Supporting Information

Dumbbell-shaped nanocircular RNAs for RNA interference

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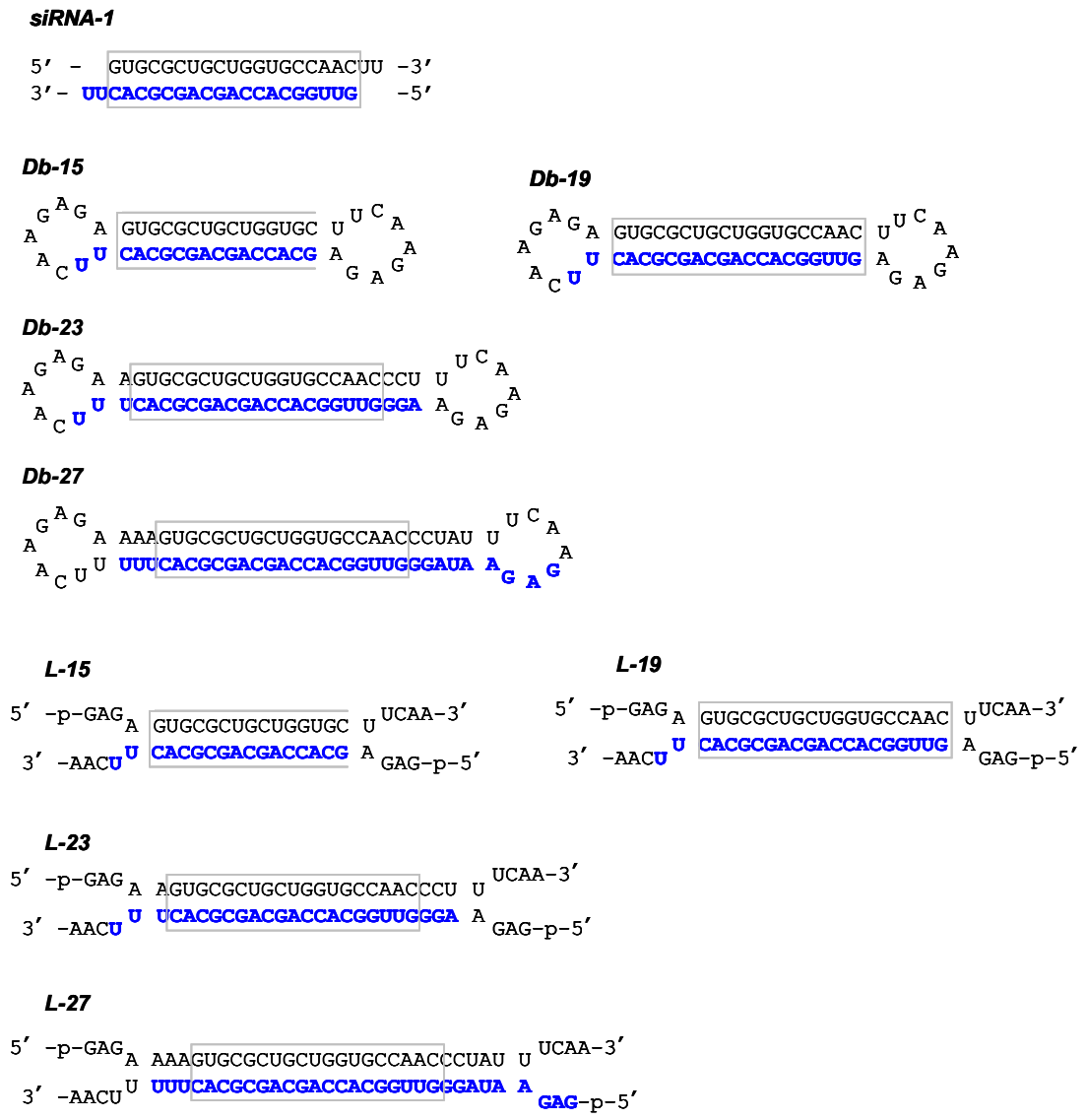

Figure S1. Sequences and structures of RNAs used in this study.
Complementary sequences to the target mRNA are shown in bold. The stem region of siRNA-1 (19 bp) and the same sequences in the dumbbells and their linear counterparts are shown in the boxes.

