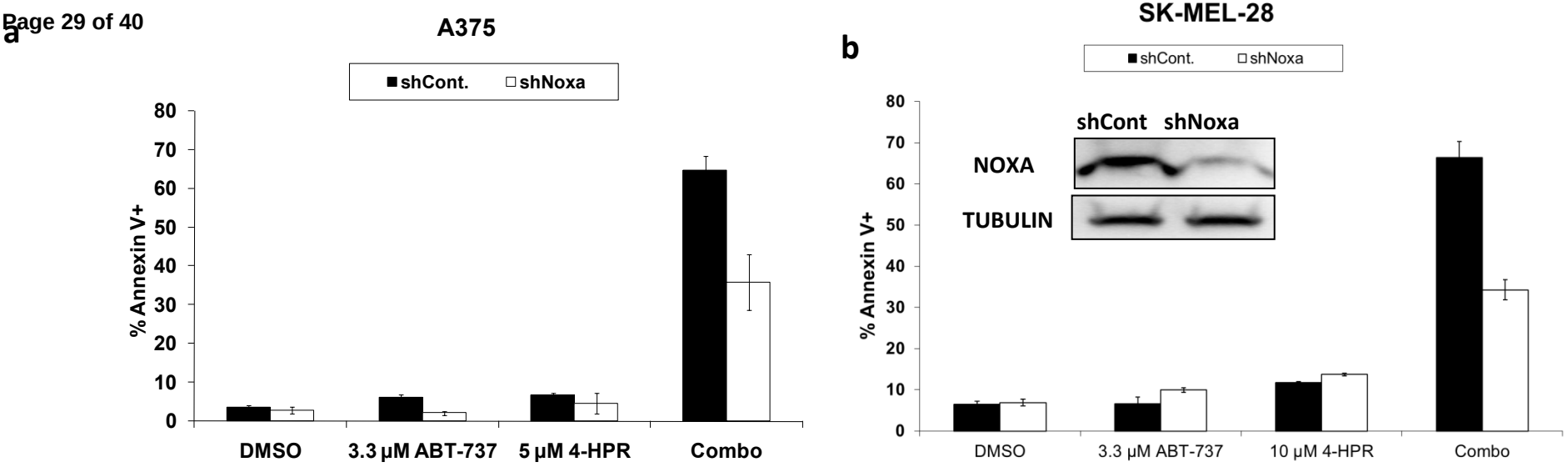
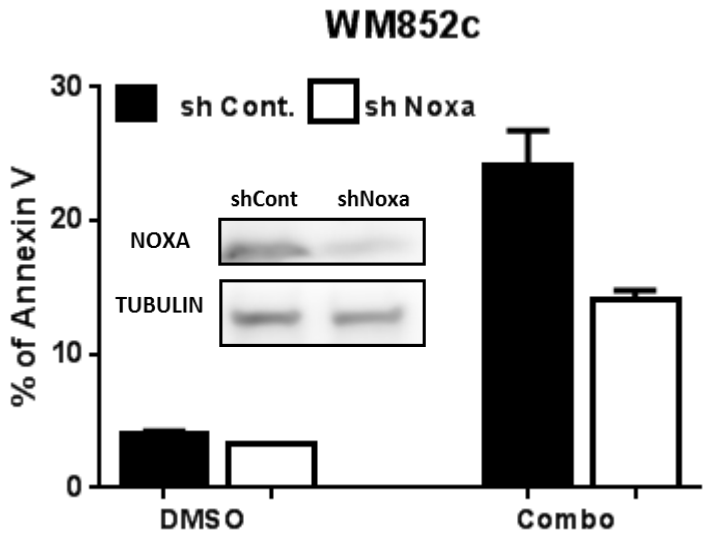
4-HPR (uM)	ABT-737 (uM)	A375	WM852c	SK-MEL-28	451Lu
1.25	**1.1**	0.28	4.692	0.181	4E+113
1.25	**3.3**	0.078	0.000	0.297	4E+113
2.5	**1.1**	0.555	0.009	0.161	8E+113
2.5	**3.3**	0.109	>0.001	0.754	2.188
5	**1.1**	0.266	>0.001	0.288	0.363
5	**3.3**	0.11	>0.001	0.222	1.023
10	**1.1**	0.249	>0.001	0.243	0.048
10	**3.3**	0.226	>0.001	0.21	0.086

