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# Synthesis, gene-silencing activity and nuclease resistance of 3'–3'-linked double short hairpin RNA

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#### ABSTRACT

To improve the nuclease resistance of siRNA while reducing its induction of an innate immune response and maintaining its biological activity for possible therapeutic application, we designed and synthesized a series of double short hairpin RNAs (dshRNAs). Each dshRNA consisted of two identical short hairpin RNAs (shRNAs) linked at their 3' ends by glycerol. The dshRNAs were synthesized on a glycerol-derivatized solid support from amidites with 2-cyanoethoxymethyl (CEM) as the 2'-hydroxyl protecting group. Synthesis was carried out in a single run on a DNA/RNA synthesizer, without the need for enzymatic ligation. The dshRNAs showed structure-dependent gene-silencing activity at the protein level, and dshRNAs in which the 3' end of the two sense regions were linked showed especially high activity. Inclusion of 2'-O-methyluridine residues in the loop region was associated with 1.6- to 2.4-fold lower induction of interferon- $\alpha$  than was siRNA, without loss of gene-silencing activity. dshRNA also showed higher exonuclease resistance than siRNA or canonical shRNA. Our studies provide a new approach to gene silencing based on the concept of linking the 3' end of the sense regions of two shRNA molecules to form a double shRNA. © 2010 Elsevier Ltd. All rights reserved.

#### 1. Introduction

In RNA interference (RNAi), the translation or transcription of mRNA is suppressed by small double-stranded RNA (dsRNA). In mammalian cells, dsRNA 21–23 base pairs (bp) in length known as small interfering RNA (siRNA) is produced by the cleavage of long dsRNA by the endoribonuclease Dicer.<sup>1</sup> The siRNA is loaded into the RNA-induced silencing complex (RISC), where the duplex is unwound and the antisense strand hybridizes with the complementary mRNA sequence, leading to cleavage of the target mRNA by the effector complex. Because siRNA can suppress protein expression in a sequence-specific manner, it is expected to have therapeutic application as a new class of drug with a high specificity for its molecular target, and such applications are under active investigation. siRNA therapy is under development for age-related macular degeneration, respiratory syncytial virus infection, and hepatitis B, among other diseases.<sup>2</sup>

However, there are major obstacles to the practical therapeutic use of siRNA, including degradation by nucleases (particularly serum nucleases) and the risk of off-target effects or an innate immune response. Chemical modification of oligonucleotides is one of a number of methods that have been used to solve these problems.<sup>3,4</sup> Several kinds of chemical modification have been used to increase nuclease resistance. For example, siRNA incorporating deoxyribonucleotides or phosphorothioate linkages,<sup>5</sup> 2'-O-methyl nucleotides,<sup>6</sup> or locked nucleic acid nucleotides,<sup>7,8</sup> has been prepared for this purpose. However, it is better to avoid excessive chemical modification of siRNA because it tends to reduce gene-silencing activity.<sup>9</sup> The major serum nuclease is a 3'-5' exonuclease,<sup>10</sup> to which the 3'-overhang region of siRNA is especially susceptible. Chemical modification of the 3' end of oligonucleotides, particularly siRNA,<sup>11-13</sup> is important to provide resistance to 3'-5' exonucleases. A duplex structure of RNA also contributes to a relatively high resistance to single-strand-specific exonucleases and endonucleases.<sup>14,15</sup>

With these considerations in mind, we set out to synthesize RNA with greater nuclease resistance and more-potent genesilencing activity than siRNA. We wanted these improvements to be, as far as possible, inherent in the molecular design of the RNA rather than the modification of its constituent nucleotides or the sugar-phosphate backbone. We hypothesized that a double short hairpin RNA (dshRNA) consisting of two identical short hairpin RNA (shRNA) molecules linked through their 3' ends would be particularly resistant to 3'–5' exonucleases because there would be no free 3' end for such nucleases to act on. However, Dicer preferentially cleaves RNA structures with 3' overhangs or open duplex termini.<sup>16,17</sup> We wanted to know whether our dshRNA, which lacks both of these features, could be processed in the cell and show targeted gene-silencing activity.





