Supporting Information

Synthesis and *in vitro* Assessment of Chemically Modified siRNAs Targeting *BCL2* that Contain 2'-Ribose and Triazole-linked Backbone Modifications

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Chemical, Nucleic Acid and Biological Procedures:

Synthesis of U_tU Phosphoramidite Dimer

The synthesis of uracil triazole-linked uracil phosphoramidite (**U_tU**) phosphoramidite was performed in accordance with the methods Efthymiou *et al.* (2012).

Procedure for Oligonucleotide Synthesis and Purification

All β-cyanoethyl 2'-*O*-TBS protected, 2'-F and 2'-*O*-Me phosphoramidites, reagents and solid supports were purchased from ChemGenes Corporation and Glen Research. All commercial phosphoramidites were dissolved in anhydrous acetonitrile to a concentration of 0.1 M. The chemically synthesized **U_tU** phosphoramidite was dissolved in 20% tetrahydrofuran in acetonitrile to a concentration of 0.1 M. All sequences were synthesized via solid support phosphoramidite chemistry using the Applied Biosystems 394 DNA/RNA synthesizer. Oligonucleotides were synthesized on 0.2 μM dT solid support except for sequences that were 3'-modified, which were synthesized on 0.2 μM Universal-III solid supports. 1.0 μM synthesis cycles were used and all reactions were performed under an inert N_2 atmosphere maintained at 55 psi. Commercially available phosphoramidites were synthesized using coupling times of 16 minutes, while chemically synthesized **UtU** coupling time was increased to 30 minutes to ensure proper coupling. Antisense sequences were chemically phosphorylated at the 5'-end by using 2-[2-(4,4'-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite. Upon completion, columns were removed from the synthesizer, dried under a stream of N_2, sealed and stored at 4°C. Oligonucleotides were removed from their solid supports upon exposure to 1.5 ml of EMAM (methylamine 40% wt. in H_2O and methylamine 33% wt. in ethanol, 1:1 (Sigma)). Columns were incubated with EMAM for 1 hour at room temperature with the solution in full contact with the controlled pore glass. The oligonucleotides were then transferred into screw cap microcentrifuge tubes and incubated overnight at room temperature in EMAM to deprotect the nitrogenous bases. The following day, samples were evaporated on a Speedvac evaporator overnight and resuspended in a solution of dimethylsulfoxide (DMSO): 3HF/triethylamine (TEA) (100 μl: 125 μl) (Sigma). The samples were then incubated at 65°C for 2.5 hours to remove the 2'-*O*-TBS protecting groups. Crude oligonucleotides were precipitated in ethanol and desalted through Millipore Amicon Ultra 3000 MW cellulose filters. Oligonucleotides were purified using a 20% denaturing polyacrylamide gel. Pure oligonucleotides were excised from the gel and were purified in a microcentrifuge tube by crushing and soaking the excised gel in an elution buffer (3 mM sodium acetate, 10 μM EDTA, pH 7). The resulting oligonucleotides were desalted a second time through Millipore Amicon Ultra 3000 MW cellulose filters. At 10 μM, equimolar amounts of complimentary RNAs were combined and dried down in a Speedvac overnight. The RNAs were then suspended in a binding buffer (75 mM KCl, 50 mM Tris-HCl, 3 mM $MgCl_2$, pH 8.3) and incubated at 95°C for 2 minutes and then cooled slowly to room temperature to generate siRNA duplexes used for biological assays.

Procedure for Characterizing Oligonucleotides through ESI Q-TOF

All single-stranded RNAs were gradient eluted through a Zorbax Extend C18 HPLC column with a MeOH/H_2O (5:95) solution containing 200 mM hexafluoroisopropyl alcohol and 8.1 mM triethylamine, and finally with 70% methanol. The eluted RNAs were subjected to ESI-MS (ES^-), producing raw spectra of multiply-charged anions and through resolved isotope deconvolution, the molecular weights of the resultant neutral oligonucleotides were confirmed. The final neutral mass of the RNAs were confirmed using this method and were within 2% of predicted mass (Table 1-1).

