Supplementary Information Appendix

Materials and Methods

Cell culture

Melanoma cell lines LOX (a gift from Dr. Oystein Fodstad, University of Oslo, Norway), A375 and SKMEL-28 (ATCC, Manassas, VA), C8161.9 (obtained from Dr. D. Welch, UAB, USA), 1205-Lu (Coriell Institute, Camden, NJ), and Ma-Mel-66a, a short-term culture (a gift from Dr. Dirk Schadendorf, University of Essen, Germany) were employed. C8161.9 cells were grown in DMEM/F12 with 5% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA); 1205-Lu cells were grown in TU2% medium; A375, SKMEL-28, Ma-Mel-66a were grown in RPMI-1640 with 5% FBS. Cell culture media was supplemented with 1X penicillin/streptomycin (Thermo Fisher Scientific) and cells were grown at 37°C and 5% CO2. All cell lines were routinely tested for mycoplasma contamination using MycoFluor Mycoplasma Detection Kit (Thermo Fisher Scientific, South San Francisco, CA) following manufacturer instructions.

RNA extraction and Quantitative RT-PCR (qRT-PCR)

RNA extraction, cDNA synthesis and qRT-PCR were performed as described previously (1). Taqman probes for GAPDH, CDK1, CCNB1, CCNB2, PLK1, VEGFA, CENPM, EEFIA2, CCNA1, PLK1, HIF1, CDC20, KIF18, POL2, KIF4A and AURKA were purchased from Thermo Fisher Scientific.

Cell viability assay

Cell viability was assessed as described (2). Briefly, melanoma cells (3000-4000) were plated in a 96 well plate and treated on the next day with bromo. Cell viability was assessed by using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) after 48 hr of drug treatment following manufacturer instructions.
**Cell cycle and Annexin V assays**

Cell cycle and Annexin V assays were performed by using the Muse cell cycle kit and Muse Annexin V apoptosis kit, respectively (EMD Millipore, Hayward, CA) as described (3).

**Western blot analysis**

Western blot analysis was performed as described previously (4, 5). Target proteins were detected by using specific antibodies against ERK1/2 (#4695), pERK1/2 (#9106), BCL-XL (#2764), CCNB1 (#4138), AURKA (#4718), pAURKA (#3079), PLK1 (#4513) and BCL2 (#15071) from Cell Signaling Technology (Danvers, MA), and CDK1 (#A700-085) from Bethyl, and GAPDH (#sc-365062), from Santa Cruz Biotechnology (Santa Cruz, CA).

**Patient-derived xenograft (PDX) models**

All tumors were initially implanted into NOD SCID gamma (NSG) mice for the development of PDX models. PDX generation and PDX cell (PDXC) culture conditions were previously described (2, 6). Briefly, PDXCs (MM300, MM302, MM313, MM337, MM358, MM386, and MM505), obtained after development of PDX models *in vivo* (Table EV1), were cultured in ultralow attachment T25 flasks with DMEM/Ham’s F12 medium containing 1X B27 supplement (Gibco), 1X pen/strep, 50ng/ml EGF, 50ng/ml FGF and no serum. Short tandem repeat analysis of PDX lines was performed by ATCC. All PDX lines were reported to be human, with no matches in the ATCC and DMFZ databases. All PDX lines tested negative for mycoplasma contamination using MycoFluor Mycoplasma Detection Kit (Thermo Fisher Scientific) following manufacturer’s instructions.
**Pharmacological studies**

Quantification of drug responses was performed as previously described (6). The drugs employed in the present screen included bromo, cobi, vem and their combinations. Bromo, vem and cobi were obtained from Selleck chemicals (Houston, TX).

**RNA Sequencing and Bioinformatics Data Analysis**

RNA extraction from flash-frozen tissue samples was performed as previously described (7-9). RNA-Seq was performed from ~500ng of total RNA processed using TruSeq polyA selection, at a target depth of 40 million paired-end, stranded reads on an Illumina 2500.

