# Reversible control of RNA interference by siRNAzos

# Matthew L. Hammill, Golam Islam and Jean-Paul Desaulniers

# **Supporting Information**

**Table of Contents** 

# **Nucleic Acid and Biological Procedures**

Procedure for Oligonucleotide Synthesis and Purification	S4
Procedure for performing CD experiments	S5
Procedure for absorbance spectra experiments	S5
Procedure for Melting Temperature of siRNA Duplexes ( <i>T<sub>m</sub></i> )	S5
Procedure for HPLC Characterization	S5
Procedure for Reduced Glutathione (GSH) Degradation Assay	S6
Procedure for Maintaining Cell Cultures of HeLa Cells	S6
Procedure for siRNA transfections	S6
Procedure for <i>in vitro</i> Dual Luciferase Assay	S6
Procedure for light inactivation of azobenzene modified siRNA (trans to cis)	S7
Procedure for light reactivation of azobenzene modified siRNA (cis to trans)	S7
Procedure for consecutive UV/Vis Light Cycling (1.5x and 2x light cycling)	S7
Procedures for Real Time PCR	S8
Procedure for XTT Assays	S10

# **Figures and Tables**

Figure S-1: CD spectra of azobenzene modified spacers replacing two nucleobases targeting luciferase mRNAs.	firefly .S11
Figure S-2: CD spectra of azobenzene modified spacers replacing two nucleobases targeting <i>BCL2</i> mRNAs	.S11
Figure S-3: Absorbance Profile of siRNA 1 when exposed to UV and Visible light	.S12
Figure S-4: Absorbance Profile of siRNA 2 when exposed to UV and Visible light	.S12
Figure S-5: Absorbance Profile of siRNA 3 when exposed to UV and Visible light	.S13
Figure S-6: Absorbance Profile of siRNA 4 when exposed to UV and Visible light	.S13
Figure S-7: Absorbance Profile of siRNA 7 when exposed to UV and Visible light	.S14
Figure S-8: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 1 UV/dark 8 h	.S14
Figure S-9: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 2 UV/dark 8 h	.S15

Figure S-10: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 3 UV/dark 8 h
Figure S-11: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 4 UV/dark 8 h
Figure S-12: Numerical bar graph showing reduction of normalized firefly luciferase expression for wt siRNA UV/dark 8 hS16
Figure S-13: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 1 UV/dark 24 hS17
Figure S-14: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 2 UV/dark 24 h
Figure S-15: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 3 UV/dark 24 h
Figure S-16: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 4 UV/dark 24 h
Figure S-17: Numerical bar graph showing reduction of normalized firefly luciferase expression for wt siRNA UV/dark 24 hS19
Figure S-18: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 1 UV/Vis/dark 8 hS19
Figure S-19: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 2 UV/Vis/dark 8 h
Figure S-20: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 3 UV/Vis/dark 8 hS20
Figure S-21: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 4 UV/Vis/dark 8 hS21
Figure S-22: Numerical bar graph showing reduction of normalized firefly luciferase expression for wt siRNA UV/Vis/dark 8 h
Figure S-23: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 1 UV/Vis/dark 24 hS22
Figure S-24: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 2 UV/Vis/dark 24 hS22
Figure S-25: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 3 UV/Vis/dark 24 h

Figure S-26: Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 4 UV/Vis/dark 24 hS23
Figure S-27: Numerical bar graph showing reduction of normalized firefly luciferase expression for wt siRNA UV/Vis/dark 24 hS24
Figure S-28: Numerical bar graph showing reduction of normalized <i>BCL2</i> expression for siRNAzo 5 UV/Vis/dark 8 hS24
Figure S-29: Numerical bar graph showing reduction of normalized <i>BCL2</i> expression for siRNAzo 5 UV/Vis/dark 8 hS25
Figure S-30: Numerical bar graph showing reduction of normalized <i>BCL2</i> expression for siRNAzo 5 UV/Vis/dark 8 h
Figure S-31: Numerical bar graph showing reduction of normalized <i>BCL2</i> expression for siRNAzo 5 UV/Vis/dark 24 hS26
Figure S-32: Numerical bar graph showing reduction of normalized <i>BCL2</i> expression for siRNAzo 5 UV/Vis/dark 24 hS26
Figure S-33: Numerical bar graph showing reduction of normalized <i>BCL2</i> expression for siRNAzo 5 UV/Vis/dark 24 hS27
Figure S-34: XTT Assay for UV exposed cell viability
Figure S-35: Comparative HPLC chromatogram of untreated siRNAzo 5 and UV light exposure
Figure S-36: Comparative HPLC chromatogram of untreated siRNAzo 6 and UV light exposure
Figure S-37: Comparative HPLC chromatogram of untreated siRNAzo 7 and UV light exposure
Figure S-38: GSH assay comparative HPLC chromatograms of untreated siRNAzo 6 and UV light exposure
Table S-1: Mass Spectrometry deconvolution results of siRNAzo sense strands 1-7
References