Procedure for Performing CD Measurements

Circular dichroism (CD) spectroscopy and UV-monitored thermal denaturation were performed on a Jasco J-815 CD equipped with temperature control. Equimolar amounts of each RNA (2.4 nmol) were annealed to their complement in 500 µL of a sodium phosphate buffer. CD measurement of each duplex were recorded in quadruplicate from 200-300 nm at 20°C with a screening rate of 10 nm/min and a 0.2 nm data pitch. The average of the four replicates was calculated using Jasco's Spectra Manager version 2 software and adjusted against the baseline measurement of the sodium phosphate buffer. All siRNA duplexes were confirmed to be in A-form helical conformation (Fig. S4).

Nuclease Stability Assay of siRNA Duplexes

At a concentration of 12 µM, 2 µl of siRNAs Bwt, B2, B3, B4, B5, and B6 were incubated with 8 µl of 13.5% fetal bovine serum at 37°C for 0.5, 1, 2, 3, 4, and 5 hours. After incubation, siRNAs were resolved via electrophoresis through the use of a non-denaturing 20% polyacrylamide gel, which ran at 60V for 24 hours. The gel was stained using 3X GelRed™ (Biotium) nucleic acid dye for 30 minutes and then visualized via Fluorchem SP (Fisher Scientific).

Waking Frozen KB Cells

Cryopreserved KB cells, stored in 1.5 ml of Eagle's Minimum Essential Medium (EMEM) (ATCC) supplemented with 5% DMSO, were removed from CryoPro® liquid nitrogen dewar (VWR) and slowly thawed to 25°C. Cells were reconstituted in 10 ml of EMEM and transferred into a 50 ml polystyrene tissue culture treated incubation flask (Falcon) containing 20 ml of EMEM. Cells were then incubated overnight in a Forma Series II CO_2 Incubator (ThermoScientific) at 37°C under 5% CO_2 atmosphere. Once cells obtained a confluency of 90%, they were passaged normally and transferred into a 250 ml polystyrene tissue culture treated incubation flask (Falcon) containing 25 ml of EMEM supplemented with 10% fetal bovine serum (FBS) (Perbio) and 1% Penicillin-Streptomycin (Sigma).

Sub-Culturing of KB Cells (Passaging)

$1x10^6$ KB cells were seeded in a 250 ml polystyrene tissue culture treated incubation flask (Falcon) containing 25 ml of EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (Perbio) and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma). Cells were incubated in a Forma Series II CO_2 Incubator at 37°C under 5% CO_2 atmosphere until 90% confluency. Once 90% confluency was reached, cells were washed 3 times with 10 ml of phosphate buffered saline (NaCl 137 mM, KCl 2.7 mM, PO_4^{3-} 10 mM, pH 7.4) (PBS). Cells were treated with 3 ml of 0.25% Trypsin (SAFC Bioscience) to disperse the cells. The cells were then pelleted and resuspended in 5 ml of EMEM for counting. They were then diluted in EMEM to a final concentration of $1x10^6$ cells/ml for further sub-culturing or biological assays. Cells were sub-cultured for no more than 10 passages for biological assays.

Cryopreservation of KB cells

KB cells were passaged normally until final resuspension of cells; cells were suspended in EMEM supplemented with 5% DMSO (Sigma) to a concentration of $1x10^7$ cells/ml. A 1 ml aliquot of cells were transferred into 2 ml Fisherbrand cryogenic vials (Fisher) and cooled to 4°C, then frozen at -20°C and finally stored in CryoPro® liquid nitrogen dewar (VWR) until use.