Supplemental Table 1: Combination Index (CI) of the data in Fig.1

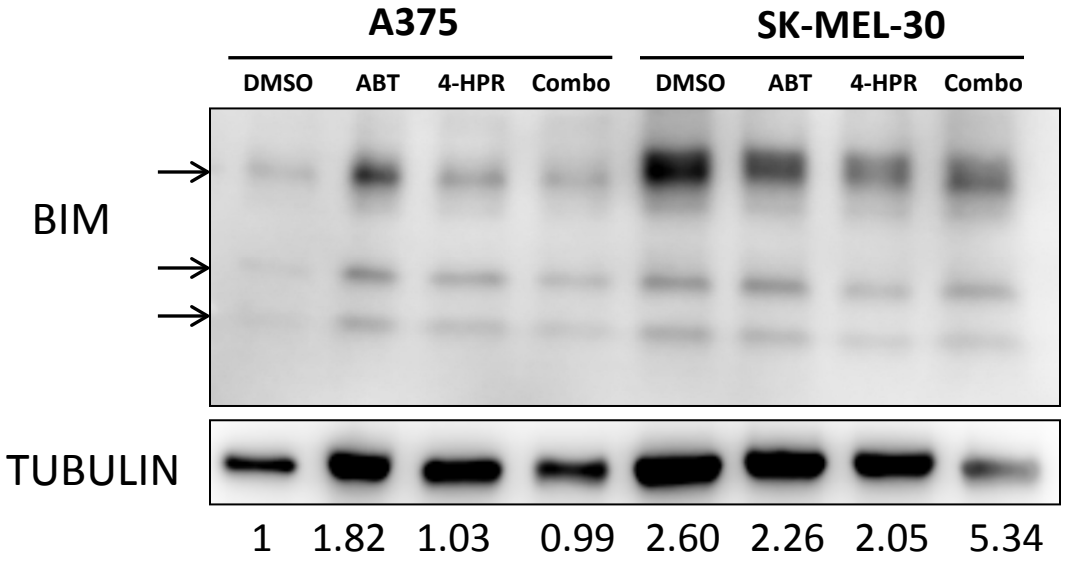
Supplemental Table 1: Combination Index (CI) of the data in Fig.1. The results from Figure 1a were used with Calcusyn to obtain CI of the drug combination. CI values < 0.9 indicate synergism. Smaller CI values indicate stronger synergy. CI values between 0.9-1.1 indicate additive. CI values > 1.1 indicate antagonism.





