Terminus-free siRNA prepared by photo-crosslinking activated via slicing by Ago2

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Supporting Information

Materials and methods

Synthesis of RNAs

Phosphoramidite monomers bearing *p*-stilbazole or *p*-cyanostilbene modified D-threoninol (B or S_{CN}, respectively) were synthesized according to a previous report.[23] All modified RNAs were synthesized using an ABI-3400 DNA synthesizer (Applied Biosystems). After the recommended work-up, the synthesized RNAs were purified through Poly-Pak cartridges and by reverse-phase HPLC. Sample handling was conducted in the dark. Synthesized oligonucleotides were characterized on a reverse-phase column and by MALDI-TOF MS using an Autoflex II (Bruker Daltonics). RNA sequences for "chemically synthesized" siRNA used as standards in gel analyses are listed below. 21S: 5'-CCUUACCCACCUCAUGUAUCU-3', 21A: 5'-AUACAUGAGGUGGGUAAGGUU-3', 5t-S_{CN}-S13nick: 5'-**S_{CN}**AACCUUACCCACC-3', 3t-S_{CN}-Snick10: 5'- UCAUGUAUCU**S_{CN}**-3', S_{CN}S7-13nick: 5'- AACCUU**S_{CN}**ACCCACC-3', S_{CN}A7-nick10: 5'-GGGU**S_{CN}**AAGGUU-3',

The MS data for synthesized oligonucleotides are summarized as follows: rS_{CN}1a: obsd 3212 (calcd for [rS_{CN}1a + H^+]: 3213). rS_{CN}1b: obsd 3252 (calcd for [rS_{CN}1b + H^+]: 3253). rS_{CN}2a: obsd 3213 (calcd for [rS_{CN}2a + H^+]: 3213). rS_{CN}2b: obsd 3253 (calcd for

[rS$_{CN}$2b + H$^+$]: 3253). rS$_{CN}$3a: obsd 3212 (calcd for [rS$_{CN}$3a + H$^+$]: 3213). rS$_{CN}$3b: obsd 3253 (calcd for [rS$_{CN}$3b + H$^+$]: 3253). rB1a: obsd 3189 (calcd for [rB1a + H$^+$]: 3189). rB1b: obsd 3229 (calcd for [rB1b + H$^+$]: 3229). rB2a: obsd 3189 (calcd for [rB2a + H$^+$]: 3189). rB2b: obsd 3229 (calcd for [rB2b + H$^+$]: 3229). rB3a: obsd 3187 (calcd for [rB3a + H$^+$]: 3189). rB3b: obsd 3227 (calcd for [rB3b + H$^+$]: 3229). t-S$_{CN}$-S: obsd 7942 (calcd for [t-S$_{CN}$-S + H$^+$]: 7942). t-S$_{CN}$-A: obsd 8285 (calcd for [t-S$_{CN}$-A + H$^+$]: 8285). S$_{CN}$S7: obsd 7543 (calcd for [S$_{CN}$S7 + H$^+$]: 7544). S$_{CN}$A7: obsd 7887 (calcd for [S$_{CN}$A7 + H$^+$]: 7887). S$_{CN}$S11: obsd 7543 (calcd for [S$_{CN}$S11 + H$^+$]: 7544). S$_{CN}$A11: obsd 7887 (calcd for [S$_{CN}$A11 + H$^+$]: 7887). 5t-S$_{CN}$-S13nick: obsd 4401 (calcd for [5t-S$_{CN}$-S13nick + H$^+$]: 4401), 3t-S$_{CN}$-Snick10: obsd 3479 (calcd for [5t-S$_{CN}$-S13nick + H$^+$]: 3481), S$_{CN}$S7-13nick: obsd 4401 (calcd for [S$_{CN}$S7-13nick + H$^+$]: 4401), S$_{CN}$A7-nick10: obsd 3638 (calcd for [S$_{CN}$A7-nick10 + H$^+$]: 3639).

