**Targeting eIF4G1 with SBI-0640756 in drug resistance and clinically unresponsive melanomas**

**Supplemental Material and Methods**

**Development of SBI-756-resistant cells and exome sequencing**. UACC903 and UACC3629 cells were treated with increasing SBI-756 concentrations from 0.01–2.5 M over 12 weeks. Single clones were isolated from 96-well plates, expanded, and genomic DNA was extracted. Exome sequencing of UACC903 SBI-756-senitive parental and resistant clones was performed by Ion Proton (Life Technologies). The library was made using the Ion AmpliSeq Exome Kit according to the manufacturer’s protocol. The exome-seq reads were aligned to the human genome (hg19) in Torrent Suit v4.2 and variants were called with the Torrent Suit Variant Caller v4.2. Exome sequencing of UACC3629 parental and SBI-756 resistant clones was performed using a HiSeq 2000 (Illumina). Exome sequence capture was performed with NimbleGen’s SeqCap EZ Human Exome Library v3.0 (Roche NimbleGen, Inc., Madison, WI, USA). The generated FASTQ files were preprocessed for high quality reads by Trimmomatic (v0.32; <http://www.usadellab.org/cms/?page=trimmomatic>), and Novoalign software ([www.novocraft.com](http://www.novocraft.com)) was used to align high quality reads to human genome (hg19). Deduplication, realignment, and recalibration was performed using GATK (v1.6; <https://www.broadinstitute.org/gatk/>), and sequence variants were called using MuTect (broadinstitute.org/cancer/cga/mutect). We further applied the following criteria to retain high-quality variants specifically appearing in SBI-756-resistant clones (but not their respective parental lines): (1) the sequencing depths for both parent and resistant lines >10, with an alternative allele depth >3; (2) variant quality score in resistant clone >30 (99.9% confidence); (3) variant was not detected in parent cell line; (4) variant is not annotated as observed in the germline by the 1,000 Genome Project database (1000genomes.org); (5) variant located within exonic or canonical splicing region with predicted effect on protein coding sequence. After filtering, all mutations were annotated by Ensembl VEP (variant effect predictor) pipeline (useast.ensembl.org/Homo\_sapiens/Tools/VEP). Pathway enrichment of mutations was subsequently analyzed using IPA (QIAGEN)

***In vitro* translation assays**. The bicistronic dual-reporter constructs that contain the firefly luciferase (FF), followed by the (encephalomyocarditis virus) HCV IRES and the Renilla reniformis (Ren) luciferase, have been previously described (1). For measurement of translation of FF-HCV-Ren mRNAs, rabbit reticulocyte lysates (RRL) (Life Technologies, Carlsbad, CA, USA) and the Dual-Glo (Promega, Madison, WI, USA) read-outs were used as previously described (2). Briefly, rabbit reticulocyte lysates mixed with Translation Mix 20x (-Met) and Translation Mix 20x (-Leu) in a ratio of 1:1 were pre-incubated with test compounds for 1 h at room temperature. Translation was started by adding capped RNA prepared by using Megascript T3 (Ambion), and incubated for 2 h at 30°C. Dual-Glo reagent 1 was added for 10 min before reading the FF luciferase activity with the EnVision plate reader (Perkin Elmer, Waltham, MA, USA). Then, Dual-Glo reagent 2 was dispensed to each well and incubated at room temperature for 10 min before reading the Ren luciferase activity on the EnVision. To exclude the possibility that test compounds interfere with any of the luciferase activities, a counter assay was performed in which the compounds were added after the completion of the *in vitro* translation.

**Microarray expression profiling of melanoma cell lines**. TotalRNA from melanoma lines was profiled per manufacturer’s protocol on Human Genome U133plus2.0 GeneChips (Affytmetrix), containing ~47,000 probe sets for 38,000 characterized human genes. GeneChips were scanned using the Affymetrix GeneChip Operating Software (GCOS) and the .CEL files were produced for downstream data analysis. The robust multi-array average (GCRMA) method (Bioconductor; bioconductor.org) was used to background-adjust and normalize expression intensity values. Probesets with present calls in less than four of the seven most SBI-756-sensitive and -resistant samples, respectively, were filtered. The limma R software (Bioconductor) package was used to identify the differentially expressed genes (DEGs). The probes with *P* < 0.05 and fold change >1.5 were used to perform IPA analysis (QIAGEN).

**Drug sensitivity profiling of melanoma cell lines**. UACC melanoma cell lines were dispensed into 384-well tissue culture treated Corning plates in a volume of 45 μL per well using a BIO-TEK μFill Microplate Dispenser. Cell seeding density ranged between 750 and 2500 cells per well, which were optimized for individual cell lines. Twelve hours after plating, 5 μL of SBI-756 dilution series were transferred to the 384-well assay plates using BioMek FX (Beckman Coulter). The 20-point dose response assay with serial 2-fold dilutions yielded final drug concentration ranges of 100 μM to 0.2 nM giving a 500-fold range of concentrations, and a final DMSO concentration of 0.5%. On all plates, vehicle control wells were also included. The cell–compound mixtures were further incubated for 72 h; afterwards, cell viability was determined by measuring the amount of ATP per well using Cell Titer Glo (Promega). Luminescence signal was measured using an Analyst GT Multimode reader (Molecular Devices). Each compound concentration was tested in triplicate within each run; and each cell line was assayed with two runs (separated by days). Raw values were normalized on a plate-by-plate basis to the median of vehicle wells. The normalized data were further applied with a nonlinear 4-parameter curve fit with Prism 5.0 (GraphPad), and IC50 values were extracted. For each cell line, an average of IC50 values between the runs was reported.