siRNA Transfections

50 µl of KB cells (total of $5x10^4$ cells) were added to each well of a 24-well plate (Falcon®) with 350 µl of growth medium and incubated at 37°C with 5% CO_2. After 24 hours of incubation, cells were treated with 1, 10 and 20 nM concentrations of siRNAs using Lipofectamine 2000 (Invitrogen) in 1X Opti-Mem (ATCC). The desired volume of siRNAs was mixed with 25 µl of Opti-Mem in a microcentrifuge tube on ice, and incubated for 5 minutes. 1 µl of Lipofectamine was mixed with 25 µl of Opti-Mem in a microcentrifuge tube and incubated for 5 minutes at room temperature. The contents of the tubes were combined and incubated for 20 minutes at room temperature and transferred into respective wells of the tissue culture treated 24-well plate (Falcon).

RNA Isolation and cDNA Synthesis

Achieving a cellular concentration of no more than 2.5×10^5 cells/well after a total incubation time of 48 hours, cells were lysed using TRIzol® Reagent (Ambion). RNA was purified following manufacturers procedure. RNA integrity was visualized using a 1% denaturing agarose gel in 1X TBE buffer. RNA was quantified by measuring absorbance at 260 nm using GENESYS™ 10S UV-Vis Spectrophotometer (Thermo). 1 µg of RNA was used for cDNA synthesis using the iScript reverse transcription kit (Biorad). Reverse transcription was performed at 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. cDNA samples were then stored at -20°C for real time quantitative polymerase chain reaction (qPCR) using a PX2 thermocycler (Thermo).

qPCR

Relative transcript levels were quantified via qPCR using CFX Connect™ Real-Time PCR Detection System (Biorad). Standard curves were generated for each gene (*BCL2*, 18s rRNA and GAPDH) to evaluate primer efficiency. The qPCR was performed in triplicate using the following conditions: 2 µl of cDNA, and 10 µl of SsoFast EvaGreen Supermix (Biorad) with respective primer concentrations and topped up to a final volume of 20 µl using DEPC treated water. 1 µl of Bcl-2 forward primer: 5'- CTGGTGGGAGCTTGCATCAC -3' and reverse primer: 5'- ACAGCCTGCAGCTTTGTTTC -3' were used for a final concentration of 500 nM. 1.6 µl of GAPDH forward primer: 5'-ACTTTGTGAAGCTCATTTCCTGGTA -3' and reverse primer: 5'- GTGGTTTGAGGGCTCTTACTCCTT -3' were used for a final concentration of 800 nM. 2 µl of 18s forward primer: 5'- CGGCTACCACATCCAAGGAAG- 3' and reverse primer: 5'- CGCTCCCAAGATCCAACTACTAC- 3' for a final concentration of 100 nM. Thermocycle conditions included an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 2 seconds, 52°C for 15 seconds, and 72°C for 5 seconds. Fluorescence was measured during the end of each extension phase. After 40 cycles, a melting curve was generated by slowly increasing the temperature from 65°C to 95°C at a rate of 0.1°C/s, while fluorescence was measured at each increment. To ensure no genomic contamination, a control lacking reverse transcriptase was used for each cDNA sample. No template controls were run for every set of primers. Normalized relative gene expression (ΔΔCq) of Bcl-2 was calculated against both reference genes (GAPDH and 18s) and corrected for primer efficiency using CFX Manager Software (BIORAD).

XTT Cellular Proliferation Assay

Cellular proliferation was determined using XTT Cell Proliferation Assay Kit (ATCC®). 2.5 x 10^3 KB cells were seeded into each well of a 96-well plate (Falcon®) with 150 µl of growth media and incubated at 37°C with 5% CO_2 for 24 hours. After 24 hours of incubation, cells were transfected in triplicate with siRNA 1 through 29 at three concentrations (1, 10 and 20 nM) using Lipofectamine 2000 in 1X Opti-Mem according to the manufacturer's protocol. Cells were then incubated for 24 hours at 37°C with 5% CO_2. After 24 hours of incubation, cells were treated with 50 µl of XTT, activated by 2% *N*-methyl dibenzopyrazine methyl sulfate and incubated for 2 hours at 37°C with 5% CO_2. After 2 hours of incubation, absorbance was measured at 475 nm and 660 nm using an xMark™ Microplate Absorbance Spectrophotometer (Biorad). Specific absorbance was calculated via: A_{475nm}(experimental)–A_{475nm}(Blank)–A_{660nm}(experimental) and results were normalized to an untreated control.

