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Introduction of 2-*O*-benzyl abasic nucleosides to the 3′-overhang regions of siRNAs greatly improves nuclease resistance

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ABSTRACT

Chemically modified siRNAs containing 2-*O*-benzyl-1-deoxy-D-ribofuranose (R^{H}_{OBn}) in their 3′-overhang region were significantly more resistant towards serum nucleases than siRNAs possessing the natural nucleoside in this region. The knockdown efficacies and binding affinities of these modified siRNAs to the recombinant human Argonaute protein 2 (hAgo2) PAZ domain were comparable with that of siRNA with a thymidine dimer at the 3′-end.

Small interfering RNAs (siRNAs) inhibit gene expression by RNA interference (RNAi) and thus have great potential as nucleic acid drugs.[1] RNAi technology is a useful strategy in the fight against cancers,[2] viral infections,[3] and other diseases.[4] However, natural RNA strands have many problems that complicate their therapeutic application, such as rapid degradation in biological media, non-specific gene silencing (off-target effects), and poor administration using existing drug delivery systems. To overcome these problems, artificially modified siRNAs have been developed extensively. An siRNA is a short (18–26 nucleotides) double-stranded RNA (dsRNA) containing a 2-nucleotide overhang at the 3′-end of each strand.[5] Once siRNAs are introduced into a cell by transfection, they are incorporated into a RNA-induced silencing complex (RISC). Each RISC contains a helicase that unwinds the siRNA helix. Upon unwinding, one of the strands, known as an antisense strand (guide strand), is retained in the RISC. This antisense RNA-RISC, called mature RISC, binds to and degrades the complementary mRNA target through base-pairing interactions. Eukaryotic translation initiation factor 2C2 (EIF2C2, Argonaute protein 2, Ago2), the core component of RISC, is considered to be the major player in RNAi. Ago2 has a conserved structure and includes PAZ, MID, and PIWI domains. The PAZ domain specifically recognizes the antisense strand of dsRNA through binding to the 3′-overhang region.[6,7] The binding site is a hydrophobic pocket composed of aromatic amino acids.[7–10]

On the basis of these findings, it has been suggested that chemical modification of the 3′-overhang region is an effective technique for improving the functionality of siRNA for RNAi-based therapy.[11–25] We previously reported the design and synthesis of various chemically modified functional RNAs bearing nucleic acid mimics at their 3′-end.[11–15,17,23] As part of our ongoing studies, we found that siRNAs containing 2-*O*-benzyl-1-deoxy-D-ribofuranose (R^{H}_{OBn}; **1**) at the 3′-ends showed high nuclease resistance and a desirable knockdown effect (Fig. 1).

Synthesis: To synthesize the desired RNAs via the conventional phosphoramidite method using a DNA/RNA synthesizer, we prepared the phosphoramidite derivative of R^{H}_{OBn} (**2**) (Scheme 1). First, 1-deoxy-D-ribofuranose (R^{H}, **3**), which can be prepared from commercially available 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose via reductive cleavage of the anomeric position,[15] was converted to TIPDS-R^{H} **4** using a disiloxane protection strategy. Subsequently, benzylation with benzaldehyde, Et_3SiH, and $FeCl_3$ in CH_3NO_2,[26] followed by desilylation by treatment with $Et_3N{\cdot}3HF$, gave **1** in moderate yield. Treatment of **1** with 4,4′-dimethoxytrityl (DMTr) chloride in pyridine gave the corresponding 5-DMTr derivative **5**, which was phosphorylated with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite to produce **2** in 97% yield. Oligonucleotides (ONs) containing R^{H}_{OBn} were synthesized using an automated nucleic acid