**Gene Expression Analyses**

The RNA-Seq data was aligned to the human reference genome (hg19) using the software STAR, followed by gene quantification in the software AltAnalyze to obtain gene-level RPKM values. Gene expression quantification (RPKM) was determined from exon-exon junctions and differential expression (fold>1.2, empirical Bayes moderated t-test p<0.05) was performed in AltAnalyze version 2.1.3 using the Ensembl 72 human database. Embedded gene-set enrichment analyses were performed using GO-Elite with default options. Hierarchical clustering was performed in AltAnalyze using HOPACH clustering for rows and weighted cosine clustering for genes. The analyses presented focused on a heat map representing the top 506 up- and 523 down-regulated genes (empirical Bayes moderated t-test, p<0.05; fold change=1.5) following pseudo-alignment with the software Kallisto in AltAnalyze.

**Comet assay**

30 µl of cell suspension at 1 X 10^5 cell per ml was combined with 300 µl of 1% LMAgarose (R&D Systems, USA) and immediately pipetted onto slides coated with 1% agarose in distilled water. Slides were stored at 4° C for 10 min, followed by incubating in Lysis solution (R&D
Systems, USA) for 1hr at 4° C. Slides were removed and allowed to equilibrate in 0.3 M sodium acetate, 0.1 M Tris, pH 9.0 for 30 min at 4° C. Neutral electrophoresis was performed at 1 V/cm in 0.3 M sodium acetate, 0.1 M Tris, pH 9.0 for 45 min at 4° C. The slides were treated with 1 M ammonium acetate, 95% EtOH for 30 min at RT followed by 70% EtOH for 30 min at RT, and then air-dried at 37° C for 10 min. 50 µl of 0.3X SYBR Gold (Invitrogen, USA), 10 mM Tris-HCl, pH 7.5, 1 mM EDTA staining solution was applied to each slide and incubated for 30 min at RT in the dark. After a short drying period at 37° C in the dark, images were acquired using a Zeiss Axio Imager Z2 microscope controlled by Axiovision software. Image analysis was performed using OpenComet software (10), an ImageJ plug-in, and % DNA in tail output was used for quantification.

**Immunofluorescence and immunohistochemistry**

Immunofluorescence was performed as previously described (11). Briefly, cells were plated and treated with bromo (6µM) and cobi (0.3µM) for 48hr. Cells were probed against RPA (#2208), H3K4 (#9751), H3K27 (#9733), HIF1A (#36169), γH2AX (#7631), RAD51 (#8875) and p21 (#2947) (1:500, Cell Signaling), CD31 (#ab28364, 1:500, abcam) and detected using a secondary antibody labeled with Alexa Fluor 594 (1:1000, Molecular Probes). Mosaic images were acquired with 20X magnification at a fixed exposure with a Zeiss Axio Imager Z2 microscope controlled by Axio Vision software. Fluorescence intensity was analyzed using ImageJ software. Immunohistochemical (IHC) analysis was performed as previously described (1, 3). The CONFIRM anti-Ki-67 (30-9) antibody (Ventana Medical Systems, Tucson, AZ) was used for staining on a Ventana Benchmark autostainer (Ventana Medical Systems). Ki-67 positively stained cells are reported as the average number of counts from 10 randomly selected tumor-containing regions.
**In vivo studies**

Six- to eight-week-old NSG or *nu/nu* mice were purchased from Jackson Laboratories, Sacramento, CA. *In vivo* studies were carried out in accordance with the National Institutes of Health guidelines, Health Research Extension Act of 1985 and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Policy), Office of Laboratory Animal Welfare assurance, and an approved Institutional Animal Care and Use Committee (IACUC) protocol. PDX cells (0.5x10^6) were mixed with 50% Matrigel for subcutaneous injections in a total volume of 100µl in the mouse flank. Once tumors were palpable, mice were randomized and divided into groups with average tumor volumes of 70-100 mm³. Mice were divided into the following treatment groups: vehicle (n=8), vem (n=8), bromo (n=8), bromo/cobi (n=12), bromo/vem (n=12), vem/cobi (n=10), and where applicable, cobi (n=8). The animals were randomly assigned to treatment groups, and the investigator performing tumor measurements was blinded to the identity of the treatment groups. No samples were excluded from the analysis.