#### **Nucleic Acid and Biological Procedures**

### Procedure for Oligonucleotide Synthesis and Purification

All standard  $\beta$ -cyanoethyl 2'-*O*-TBDMS protected phosphoramidites, reagents and solid supports were purchased from Chemgenes Corporation and Glen Research. Wild-type luciferase strands including the sense and 5'-phosphorylated antisense strand were synthesized. All commercial phosphoramidites were dissolved in anhydrous acetonitrile to a concentration of 0.10 M. The chemically synthesized (azobenzene derivative) phosphoramidites were dissolved in 3:1 (v/v) acetonitrile:THF (anhydrous) to a concentration of 0.10 M. The reagents that were used for the phosphoramidite coupling cycle were: acetic anhydride/pyridine/THF (Cap A), 16% *N*-methylimidazole in THF (Cap B), 0.25 M 5-ethylthio tetrazole in ACN (activator), 0.02 M iodine/pyridine/H<sub>2</sub>O/THF (oxidation solution), and 3% trichloroacetic acid/dichloromethane. All sequences were synthesized on 0.20  $\mu$ M or 1.00  $\mu$ M dT solid supports. The entire synthesis ran on an Applied Biosystems 394 DNA/RNA synthesizer using 0.20  $\mu$ M or 1.00  $\mu$ M cycles kept under argon at 55 psi. Standard and synthetic phosphoramidites ran with coupling times of 999 seconds.

Antisense sequences were chemically phosphorylated at the 5'-end by using 2-[2-(4,4'dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite. At the end of every cycle, the columns were removed from the synthesizer, dried with a stream of argon gas, sealed and stored at 4 °C. Cleavage of oligonucleotides from their solid supports was performed through oncolumn exposure to 1.50 mL of EMAM (methylamine 40% wt. in H<sub>2</sub>O and methylamine 33% wt. in ethanol, 1:1 (Sigma-Aldrich)) for 1 hour at room temperature with the solution in full contact with the controlled pore glass. The oligonucleotides were then incubated overnight at room temperature in EMAM to deprotect the bases. On the following day, the samples were concentrated on a Speedvac evaporator overnight, resuspended in a solution of DMSO:3HF/TEA (100 μL:125 μL) and incubated at 65 °C for 3 hours in order to remove the 2'-O-TDBMS protecting groups. Crude oligonucleotides were precipitated in EtOH and desalted through Millipore Amicon Ultra 3000 MW cellulose. Oligonucleotides were separated on a 20% acrylamide gel and were used without further purification for annealing and transfection. Equimolar amounts of complimentary RNAs were annealed at 95 °C for 2 min in a binding buffer (75.0 mM KCl, 50.0 mM Tris-HCl, 3.00 mM MgCl<sub>2</sub>, pH 8.30) and this solution was cooled slowly to room temperature to generate siRNAs used for biological assays. A sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.00 mM EDTA, pH 7.00) was used to anneal strands for biophysical measurements.

### **Procedure for Performing CD Experiments**

Circular Dichroism (CD) spectroscopy was performed on a Jasco J-815 CD equipped with temperature controller. Equimolar amounts of each siRNA (10  $\mu$ M) were annealed to their compliment in 500  $\mu$ L of a sodium phosphate buffer by incubating at 95 °C for two minutes and allowing to cool to room temperature. CD measurement of each duplex were recorded in triplicate from 200-500 nm at 25 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. The average of the three replicates was calculated using Jasco's Spectra Manager version 2 software and adjusted against the baseline measurement of the sodium phosphate buffer.