Time Course XTT Cellular Proliferation

Cellular proliferation was determined using XTT Cell Proliferation Assay Kit (ATCC®). Cells were seeded at concentrations required to reach 90% confluency at each end point (1, 2, 3 and 4 days post-transfection). After 24 hours of incubation, cells were transfected in triplicate with 20

nM of respective siRNAs using Lipofectamine 2000 in 1X Opti-Mem according to the manufacturer's protocol. Cells were incubated at 37°C with 5% CO_2. Cells were transfected again following the same procedure on day 2 and day 4. At each end point, 50 µl of XTT was added to siRNA-treated cells, activated by 2% *N*-methyl dibenzopyrazine methyl sulfate and incubated for 2 hours at 37°C with 5% CO_2. After 2 hours of incubation, absorbance was measured at 475 nm and 660 nm using an xMark™ Microplate Absorbance Spectrophotometer (Biorad). Specific absorbance was calculated via A_{475nm}(experimental)–A_{475nm}(Blank)–A_{660nm}(experimental) and results were normalized to an untreated control.

Detection of Human Interferon Alpha in PBMC Cultures

After ethical approval from the University Of Ontario Institute Of Technology's Research Ethics Board, human blood was obtained from consenting normal healthy donors (University of Ontario Institute of Technology, Oshawa, Ontario). Human blood (9 volumes; 30 ml) was collected in 50 ml syringes prefilled with 3.2% (w/v) Trisodium citrate as an anti-coagulation agent. Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation using Ficoll™ separating solution (GE Healthcare). Isolated PBMC cells were counted and 4.0×10^5 cells were seeded in a 96-well plate containing 200 µl RPMI 1640 (ATCC) supplemented with 10% (v/v) FBS, 1 mM L-Glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Once seeded in 96-well plate the PBMC cells were immediately stimulated in triplicate using 20 nM of siRNA following standard transfection procedures using Lipofectamine. 24-hours post siRNA transfection, culture plates were centrifuged at 400g for 10 minutes. The supernatant was collected and directly processed with Verikine ELISA kits (PBL). The supernatant was diluted 1:1 with supplied diluent from the kit and interferon-α (IFN-α) levels were measured according to the manufacturer's protocol (PBL) using prufied IFN- α to construct standard curves.

Figures and Tables:

Table S1: The predicted and Recorded Masses of Oligonucleotides. Masses of Chemically Modified siRNA Molecules Recorded Using Quantitative Time-Of-Flight Spectrometry