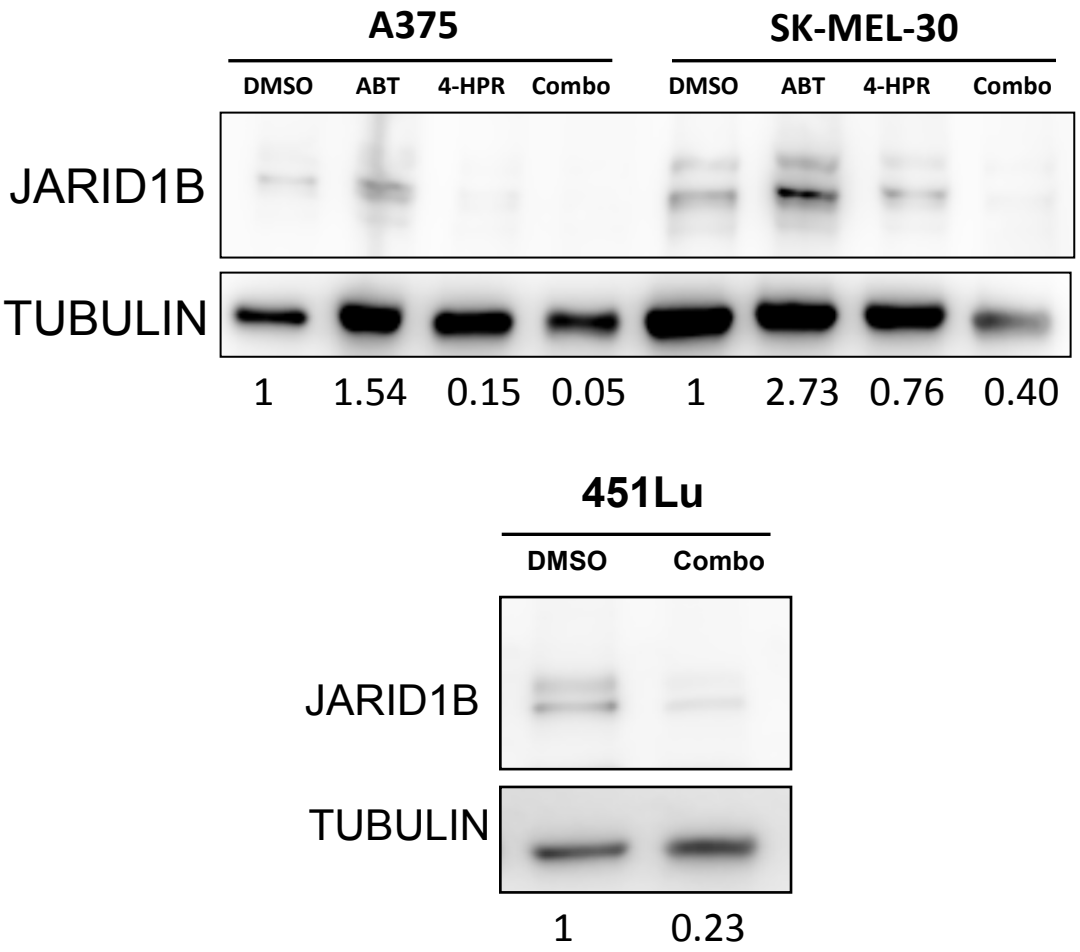
Supplemental Figure S1. Knockdown of NOXA decreased drug-induced cell death. Annexin V assays with NOXA knockdown (shNoxa) or control (shCont.) derived from A375 (a), SK-MEL-28 (b), or WM852c(c) after being treated with indicated drugs for 48 h. The insets show immunoblots of lysates from the respective cell lines used in that graph indicating efficient knockdown of NOXA. shNoxa was significantly different from shCont upon the combination treatment for all three cell lines ($p < 0.05$). There was no statistically significant differences between the shCont and shNoxa for the vehicle (DMS0) or the single drug treatments for all the cell lines tested.

Supplemental Figure S1



Supplemental Figure S2. Higher overall expressions of BIM in SK-MEL-30 than in A375 cells. Immunoblot of cell lysates treated with either vehicle control (DMSO), 3.3μM ABT-737 (ABT), 10μM 4-HPR (4-HPR), or the combination of the two drugs (Combo). SK-MEL-30 exhibits higher level of BIM than A375 in vehicle (DMSO) treated cells. Combination treatment did not significantly affect BIM expression in either cell lines.

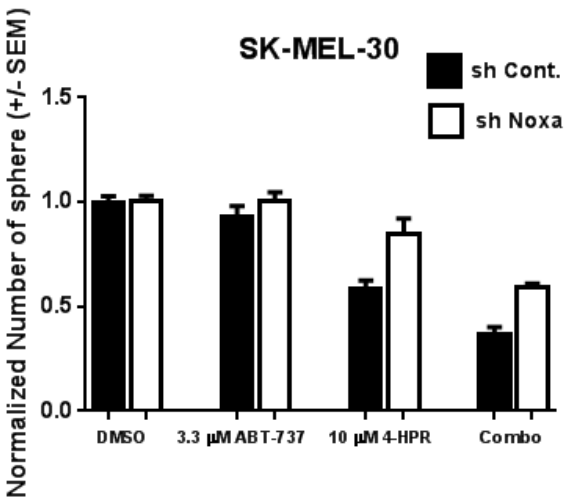
Supplemental Figure S2



Supplemental Figure S3. The combination treatment of ABT-737 plus 4-HPR decreased JARID1B expression. Immunoblot of cell lysates treated with indicated treatment: vehicle control (DMSO), 3.3μM ABT-737 (ABT), 10μM 4-HPR (4-HPR), or the combination of the two drugs (Combo). The Combination treatment reduced the expression of JARED 1B compared to the control in all the cell lines tested.