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**Figure 1.** Strategy for solid-phase synthesis of dshRNA. (i) DMTr protection of glycerol. DMTrCl (2.1 equiv) in tetrahydrofuran/pyridine (1:1 [v/v]), rt, 1.5 h. (ii) Attachment of succinyl linker to glycerol. Succinic anhydride (3.0 equiv) and *N*,*N*-dimethyl-4-aminopyridine (0.4 equiv) in pyridine, 35 °C, 3 h. (iii) Attachment of glycerol to long-chain alkylamine CPG solid support through succinyl linker. *N*,*N*-Dimethyl-4-aminopyridine (0.5 equiv), water-soluble carbodiimide (7 equiv), and CPG (0.1 equiv) in pyridine, rt, three days. (iv) Oligonucleotide synthesis on DNA/RNA synthesizer (X, nucleotide in duplex region; Y, nucleotide in loop region). (v) Cleavage from solid support and deprotection.

Recognition of siRNA or dsRNA by Toll-like receptors (TLRs) induces an innate immune response.<sup>18</sup> Although our main purpose in designing dshRNA was to improve nuclease resistance without nucleoside modification, we also wished to include structural features that would prevent its recognition by TLRs.

In the present study, we synthesized a series of dshRNA molecules in which two identical shRNA elements were linked through their 3' ends by glycerol and evaluated their gene-silencing activity at the protein level. Activity was observed, and the highest activity was obtained when the 3' ends of the antisense sequences were far apart. Compared to typical siRNA, dshRNA also showed higher exonuclease resistance and, when 2'-O-methyluridine residues were included in the loops, lower immunostimulatory activity.

#### 2. Results and discussion

#### 2.1. Synthesis

We have previously developed a new methodology for the synthesis of RNA from amidites with a 2-cyanoethoxymethyl (CEM) 2'-O-protecting group.<sup>19,20</sup> The new method allows efficient synthesis of RNA oligomers up to at least 170 nucleotides in length.<sup>21</sup> In the present study, we used the CEM method to synthesize a series of dshRNAs. Each dshRNA was synthesized in a single run on a DNA/RNA synthesizer without the need for enzymatic ligation. Although 3'-3'-linked single-stranded DNA oligomers with no duplex regions have been synthesized as immunomers with enhanced immunostimulatory activity,<sup>22</sup> our study provides the first reported example of 3'-3'-linked shRNAs. Synthesis was carried out on a solid support derivatized with glycerol (Fig. 1), and the final products (Figs. 2 and 4) were identified by electrospray ionization (ESI) mass spectrometry. The dshRNAs were all targeted to the same mRNA sequence corresponding to a part of the antiapoptotic protein B-cell lymphoma 2 (Bcl-2).<sup>23</sup>

#### 2.2. Structure and gene-silencing activity

We tested the gene-silencing activity of our dshRNAs by measuring their suppression of Bcl-2 protein production in A431 cells. First, we measured the gene-silencing activity of dsh1–5 at concentrations of 3 and 10 nM (Fig. 3). The positive control was B717, an siRNA with known Bcl-2-suppressing activity.<sup>23</sup>

At 10 nM, weak suppression of Bcl-2 was observed with dsh2, in which the 3' end of the core 19-nt antisense sequence was directly attached to the glycerol linker. However, when the 3' end of the core 19-nt antisense sequence was separated from the glycerol



**Figure 2.** Sequences and structures of synthetic dshRNAs (first series; dsh1–5). dsh1: 22-bp duplex, 2-nt single-stranded link between duplex and glycerol, attachment of shRNA to glycerol through antisense region. dsh2: 22-bp duplex, attachment to glycerol through antisense region. dsh3: 22-bp duplex, attachment to glycerol through antisense region. dsh4: 19-bp duplex, 2-nt single-stranded link between duplex and glycerol, attachment to glycerol through antisense region. dsh5: 22-bp duplex, 4-nt single-stranded link between duplex and glycerol, attachment to glycerol through antisense region. dsh5: 22-bp duplex, 4-nt single-stranded link between duplex and glycerol, attachment to glycerol through antisense region.