**Synthesis of BI-69A11 derivatives**. All reactions involving air and moisture-sensitive reagents and solvents were performed under a nitrogen atmosphere using standard chemical techniques. Anhydrous solvents were purchased from Sigma-Aldrich or EMD Biosciences and used when fresh. All organic reagents were used as purchased. All synthesized final compounds were determined to be ≥ 95% pure by LC-MS. Compound identity was verified by 1H NMR.

**General procedure for the synthesis of SBI-756**. Step 1: A mixture of (2-amino-5-chloro-phenyl)-phenyl-methanone (2.0 g, 8.70 mmol) and 3-oxo-butyric acid ethyl ester (1.13 g, 8.70 mmol) was heated to 160°C with stirring for 3 h. The reaction was cooled to room temperature and the yellow solid was recrystallized from EtOH to afford 3-acetyl-6-chloro-4-phenyl-1H-quinolin-2-one (1.30 g, yield: 50%) as a yellow solid. Step 2: 3-Acetyl-6-chloro-4-phenylquinolin-2(1*H*)-one (1 eq), 5-fluoronicotinaldehyde (1.1 eq) and NaOH (1.1 eq) was dissolved in EtOH. The mixture was stirred for at 40°C for 1 h. After cooling to room temperature, the mixture was diluted with water and the pH was adjusted to 8. The resulting solid was collected by filtration and purified by prep-TLC to afford 6-Chloro-3-[3-(5-fluoro-pyridin-3-yl)-acryloyl]-4-phenyl-1H-quinolin-2-one (yield: 53%) as a yellow solid. 1H NMR (300 MHz, DMSO-*d6*): δ = 12.39 (s, 1H), 8.71 (s, 1H), 8.59 (s, 1H), 8.12 (d, *J* = 9.0 Hz, 1H), 7.69-7.43 (m, 6H), 7.34-7.31 (m, 2H), 6.99 (t, *J* = 8.2 Hz, 2H) . MS: m/z 405.1 (M+H+).

**High-throughput screen**. Melanoma lines WM793, Lu1205, WM1346, and WM1366 were plated in 384-well plates at 1500cells/well. After incubation at 37°C overnight, relevant compounds dissolved in DMSO were serially diluted 2-fold from stock solutions and added to wells using an Echo555 acoustic dispenser (Labcyte); the highest drug concentration was 10 µM and that of DMSO was 0.1%. Cell viability was assessed using ATPlite after a 48-h incubation. Cell growth inhibition was calculated as a percentage of DMSO-treated controls. IC50 values were calculated using GraphPad Prism.

**Reagents and drug treatment protocol**. BI-69A11 and derivatives were synthesized and purified by SBMRI as described and reported above. All compounds were maintained as 10 mM DMSO stocks. PLX4032 was purchased from ChemieTek (Indianapolis, IN, USA) and PD0325901 from Selleck Chemicals (Houston, TX, USA).

To determine the effect of BI-69A11 and derivatives, cells were plated to 75% confluency and grown overnight in 6-well plates. Compounds at different concentrations were added to the plate, cells were then incubated for 6, 12 or 24 h, and lysates were then prepared for Western blot analysis.

**Proliferation assay**. WM793, Lu1205, WM1346 and WM1366 melanoma cells were seeded at 1500 cells in 50 μL per well in 384-well plates. Cells were allowed to attach overnight. BI-69A11 or derivatives were serially diluted 2-fold with media from stock solutions and added to cells. Tests were performed in triplicate, and each microplate included media and DMSO control wells. Cell viability was assessed using ATPlite after 48 or 72 h, according to the manufacturer’s protocol. Cell growth inhibition was calculated as a percentage of DMSO-treated controls and plotted against the log drug concentration. IC50 values were interpolated from the resulting linear regression curve fit (GraphPad Prism 6).

For the bromodeoxyuridine (BrdU) incorporation assay (Cell Proliferation ELISA BrdU Kit from Roche), WT or 4E-BP DKO cells were seeded in 96-well plates (1,000 cells/well). Tests were performed in three biological replicates each of which was carried out in a triplicate. Absorbance at 370 nm (corrected for absorbance at the reference wavelength of 492 nm) was measured using a microplate reader (Benchmark Plus microplate reader; Bio-Rad) according to a manufacturer’s instructions. BrdU incorporation is expressed as a percentage of a vehicle (DMSO) treated control.