### **Procedure for Absorbance Spectra Experiments**

All absorbance spectra measurements were done on a Jasco J-815 CD with temperature controller. Measurement was recorded from 200-500 nm at 10 °C at least 3 times. UV treated samples were placed under a UVP UVL-23RW Compact UV lamp 4.00 W 365nm for the indicated time. Visible light treated samples were placed under a 60.0 W daylight bulb from NOMA in standard desk lamp.

### Procedure for Melting Temperature of siRNA Duplexes (T<sub>m</sub>)

The siRNA duplexes annealed as above were placed in the Jasco J-815 CD spectropolarimeter and then UV absorbance was measured at 260 nm against a temperature gradient of 10 °C to 95 °C at a rate of 0.5 °C per minute with absorbance being measured at each 0.5 °C increment. Absorbance was adjusted to baseline by subtracting absorbance of the buffer. The  $T_m$  values were calculated using Meltwin v3.5 software. Each siRNA result was the average of 3 independent experiments and the reported values were calculated using Meltwin v3.5 assuming the two-state model.<sup>2</sup>

### **Procedure for HPLC Characterization**

HPLC chromatograms were obtained on a Waters 1525 binary HPLC pump with a Waters 2489 UV/Vis detector using the Empower 3 software. A C18 4.6 mm x 150 mm reverse phase column was used. Conditions were 5% acetonitrile in 95% 0.1 M TEAA (Triethylamine-Acetic Acid) buffer up to 100% acetonitrile over 40 min.

### **Procedure for Reduced Glutathione (GSH) Degradation Assay**

The GSH assay was performed on the sense strand of siRNA 6 in a 96 well plate at 37 °C. A concentration of 2.7  $\mu$ M of siRNA was added to 10 mM glutathione and 5 mM TCEP in PBS to a final volume of 100  $\mu$ L. Dark experiments were performed with no additional treatment at 0, 8, and 24 h time points after which the entire 100  $\mu$ L was sample was injected into the HPLC and characterized (same conditions as above) to afford the HPLC traces at the different time points. UV treated siRNA was exposed to 5 min of UV light before incubation at 37 °C, and kept in the dark until injection for 0, 8, and 24 h time points. The UV 0 h time point was exposed to UV light and then injected immediately onto the HPLC.

### Procedure for Maintaining Cell Cultures of HeLa Cells

For biological analysis of these siRNAs in a live environment, human epithelial cervix carcinoma cells were used (HeLa cells). They were kept in 250 mL vented culture flasks using 25.0 mL of DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma) in an incubator set for 37 °C @ 5%  $CO_2$  humidified atmosphere.

Once cell lines became confluent (80-90%) they were passaged by washing 3 times with 20 mL of phosphate buffered saline (NaCl 137 mM, KCl 2.70 mM,  $PO_4^{3-}$  10.0 mM, pH 7.40) and incubated with 5.00 mL of 0.25% trypsin (SAFC bioscience) for 4 min @ 37 °C to detach the cells. The cells were transferred to a 50.0 mL centrifuge tube after the addition of 10.0 mL of DMEM solution and pelleted at 2000 rpm for 5 minutes. The supernatant was discarded and the pellet resuspended in 5.0 mL DMEM with 10% FBS.

A standard haemocytometer was used to obtain cell counts, after which the cells were diluted to a final concentration of  $1.00 \times 10^6$  cells/mL for subsequent assays. To continue the cell line 1.00 mL of freshly passaged cells was added to 24.0 mL of DMEM/10% FBS and 1% penicillin-streptomycin at 37 °C in a new culture flask while the rest were used for assays.