UV irradiation for photo-crosslinking of dsRNAs

For photo-crosslinking, a xenon light source (MAX-301, Asahi Spectra) equipped with interference filters at 340 nm was used. The sample (2.0 μM) in 10 mM sodium phosphate (pH 7.0), 100 mM NaCl was irradiated at 20 ^{o}C by using a programmable temperature controller.

UV-VIS spectra measurements

UV spectra were measured on a JASCO model V-530 equipped with a programmed temperature controller; 10-mm quartz cells were used. The melting curves were obtained with a JASCO model V-5360 by measuring the change in absorbance at 260 nm versus temperature. The temperature ramp was 1.0 °C min^{-1}. Photo-crosslinking ratios of each homodimer were plotted on the bases of UV-Vis spectra data, and reaction kinetics were determined.

HPLC analyses and purification of photocrosslinked siRNA

A Merck LiChrospher 100 RP-18(e) column was used for HPLC analyses. The flow rate was 0.5 mL/min. A solution of 50 mM ammonium formate (solution A) and a mixture of 50 mM ammonium formate and acetonitrile (50/50, v/v) (solution B) were used as mobile phases. A linear gradient of 30–50% solution B over 30 min was employed. HPLC chromatograms were monitored at 260 nm absorption.

Cell culture

293 FT and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% fetal bovine serum, 80 μg/ml penicillin, 90 μg/ml streptomycin. Cells were cultured at 37 °C with 5% CO_2 in humidified air.

Preparation of cell lysate

293 FT or HeLa cells were cultured in 10-cm dishes to confluence. Cells were washed with PBS and lysed in 1 ml of buffer containing 10 mM Pipes-NaOH (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100. Cells were homogenized using 2.5-G syringe needle, centrifuged at 16,000 g for 20 min at 4 °C, and then the supernatants were collected.

siRNA cleavage assay with cell lysate

siRNA (10 μM) was annealed in the 10 mM Tris-acetate (pH 8.3) buffer containing 0.25 mM EDTA. The siRNA solution (5 μl) was mixed with lysate solution (45 μl) and incubated at 37 °C. Aliquots were removed at indicated times, added to loading buffer containing EDTA (50 mM) and bromophenol blue (0.025%), and subjected to electrophoresis on a 20% polyacrylamide gel at 750 CV for 120 min. Gels were stained with SYBR® Green II and analyzed with a Typhoon FLA 9500 (GE Healthcare).

Dual luciferase assay

The dual luciferase assays were performed as described in previous reports[14, 24, 25] In this assay, two types of reporter plasmids, pGL3-Fw and pGL-Rv, were used. The target sequence (5'-AACCTTACCCACCTCATGTATCT-3' for pGL3-Fw or 5'-AGATACATGAGGTGGGTAAGGTT-3' for pGL3-Rv) was inserted into the 3'-UTR region of the gene encoding the firefly luciferase in pGL3. Co-transfections of 293FT cells with siRNA (final concentrations 17 nM), 100 ng of pGL3, and 0.5 ng of *Renilla* luciferase expression vector, which is used as an internal control, were performed using Lipofectamine™ 2000 (Invitrogen) in 96-well plates according to the manufacturer's instructions. After incubation at 37 °C for 48 h, 75 μl of medium was removed, and 75 μl of Dual-Glo® regent (Promega) was added. Firefly luciferase luminescence was measured on a Multi-label Plate Reader (EnSpire™, Perkin Elmer). Subsequently 75 μl of Dual-Glo® Stop & Glo® reagent was added, and *Renilla* luciferase luminescence was measured. The relative luciferase activity (firefly/Renilla) was determined. The level of luciferase activity in the absence of siRNA was taken as 1.0.

Dicing assay

siRNA (6.0 μM) was incubated with Dicer (0.1 U/μl, Genlantis) in Dicer Reaction

Buffer (Genlantis) supplemented with 1.0 mM ATP and 2.5 mM $MgCl_2$. Aliquots removed at indicated times were added to loading buffer containing EDTA (50 mM) and bromophenol blue (0.025%) and were subjected to electrophoresis on a 20% polyacrylamide gel. Gels were stained with SYBR® Green II and analyzed with Typhoon FLA 9500 (GE Healthcare). The sequences of "Chemically synthesized" siRNA in Figure 3a are S/A, S/21A, 21S/A, 21S/21A, and before and after photocrosslinking of t-S_{CN}-S/t-S_{CN}-A.