Oligonucleotide	Predicted Mass	Observed Mass	Oligonucleotide Sequence
1	6618.84	6618.83	5'- GCCUUCUUUGAGUUCGGUGtt -3'
2	6586.05	6586.02	5'- GCCUUCUUUGAG**U_tU**CGGUGtt -3'
3	6604.02	6604.00	5'- GCCUUCUUUGAGUUCGGUG**U_tU** -3'
4	6725.96	6725.97	5'- GCCUUCUUUGAGUUCGGUGtt -3'
5	6712.29	6712.14	5'- GCCUUCUUUGAG**U_tU**CGGUGtt -3'
6	6744.29	6744.05	5'- GCCUUCUUUGAGUUCGGUG**U_tU** -3'
7	6841.37	6841.08	5'- GCCUUCUUUGAGUUCGGUGtt -3'
8	6851.23	6851.27	5'- GCCUUCUUUGAG**U_tU**CGGUGtt -3'
9	6856.50	6856.17	5'- GCCUUCUUUGAGUUCGGUG**U_tU** -3'
10	6629.81	6629.80	5'- GCCUUCUUUGAGUUCGGUGtt -3'
11	6616.01	6616.04	5'- GCCUUCUUUGAG**U_tU**CGGUGtt -3'
12	6603.93	6603.82	5'- GCCUUCUUUGAGUUCGGUG**U_tU** -3'
13	6652.79	6652.77	5'- GCCUUCUUUGAGUUCGGUGtt -3'
14	6783.88	6784.83	5'- GCCUUCUUUGAG**U_tU**CGGUGtt -3'
15	6683.940	6684.11	5'- GCCUUCUUUGAGUUCGGUG**U_tU** -3'
16	6685.87	6685.86	5'- GCCUUCUUUGAGUUCGGUGtt -3'
17	6678.99	6678.17	5'- GCCUUCUUUGAG**U_tU**CGGUGtt -3'
18	6672.07	6672.83	5'- GCCUUCUUUGAGUUCGGUG**U_tU** -3'
19	6697.18	6697.01	5'-ph-CACCGAACUCAAAGAAGGCtt-3'
20	6792.01	6791.94	5'-ph-CACCGAACUCAAAGAAGGCtt-3'
21	6771.08	6771.06	5'-ph-CACCGAACUCAAAGAAGGCtt-3'

ESI Q-TOF were recorded in a negative electrospray mode after HPLC elution using two mobile phases; MeOH/H_2O 5:95 (v/v) with 200 mM hexafluoroisopropyl alcohol and 8.1 mM triethylamine, and 70% MeOH.

Table S2: Sequences of anti-*BCL2* siRNAs.

Anti-BCL2 siRNA	siRNA duplex
Bwt	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B2	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B3	5'- GCCUUCUUUGAGUUCGGUGU_tU -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B4	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B5	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B6	5'- GCCUUCUUUGAGUUCGGUGU_tU -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B7	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B8	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B9	5'- GCCUUCUUUGAGUUCGGUGU_tU -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B10	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B11	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B12	5'- GCCUUCUUUGAGUUCGGUGU_tU -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B13	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B14	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B15	5'- GCCUUCUUUGAGUUCGGUGU_tU -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B16	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B17	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B18	5'- GCCUUCUUUGAGUUCGGUGU_tU -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B19	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B20	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B21	5'- GCCUUCUUUGAGUUCGGUGU_tU -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B22	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B23	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B24	5'- GCCUUCUUUGAGUUCGGUGU_tU -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B25	5'- GCCUUCUUUGAGUUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5
B26	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B27	5'- GCCUUCUUUGAGU_tUCGGUGtt -3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
NC	5'- CUUACGCUGAGUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'

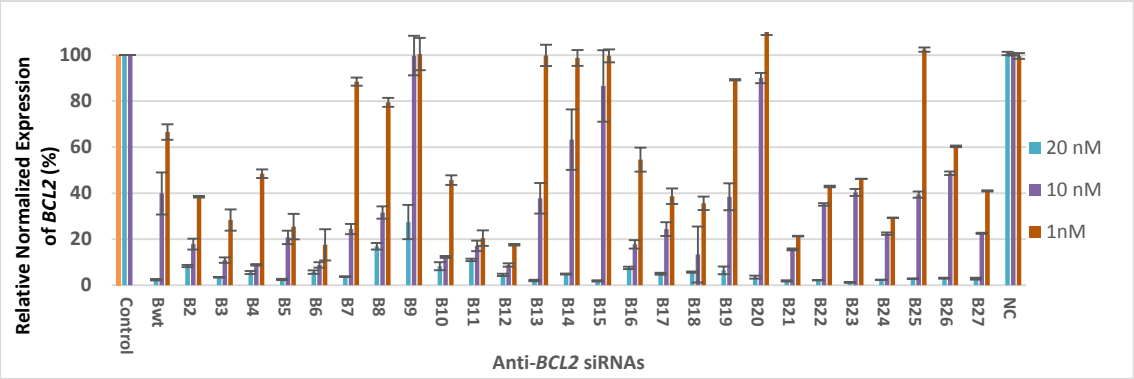

FIGURE S1: Relative gene expression of *BCL2* in KB cells 24 hours post anti-*BCL2* siRNA transfections at 1 nM, 10 nM and 20 nM. Gene expression was measured using qPCR and expression was normalized to an untreated control using reference genes 18s rRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

