. Similar antisense strand modification (siBNA11 and siRNA^{NC}11) also showed insignificant inhibitory activity. Interestingly, siBNA12 and siBNA^{NC}12 with five modifications sliding one base to the right (compare siBNA10 and siBNA^{NC}10 with siBNA12 and siBNA^{NC}12) showed usual knockdown property. The T_ms of siBNA12 and

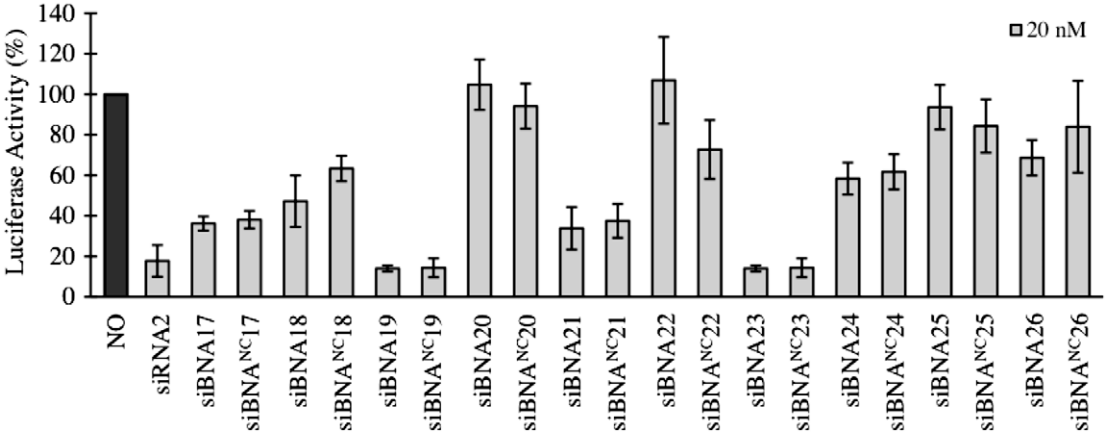

Figure 6. Effect of 2′,4′-BNA- and 2′,4′-BNA^{NC}-modified siRNAs (20 nM concentration) on luciferase activity of CHO-luc cells. Data are shown in mean and SD which were derived from three or more experiments. 'NO' means the luciferase expression in the absence of siRNA.

siBNA^{NC}12 are 16 °C and 17 °C higher than that of natural siRNA1 (Table 1). Therefore, introduction of 2′,4′-BNA or 2′,4′-BNA^{NC} can substantially stabilize siRNA analogues without any loss of RNAi activity.

To understand further the influence of 2′,4′-BNA or 2′,4′-BNA^{NC} substitution on the Ago2 cleavage site of siRNAs, we studied the comparative inhibitory profiles of luciferase expression by 2′,4′-BNA-modified siRNAs, and the results are shown in Figure 5. As described above, siBNA10 with five 2′,4′-BNA residues including modification at the 10th position from the 5′-end of the sense strand is ineffective for gene silencing. Exclusion of modification only at the 10th position of siBNA10 (siBNA13) or retaining modification at the 10th position while excluding modifications at other positions (siBNA14) rendered the siBNAs somewhat effective in gene silencing. Changing the site of modifications of siBNA10 one base to the right (siBNA12) also showed the usual knockdown property (Fig. 4). In contrast, changing the site of modifications one nucleotide to the left (modifications included at the 9th position from the 5′-end, as in siBNA15) lost the inhibitory activity markedly. However, modification only at the 9th position (siBNA16) showed significant suppression of luciferase expression (Fig. 5). Therefore, although modification only at the Ago2 cleavage site is tolerable by Ago2 protein to some extent, modifications at many places including the Ago2 cleaving sites may not be acceptable. However, if modification is not performed at the cleavage sites, siRNA can accommodate a number of BNA modifications in the sense strand.