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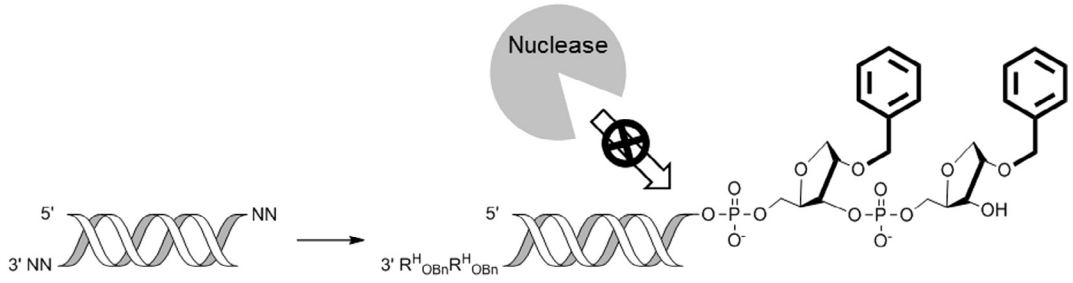
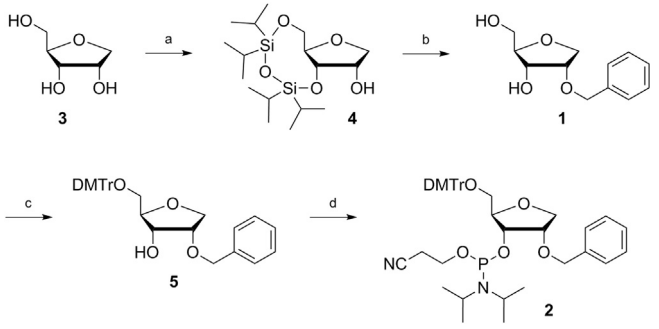


Fig. 1. Conceptual diagram of this study.



Scheme 1. Reagents and conditions: (a) $TIPDSCl_2$, imidazole, DMF, rt, 82%; (b) i) PhCHO, Et_3SiH, $FeCl_3$, $MeNO_2$, rt; ii) $Et_3N{\cdot}3HF$, THF, rt, 47%; (c) DMTrCl, pyridine, rt, 67%; (d) $(i\text{-}Pr_2N)P(Cl)O(CH_2)_2CN$, $i\text{-}Pr_2NEt$, THF, rt, 97%.

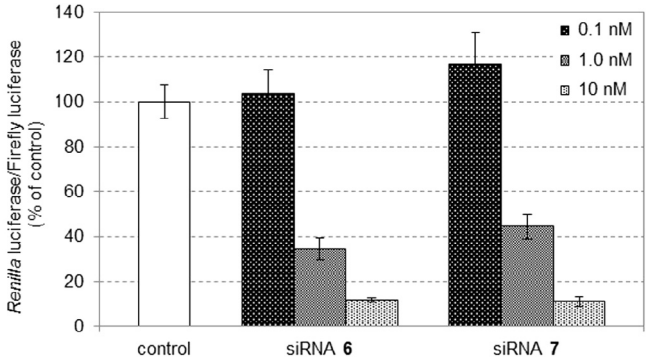


Fig. 2. Dual-luciferase assay.

synthesizer with phosphoramidite derivative **2** (Table 1). The fully protected ONs linked to a solid support were treated with NH_4OH/$MeNH_2$ (40% in H_2O), 1:1 (v/v) at 65 °C for 30 min and with $Et_3N{\cdot}3HF$ in DMSO at 65 °C for 2.5 h. The crude product can be precipitated by adding 3 M NaOAc, followed by *n*-BuOH. The mixture was cooled at −80 °C for 12 h and centrifuged at 4 °C, 12,500 rpm, for 30 min. After removal of the supernatant, 70% EtOH was added to the pellet. The resulting mixture was centrifuged at 4 °C, 12,500 rpm, for 15 min. The supernatant was removed, and the washing step was repeated. Deprotected ONs were purified by denaturing 20% polyacrylamide gel electrophoresis (PAGE) to isolate the desired ONs bearing the R^H_{OBn} modification. These ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and the observed molecular weights were in good agreement with their expected structures.[27]