### **Procedure for siRNA Transfections**

100  $\mu$ L of cells (total 1.00 x 10<sup>5</sup>) were transfected into 12 well plates (Falcon<sup>®</sup>) with 1 mL of DMEM (10% FBS, 1% penicillin-streptomycin) and incubated at 37 °C with 5% CO<sub>2</sub>. After 24 hours the cells were transfected with various concentrations of siRNAs, along with both pGL3 (Promega) and pRLSV40 luciferase plasmids using Lipofectamine 2000 (Invitrogen) in Gibco's 1X Opti-Mem reduced serum media (Invitrogen) according to the manufacturer's instructions. 1.00  $\mu$ L of siRNA was added along with 2.00  $\mu$ L (pGL3 200 ng) and 0.50  $\mu$ L pRLSV40 (50.0 ng) to 100  $\mu$ L of 1X Opti-Mem in a microcentrifuge tube and

kept on ice for 5 min. In a different microcentrifuge tube 1.00  $\mu$ L of Lipofectamine 2000 (Invitrogen) was mixed with 100  $\mu$ L of Gibco's 1X Opti-Mem reduced serum media (Invitrogen) and incubated at room temperature for 5 min. After 5 minutes the tubes were mixed and incubated at room temperature for 20 min and then the entire contents transferred to the wells of the 12 well plate.

# Procedure for *in vitro* Dual-Reporter<sup>®</sup> Luciferase Assay

100 µL of cells (total of 1.00 x 10<sup>5</sup> cells) were added to 12 well plates (Falcon<sup>®</sup>) with 1 mL of growth media (DMEM 10% FBS, 1% penicillin-streptomycin) and incubated at 37 °C with 5% CO<sub>2</sub>. After 24 hours the cells were transfected with 160, 400 and 800 pM concentrations of siRNAs, along with both pGL3 (Promega) and pRLSV40 luciferase plasmids using Lipofectamine 2000 (Invitrogen) in Gibco's 1X Opti-Mem reduced serum media (Invitrogen) according to the manufacturer's instructions. After a set amount of time (8 or 24 h) the cells were incubated at room temperature in 1X passive lysis buffer (Promega) for 20 minutes. The lysates were collected and loaded onto a 96 well, opaque plate (Costar). With a Dual-Luciferase reporter Assay kit (Promega), Lar II and Stop & Glo<sup>®</sup> luciferase substrates were sequentially added to the lysates and enzyme activity was measured through luminescence of both *firefly/Renilla* luciferase on a Synergy HT (Bio-Tek) plate luminometer. The ratio of *firefly/Renilla* luminescence is expressed as a percentage of reduction in *firefly* protein expression to siRNA efficacy when compared to untreated controls. Each value is the average of at least 3 different experiments with standard deviation indicated.

### Procedure for Light Inactivation of Azobenzene Modified siRNA (trans to cis)

The cell culture plates were exposed to a 4.00 W 365 nm UV lamp (UVP) 2 hours after transfection for 8 h assays (1 exposure total) and every 4 h thereafter for 24 h assays (6 exposures total). Luciferase assays were then performed as indicated above at the desired time points.

# Procedure for Light Reactivation of Azobenzene Modified siRNA (cis to trans)

4 hours after the transfection procedure, the plate was exposed to a 60.0 W daylight bulb (NOMA) and left under the visible lamp for the rest of the time before the cells were lysed and the plate read as above.

### Procedure for consecutive UV/Vis Light Cycling (1.5x and 2x light cycling)

The first cycle was performed normally: UV inactivation after 2 hours for 45 min, and then visible light reactivation at 4 hours for 30 min. We then observed continued exposure to UV light

every 4 hours for 45 min, as per the normal procedure up to 24 h (1.5x, 5 exposures total) to keep the siRNA inactive. The 2x procedure was identical except after the second UV exposure (45 min), a 1 h 15 min resting period of darkness to let the cells recover was observed after which the cells were re-exposed to visible light for 30 min which restored the siRNAzo's activity.

### **Procedures for Real Time PCR**

### Transfection of HeLa cells with Lipofectamine 2000

Cell transfection procedure was identical to the dual assay procedure, above.

### HeLa Cell Reverse Transcription (RT) Preparation

After the expired transfection period the growth medium was removed and the plate was washed twice with PBS. The cells were removed from the plate using 250  $\mu$ L of 0.25% trypsin to each well and incubated for 4 minutes at 37 °C with 5% CO<sub>2</sub>. The cell suspension was then added to 1 mL of growth medium to inactivate the trypsin and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the cells were resuspended in 500  $\mu$ L of fresh media. The cells in each tube were then counted to ensure a total number of cells between 100-200k per sample. After counting the cells were repelleted as above and washed with 500  $\mu$ L of ice-cold PBS and then repelleted and the pellet was placed on ice.