Supplemental Figure S3

a



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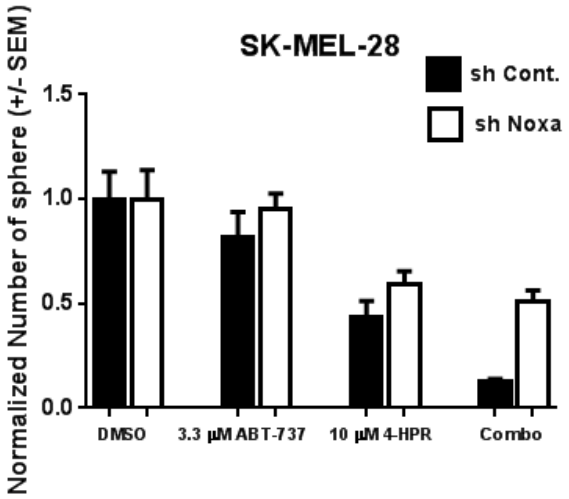

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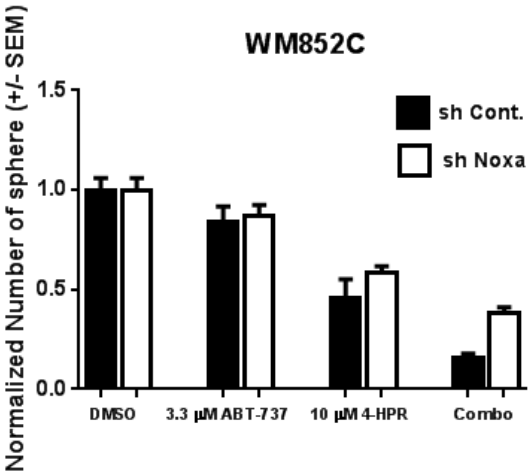
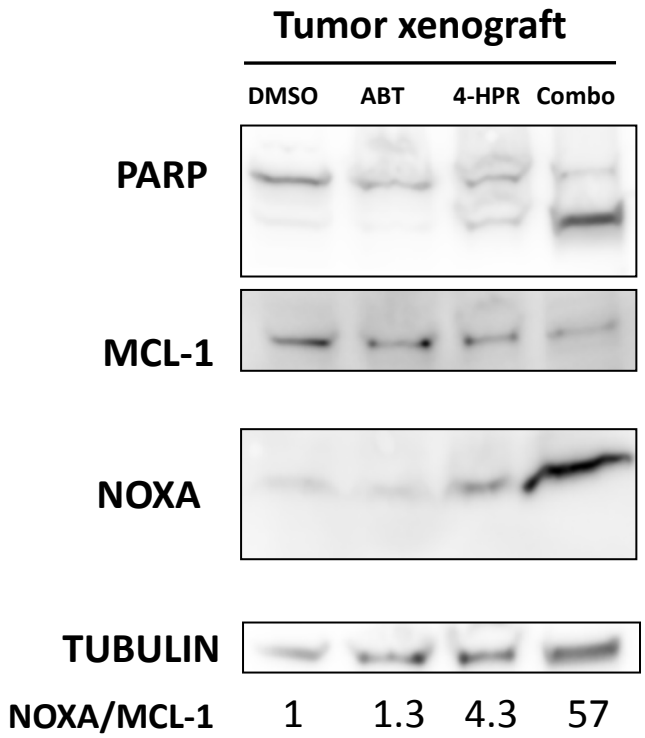


Supplemental Figure S4. Knock-down of NOXA lessens the effects of combining 4-HPR and ABT-737 on sphere-forming capacity of melanoma cells. Primary sphere assays with NOXA knockdown (shNoxa) or control (shCont.) derived from SK-MEL-30 (a), SK-MEL-28 (b), or WM852c (c) after being treated with indicated drugs. Knockdown of NOXA significantly protected cells from the combination-induced disruption of spheres compared to the control in all the cell lines tested ($p < 0.01$) . There were no statistical differences between the shCont. and shNoxa for control or the single drug treatments in any of the cell lines, except 4-HPR treatment in SK-MEL-30 ($p < 0.01$).