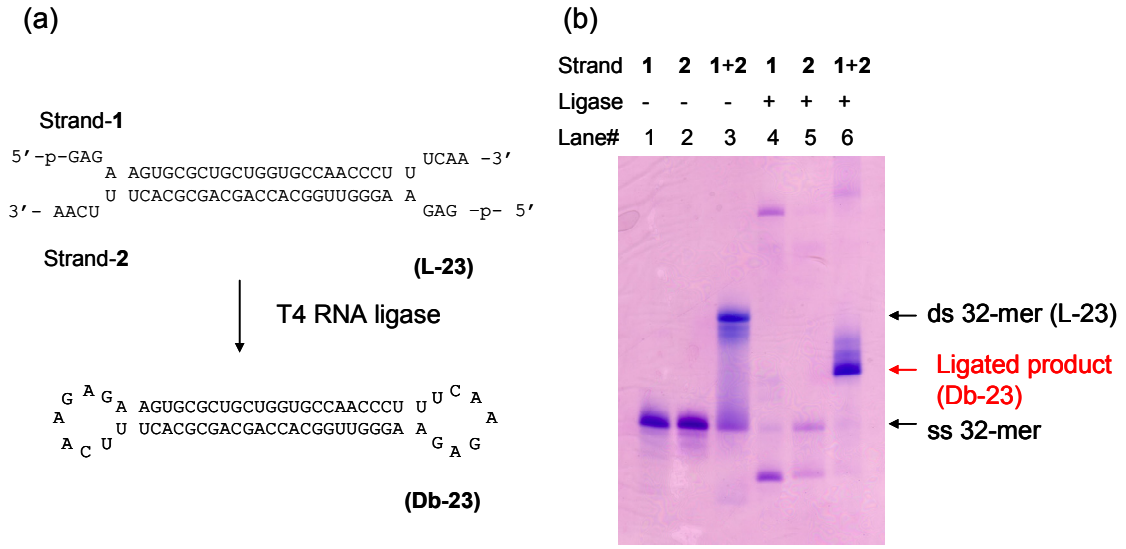

Figure S2. Synthesis of an RNA dumbbell (Db-23) using T4 RNA ligase. (a) Sequence and structure of the starting material (L-23) and the reaction product (Db-23). 'p' means 5′-phosphorylated. (b) Denaturing PAGE (10% PAGE, 25% formamide, 7 M urea in 1× TBE) analysis of the ligation reaction used in making the RNA dumbbell Db-23. Stained with Stains-All. Lanes 1 to 3, T4 RNA ligase (−); lanes 4 to 6, T4 RNA ligase (+). Lanes 1 and 4, Strand-1 only; lanes 2 and 5, Strand-2 only; lanes 3 and 6, Strand-1 + Strand-2.