### RT-PCR with the Invitrogen cells to cDNA kit II

The following protocol uses reagents found in the Cells-to-cDNA kit purchased from Invitrogen. The pellet on ice was resuspended in 100  $\mu$ L of ice cold Cell Lysis II Buffer and each sample was mixed by vortex. The samples were then immediately transferred to a pre-heated 75 °C heat block and left to denature for 10 minutes. The samples were then removed from the water bath and placed on ice. To each centrifuge tube, 2  $\mu$ L of DNase I (2 U/ $\mu$ L stock) was added and these mixtures were gently vortexed followed by a brief centrifugation to concentrate the sample. A genomic wipeout was accomplished by incubating the DNase I reaction at 37°C for 30 minutes. The samples were then heated to 75 °C for 10 minutes to deactivate the DNase I. To new nuclease-free microfuge tubes, was added 5  $\mu$ L of cell lysate (RNA), 4  $\mu$ L of dNTP mix (2.5 mM stock for each dNTP), 2  $\mu$ L of random decamers (50  $\mu$ M stock) and 9  $\mu$ L of nuclease-free water. The resulting mixture was then heated to 70 °C for 5 minutes to denature the RNA template, placed on ice for 1 minute, flash centrifuged, and placed on ice. The remaining RT reagents including: 2  $\mu$ L of 10X RT Buffer, 1  $\mu$ L of M-MLV Reverse Transcriptase (or 1  $\mu$ L of nuclease-free H2O for a no reverse transcription (NRT) Control) and 1  $\mu$ L of RNase inhibitor (10 U/ $\mu$ L stock) were added, mixed and centrifuged briefly. Reverse transcription was initiated by warming the samples to 42 °C using a

thermal cycler for 60 minutes. Reverse transcriptase was inactivated by incubating the samples at 95°C for 10 minutes. This lysate can be stored for up to 2 weeks at -20 °C.

### RNA extraction, cDNA synthesis and RT-qPCR

HeLa cells were transfected with anti-*BCL2* siRNAs as described. RNA extraction, cDNA production and RT-qPCR. Prior to the RNA extraction, each well of the 24-well plate washed twice with 1X PBS. Total RNA was extracted from the Hela cells using the manufacturer's instructions of the Total RNA Purification Plus Kit (Cat#: 48400. Norgen BioTek Corp, Thorold, ON, Canada). In addition, an on-column DNA digestion was performed using RNase Free DNase I Kit (Cat#:25710. Norgen BioTek Corp, Thorold, ON, Canada). Two microliter of each extracted RNA sample was used to measure the concentration and RNA integrity (A260/280) on the BioDrop Duo Plus (UK), and the presence of the RNA was confirmed by gel electrophoresis on a 1% (w vol-1) agarose gel. Three biological replicates were completed for each Azo-Modified SiRNA. The SiRNAs were inactivated and reactivated using the exact same procedures as listed above for the firefly/ *Renilla Luciferase* assays.

The RT reaction was performed using the IScript cDNA synthesis kit (Cat #: 1708891. Bio-Rad, Hercules, California) in a total reaction volume of 20µL. The reaction mixture contained 400 ng of total RNA, M-MLV reverse transcriptase, oligo (DT) and random primers. Two negative controls were performed with all reactions. The first control contained the RNA template and all DNAse/RT reagents, except for the final addition of the RT enzyme. A second control contained no template (water only) to ensure that all reagents were free from possible contaminants. RT reactions were placed in 200 µL PCR tubes and incubated within a BIORAD T100 Thermal Cycler for 5 min at 25°C followed by 20 min at 46°C, 1 min at 95°C and then held at 4°C. Once cDNA was produced, the products could be amplified (RT-qPCR).