Preparation of Argonaute 2

pcDNA4-Ago2 was generated by cloning the hAgo2 gene from pFN21AE9257 (Kazusa DNA Res. Inst.) into pcDNA4 vector (Invitrogen) using *Hind III* and *EcoRI*. 293FT cells were cultured in 10-cm dish to 90% confluence, and cells were transfected with 14 μg of pcDNA4-Ago2 using Lipofectamine™ 2000 (Invitrogen) with Opti-MEM according to the manufacturer's instructions. After culture at 37 °C for 4 h, medium was changed with DMEM and cells were further cultured for 20 h at 37 °C. Then cells were washed with PBS and lysed in 1 ml of buffer containing 10 mM Hepes-NaOH (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 0.1% (v/v) Tween-20, 5 mM dithiothreitol (DTT), and EDTA-free protease inhibitor cocktail (SIGMA). Cells were homogenized using a 2.5-G syringe needle, centrifuged at 16,000 g for 20 min at 4 °C, and then the supernatant was collected. The poly-His fusion Ago2 were purified from the supernatant with a Ni^{2+}-sepharose high performance column (GE Healthcare) by using 50 mM Tris-HCl (pH 7.4), 5 mM DTT, 5 ~500 mM Imidazole.

Slicing assay of siRNA by Argonaute 2

Slicing assays were performed using 50 μM recombinant hAgo2 dissolved in 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.5 mM $MgCl_2$, and EDTA-free protease inhibitor cocktail. Crosslinked siRNAs (1 μM) were mixed with hAgo2 and incubated at 37 °C. Aliquots were removed at indicated times and added to loading buffer containing 80% formamide, 50 mM EDTA, and 0.025% bromophenol blue. The mixture was subjected to electrophoresis on a 15% polyacrylamide gel including 8 M urea and 10% (v/v) glycerol at 700 CV for 90 min at 60 °C. Gels were stained with SYBR® Green II and analyzed with a Typhoon FLA 9500 (GE Healthcare) and ImageQuantTL (GE Healthcare).

The sequences of "Chemically synthesized" siRNA are photocrosslinked t-S_{CN}-S/t-S_{CN}-A and 5t-S_{CN}-S13nick/3t-S_{CN}-Snick10/t-S_{CN}-A in Figure 3b, photocrosslinked S_{CN}S7/S_{CN}A7, S_{CN}S7-13nick/S_{CN}A7, S_{CN}S7/S_{CN}A7-nick10, and S_{CN}S11/S_{CN}A11 in Figure 4c