**Figure 3.** Suppression of Bcl-2 gene expression by dsh1–5. Western blot analysis of the expression of Bcl-2 protein in A431 cells 72 h after transfection with dshRNA complexed with LIC-101. Actin was used as an internal control. Luciferase GL3 siRNA was used as the negative control and B717 siRNA as the positive control GL3 sense strand, 5'-CUUACGCUGAGUACUUCGAdTdT-3'; antisense strand, 5'-UCGAA-GUACUCAGCGUAAG-3'. B717 sense strand, 5'-GUGAAGUCAACUGCCUGCdTdT-3'; antisense strand, 5'-GCAGGCAUGUUGACUUCACCTdT-3'.

linker by two (dsh1) or four (dsh5) uridine residues, strong activity was observed at 10 nM. Therefore, a number of nucleotides intervening between the 3' end of the core antisense sequence and the linker was required for strong activity. When the duplex region of dsh1 was shortened by 3 bp to give dsh4, no activity was observed at 3 nM, although moderate activity was observed at 10 nM, so that a duplex of more than 19 bp was required for strong activity. When the shRNA elements were linked through their sense instead of their antisense sequences, as in dsh3, Bcl-2 was almost completely suppressed at only 3 nM. This activity was even higher than that of the positive-control siRNA, B717.

Because dshRNAs contain twice the number of mole equivalents of the antisense sequence per molecule as the corresponding siRNA available for participation in gene silencing, we judged that, among dsh1–5, only dsh3 showed gene-silencing activity comparable to that of siRNA. dsh3 has the same 22-bp stem duplex as dsh2. The main difference between the highly active dsh3 and the inactive dsh2 is whether the 3' end of the antisense sequence is attached

to the loop region (dsh3) or to glycerol (dsh2). In siRNAs, the integrity of the 5' end, but not the 3' end, of the antisense sequence is important for the initiation of RNAi.<sup>24–26</sup> For example, siRNAs conjugated to peptides through the 3' end of the antisense sequence show potent gene-silencing activity.<sup>27</sup> In contrast, our results with dshRNA show that direct linking of the 3' ends of the two antisense sequences through glycerol reduced the activity of the antisense sequences, whereas similarly linking the 3' ends of the two sense sequences preserved the activity of the antisense sequences. These results suggest that whichever sequence is directly linked to glycerol cannot participate in RNAi-mediated gene silencing, so that proper design of dshRNAs may help decrease off-target effects of the sense sequence. To investigate possible differences among fragments of dsh1-5 obtained after processing by Dicer, we treated these dshRNAs with purified recombinant Dicer. However, no fragments were observed under conditions under which the corresponding shRNA, with its 2-nt 3' overhang, was completely digested (data not shown). This may be due to the absence of both a 3' overhang and an open duplex terminal structure on dshRNAs, either of which is necessary for efficient cleavage of dsRNAs by Dicer.17,28

To further explore the relationship between the structure of dshRNAs and their gene-silencing activity, we synthesized a series



**Figure 5.** Suppression of Bcl-2 gene expression by dsh2, dsh3, and dsh6–10. dshRNAs were diluted twofold relative to siRNA. See the legend to Fig. 3 for further details.



**Figure 4.** Sequences and structures of synthetic dshRNAs (second series; dsh6–10). In all these dshRNAs, attachment of shRNA to glycerol is through the sense region. dsh6: 22-bp duplex, 2-nt single-stranded link between duplex and glycerol. dsh7: 24-bp duplex. dsh8: 22-bp duplex, 3-nt 5' tails. dsh9: 22-bp duplex, 1,3-dihydroxypropyl phosphate spacer (w) between duplex and glycerol. dsh10: 22-bp duplex, loop contains 2'-O-methyluridine (U).