### **Quantitative RT-PCR**

Real-time PCR was performed in a total reaction volume of 20 µL containing 10 uL SsoFast EverGreen Supermix (Bio-Rad, Hercules, California) containing Sso 7-d Fusion Polymerase, 0.5 µM forward primer and reverse primer and 2 µL cDNA template. In the final reaction, cDNA was diluted 40x to produce the best results. Pre-designed primers *BCL2*F 5'-CTG GTG GGA GCT TGC ATC AC-3' and *BCL2*R-5' GCC TGC AGC TTT GTT TC-3' were purchased to target the *BCL2* gene and yielding a 150-bp amplicon and 18S-F 5'-CGG CTA CCA CAT CCA AGG AAG-3' and 18S-R 5'-CGC TCC CAA GAT CCA ACT ACT3' (Integrated DNA Technologies Inc, San Diego, California) were used to target the 18s gene in Hela cells and yielding a 247bp amplicon. Reactions were incubated in the Bio-Rad CFX 96 Real-Time Detection System using the

following cycle conditions: 50°C for 10 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Reaction specificity was assessed by melting curve analysis immediately after the qPCR experiment. The efficiency of each primer set for RT-qPCR was determined to be between 95 and 100% using the standard curve method. NRT controls were performed during standard curve analysis to confirm that amplification of the PCR product was cDNA and not genomic DNA. NTC controls were also performed to ensure that amplification of the PCR product was not a result of primer- dimer. Results were analyzed using the Bio-Rad CFX manager 3.1 software where the *BCL2* expression data was normalized against 18s gene as the reference and expression profiles were generated using the comparative Delta-CT method of analysis. The repeatability of the RT-qPCR was assessed by measuring the imprecision of the standard deviations of Cq values compiled from three biological replicates for each treatment and three technical replicates having the same input RNA.

### **Procedure for XTT Assays**

XTT reagents were allowed to thaw in the incubator at 37 °C. Once a consistent liquid with no particles was obtained after thawing, 2.5 mL of XTT Reagent was combined with 0.05 mL of Activation Reagent. 200  $\mu$ L of this mixture was added to each well and the plates were placed back in the incubator for at least one hour. Plates were read using a BioTek plate reader (Fischer Scientific).All blanks and samples were averaged at both wavelengths. Specific absorbance for UV and non-UV samples were calculated using the following equation:

### Specific Absorbance at 475 nm = Test Avg<sub>475nm</sub> - Blank Avg<sub>475nm</sub> - Test Avg<sub>660nm</sub>

Cell viability was then assessed and error bars were placed based on standard deviation.

### **Figures and Tables**



**Figure S-1.** CD spectra of azobenzene modified spacers replacing two nucleobases targeting firefly luciferase mRNAs. Wildtype and modified anti-*firefly* luciferase siRNAs (10  $\mu$ M/duplex) were suspended in 500  $\mu$ L of a sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.00 mM EDTA, pH 7.00) and scanned from 200-300 nm at 25 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. All scans were performed in triplicate and averaged using Jasco's Spectra Manager version 2.



**Figure S-2.** CD spectra of azobenzene modified spacers replacing two nucleobases targeting *BCL2* mRNAs. Wildtype and modified anti-*BCL2* siRNAs (10  $\mu$ M/duplex) were suspended in 500  $\mu$ L of a sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.00 mM EDTA, pH 7.00) and scanned from 200-300 nm at 25 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. All scans were performed in triplicate and averaged using Jasco's Spectra Manager version 2. Updated with siRNAs 5 and 6



**Figure S-3.** Absorbance Profile of siRNA 1 when exposed to UV and Visible light in 500  $\mu$ L of a sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.00 mM EDTA, pH 7.00) and scanned from 200-400 nm at 10 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. Inset: Zoomed in portion of 320-380 nm highlighting azobenzene changes



**Figure S-4.** Absorbance Profile of siRNA 2 when exposed to UV and Visible light in 500  $\mu$ L of a sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.00 mM EDTA, pH 7.00) and scanned from 200-400 nm at 10 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. Inset: Zoomed in portion of 320-380 nm highlighting azobenzene changes



**Figure S-5.** Absorbance Profile of siRNA 3 when exposed to UV and Visible light in 500  $\mu$ L of a sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.00 mM EDTA, pH 7.00) and scanned from 200-400 nm at 10 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. Inset: Zoomed in portion of 320-380 nm highlighting azobenzene changes







**Figure S-7.** Absorbance Profile of siRNA 7 when exposed to UV and Visible light in 500  $\mu$ L of a sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.00 mM EDTA, pH 7.00) and scanned from 200-400 nm at 10 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. Inset: Zoomed in portion of 320-380 nm highlighting azobenzene changes. SiRNAs 5 and 6 have similar profiles (data not shown).