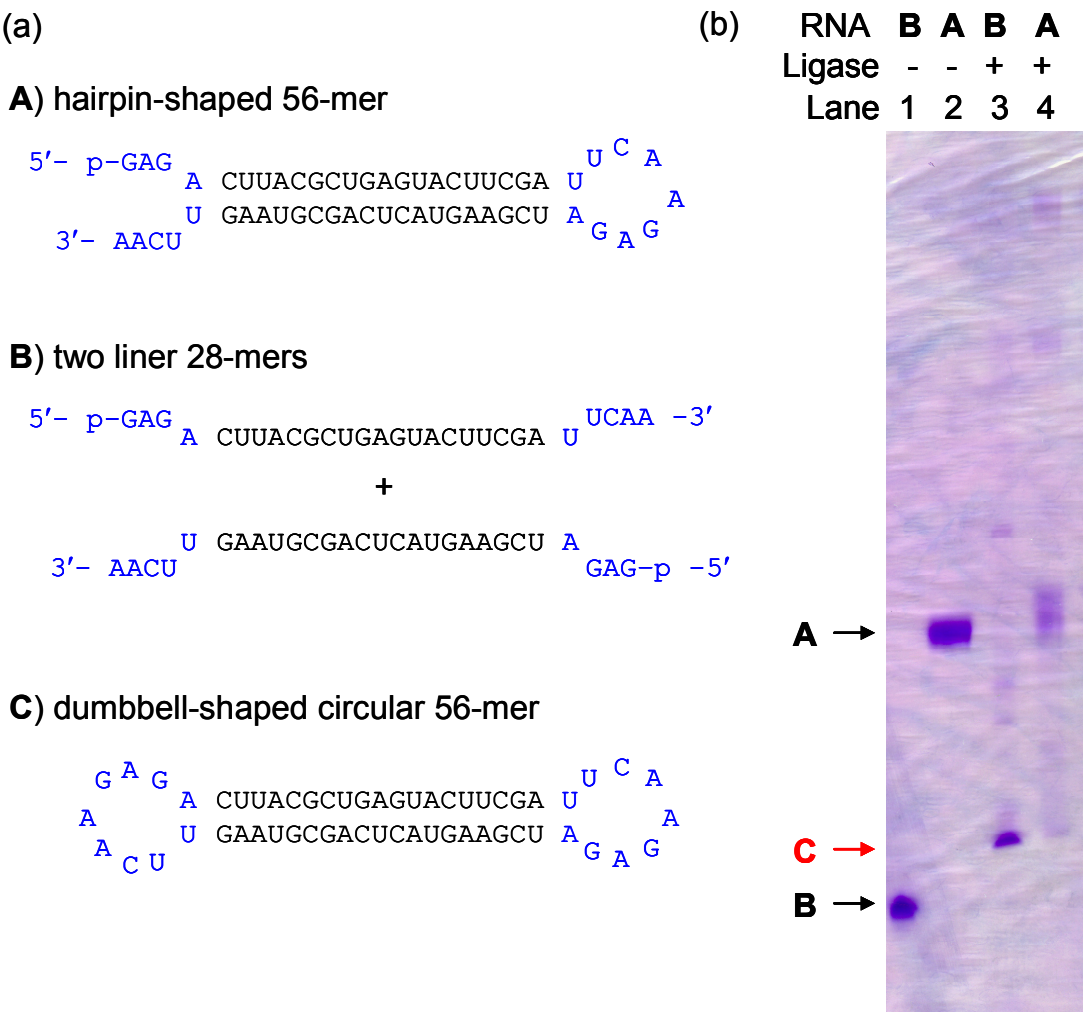

Figure S3. Synthesis of an RNA dumbbell (**C**) using T4 RNA ligase. (a) Sequence and structure of RNA used. 'p' means 5′-phosphorylated. (b) Denaturing PAGE (10% PAGE, 25% formamide, 7 M urea in 1× TBE) analysis of the ligation reaction used in making the RNA dumbbell **C**. Lane 1, **B**; lane 2, **A**; lane 3, **B** + T4 RNA ligase; lane 4, **A** + T4 RNA ligase. Stained with Stains-All.

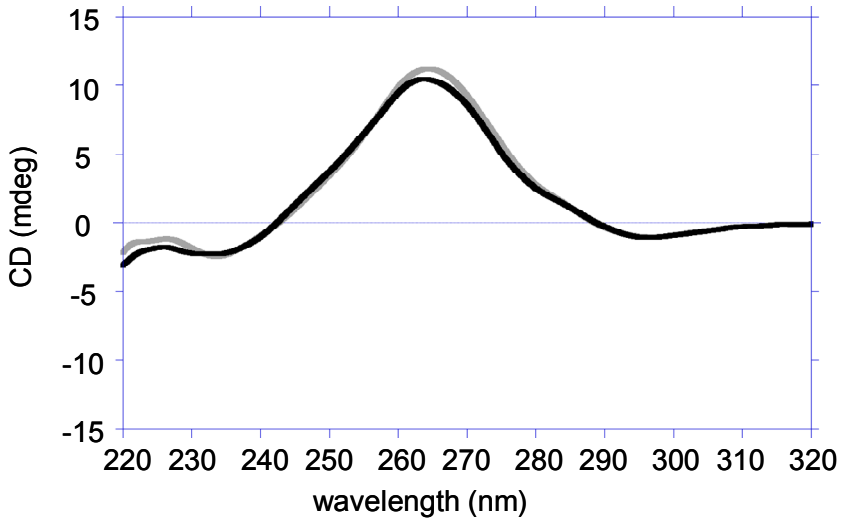

Figure S4. Circular dichroism (CD) spectra of an RNA dumbbell (Db-23, black) and its linear counterpart (L-23, gray) at 20 °C. RNA (0.5 μM) in 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, 1 mM EDTA.