Toxicity studies were performed initially to determine the optimal tolerable dose for single agents and drug combinations. The optimal dose of bromo (40mg/kg) and cobi (5mg/kg) was well tolerated, producing a mild reduction in weight loss that was stable across multiple *in vivo* studies. Mice treated with various drug combinations were provided with supportive care for the duration of the study, following IACUC guidelines. For the bromo/cobi treated group, laboratory analysis, including complete blood count and comprehensive serum chemistry, was performed at the Clinical Pathological Laboratory (UC Davis, Davis, CA). All drugs were administered intraperitoneally (i.p.) at the following doses: bromo (40 mg/kg), cobi (5 mg/kg) and vem (20 mg/kg). Bromo and cobi were administered five times a week, whereas vem was administered twice weekly. Tumors were measured by caliper and volumes were calculated as a product of
Mice were sacrificed and tumors collected and processed for immunofluorescence, immunohistochemistry and RNA/protein extraction.

**Statistical analysis**

All quantified data represent an average of at least triplicate samples or as indicated. Statistical significance was determined using the Student’s t-test, Mann-Whitney test, ANOVA, or Kolmogorov-Smirnov test, and P values <0.05 were considered significant. PRISM graphing software (GraphPad Software) was used to determine IC50 values. In the in vivo drug efficacy studies, sample sizes were determined prospectively, using a type I error rate of 0.05 and power of 0.8 to detect differences in means of at least 30%.

**References**

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Figure S1

A) Cell Survival (%)

B) Tumor volume (mm³)

C) DNA Content Index

D) DNA Content Index

E) Viability

F) Viability

G) Viability

H) Protein Western Blot

I) Tumor volume (mm³)

* p<0.002
Figure S3

A) MM337

Counts

DNA content

Vehicle
Bromo
Bromo + Cobi

B) MM358

Counts

DNA content

Vehicle
Bromo
Bromo + Cobi

C) MM337

Viability

Annexin V

Vehicle
Bromo
Bromo + Cobi

D) MM358

Viability

Annexin V

Vehicle
Bromo
Bromo + Cobi

E) MM358

pERK
ERK
BCL2
BCL-XL
GAPDH

Vehicle
Bromo
Bromo + Cobi

F) MM300

pERK
ERK
BCL2
BCL-XL
GAPDH

# 18 19 43 2 48 51 31 14 56

Vehicles
Bromo
Bromo + Cobi
Vem
+ Cobi

G) MM300

Vehicle
Bromo + Cobi

H) MM300

Number of Ki-67 positive cells

Vehicle
Bromo + Cobi
Figure S5

A) Cumulative fraction of mean pixel intensity for RPA with Vehicle vs. Bromo+Cobi treatment. The p-value is <0.0005.

B) Composite, RPA, and DAPI images showing difference in staining between Vehicle and Bromo+Cobi treatments.

C) Cumulative fraction of mean pixel intensity for RPA with Vehicle vs. Bromo+Cobi treatment. The p-value is <0.0005.

D) Cumulative fraction of mean pixel intensity for RAD51 with Vehicle vs. Bromo+Cobi treatment. The p-value is <0.0005.

E) Composite, RAD51, and DAPI images showing difference in staining between Vehicle and Bromo+Cobi treatments.

F) Cumulative fraction of mean pixel intensity for RAD51 with Vehicle vs. Bromo+Cobi treatment. The p-value is <0.0005.

G) Relative mRNA expression (Fold) for LOX with Vehicle vs. Bromo+Cobi treatment.

H) Relative mRNA expression (Fold) for MM358 with Vehicle vs. Bromo+Cobi treatment.
Figure S6

A) Relative mRNA expression

B) Composite p21 DAPI

C) Composite CD31 DAPI

D) Cumulative fraction of p21

E) Cumulative fraction of p21

F) Composite pHH3 DAPI

G) Cumulative fraction of HIF1α

H) Composite HIF1α DAPI

I) Cumulative fraction of HIF1α

J) Composite CD31 DAPI
Figure S7

**Replication catastrophe**

- DNA damage
  - γH2AX
  - H3K4me3
  - H3K27me3
  - RAD51
  - RPA
  - Replication catastrophe

**DNA damage**

- Transcriptional repression
  - CDK1
  - CCNB1
  - AURKA
  - PLK1
  - p21
  - pH3
  - Cell cycle arrest
    - BCL2
    - Apoptosis

**Transcriptional repression**

- Bromosporine and Cobimetinib
  - VEGFA
  - HIF1A
  - Angiogenesis inhibition
Supplementary Figure Legends