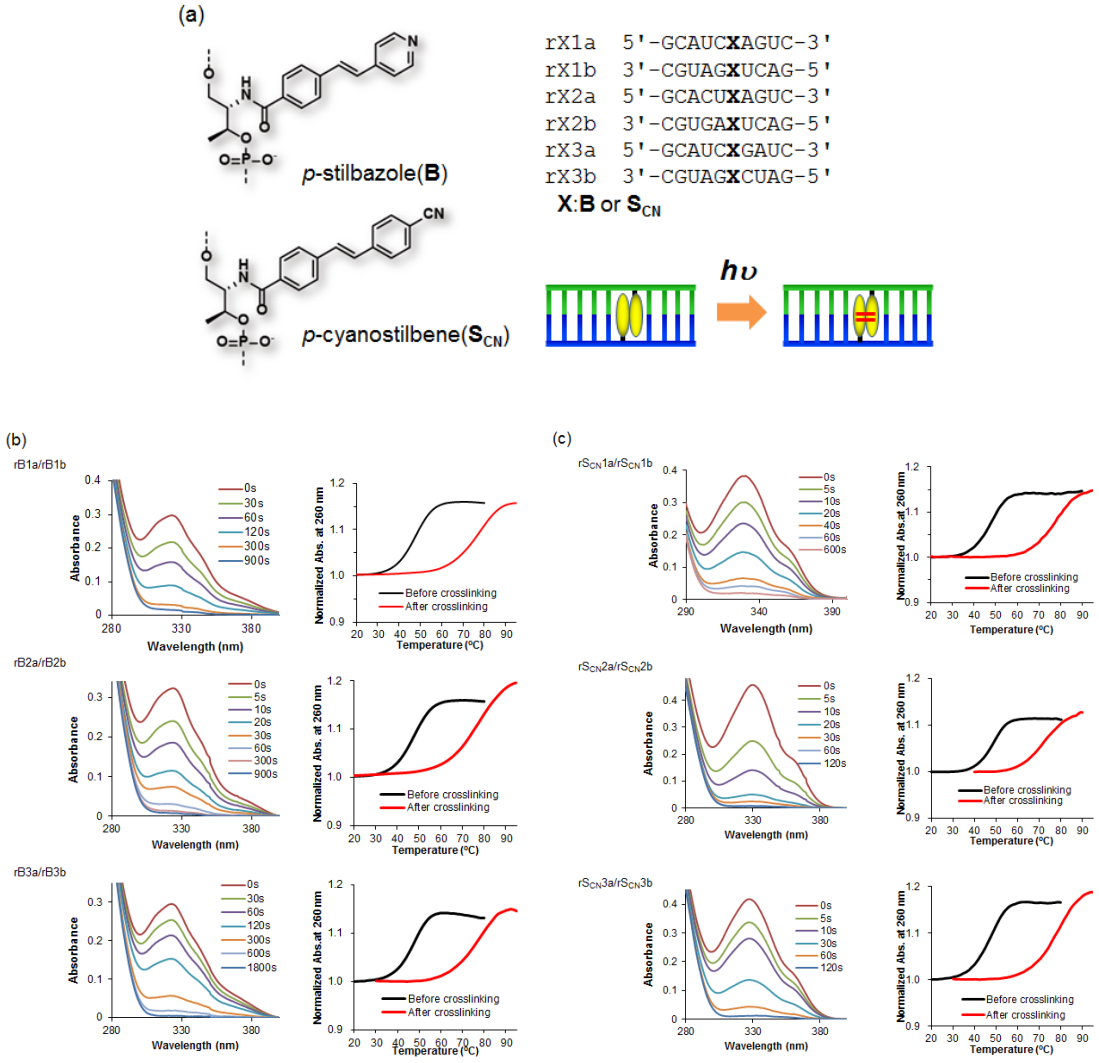
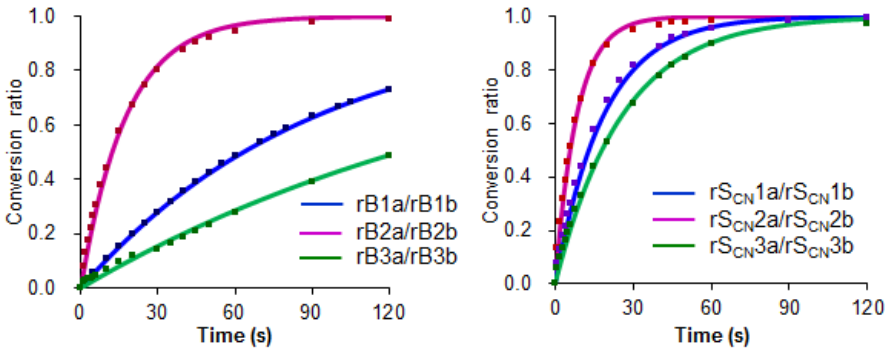


Figure S1. (a) Photo-crosslinking of *p*-stilbazoles or *p*-cyanostilbenes in rX1a/rX1b, rX2a/rX2b, and rX3a/rX3b. UV-Vis spectra of dsRNA with **B**s (b, left) or **S**$_{CN}$s (c, left) irradiated by UV irradiation at 340 nm. Melting curves of dsRNA with **B**s (b, right) or **S**$_{CN}$s (c, right) before (black) and after photo-irradiation (red). Solution conditions for photo-crosslinking were 2.0 μM dsRNA, 100 mM NaCl, 10 mM phosphate buffer (pH 7.0), 20 °C.