Table 2 shows modified siRNAs bearing a different sequence targeted to a different site of the luciferase gene. In this case, siBNA^{NC}17–19 contains only single 2′,4′-BNA^{NC} residues (the other modified residues are 2′,4′-BNA), and the remaining 2′,4′-BNA^{NC}-modified siRNAs, that is, siBNA^{NC}20–26 contain only 2′,4′-BNA^{NC} and natural RNA residues. As shown in Figure 6, siBNA17 and siBNA^{NC}17 with five modifications at the sense strand, including modification at the 9th position from the 5′-end, showed moderate knockdown property. Similar siBNAs having modification at the 10th position (siBNA18 and siBNA^{NC}18) exhibited no or very low silencing property. By contrast, siBNA19 and siBNA^{NC}19 having modification at the 11th position from the 5′-end are highly effective siBNAs. These results are consistent with the results obtained by siBNA12 and siBNA^{NC}12 (Table 1, Fig. 4) containing modified residues in several places, including the 11th position. Therefore, it can be concluded that modification at the 11th position of the sense strand does not affect the silencing property of siRNAs, as does modification at the 9th or 10th position. To confirm the effect at the 9th and 10th positions, a number of siBNAs (siBNA20–23 and siBNA^{NC}20–23) were examined. The 2′,4′-BNA- and 2′,4′-BNA^{NC}-modified siRNAs with six modifications in the sense strand including modification at both the 9th and 10th positions (siBNA20 and siBNA^{NC}20, respectively, Table 2) were found to be totally ineffective in RNAi machinery (Fig. 6) consistent with our observation of other siBNAs having modifications at the 10th and 9th positions (Figs. 4 and 5). Interestingly, while excluding the modification only at the 10th position and retaining modification at the 9th position (siBNA21 and siBNA^{NC}21) regained inhibitory activity to about 60%, exclusion of modification at the 9th position, that is, retaining modification at the 10th position (siBNA22 and siBNA^{NC}22) was found to be ineffective. This result contradicted the results obtained by siBNA15 (Fig. 5), whereby moderately modified (five modifications) siBNAs with a modified 9th position were found to be ineffective. On the other hand, excluding modifications from both the 9th and 10th positions (siBNA23 and siBNA^{NC}23) retained usual gene silencing property. Thus, these results further confirm that modification at the 10th position is responsible for the loss of inhibitory activity of siBNA20 and 22, and siBNA^{NC}20 and 22. Two examples in previous reports by Elmén et al.[32] and Bramsen et al.[20] with 2′,4′-BNA modifications at the 9th position were shown to be effective. Taken together, although modification at the 9th position in a moderately modified siRNA might be tolerable in RNAi technology, modification of the 10th position is not acceptable. Efficacy of modified siRNAs having modifications at the 9th position of sense strand might depend on the site of other modifications or siRNA composition.

Finally, the effect of modification on antisense strands of siRNA2 was examined. Modification of the antisense strand by four or more BNA residues in different positions and arrangements (separately or consecutively) (siBNA24–26 and siBNA^{NC}24–26) diminished the usual RNAi property of siRNAs. Similar results were obtained with RNA antisense strands with 2′,4′-BNA modification as described by Corey and co-workers[22] Generally, increased modification at the antisense strand is unfavorable for RNAi activity. However, tri-molecule constructed siRNA or sisiRNA can accommodate a number of modifications in the antisense strand as described by Bramsen et al.[41]

3. Conclusions

In conclusion, we found that 2′,4′-BNA- and 2′,4′-BNA^{NC}-modified siRNAs are equally compatible with RNAi machinery similar to that observed for natural siRNA. To improve siRNA biostability, a number of bridged nucleotide moieties can be incorporated in the sense strand without loss of the usual gene silencing property. Thermally stable functional siRNAs can also be obtained by slightly modifying the middle of the sense and antisense strands together (as in siBNA4 and 5). Unlike the 3′-overhang modification, this modification increased T_m satisfactorily and contains an antisense strand with BNA residues which might be more efficacious in gene silencing. Modification at the Ago2 cleavage site (9–11th positions) produced variable results based on siRNA composition and sequence; usually the modification at the 10th position of the sense strand is more sensitive. Modification at the 11th position of the cleavage site is safer than that of the 10th or 9th position. For the first time, this study as a whole shows the utility and capability of 2′,4′-BNA^{NC}, a highly stable and efficient nucleic acid derivative in RNAi technology, and also gives some new ideas about designing biostable, functional siRNAs consisting both of 2′,4′-BNA and 2′,4′-BNA^{NC} residues.