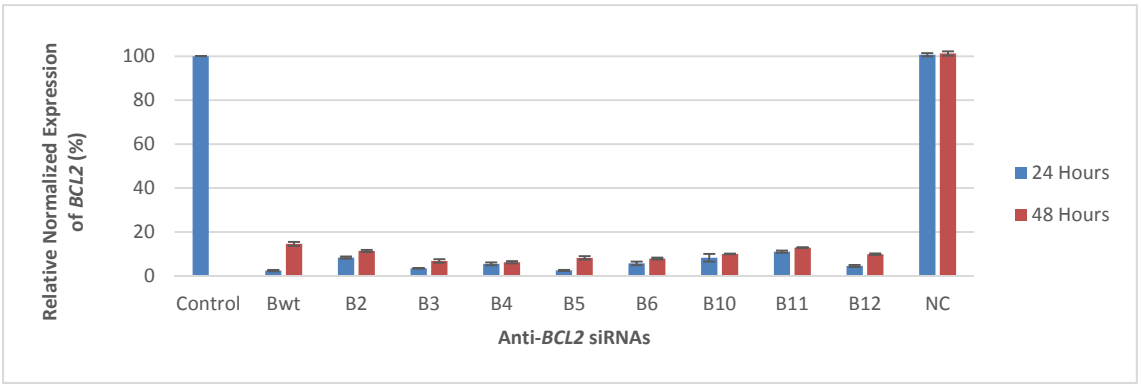

FIGURE S2: Relative gene expression of *BCL2* in KB cells 24 and 48 hours post anti-BCL2 siRNA transfections at 20 nM. Gene expression was measured using qPCR and expression was normalized to an untreated control using reference genes 18s rRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