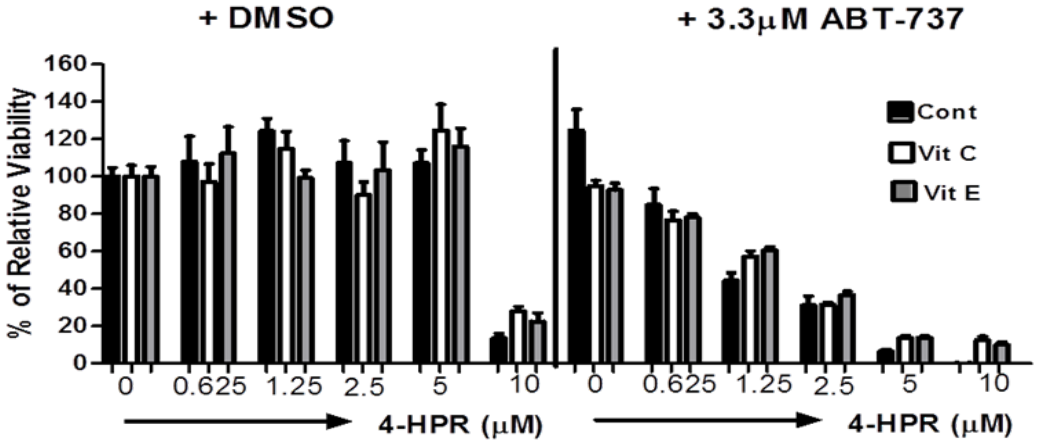
Supplemental Figure S4



Supplemental Figure S5. The effects of combination treatment of ABT-737 plus 4-HPR on the tumor samples harvested from *in vivo* experiments of Figure 6c. Immunoblot of cell lysates from the tumor samples harvested at the end of the *in vivo* xenograft experiment of Figure 6c post treatments of with indicated drugs: vehicle control (DMSO), ABT-737 (ABT), 4-HPR or the combination of the two drugs (Combo).

Supplemental Figure S5

a



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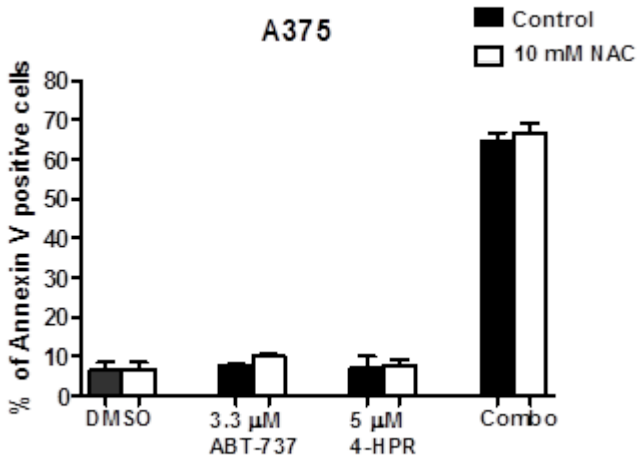