4. Experimental

4.1. Synthesis of oligonucleotides and siRNAs

All siRNAs used in this study are listed in Tables 1 and 2. Unmodified RNA oligonucleotide and 2′,4′-BNA- and 2′,4′-BNA^{NC}-modified RNA oligonucleotides were synthesized on 0.2-μmol scale and purified by Gene Design Inc. Synthesized oligonucleotides were characterized by MALDI-TOF MS (negative mode) measurements. MALDI-TOF MASS data for the oligonucleotides containing the 2′,4′-BNA^{NC} residues: siBNA^{NC}6 (sense): calcd for 6751.17, found 6748.15, siBNA^{NC}7 (sense): calcd for 6779.19, found 6783.88, siBNA^{NC}8 (antisense): calcd for 6835.31, found 6832.46, siBNA^{NC}9 (antisense): calcd for 6809.24, found 6806.35, siBNA^{NC}10 (sense): calcd for 6723.15, found 6721.37, siBNA^{NC}11 (antisense): calcd for 6837.27, found 6840.68, siBNA^{NC}12 (sense): calcd for 6751.17, found 6754.10, siBNA^{NC}17 (sense): calcd for 6773.20, found 6775.39, siBNA^{NC}18 (sense): calcd for 6773.20, found 6773.79, siBNA^{NC}19 (sense): calcd for 6801.24, found 6800.21, siBNA^{NC}20 (sense): calcd for 6917.31, found 6921.83, siBNA^{NC}21 (sense): calcd for 6876.27, found 6879.70, siBNA^{NC}22 (sense): calcd for 6876.27, found 6880.67, siBNA^{NC}23 (sense): calcd for 6835.23, found 6835.42, siBNA^{NC}24 (antisense): calcd for

6778.23, found 6778.03, siBNA^{NC}25 (antisense): calcd for 6860.31, found 6864.85, siBNA^{NC}26 (antisense): calcd for 6819.27, found 6824.30. Natural and modified siRNAs at 0.2 nM and 20 nM strengths were obtained by annealing an appropriate amount of respective sense and antisense strands.

4.2. UV melting experiment

UV melting experiments were carried out on a Beckman DU-650 spectrometer equipped with a T_m analysis accessory. siRNAs or siBNAs (siBNA and siBNA^{NC}) were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to provide a final strand concentration of 1 μM. The samples were then annealed in boiling water for 3 min, followed by slow cooling to room temperature. The melting profiles were recorded at 260 nm from 25 °C to 90 °C at a scan rate of 0.5 °C/min. Melting temperature (T_m) was calculated as the temperature at which the duplexes were half dissociated, determined by taking the first derivative of the melting curve.

4.3. Serum stability

Duplexes of siRNA, siBNA and siBNA^{NC} (20 pmol) were incubated at 37 °C in 10% FBS (Invitrogen), diluted in phosphate buffered saline containing 5% CO_2. At various time points, 10 μl aliquots of samples were withdrawn and immediately mixed with 2 μl loading buffer (TAE buffer containing 6.7 μM Tris, 3.3 mM NaOAc, and 1 mM EDTA). Samples were subjected to 15% polyacrylamide gel electrophoresis under non-denaturing conditions, stained by ethidium bromide and visualized under UV.

4.4. Culture and transfection of cells

Chinese hamster ovary cells expressing luciferase protein (CHO-luc) were generously provided by Otsuka Pharmaceutical Co., Ltd. These cells were maintained at 37 °C under 5% CO_2 atmosphere in Ham's F-12 medium (ICN Biomedicals, Inc.) supplemented with 10% FBS, 600 μg/ml hygromycin B (Nacalai Tesque). Cells were seeded in 96-well plates at a density of about 30,000 cells per well 24 h before transfection, allowing them to attach on the well surface before transfection. Transfection of siRNA samples of two different concentrations (0.2 nM and 20 nM) were carried out with the aid of Lipofectamine 2000 (Invitrogen). A transfection mixture containing various amounts of siRNAs and 1 μl Lipofectamine 2000 in 50 μl serum-reduced OPTI-MEM (Invitrogen) buffer was added to each well. Cells were incubated in the transfection mixture for 4 h and further cultured in Hams F-12 medium and incubated at 37 °C for an additional 20 h (total incubation time = 24 h).

4.5. Assay of luciferase activity

After 24 h post-transfection, luciferase activity was assessed using Luciferase Assay System (Promega) according to the manufacturer's protocol. The 96-well plates containing the transfected cells were washed with phosphate buffer saline, and the cell extract was prepared using passive lysis buffer (Promega). The firefly luciferase activity was then measured by Mithras LB940 luminometer (Berthold, Japan) by treating cell lysates with luciferase assay reagent and simultaneous recording the light intensity. Results were confirmed by at least three independent transfection experiments.

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