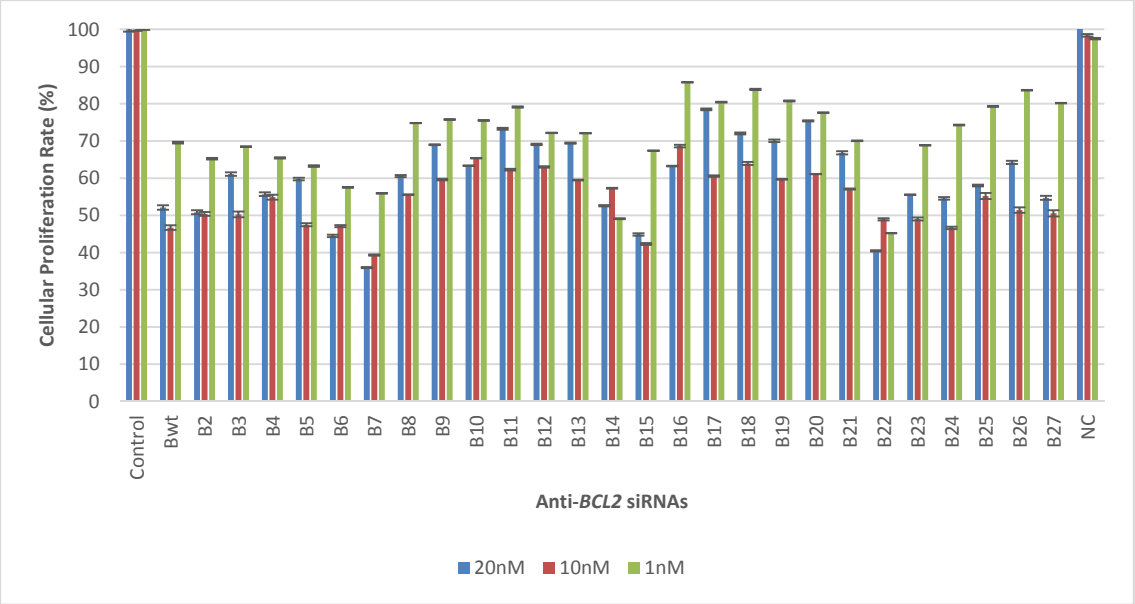

FIGURE S3: Cellular Proliferation Assay. Relative cellular proliferation rates of KB cells treated with 1 nM, 10 nM and 20 nM concentrations of anti-*BCL2* siRNAs compared to untreated KB cells. Cellular proliferation rates were measured using XTT reagent 24 hours post siRNA transfection.

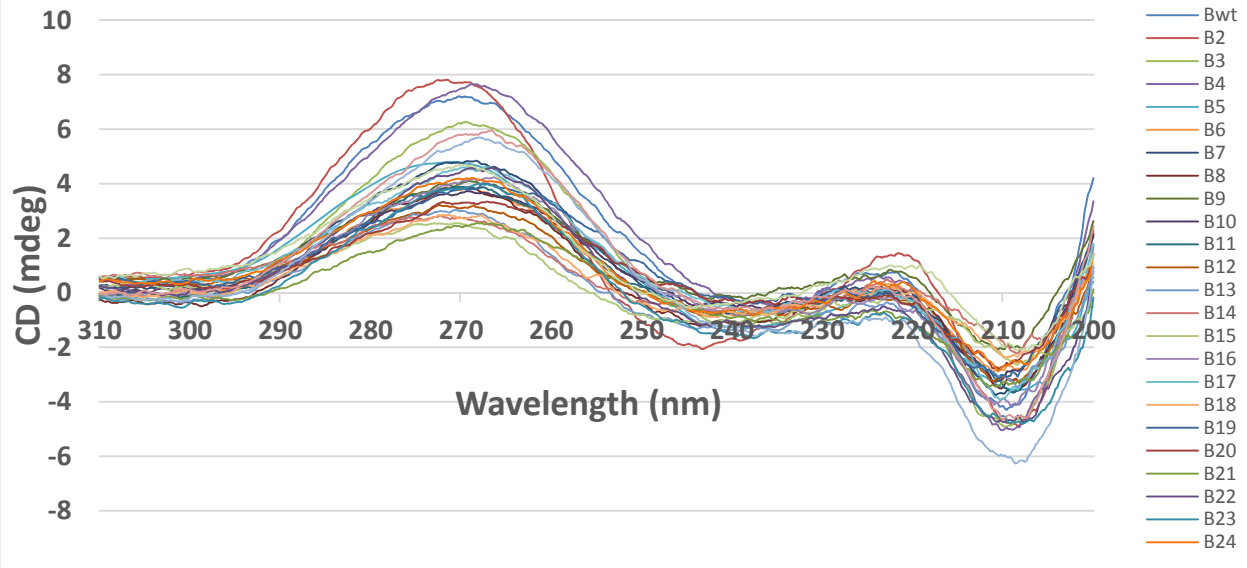

Figure S4: RNA duplex conformation of all anti-*BCL2* siRNAs displayed through circular dichroism spectroscopy. Wild-type and modified anti-*BCL2* siRNAs (10 µmol/duplex) were suspended in 500 µl of a sodium phosphate buffer (90 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7) and the solution was scanned from 200–310 nm at 20°C. All scans were performed in quadruplicate and averaged using version 2 of Jasco's Spectra Manager software.

References:

1. T.C. Efthymiou, V. Huynh, J. Oentoro, B. Peel and J.-P. Desaulniers. "Efficient Synthesis and Cell-Based Silencing Activity of siRNAs that Contain Triazole Backbone Linkages" *Bioorg. Med. Chem. Lett.* **2012,** *22*, 1722-1726.