of dshRNAs based on dsh3 with modifications to the loop or 5' ends and including a conserved 22-bp duplex (dsh6-10; Fig. 4), and tested their activity (Fig. 5). In these experiments, the dshRNAs were diluted twofold relative to the siRNA B717 to allow for the fact that they have twice as many antisense sequences per molecule. dsh8, which is identical to dsh3 except for a 3-nt uridine tail at the 5' end of the antisense region of each 22-bp duplex, showed weaker activity than dsh3. This demonstrates that activity is sensitive to the structure around the 5' end of the antisense region of the duplex, and that even a short extension of the 5' end can reduce activity. In contrast, dsh6, which is identical to dsh3 except for a 2-nt extension at the 3' end of the sense region of the duplex, showed almost the same potency as dsh3. The insertion of a 1,3-dihydroxypropyl phosphate spacer between the 3' end of each sense region and the glycerol linker of dsh3 to give dsh9 did not affect the activity, showing that activity was not sensitive to the distance of the duplexes from the glycerol linker. This is also consistent with the similar activity observed for dsh1 (2-nt single-stranded link) and dsh5 (4-nt single-stranded link). In contrast, dsh7, whose duplex is 2 bp longer than that of dsh3, showed no detectable activity, indicating that the length of the stem duplexes strongly affects the potency.

In designing dsh10 with 2'-O-methylated loop residues to suppress the innate immune response, we assumed that two natural nucleotides would be needed to form an overhang at the 3' end of the 19-nt antisense sequence after enzymatic processing in the cell for high silencing activity of the antisense sequences. Therefore, we designed the dshRNA to include two natural nucleotides at the 3' end of the 19-nt antisense sequence and four 2'-Omethyluridines as the loop components. dsh10 was fully active, so that activity was not affected by loop size or the nature of the loop components. Dicer is known to cleave long dsRNA or shRNA into 21-23-bp dsRNA, and it is unable to cleave dsRNA or shRNA stems less than 19 bp in length.<sup>29</sup> The fact that we observed strong activity only at a duplex length of 22 nt is consistent with the interpretation that the gene-silencing activity of dshRNA was mediated by the RNAi pathway. This is because the effective duplex length for RNAi is around 21–23 nt. with activity falling off for shorter or longer duplexes, as observed in our experiments. Although we did not observe dshRNA cleavage in vitro by purified Dicer, it is probable that, in cells, the action of another nuclease on the single-stranded part of the dshRNAs produced a 3' overhang or an open duplex terminal structure, allowing subsequent cleavage by Dicer. We hope to directly investigate the mechanism of dshRNA processing in cells in a future study.

#### 2.3. Induction of interferon- $\alpha$ (IFN- $\alpha$ )

We assessed the ability of dsh3 and dsh6-10 to induce an innate immune response as measured by the production of IFN- $\alpha$ by human peripheral blood mononuclear cells (Fig. 6). dsh3 and dsh6–9 showed 1.1- to 2.6-fold higher induction of IFN- $\alpha$  than did siRNA. In contrast, dsh10, which differs from dsh3 only in the loop region, showed 1.6- to 2.4-fold lower induction of IFN- $\alpha$  than siRNA, and this lower induction of IFN- $\alpha$  was achieved with no loss of gene-silencing activity. The lower induction of IFN- $\alpha$  is probably attributable to the use of 2'-O-methyluridine residues in the loop. It has previously been shown that the inclusion of 2'-O-methyluridine residues in an siRNA sequence can suppress immune activation,<sup>30</sup> but there are no reports of the suppression of cytokine induction by the inclusion of 2'-O-methyluridine residues in shRNA. We initially thought that dshRNA would not be recognized by TLRs<sup>31</sup> because of the reversal of the direction of the double helix within the molecule. However, our results suggest that dshRNA is indeed recognized by TLRs, except when the loop is modified. Modification of the loop region of shRNA may be a promising general strategy for suppressing the induction of cytokines.



**Figure 6.** Induction of IFN- $\alpha$  by dshRNA and siRNA. Human peripheral blood mononuclear cells were treated for 24 h with dshRNA complexed with LIC-101 and IFN- $\alpha$  levels were measured by ELISA. Maltose solution (10%) was used as the negative control and B717 siRNA as the positive control.

#### 2.4. Nuclease resistance

We assessed the resistance of dsh3 to the 3'-5' exonuclease snake venom phosphodiesterase (SVPD). Before incubation with SVPD, RNA samples were annealed to form the duplex structures. After incubation, the percentage of intact full-length RNA remaining was determined by high performance liquid chromatography (HPLC). siRNA was completely digested after incubation for 2 h (Fig. 7). shRNA, with a two-nucleotide 3' overhang, was digested to the extent of 56% under the same conditions, while dsh3, which is 3'-3'-linked through glycerol, was only digested to the extent of 16%. Thus, the rank order of SVPD resistance was dshRNA > shRNA > siRNA, with dshRNA being the most resistant of all the RNA structures tested.