**Figure S-8.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 1 at 160, 400 and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min (1 exposure total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-9.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 2 at 160, 400 and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min (1 exposure total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-10.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 3 at 160, 400 and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min (1 exposure total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-11.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 4 at 160, 400 and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min (1 exposure total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-12.** Numerical bar graph showing reduction of normalized firefly luciferase expression for wt siRNA at 160, 400 and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min (1 exposure total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-13.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 1 at 160, 400 and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min and then for every 4 h thereafter (6 exposures total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-14.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 2 at 160, 400 and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min and then for every 4 h thereafter (6 exposures total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.

siRNA 3 24 h



**Figure S-15.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 3 at 160, 400 and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min and then for every 4 h thereafter (6 exposures total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-16.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 4 at 160, 400 and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min and then for every 4 h thereafter (6 exposures total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-17.** Numerical bar graph showing reduction of normalized firefly luciferase expression for wt siRNA at 160, 400 and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min and then for every 4 h thereafter (6 exposures total). 2) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



siRNA 1 8 h

**Figure S-18.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 1 at 160, 400, and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min. 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-19.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 2 at 160, 400, and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min. 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



siRNA 38h

**Figure S-20.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 3 at 160, 400, and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min. 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-21.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 4 at 160, 400, and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min. 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-22.** Numerical bar graph showing reduction of normalized firefly luciferase expression for wt siRNA at 160, 400, and 800 pM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min. 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.





**Figure S-23.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 1 at 160, 400, and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min, and for an additional 45 min of UV exposure every 4 hours (6 exposures total) 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-24.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 2 at 160, 400, and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min, and for an additional 45 min of UV exposure every 4 hours (6 exposures total) 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.





**Figure S-25.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 3 at 160, 400, and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min, and for an additional 45 min of UV exposure every 4 hours (6 exposures total) 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-26.** Numerical bar graph showing reduction of normalized firefly luciferase expression for siRNAzo 4 at 160, 400, and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min, and for an additional 45 min of UV exposure every 4 hours (6 exposures total) 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.





**Figure S-27.** Numerical bar graph showing reduction of normalized firefly luciferase expression for wt siRNA at 160, 400, and 800 pM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min, and for an additional 45 min of UV exposure every 4 hours (6 exposures total) 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-28.** Numerical bar graph showing reduction of normalized *BCL2* expression for siRNAzo 5 at 1, 10, and 20 nM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min. 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-29.** Numerical bar graph showing reduction of normalized *BCL2* expression for siRNAzo 6 at 1, 10, and 20 nM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min. 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-30.** Numerical bar graph showing reduction of normalized *BCL2* expression for siRNAzo 7 at 1, 10, and 20 nM in HeLa cells monitored 8 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min. 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-31.** Numerical bar graph showing reduction of normalized *BCL2* expression for siRNA 5 at 1, 10, and 20 nM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min, and for an additional 45 min of UV exposure every 4 hours (6 exposures total) 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-32.** Numerical bar graph showing reduction of normalized *BCL2* expression for siRNA 6 at 1, 10, and 20 nM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min, and for an additional 45 min of UV exposure every 4 hours (6 exposures total) 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-33.** Numerical bar graph showing reduction of normalized *BCL2* expression for siRNA 7 at 1, 10, and 20 nM in HeLa cells monitored 24 hours post-transfection. 1) UV corresponds to the siRNA being exposed under a 365nm UV lamp for inactivation 2 h post transfection for 45 min, and for an additional 45 min of UV exposure every 4 hours (6 exposures total) 2) Vis corresponds to visible light exposure 4 h after transfection. 3) Dark corresponds to siRNAs being transfected in HeLa cells in the absence of UV light.



**Figure S-34**: HeLa cells were exposed to UV Light under a 365nm UV lamp 2 h post transfection for 45 min, (8 h assay) and for an additional 45 min of UV exposure every 4 hours (6 exposures total) (24 h assay). Data showed no significant changes in cell viability when compared to cells not exposed to UV light. The cell viability was determined using an XTT Assay (Promega).