Gene silencing of Renilla luciferase: The silencing activities of the siRNAs were examined by a dual-reporter assay using the psi-CHECK-2 vector in HeLa cells. This vector contains the *Renilla* and firefly luciferase genes, and the siRNA sequences were designed to target the *Renilla* luciferase gene. HeLa cells were co-transfected with the vector and the indicated amount of each siRNA. The signal levels of *Renilla* luciferase were normalized to those of firefly luciferase. Fig. 2 shows the silencing activities of the siRNAs. *Renilla* luciferase was suppressed by each siRNA in a dose-dependent manner. At 1.0 nM and 10 nM, the silencing activity of siRNA **7** with R^H_{OBn} dimers was almost equal to that of siRNA **6** with natural thymidines.

Nuclease resistance: The susceptibilities of the ONs to snake venom phosphodiesterase (SVPD), a highly active 3′-exonuclease, were examined. Thymidine-modified ON **14** and R^H_{OBn}-modified ON **15**, each labeled with fluorescein at their 5′-end, were incubated with SVPD and the reactions were analyzed by PAGE under denaturing conditions. The half-life ($t_{1/2}$) of ON **14** was <4 min, and that of ON **15** carrying a R^H_{OBn} dimer was 19 min. ON **15** was at least 5 times more resistant to the enzyme than thymidine-modified ON **14** (Fig. 3). Furthermore, the R^H_{OBn}-modified ON **15** was more resistant to nucleolytic hydrolysis by SVPD than the corresponding modified

Table 1
Sequences of ONs and siRNAs used in this study.

No. of siRNA	No. of ON	Sequence
siRNA **6**	ON **10**	5′-GGCCUUUCACUACUCCUAC**tt**-3′
	ON **11**	3′-**tt**CCGGAAAGUGAUGAGGAUG-5′
siRNA **7**	ON **12**	5′-GGCCUUUCACUACUCCUAC$\mathbf{R^H_{OBn}R^H_{OBn}}$-3′
	ON **13**	3′-$\mathbf{R^H_{OBn}R^H_{OBn}}$CCGGAAAGUGAUGAGGAUG-5′
–	ON **14**	F-5′-GUAGGAGUAGUGAAAGCC**tt**-3′
–	ON **15**	F-5′-GUAGGAGUAGUGAAAGCC$\mathbf{dR^HdR^H}$-3′
asiRNA **8**	ON **16**	5′-GGCCUUUCACUACUCCUAC-3′
	ON **11**	3′-**tt**CCGGAAAGUGAUGAGGAUG-5′
asiRNA **9**	ON **16**	5′-GGCCUUUCACUACUCCUAC-3′
	ON **13**	3′-$\mathbf{R^H_{OBn}R^H_{OBn}}$CCGGAAAGUGAUGAGGAUG-5′

[a]Capital letters indicate ribonucleosides and small letters show 2′-deoxyribonucleosides.
[b]F denotes fluorescein.

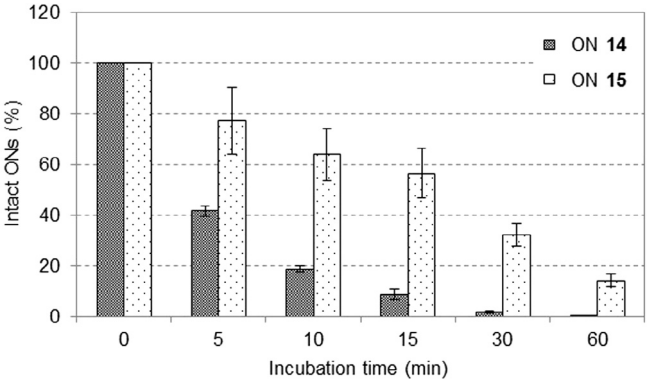


Fig. 3. Nuclease resistance of ONs against SVPD.