Fig. S1. Effects of single or combination drug treatments in various melanoma models.
A) Cell survival analysis of six melanoma cell lines treated with bromo. B-D) Cell cycle analysis of Ma-Mel-66a, LOX and 1205-Lu melanoma cells, respectively, following bromo treatment. Bar graphs showing the proportion of melanoma cells in the different cell cycle phases. E-G) Apoptotic index of Ma-Mel-66a, LOX, and 1205-Lu melanoma cells, respectively, after bromosporine treatment. Bar graphs showing percentage of apoptotic cells. H) Western blot analysis indicating expression of various proteins following bromo treatment in Ma-Mel-66a, LOX, and 1205-Lu cells. I) *In vivo* tumor growth of LOX cells following bromo treatment. *P<0.05.

Fig. S2. Effects of bromo and/or cobi treatment on melanoma PDX lines.
A) Cell cycle analysis of MM-358 cells following treatment with bromo alone and in combination with cobi. Bar graph showing the proportion of melanoma cells in the different cell cycle phases. B) Apoptotic index of MM-358 cells treated with bromo alone and in combination with cobi. Bar graph showing the percentage of apoptotic cells. C) Western blot analysis of MM-358 cells showing expression of target proteins following bromo/cobi treatment in culture. D) Western blot analysis showing expression of target proteins in MM-300 tumors *in vivo* following treatment with various drug combinations. E) Ki-67 staining of MM-300 tumors *in vivo* following vehicle or bromo/cobi treatment. F) Bar graph showing quantification of Ki-67 staining. Scale bar=100 µm; *P<0.05.

Fig. S3. Expression of various proteins following bromo/cobi treatment of MM-358 cells.
A) Quantification of expression of γH2AX in LOX cells. B-C) Qualitative immunofluorescence and quantification of expression of γH2AX in MM-358 cells. D) Comet assay illustrating increased DNA strand breaks in MM-358 cells treated with bromo/cobi versus vehicle. E-F) Quantification of expression of H3K4me3 and H3K27me3 in LOX cells. G-J) Qualitative immunofluorescence and quantification of expression of H3K4me3 and H3K27me3 in MM-358 cells. Scale bar=20µm.

Fig. S4. Quantitative immunofluorescence analysis following bromo/cobi treatment of various melanoma cell lines.

A) Quantification of expression of RPA in LOX cells. B-C) Qualitative immunofluorescence and quantification of expression of RPA in MM-358 cells. D) Quantification of expression of RAD51 in LOX cells. E-F) Qualitative immunofluorescence and quantification of expression of RAD51 in MM-358 cells. G-H) Quantitative RT-PCR analysis showing expression of RAD51 and RPA following bromo/cobi combination in LOX and MM-358 cells. Scale bar=20µm.

Fig. S5. Effects of bromo/cobi treatment on expression of target proteins.

Quantitative RT-PCR analysis of expression of various genes in MM-337 (A) and MM-358 (B) cells following bromo/cobi treatment. C) Western blot analysis showing expression of target proteins following bromo/cobi treatment of MM-337 and MM-358 cells. D) Quantification of expression of p21 in LOX cells treated with bromo/cobi. E) Quantification of expression of p21 in MM-358 cells following bromo/cobi treatment. F) Qualitative and quantitative immunofluorescence analysis of expression of pHH3 in MM-337 tumors in vivo. Bar graph showing quantification of pHH3 staining. G) Quantification of expression of HIF1α in LOX cells treated with bromo/cobi. H) Qualitative immunofluorescence of expression of HIF1α in
MM-358 cells following bromo/cobi treatment. I) Quantification of expression of HIF1α in MM-358 cells following bromo/cobi treatment. J) Microvessel density, as determined by mean CD31 pixel intensity, of MM-337 tumors treated with bromo/cobi or vehicle. Representative images provided of immunofluorescence analysis of treatment and control groups. Scale bar=20µm.

Fig. S6. Signal transduction and growth-promoting pathways altered following bromo/cobi treatment of melanoma cells.

Dataset S1: Clinical and molecular features of featured melanoma PDX models.

Dataset S2. Laboratory analysis of NSG mice following bromo/cobi treatment

Dataset S3: Gene ontology analysis of RNA-Seq data of MM-300 tumors treated in vivo with bromo/cobi or vehicle.