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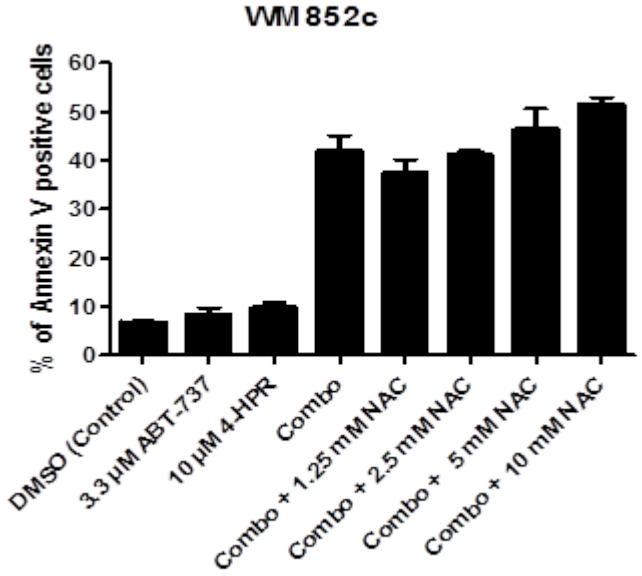
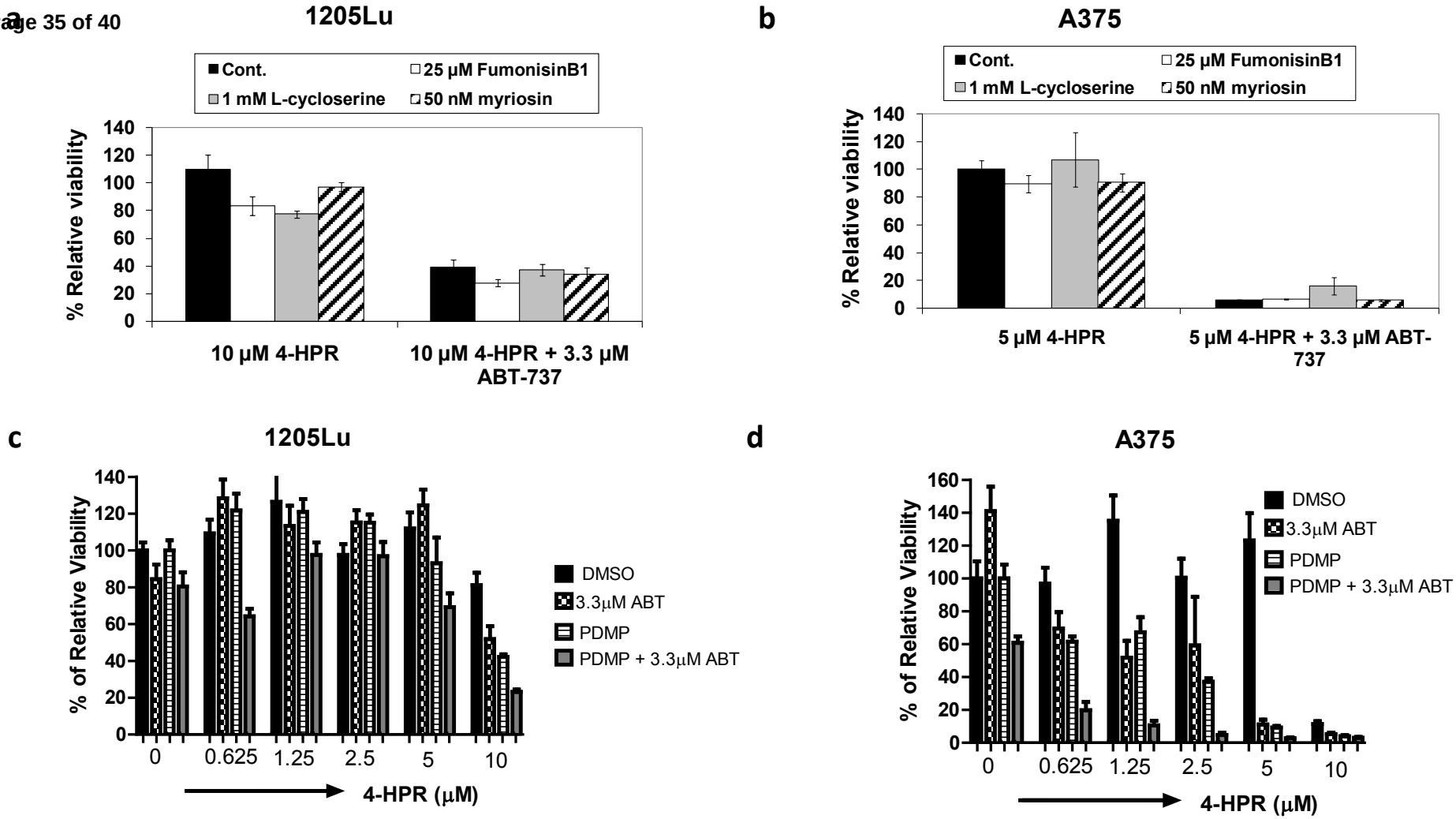


Supplemental Figure S6. Pretreatment with antioxidants does not abrogate the effects of combining 4-HPR and ABT-737. (a) MTS assays with A375 cells pretreated with vehicle, 100 μM vitamin C, or 1 mM vitamin E for 2 h prior to the addition of varying concentrations of 4-HPR (0.625-10 μM) with or without 3.3 μM ABT-737 show no difference in sensitivity to the drug combination after 48 h. (b) Annexin V assays of A375 cells pretreated with 10 mM NAC or vehicle for 2 h prior to the addition of 5 μM 4-HPR and 3.3 μM ABT-737 show no difference in sensitivity between control and NAC treatment. (c) WM852c cells pretreated with varying concentrations of NAC (1.25-10 mM) for 2 h prior to the addition of 10 μM 4-HPR and 3.3 μM ABT-737 show no difference in sensitivity to the drug combination, but there is a tendency for higher NAC concentrations to promote more cell death.