(a)



(b)

Sequence	k_{obs} (min^{-1})	
	p-stilbazole	*p*-cyanostilbene
rX1a/rX1b	0.511	3.21
rX2a/rX2b	3.19	7.17
rX3a/rX3b	0.337	2.32

Figure S2. Photo-reactivaties of *p*-stilbazole and *p*-cyanostilbene in RNA duplexes. (a) Photo-crosslinking ratios of each homodimer in each dsRNA were plotted on the bases of UV-Vis spectra data (Figure S1). (b)Observed rate constants determined from initial slope of Figure S2a.

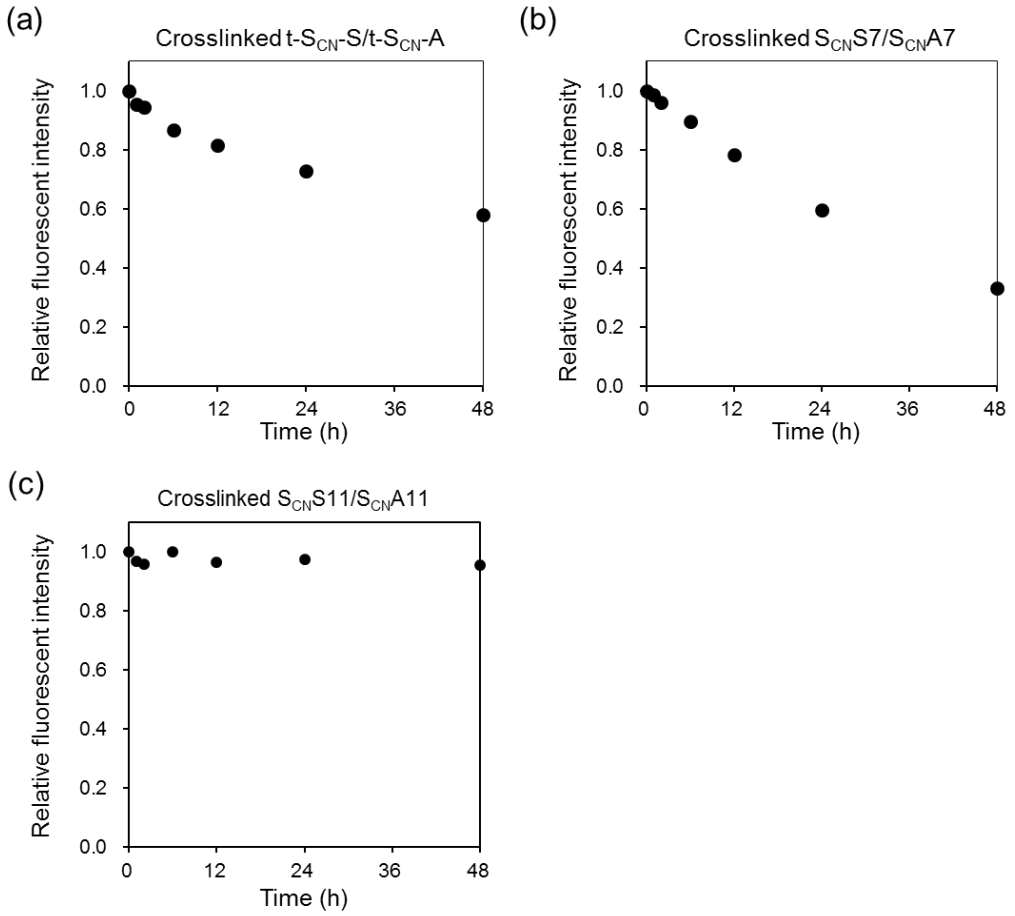


Figure S3. The fraction of remaining crosslinked t-SCN-S/t-SCN-A (a), crosslinked SCNS7/SCNA7 (b), and crosslinked SCNS11/SCNA11 were plotted on the bases of denaturing-PAGE data in the Ago2-slicing assay (Figure 3b and 4c).