#### 3. Conclusion

We have synthesized a series of double short hairpin RNAs (dshRNAs) consisting of two identical short-hairpin elements linked by glycerol through their 3' ends. The dshRNAs were synthesized from the CEM amidites entirely on a DNA/RNA synthesizer without the need for enzymatic ligation. Some of the dshRNAs showed gene-silencing activity as potent as that of siRNA with the same antisense sequence, and activity was highly dependent on the structure of the dshRNA. In particular, when the core



**Figure 7.** Digestion of siRNA, shRNA, and dshRNA by snake venom phosphodiesterase. siRNA, B717; shRNA, 5'- GUGAAGUCAACAUGCCUGCCCAACUUUCUGGGCA GGCAUGUUGACUUCACUU-3'; dshRNA, dsh3. Intact, full-length RNA remaining was determined by reverse-phase HPLC.

effector duplex was identical, linking the 3' end of the two sense regions with glycerol was associated with especially high genesilencing activity, whereas directly linking the 3' ends of the two antisense regions with glycerol resulted in a total loss of activity. dshRNA with 2'-O-methyluridine residues in the loop showed lower induction of IFN- $\alpha$  than did siRNA. dshRNA also showed higher exonuclease resistance than siRNA or canonical shRNA. Our results suggest a new approach to gene silencing based on the concept of linking the 3' ends of the sense regions of two shRNA molecules to form a double shRNA. Our designed dshRNAs may represent a new class of therapeutic RNA with high gene-silencing activity and exonuclease resistance yet low cytokine induction.

#### 4. Experimental

#### 4.1. General methods

<sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR spectra were obtained on a Bruker DRX500 or DPX300 spectrometer, and chemical shifts are reported relative to tetramethylsilane and referenced to the residual proton signal of CDCl<sub>3</sub> (7.25 ppm) for <sup>1</sup>H NMR, CDCl<sub>3</sub> (77.0 ppm) for <sup>13</sup>C NMR, and external 85% phosphoric acid for <sup>31</sup>P NMR. UV spectra were measured with a Hitachi U-2810 double-beam spectrophotometer. ESI mass spectra were recorded on a JEOL JMS-SX 102 mass spectrometer, and the calculated and observed masses of products were equal within a tolerance of about 0.12%. Analytical HPLC was performed on a Shimadzu system equipped with a Waters XTerra (MS C18, 2.5 µm, 4.6 mm × 50 mm) or XBridge (C18, 2.5 µm, 4.6 mm × 50 mm) column. Column chromatography was performed with Wako silica gel C-200, and thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F<sub>254</sub> TLC aluminum sheets.

#### 4.2. Materials

Reagents and solvents were purchased from commercial suppliers. Long-chain alkylamine controlled-pore-glass (CPG) beads (pore size, 1000 Å or 2000 Å) were from 3Prime (Aston, PA, USA), anhydrous solvents were from Kanto Kagaku (Tokyo, Japan), and snake venom phosphodiesterase was from Worthington Biochemical (Lakewood, NJ, USA).