Supplemental Figure S6



Supplemental Figure S7. Pretreatment with inhibitors of ceramide biosynthesis does not abrogate the effects of combining 4-HPR and ABT-737. MTS Assays of 1205Lu (a) and A375 (b) melanoma cell lines, pretreated with various inhibitors of ceramide biosynthesis for 2 h prior to the administration of indicated doses of 4-HPR and ABT-737 for 48 h. MTS Assays of 1205Lu (c) and A375 (d) melanoma cell lines, pretreated with an inhibitor of glucosylceramide synthase, PDMP prior to the administration of indicated doses of 4-HPR and ABT-737 for 48 h. L-cycloserine, myriocin and Fumonisin B1 are inhibitors of the de novo ceramide synthesis pathway. Desipramine and GW4869 are inhibitors of sphingomyelinase pathway. MTS assays show that inhibitors of the *de novo* and salvage ceramide synthesis pathways did not affect sensitivity to the drug combination compared to vehicle, but an inhibitor of glucosylceramide synthase, PDMP, sensitized melanoma cells to 4-HPR.

Supplemental Figure S7

SUPPLEMENTAL MATERIALS AND METHODS

Reagents

N-(4-Hydroxyphenyl) retinamide (>95%; a.k.a. retinoic acid, p-hydroxyanilide, fenretinide, 4-HPR), was obtained from Sigma-Aldrich (St. Louis, MO) or L.C. Labs (Woburn, MA). ABT-737 was kindly provided by Abbott Laboratories (Abbott Park, IL). General caspase inhibitor (z-VAD-FMK) was obtained from R&D systems (Minneapolis, MN).

Cell lines and culture conditions

Human melanoma metastatic cell lines with BRAF mutations (A375, 1205Lu, SK-MEL-28, 451Lu, and HT-144) and cell lines with NRAS mutations (SM-MEL-2, SK-MEL-30, and HT852c) were obtained from ATCC (Manassas, VA), and cell line with NRAS mutations (WM852c) was kindly provided by Dr. Meenhard Herlyn. Cells were maintained in RPMI 1640 media (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Gemini Bio-Products, Inc., West Sacramento, CA). Primary melanocytes HEM_NLP2 were obtained from Life Technologies (Carlsbad, CA), and immortalized melanocytes PIG1 were kindly provided by Dr. Le Poole (Le Poole *et al.*, 1997). Melanocytes were maintained in Medium 254 with Human Melanocyte Growth Supplement-2 (Life Technologies, Carlsbad, CA). To mimic melanoma culture conditions, 10% FBS was added for drug assays.

Measurement of cell proliferation, apoptosis, and ALDH activity

The Cell Titer 96™ Aqueous One solution cell proliferation assay (MTS assay; Promega Corp., Madison, WI) was used to quantify cell viability as previously described (Shellman *et al.*, 2005). The Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA) was used to quantify apoptosis according to the manufacturer's protocol. Cells were analyzed by flow cytometry using a Beckman Coulter FC500 with CXP software (Hialeah, FL) in the University of Colorado Cancer Center Flow Cytometry Core.

The Aldefluor kit (Stem Cell Technologies, Vancouver, Canada) was used to detect the ALDH activity according to the manufacturer's instructions. Aldefluor staining was detected using the FITC channel and analyzed at the University of Colorado Cancer Center Flow Cytometry Core. At least three repeats were done for each cell line. The data was normalized as the relative fold in order to visualize the change of ALDH positive cells compared to the DMSO control, with the percentage of $ALDH^{high}$ cells in the DMSO condition set as "1".

Creation of short hairpin RNA transduced cell lines

Short hairpin RNA (shRNA) expressing cell lines against various BCL2 family members were created as described previously (Bhandarkar *et al.*, 2009; Reuland *et al.*, 2011). Knockdown of genes of interest was measured by immunoblotting of cell lysates.