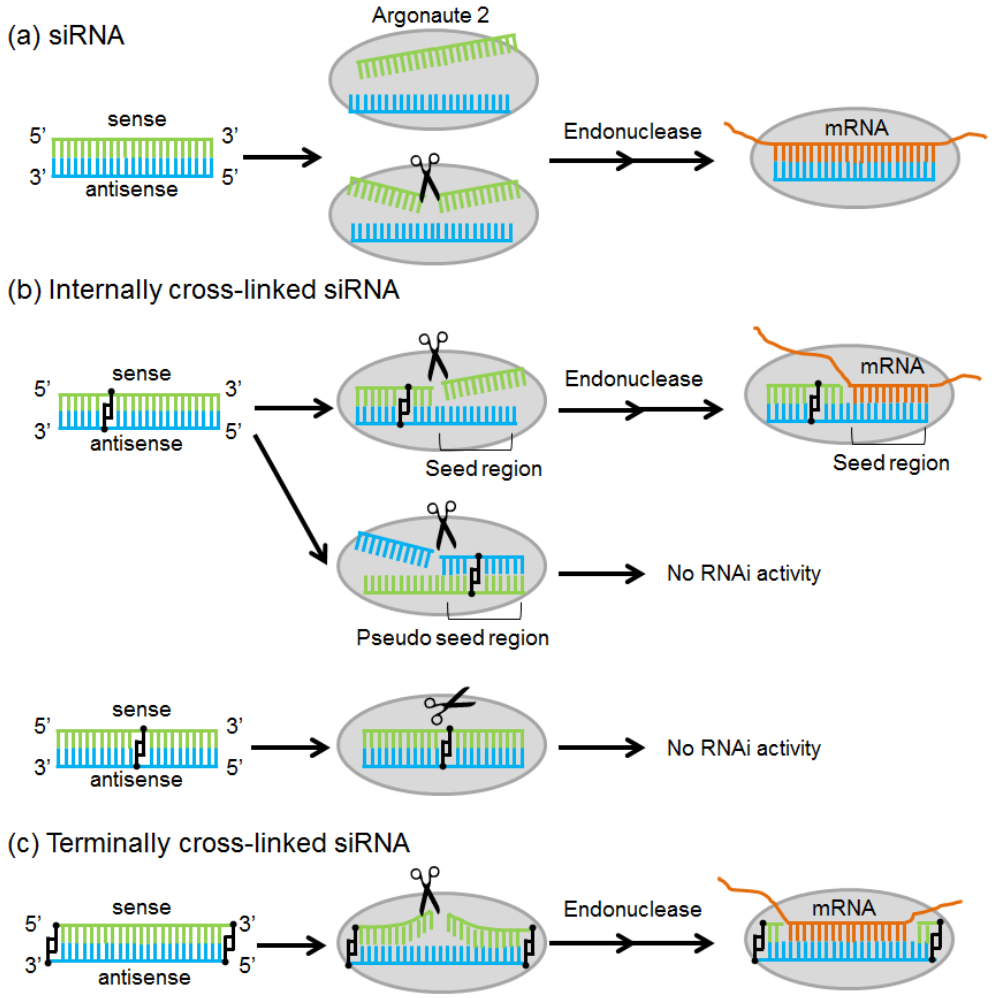


Figure S4. Our hypotheses for mechanisms of sense strand removal for RISC activation. (a) Removal of sense strand by slicing and/or unwinding of siRNA from RISC promotes the interaction between antisense strand and the target mRNA. (b) If the internal crosslinking moiety does not perturb slicer activity of Ago2, the sliced sense strand of siRNA can be removed from RISC and digested by an endonuclease. This allows RISC to capture target mRNA through an interaction with the seed region of antisense strand. (c) Terminally crosslinked siRNA can be sliced in RISC by Ago2 allowing RISC activation.