#### 4.3. Preparation of derivatized solid support

#### 4.3.1. Preparation of 1,3-bis(4,4'-

#### dimethoxytriphenylmethyloxy)propan-2-ol (1)<sup>32</sup>

1,2,3-Propanetriol (1 g) was co-evaporated with pyridine twice and dried in vacuo for 0.5 h. The residue was dissolved in pyridine:tetrahydrofuran (80 mL; 1:1 [v/v]), then molecular sieves 4A (1 g) was added and the mixture was stirred for 5 min. 4,4'-Dimethoxytriphenylmethyl chloride (2.1 equiv) was added in three equal portions at a rate of one portion every 0.5 h. MeOH (5 mL) was added and the mixture was stirred for 5 min and filtered. The filtrate was evaporated to dryness at 35 °C and the residue was dissolved in ethyl acetate (100 mL) and washed with ice-cold NaHCO<sub>3</sub> aq. After re-extraction of the aqueous layer with ethyl acetate (100 mL), the organic layers were combined, washed with water and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The residue was chromatographed on silica gel (hexane:ethyl acetate:triethylamine, 80:20:0.5 [v/v/v], followed by hexane:ethyl acetate:acetone:triethylamine, 66:17:17:0.5 [v/v/v] to give a pale yellow foam (7.3 g, 96% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>,  $\delta$ ): 2.3 (d, 1*H*); 3.20-3.35 (m, 4H); 3.75 (s, 12H); 3.88-3.95 (m, 1H); 6.73-7.40 (m, 26*H*). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>,  $\delta$ ): 55.1, 64.2, 70.2, 85.9, 113.0, 126.7, 127.7, 128.1, 130.0, 135.9, 136.0, 144.8, 158.4.

#### 4.3.2. Preparation of 4-(1,3-bis(4,4'-

## dimethoxytriphenylmethyloxy)propan-2-yloxy)-4-oxobutanoic acid (2)<sup>32</sup>

1,3-Bis(4,4'-dimethoxytriphenylmethyloxy)propan-2-ol (1 g)was co-evaporated with pyridine and dried in vacuo for 0.5 h. The residue was dissolved in pyridine (2 mL) and treated with N,N-dimethylaminopyridine (70 mg; 0.4 equiv) and succinic anhydride (430 mg; 3.0 equiv) for 3 h at 35 °C. After confirmation of the disappearance of the starting material by TLC, MeOH (5 mL) was added and the mixture was evaporated to dryness. The residue was dissolved in ethyl acetate (20 mL) and washed with ice-cold 0.5 M KH<sub>2</sub>PO<sub>4</sub> aq (20 mL), and the aqueous layer was again extracted with ethyl acetate (20 mL). The combined organic layers were washed successively with ice-cold 0.5 M KH<sub>2</sub>PO<sub>4</sub> aq  $(2 \times 20 \text{ mL})$ , water, and brine, dried over MgSO<sub>4</sub>, and concentrated to afford the crude product as a white foam (1.14 g; quant.). <sup>1</sup>H NMR (300 MHz, CDCl<sub>2</sub>,  $\delta$ ): 2.65 (s. 4*H*): 3.25–3.30 (m. 4*H*): 3.75 (s, 12H); 5.26-5.29 (m, 1H); 6.73-7.36 (m, 26H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, δ): 28.9, 29.2, 55.1, 61.8, 72.5, 85.8, 113.0, 126.6, 127.7, 128.1, 130.0, 135.9, 144.8, 158.4, 171.5, 176.8.

#### 4.3.3. Derivatization of CPG solid support (3)

The solid support was derivatized with glycerol as previously described.<sup>32</sup> Briefly, pyridine (9.8 mL) and triethylamine (151  $\mu$ L) were added to a mixture of 4-(1,3-bis(4,4'-dimethoxytriphenylmethyloxy)propan-2-yloxy)-4-oxobutanoic acid (710 mg), *N*,*N*-dimethyl-4-aminopyridine (54 mg), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (water-soluble carbodiimide; 1.19 g), and CPG solid support, and the mixture was shaken at room temperature for three days. After filtration, the solid support was washed successively with pyridine, MeOH, and CH<sub>2</sub>Cl<sub>2</sub>, and dried in vacuo for 1 h. The solid support was transferred to a vial containing capping solution (Ac<sub>2</sub>O, 1.0 mL; 2,6-lutidine, 1.4 mL; tetrahydrofuran, 20 mL) and shaken for 2 h, then filtered, washed successively with pyridine, MeOH, and CH<sub>2</sub>Cl<sub>2</sub>, and dried in vacuo for 2 h. The loading was estimated by the absorbance of the trityl cation at 503 nm ( $\varepsilon$ , 76,000 M<sup>-1</sup> cm<sup>-1</sup>).