Immunoblot

Cells, both floating and adherent, were harvested with 1x Laemmli Sample Buffer (Bio-Rad, Hercules, CA). Samples were used in the standard western blot analysis protocol as described previously (Ruth *et al.*, 2006). The following antibodies were used at suggested dilutions from the manufacturers: PARP1 (PARP), BID (Bid), TP53 (p53), BBC3 (PUMA, Puma), and TUBB2A (α/β Tubulin) (Cell Signaling Technology, Danvers, MA); PMAIP1 (NOXA, EMD Biosciences, Inc. San Diego, CA); MCL1 (BD Biosciences, San Jose, CA); BIM (Bim) (Millipore, Billerica, MA), JARID1B (Novus Biologicals, Littleton, CO, Cat # NB100-97821), and HRP-conjugated goat anti-mouse and anti-rabbit antibodies (Jackson Immuno-Research, West Grove, PA). Immunoblots were typically performed 2-3 times for each cell line, and representative examples are shown. Immunoblot data was quantified using Image Studio Ver. 2.0 (LI-COR, Lincoln, NE).

Melanoma patient samples

Melanoma patient samples were maintained in a Patient-Derived Tumor Xenograft model (PDX)

as described in (Luo *et al.*, 2012; Luo *et al.*, 2013). Xenografted tumors of F2-F4 generations were harvested and frozen as single-cell suspensions before being used in this study, and were prepared according to the method described in (Luo *et al.*, 2013). The study was conducted according to Declaration of Helsinki Principles.

Mouse Xenograft studies

Female NCRNU nude mice, aged 4 weeks, were used for the study. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado Denver (protocol number 88512(11)1E). Each mouse was subcutaneously injected on each flank with 1 million 1205Lu cells in a 100 µl volume consisting of 50% BD Matrigel Matrix (BD Biosciences) prepared according to the manufacturer's protocol. Drug treatments began after tumors reached approximately 100 mm^3. Mice were randomly divided into four treatment groups consisting of at least 10 tumors each group: 1) vehicle only, 2) ABT-737 only, 3) 4-HPR only, 4) 4-HPR plus ABT-737. 4-HPR and ABT-737 were administered at 60 mg/kg and 100 mg/kg, respectively. All mice received either a single drug, both drugs, or vehicle. ABT-737 was prepared according to the protocol described in (Reuland *et al.*, 2011; Reuland *et al.*, 2012). ABT-737 or vehicle was administered daily for 21 days via intraperitoneal (i.p.) injection. 4-HPR was prepared according to the protocol described in (Formelli and Cleris, 1993; Gopal *et al.*, 2004). 4-HPR or vehicle was administered via i.p. injection on a schedule of 5 days on and 2 days off. On days when both drugs were administered, ABT-737 was administered at least 6 h prior to 4-HPR. Mice were weighed daily and tumor volume was measured every alternate day with digital calipers. Tumor volume was calculated with the formula: tumor volume (mm^3) = (length × $width^2$)/2. To normalize the data, the tumor volumes were calculated as a percentage compared to day 1, with day 1 equal to 100%. Mice were sacrificed at the end of the experiment and tumors were collected and dissociated into single cell suspensions to perform sphere assays and immunoblot.

Statistical analysis

The data for the anti-proliferative effects obtained from the MTS assay were entered into Calcusyn software (Biosoft, Ferguson, MO) to calculate Combination Index values (CI), and used to determine whether the combination treatments had synergistic, additive, or antagonistic effects using the Chou-Talalay method (Chou and Talalay, 1984). When the CI value is less than 1, it indicates synergistic effects, and the lower the CI, the stronger the synergism.

All graphs and statistical analyses for the MTS assays, Annexin assays, sphere-forming assays, and ALDH assays were created and conducted with GraphPad Prism 5 software. Specifically, one-way analysis of variance (ANOVA) was used to evaluate if there were any statistically significant differences among all the conditions within each experiment. Tukey's post-hoc test was then performed to determine which comparisons among the conditions were statistically significantly different. Analyses with p-values of 0.05 and below were considered significant.

Statistical analysis for tumor growth data was conducted using a mixed model followed by a simple effect test for pairwise comparisons of mean fold change in tumor volume between treatment groups using SPSS software (IBM, SPSS Statistics). A p-value less than 0.05 was considered significant.

References for Supplemental Methods

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