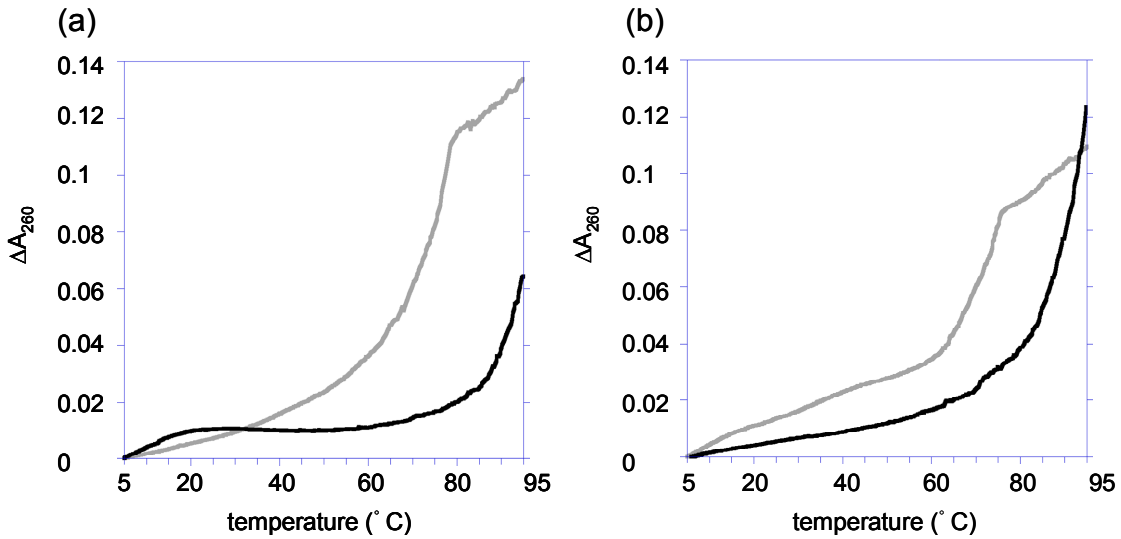

Figure S5. UV melting curves of an RNA dumbbell (Db-23, black) and its linear counterpart (L-23, gray). RNA concentration was 0.5 μM. (a) 10 mM sodium phosphate (pH 7.0), 10 mM NaCl, 1 mM EDTA. (b) 10 mM sodium phosphate (pH 7.0), 1 mM EDTA.

Experimental details

RNA synthesis and purification methods.

RNA oligonucleotides were synthesized using 2′-*O*-TBDMS or 2′-*O*-TOM protected β-cyanoethyl phosphoramidites. 5′-Phosphorylation was performed on the synthesizer using Chemical Phosphorylation Reagent (Glen Research, VA). Deprotected oligonucleotides were purified by 15% denaturing PAGE, isolated by the crush and soak method (elution buffer: 0.2 M NaCl, 10 mM EDTA, pH 8.0). Desalted RNAs were precipitated with sodium acetate (pH 5.2) and 2-propanol.

siRNA targeting GL3 luciferase (siRNA-1) corresponded to the coding region nucleotides 849–869 relative to the first nucleotide of the start codon.[6b] All RNA dumbbells targeted the same region.

Enzymatic synthesis of RNA dumbbells using T4 RNA ligase.

Final composition of the reaction mixture was as follows; 2 μM 5′-phosphorylated-dsRNA, 0.4 units/μL T4 RNA ligase (Takara Bio, Japan), 25% PEG6000, 0.006% bovine serum albumin (BSA), 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP. After 5′-phosphorylated RNAs had been annealed, PEG6000, BSA, and T4 RNA ligase were added to the concentrations described above and incubated at 16 °C overnight. After extraction with chloroform, the RNA was precipitated by the addition of sodium acetate (pH 5.2) and 2-propanol. Ligated products were purified by preparative (1 mm thick) denaturing PAGE (10% PAGE, 25% formamide, 7 M urea in 1× TBE)*. Bands were visualized by UV shadowing, and crushed and extracted with 0.2 M NaCl, 10 mM EDTA (pH 8.0). The eluate was desalted using a Sep-Pak cartridge (Waters, MA). The RNA dumbbells were then precipitated with sodium acetate (pH 5.2) and 2-propanol.

(*Sambrook, J., and Russell, D. W. *Molecular Cloning: a Laboratory Manual, 3rd Ed.* CSHL Press, **2001**.)

Enzymatic synthesis of an RNA dumbbell using T4 RNA ligase (Figure S3).

RNAs (50 pmol, 2 μM) were incubated with 0.4 units/μL T4 RNA ligase in 25% PEG6000, 0.006% BSA, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP at 11 °C overnight. After extraction with chloroform, the RNA was precipitated by the addition of sodium acetate (pH 5.2) and 2-propanol in the presence of glycogen (10 μg). The reactions were analyzed by 10% PAGE, 25% formamide, 7 M urea, 1× TBE. Bands were visualized by staining with Stains-All (Sigma-Aldrich, MO).

CD spectra of Db-23 and L-23.

RNAs (0.5 μM) were dissolved in buffer (10 mM sodium phosphate [pH 7.0], 100 mM NaCl, 1 mM EDTA) and the solutions were heated at 90 °C for 3 min, then slowly cooled to room temperature. The solutions were incubated further at 4 °C overnight. CD spectra were measured on a JASCO J-720 spectropolarimeter (JASCO, Japan) in quartz cuvettes (1 cm pathlength), at 350–200 nm, using 10 scans at 50 nm min^{-1}, 0.25 s response time, 1.0 nm band width, 0.1 nm resolution, 50 mdeg sensitivity at 20 °C. All spectra were noise-reduced by the software provided in that system.