#### 4.4. Synthesis of spacer

#### 4.4.1. Preparation of 3-(4,4'dimethoxytriphenylmethyloxy)propan-1-ol<sup>33</sup>

1,3-Propanediol (3 g) was co-evaporated with pyridine and dried in vacuo for 0.5 h. The residue was dissolved in a mixture of pyridine (80 mL) and tetrahydrofuran (100 mL), after which 4,4'-dimethoxytriphenylmethyl chloride  $(3 \times 0.35 \text{ equiv})$  was added in three portions at a rate of one portion every hour. MeOH (20 mL) was added and the mixture was stirred for 5 min and evaporated to dryness at 35 °C. The residue was dissolved in ethyl acetate (100 mL) and washed with ice-cold NaHCO3 aq. The aqueous layer was re-extracted with ethyl acetate (100 mL) and the combined organic layers were washed with water and brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The residue was chromatographed on silica gel (hexane:ethyl acetate:pyridine, 80:20:0.1 [v/v/v] and then hexane:ethyl acetate:acetone:pyridine, 66:17:17:0.1 [v/v/v/ v]) to give a yellow oil (7.8 g, 52% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, δ): 1.81–1.89 (m, 2H); 3.25–3.29 (m, 2H); 3.74–3.78 (m, 2H); 3.79 (s, 6*H*); 6.81–7.68 (m, 13*H*).

#### 4.4.2. Preparation of 3-(4,4'-dimethoxytriphenylmethyloxy)propyl 2-cyanoethyl *N*,*N*-diisopropylphosphoramidite<sup>33</sup>

3-(4,4'-Dimethoxytriphenylmethyloxy)propan-1-ol (2 g) was co-evaporated with dry acetonitrile. After having being dried in vacuo for 0.5 h, the residue was dissolved in dry acetonitrile (21 mL), then treated with diisopropylamine tetrazolide (0.9 g; 1.0 equiv) and bis(*N*,*N*-diisopropyl)cyanoethoxyphosphite (1.75 g; 1.1 equiv) for 2.5 h at 40 °C. After confirmation of the disappearance of the starting material by TLC, the mixture was evaporated to dryness and the phosphoramidite was dissolved in CH<sub>2</sub>Cl<sub>2</sub>. The mixture was transferred directly to the top of a silica gel column (hexane:ethyl acetate:pyridine, 17:83:0.1 [v/v/v]) and chromatographed to give the product as an oil (2.8 g, 91% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>,  $\delta$ ): 1.10–1.21 (m, 12H), 1.85–1.95 (m, 2H); 2.52–2.57 (m, 2H); 3.14–3.18 (m, 2H); 3.49–3.82 (m, 6H); 3.76 (s, 6H); 6.79–7.44 (m, 13H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>,  $\delta$ ): 14.2, 20.2, 20.3, 21.0, 24.5, 24.6, 31.8, 31.9, 42.8, 43.0, 55.2, 58.2, 58.4, 60.1, 60.4, 60.7, 60.9, 85.8, 112.9, 117.6, 126.6, 127.7, 128.2, 130.0, 136.5, 145.2, 158.3. <sup>31</sup>P NMR (202 MHz, CDCl<sub>3</sub>,  $\delta$ ): 149.4.

#### 4.5. Synthesis of dshRNA

We synthesized a series of dshRNAs on an Applied Biosystems Expedite Model 8909 nucleic acid synthesizer from CEM amidites prepared as previously described.<sup>19,20</sup> The two shRNA elements of the dshRNA were built up simultaneously on either end of the glycerol molecule attached to the solid support (Fig. 1). 2'-O-Methyluridine residues were incorporated by the use of commercially available 2'-O-methyluridine amidites. Base deprotection and cleavage from the solid support were accomplished in a single step by treatment with 28% ammonia solution and EtOH (3:1 [v/v])at 40 °C for 3 h and the CEM protecting group was then removed by treatment with 0.67 M tetrabutylammonium fluoride in DMSO containing 0.67% nitromethane at room temperature for 5-7 h. After quenching of the deprotection reaction with Tris-HCl buffer, pH 7.5, crude dshRNA was obtained by EtOH precipitation. The dshRNA was purified by reverse-phase HPLC on an XTerra column at 60 °C or an XBridge column at 80 °C in the 4,4'-dimethoxytrityl (DMTr)-off mode to give dsh1-10. dshRNA concentrations were determined by measuring the absorbance at 260 nm in 1 M Tris-HCl buffer, pH 7.5, containing 7 M urea at 90 °C to reduce the effects of secondary structure. The extinction coefficient of each dshRNA was separately calculated by the nearest-neighbor approximation method.<sup>34</sup>