**Figure S-35.** HPLC chromatogram of sense strand of the sense strand of siRNA 5 showing both *cis* (left) and *trans* (right) peaks. Background (blue): The same sample of siRNA 5 collected and exposed to 60 min UV light. Foreground (red): untreated sense strand of siRNA 5 spectra. Conditions were 5% acetonitrile in 95% 0.1 M TEAA (Triethylamine-Acetic Acid) buffer up to 100% acetonitrile over 30 min. Spectra were processed using the Empower 3 software.



**Figure S-36.** HPLC chromatogram of the sense strand of siRNA 6 showing both *cis* (left) and *trans* (right) peaks. Background (blue): The same sample of siRNA 5 collected and exposed to 60 min UV light. Foreground (red): untreated sense strand of siRNA 5 spectra. Conditions were 5% acetonitrile in 95% 0.1 M TEAA (Triethylamine-Acetic Acid) buffer up to 100% acetonitrile over 30 min. Spectra were processed using the Empower 3 software.



**Figure S-37.** HPLC chromatogram of the sense strand of siRNA 7 showing both *cis* (left) and *trans* (right) peaks. Background (blue): The same sample of siRNA 5 collected and exposed to 60 min UV light. Foreground (red): untreated sense strand of siRNA 5 spectra. Conditions were 5% acetonitrile in 95% 0.1 M TEAA (Triethylamine-Acetic Acid) buffer up to 100% acetonitrile over 30 min. Spectra were processed using the Empower 3 software.



**Figure S-38**. GSH assay results of the sense strand of the sense strand of siRNAzo 6 showing minimal degradation due to the presence of GSH. UV treated RNA was exposed to 5 min of UV light and then kept at 37 °C until injection. Conditions were 5% acetonitrile in 95% 0.1 M TEAA (Triethylamine-Acetic Acid) buffer up to 100% acetonitrile over 30 min. Spectra were processed using the Empower 3 software.

siRNAzo	siRNAzo Duplex <sup>[a]</sup>	Predicted Mass of Sense Strand	Actual Mass of Sense Strand <sup>[b]</sup>	Target
1	5'- CUUACGC <u>Az2</u> AGUACUUCGAtt -3' (s) 3'- ttGAAUGCGACUCAUGAAGCU-5'(as)	6367.91	6366.89	luciferase
2	5'- CUUACGCU <u>Az2</u> GUACUUCGAtt -3' (s) 3'- ttGAAUGCGACUCAUGAAGCU-5'(as)	6344.86	6344.91	luciferase
3	5'- CUUACGCUG <u>Az2</u> UACUUCGAtt -3' (s) 3'- ttGAAUGCGACUCAUGAAGCU-5'(as)	6344.86	6344.86	luciferase
4	5'- CUUACGCUGA <u>Az2</u> ACUUCGAtt -3' (s) 3'- ttGAAUGCGACUCAUGAAGCU-5'(as)	6367.91	6366.95	luciferase
5	5'-GCCUUCU <u>Az2</u> GAGUUCGGUGtt-3' (s) 3'-ttCGGAAGAAACUCAAGCCAC-5' (as)	6336.91	6336.86	BCL2
6	5'-GCCUUCUU <u>Az2</u> AGUUCGGUGtt-3'(s) 3'-ttCGGAAGAAACUCAAGCCAC-5'(as)	6377.82	6377.81	BCL2
7	5'-GCCUUCUUUG <u>Az2</u> UUCGGUGtt-3' (s) 3'-ttCGGAAGAAACUCAAGCCAC-5'(as)	6369.76	6369.76	BCL2

Table S-1 Sequences of anti-luciferase and anti-BCL2 siRNAzos, predicted and recorded mass

References

[a] <u>Az2</u> corresponds to the azobenzene derivative synthesized from 4-nitrophenylethyl alcohol; the top strand corresponds to the sense strand; the bottom strand corresponds to the antisense strand. In all duplexes, the 5'-end of the bottom antisense strand contains a 5'-phosphate group. [b] Deconvolution results for siRNAzos. ESI-HRMS (ES<sup>+</sup>) m/z calculated for siRNAzos 1-7  $[M+H]^+$ 

1. M. L. Hammill, C. Isaacs-Trepanier and J. P. Desaulniers, *ChemistrySelect*, **2017**, 2, 9810-9814.

2. McDowell, J.A.; Turner, D. H. *Biochemistry* **1996**, *35*, 14077.