UV melting curves of Db-23 and L-23.

RNAs (0.5 μM) were dissolved in buffer (see legend of Figure S5) and the solutions were heated at 90 °C for 3 min, then slowly cooled to room temperature. The solutions were further incubated at 4 °C overnight before the measurements were made. UV absorbance at 260 nm was

monitored on a JASCO spectrophotometer V-550 (JASCO, Japan) at a heating rate of 0.5 °C/min from 5 °C to 95 °C.

Stability of RNAs against snake venom phosphodiesterase.

Annealed RNAs (2 μM) were incubated with 2×10^{-4} units/μL Phosphodiesterase I from *Crotalus adamanteus* venom (Worthington Biochemical, NJ) in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 15 mM $MgCl_2$ at 37 °C. After 0.5, 1 and 2 h, aliquots (3 μL) were taken from the mixture and the reaction stopped by mixing with 5.3 μL of 0.5 M EDTA (pH 8.0) and frozen. They were analyzed by 15% native PAGE. The total amount of RNA loaded was adjusted according to the stem length to obtain a uniform intensity for the bands. siRNA Ladder Marker (Takara Bio, Japan) was used as the size marker for dsRNA. The gel was stained with SYBR Green I (Cambrex, ME), and visualized by scanning on a BioRad Molecular Imager FX (BioRad, CA).

Stability of RNAs in human serum.

Db-23, L-23 or siRNA-1 (5 μM) was annealed in 1× annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) and diluted with an equivalent volume of 1× phosphate-buffered saline (pH 7.2; GIBCO). Normal human serum (Chemicon, CA) was added to a final volume of 20% and was incubated at 37 °C. After 0.5, 1 and 2 h, aliquots (6 μL) were taken from the mixture, mixed with 2 μL of 0.5 M EDTA (pH 8.0) and frozen. They were analyzed by 15% native PAGE. The total amount of RNA loaded was adjusted according to the stem length to obtain a uniform intensity for the bands. siRNA Ladder Marker (Takara Bio, Japan) was used as the size marker for dsRNA. The gel was stained with SYBR Green I (Cambrex, ME), and visualized by scanning on a BioRad Molecular Imager FX (BioRad, CA).

Dicer cleavage reaction of RNA dumbbells and their linear counterparts.

Annealed RNAs (2.5 μM) were incubated with 0.1 units/μL ColdShock-DICER (TmCspB and fragment of h-Dicer; Takara Bio, Japan) in 20 mM Tris-HCl (pH 8.5), 150 mM NaCl, 2.5 mM $MgCl_2$ at 37 °C. After 1, 6, and 20 h, aliquots (4.5 μL) were taken from the mixture and the reaction stopped by mixing with 1.5 μL of 0.5 M EDTA (pH 8.0) and frozen. They were analyzed by 15% native PAGE. siRNA Ladder Marker (Takara Bio) was used as the size marker for dsRNA. The total amount of RNA loaded was adjusted according to the stem length to obtain a uniform intensity for the bands. The gels were stained with SYBR Green I (Cambrex, ME), and visualized by scanning on a BioRad Molecular Imager FX (BioRad, CA).

We note that smearing of the bands was seen for all dumbbells and in L-15 after treatment with Dicer enzyme (Figure 3). Phenol extraction of the reaction mixture removed this smearing, so these smears are attributable to the interaction of the RNAs with the Dicer enzyme.

Measurements of RNAi activity.

All RNAs used were annealed at 5 μM concentrations in 1× annealing buffer by heating at 90 °C for 3 min, then cooled slowly to room temperature. The solutions were further incubated at 4 °C overnight.

NIH/3T3 cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS; Israel Beit Haemek, Israel). Cells were regularly passaged to maintain exponential growth. One day before transfection at 70% confluency, cells were plated in 96-well plates (1.6×10^4 cells/100 μL per well). Cotransfection of reporter plasmids and RNAs was carried out with GeneSilencer (Genlantis, CA) as described by the manufacturer for adherent cell lines. Just before cotransfection, the culture medium was replaced with 56 μL of DMEM without FCS, and 0.2 μg of pGL3-Control (Promega, WI), 0.2 μg of pRL-TK (Promega), and 2.5 pmol of RNA formulated into liposomes

(44 μL) were added to each well. Four hours after the transfection, 100 μL of 20% FCS in DMEM was added to each well. Luciferase expression was subsequently monitored with the Dual-Luciferase Reporter Assay System (Promega) on a Wallac ARVO SX (PerkinElmer, MA).