**Colony formation**. For the colony formation assay, cells were plated in triplicate at 500 cells/well in 6-well plates and grown for 16 h before compounds were added for 1–2 weeks, depending on cell line. Colonies were stained with Accustain Crystal Violet solution (Sigma-Aldrich) for 30 min. Plates were rinsed with water, and colonies (>50 cells per clone) were counted. Colony formation efficiency was calculated relative to the number of colonies in control (DMSO)-treated wells.

**Reverse phase Protein Array Analysis (RPPA)**. UACC903 and WM1346 cells were treated with DMSO (controls) or 1 µM SBI-756 for 24 h in triplicate. Cells were lysed with lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na3VO4, 10% glycerol, with freshly added protease and phosphatase inhibitors) and centrifuged at 13,000g for 20 minutes. Denatured protein lysates containing the same amount of protein were then subjected to RPPA analysis at the RPPA Core Facility, MD Anderson Cancer Center, as previously described ([3](#_ENREF_1)).

**LC/MS/MS**. UACC903 cell lysates (1 mg protein) were first incubated with 10 µM biotin or 10 µM biotinylated-BI-69A11 or 100 µM BI-69A11 for 30 min at room temperature on a rotating wheel. Pierce Monomeric Avidin Agarose beads (100 µl) were then added to each sample, which was incubated another 30 min and then washed five times with PBS. One tenth of the bead mixture was run on SDS PAGE, which was then silver-stained, while the rest of the bead mix was subjected to LC/MS/MS analysis by the SBMRI Proteomics Facility. For tryptic digestion and 1D LC/MS/MS analysis, samples of beads plus protein were re-suspended in 100 ul of 50 mM ammonium bicarbonate, and 2 ul of 0.5M Tris (2-carboxyethyl) phosphine (TCEP) was added to 200 µl of the beads/protein mix at 40°C for 30 min to reduce proteins. 4 ul of 0.5 M iodoacetamide was added, and proteins were alkylated at room temperature in the dark for 30 min. Mass spectrometry grade trypsin (Promega) was added at a 1:20 ratio to beads for overnight digestion at 37°C using an Eppendorf Thermomixer at 800 rpm. Digested peptides were separated from beads by centrifugation and transferred to a new tube. Formic acid was added to the peptide solution (to 2%), followed by desalting using a Microtrap (Thermo) and on-line analysis of peptides by high-resolution, high-accuracy LC-MS/MS, which consisted of an EASY-nLC 1000 HPLC Acclaim PepMap peptide trap, a 15-cm 3um Easy-Spray C18 column, an Easy Spray Source, and a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific).

**Pharmacology analysis**. SBI-756 and its analogues were evaluated in a detailed *in vitro* pharmacology screen (absorption, distribution, metabolism, excretion, toxicity (ADME/T)) as shown in Table S1. SBI-756 is moderately soluble across a range of pHs and exhibits high permeability in a standard parallel artificial membrane permeability assay (PAMPA). Lastly, SBI-756 shows moderate stability in human and mouse liver homogenates. In all cases (with the exception of partially diminished microsomal stability), SBI-756 represents an improvement on the original lead BI-69A11, as reflected by improved rodent pharmacokinetics.

**SBI-756 pharmacokinetics in mouse**. Fasted male C57Bl/6J mice were dosed with a 1 mg/mL solution of SBI-756 in 10% DMSO 10% Tween-80 and 80% water by intravenous injection (2 mg/kg, as a 0.4 mg/mL solution), oral gavage (10 mg/kg as a 1 mg/mL solution) or intraperitoneal injection (10 mg/kg as a 1 mg/mL solution). Plasma was collected at various time points and analyzed by LC/MS/MS to determine compound levels.

**Xenograft tumors**. All animal studies were conducted in the SBMRI Animal Facility in accordance with the Institutional Animal Care and Use Committee guidelines. Six-week-old female nu/nu mice were purchased Harlan Laboratories (Indianapolis, IN) and allowed to acclimatize for 1 week. A375 cells (1×106, suspended in 200 μl sterile PBS) were injected into subcutaneous flank tissue. When the xenograft size reached ~250 mm3, mice were sorted into different groups. For PLX4720 and SBI-756 combination experiments, mice were either fed PLX4720-containing chow alone (AIN76A Roden Diet with 417mg PLX4720/kg from Open Source Diets) for the control group or PLX4720-containing chow plus an IP injection of 1 mg/kg SBI-756 twice a week.

Mice were maintained in a pathogen-free environment with free access to food. Body weight and tumor volume were measured twice a week. Tumor size was measured with linear calipers and calculated using the formula: ([length in millimeters × (width in millimeters)2]/1). Mice were sacrificed after 4 weeks and tumors were fixed in Z-Fix (Anatech, Battle Creek, MI) and embedded in paraffin for immunohistochemistry.

**Statistical analysis**. Most results are shown as an average ± standard deviation. Significance was determined using a two-tailed Student’s *t*-test. A value of *p* < 0.05 was considered significant. Specific tests are indicated in figure legends.

**Supplementary Reference**

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