#### 4.6. Calculated and observed masses of dshRNA

dsh1: calculated, 33349.5; observed, 33351.6. dsh2: calculated, 32124.8; observed, 32125.8. dsh3: calculated, 33349.5; observed, 33349.6. dsh4: calculated, 29477.2; observed, 29478.4. dsh5: calculated, 34574.1; observed, 34576.5. dsh6: calculated, 34574.2; observed, 34576.5. dsh7: calculated, 35891.0; observed, 35894.5. dsh8: calculated, 34574.2; observed, 34577.6. dsh9: calculated, 33625.7; observed, 33629.5. dsh10: calculated, 32195.0; observed, 32198.0.

#### 4.7. Western blotting

A431 cells were seeded at a density of  $3 \times 10^5$  cells per 6-cm dish in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO<sub>2</sub>. The next day, the medium was changed and the cells were transfected with dshRNA complexed with the cationic liposome LIC-101 as previously described.<sup>23</sup> Seventy-two hours after transfection, protein was extracted from the transfected cells with lysis buffer (50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor cocktail [Roche, Basel, Switzerland]). Cell extract containing 15 µg of protein was resolved by electrophoresis on a sodium dodecyl sulfate 5–20% polyacrylamide gradient gel and transferred to polyvinylidene fluoride membrane filters (Millipore, Billerica, MA, USA). Bcl-2 was detected with a mouse anti-Bcl-2 antibody and horseradish-peroxidase-labeled anti-mouse IgG

(Dako, Glostrup, Denmark).  $\beta$ -Actin was detected with a goat polyclonal anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish-peroxidase-labeled anti-goat IgG (Dako). Immunoreactive bands were visualized with the ECL Plus Western blotting detection system (GE Healthcare, Piscataway, NJ, USA) and analyzed with the ChemiDoc imaging system (Bio-Rad, Hercules, CA, USA).

## 4.8. Determination of IFN-α in human peripheral blood mononuclear cells (PBMCs)

Fresh human blood samples taken from three people were mixed and Heparin Sodium Injection (Ajinomoto, Tokyo, Japan; 1 mL for every 10 mL of blood) and an equal volume of phosphate-buffered saline were added. The diluted blood was gently lavered onto Ficoll-Pague<sup>™</sup> PLUS (GE Healthcare: 3 mL for every 10 mL of diluted blood), care being taken not to disturb the interface. After centrifugation at 400g for 30 min at room temperature, the PBMCs, which were located at the interface between the Ficoll-Paque and the plasma, were collected, washed twice with phosphate-buffered saline, and resuspended in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were seeded in 48-well plates at a density of  $6 \times 10^5$  cells per well, and after incubation for 4 h at 37 °C in 5% CO<sub>2</sub> the cells were transfected with dshRNA complexed with LIC-101. Twenty-four hours after transfection, the culture supernatant was collected and IFN- $\alpha$  was determined with the Human Interferon Alpha enzyme-linked immunosorbent assay (ELISA) Kit (PBL Biomedical Laboratories, Piscataway, NJ, USA) according to the manufacturer's protocol.

#### 4.9. Digestion with snake venom phosphodiesterase (SVPD)

Each 14- $\mu$ L RNA sample (10  $\mu$ M) was diluted with 56  $\mu$ L of 50 mM Tris–HCl buffer, pH 8.0, and the diluted sample was heated at 99 °C for 2 min and cooled slowly to room temperature to allow annealing to take place. SVPD (2  $\mu$ L; 22.4 U) was added to give a total volume of 72  $\mu$ L, the mixture was incubated at 37 °C, and 10- $\mu$ L samples were taken at 0, 0.5, 1, 1.5, 2, and 24 h. After inactivation of the enzyme by heating at 99 °C for 1 min, samples were analyzed by reverse-phase HPLC.

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