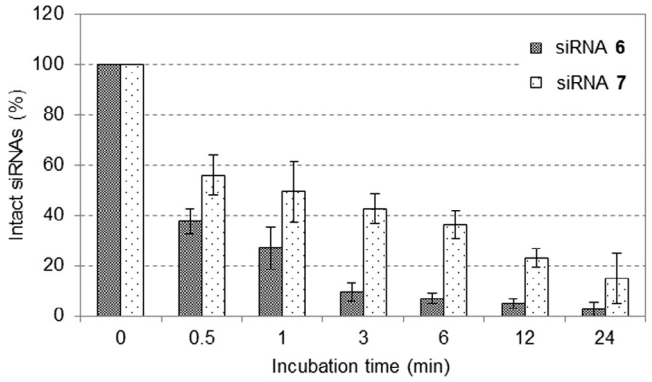


Fig. 4. Serum stability of siRNAs.

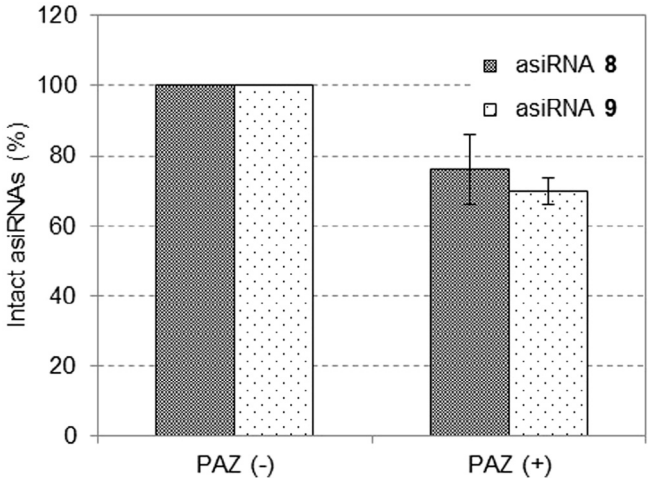


Fig. 5. Binding affinities of asiRNAs to the recombinant hAgo2 PAZ domain.

ON bearing the parent abasic ribo- and deoxyribonucleoside (Fig. S1).[15,23]

Serum stability: Next, we tested the stability of chemically modified siRNA with R^{H}_{OBn} in 50% bovine serum. At various incubation times, aliquots of each siRNA were analyzed by 20% native PAGE to detect any degradation products. As shown in Fig. 4, siRNA **7** possessing R^{H}_{OBn} was more resistant to serum-derived nucleases than unmodified siRNA **6** with natural thymidines. Unmodified siRNA **6** was largely degraded within 1 h, whereas less than 40% of modified siRNA **7** was degraded after 3 h and approximately 15% remained intact after 24 h incubation (Fig. S2).

Binding affinity with hAgo2 PAZ: We further investigated the binding affinities of a recombinant hAgo2 PAZ domain protein to the chemically modified siRNAs (Fig. 5).[20] In order to simplify the study, we used asymmetric siRNAs (asiRNAs) with an overhang region at the 3′-end of the antisense strand and a blunt end structure at the 3′-end of the sense strand (ON **16**). The asiRNA **8** consists of ON **11** and ON **16**, and the asiRNA **9** was duplex of ON **13** and ON **16**. The addition of hAgo2 PAZ protein resulted in a slight decrease in the absorbance at 312 nm. The binding affinity of asiRNA **9** possessing R^{H}_{OBn} is almost the same as that of asiRNA **8** carrying a thymidine dimer (Fig. S3). This result suggests that asiRNA **9** with R^{H}_{OBn} can bind the hAgo2 PAZ domain in a very similar manner to that of asiRNA **8** binding with natural thymidine dimers.

In conclusion, we demonstrated the synthesis of chemically modified siRNAs bearing a 2-*O*-benzylated abasic nucleoside in their 3′-overhang region. It was found that introduction of the abasic nucleoside at the 3′-end of an siRNA greatly improves resistance towards various nucleases. Furthermore, the knockdown efficacies and binding affinities of these modified siRNAs to recombinant hAgo2 PAZ domain were comparable with that of siRNA with a thymidine dimer. We believe that this modification method is a useful technique for increasing the duration of silencing *in vivo*.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.10.070.

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