

ABT-737 synergizes with Bortezomib to kill melanoma cells

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Summary

The BH3 mimetic ABT-737 is a potent inhibitor of the anti-apoptotic proteins Bcl-2, Bcl-X_L, and Bcl-w. The Bcl-2 family modulates sensitivity to anticancer drugs in many cancers, including melanomas. In this study, we examined whether ABT-737 is effective in killing melanoma cells either alone or in combination with a proteasome inhibitor already in clinical use (Bortezomib) *in vitro* and *in vivo*, and further evaluated the mechanisms of action. Results showed that ABT-737 alone induced modest cytotoxicity in melanoma cells, but only at higher doses. Knock-down of the anti-apoptotic proteins Bcl-2, Bcl-X_L, or Mcl-1 with siRNAs demonstrated that Mcl-1 is the critical mediator of melanoma's resistance to ABT-737 treatment. However, ABT-737 displayed strong synergistic lethality when combined with Bortezomib. Immunoblot analyses demonstrated that Bortezomib increased expression of Noxa, a pro-apoptotic Bcl-2 member that antagonizes Mcl-1. Additionally, siRNA-mediated inhibition of Noxa expression protected melanoma cells from cytotoxicity induced by the

combination treatment. These results demonstrate that Bortezomib synergizes with ABT-737 by neutralizing Mcl-1's function via increased levels of Noxa. In a xenograft mouse model, although drug doses were limited due to toxicity, ABT-737 or Bortezomib slowed melanoma tumor growth compared to the control, and the drug combination significantly decreased growth compared to either drug alone. These data imply that less toxic drugs fulfilling a function similar to Bortezomib to neutralize Mcl-1 are promising candidates for combination with ABT-737 for treating melanomas.

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Key words: Proteasome inhibitor, Mcl-1, Bcl-2 inhibitor, Noxa, ABT-737, Bortezomib, melanoma

Introduction

Malignant melanoma is a devastating disease since it metastasizes early and is highly resistant to all conventional treatments including chemo-, immuno-, or radiation therapy. Virtually no progress was made over the past thirty years until the recent advent of *BRAF* inhibitors and new immunotherapies (Buzaid, 2004; Cummins et al., 2006; Gogas et al., 2007; Eggermont, 2010; Natarajan et al., 2011). However, as promising as these new therapies are, remission and resistance are inevitable, and thus there is still a pressing need for new treatments. These recent advances are an example of how anticancer strategies have evolved from using non-specific cytotoxic agents to rationally designed drugs that target specific signaling pathways involved in tumorigenesis. This molecular-targeted therapeutic approach holds the promise of providing new and more effective treatment options with minimal toxicity (Weinstein and Joe, 2006).

The high frequency of activating mutations in *NRAS* and *BRAF* in melanoma samples, and the clinical effectiveness of *BRAF* inhibition, suggests that the Ras/Braf/MEK/ERK signaling pathway plays important roles in melanoma tumorigenesis, progression, and development (Chudnovsky et al., 2005; Miller and Mihm, 2006; Gray-Schopfer et al., 2007). We and others

have shown that *NRAS*^{Q61K} and *BRAF*^{V600E} mutations contribute to melanoma's resistance to apoptosis in part by down-regulating BH3 (Bcl-2 homolog domain 3)-only pro-apoptotic Bcl-2 family members such as Bim and Bad (Wang et al., 2007; Boisvert-Adamo and Aplin, 2008; Cartledge et al., 2008; Goldstein et al., 2008; Hendrickson et al., 2008). These studies suggest that BH3-only pro-apoptotic Bcl-2 family members are possible treatment targets for overriding melanoma's inherent defenses against cell death.

Application of BH3 mimetics to activate the intrinsic apoptotic pathway is a promising approach to treating various cancers (Labi et al., 2008). Using a BH3 mimetic bypasses the need to induce endogenous expression of BH3-only proteins, an ability which is often strongly inhibited in many cancers, including melanomas. One promising BH3 mimetic is ABT-737 (developed by Abbott). ABT-737 is a mimetic of the BH3-only pro-apoptotic protein Bad, and is a potent small molecule inhibitor of the anti-apoptotic proteins Bcl-2, Bcl-X_L, and Bcl-w with an affinity 2–3 orders of magnitude higher than any previously reported compounds (Letai, 2005; Oltersdorf et al., 2005). It acts like a BH3-only protein to antagonize anti-apoptotic Bcl-2 family members, thereby diminishing their ability to inhibit apoptosis (Oltersdorf et al., 2005). Many groups have reported on the high efficacy of ABT-737 either as a

single agent or as a chemo-potentiator in combination with other chemotherapeutic agents to treat multiple types of cancers (Adams et al., 2005; Oltersdorf et al., 2005; Certo et al., 2006; Konopleva et al., 2006; Shoemaker et al., 2006; van Delft et al., 2006; Chauhan et al., 2007; Chen et al., 2007; Kang et al., 2007; Kohl et al., 2007; Olberding et al., 2010; Reynoso et al., 2010; Song et al., 2010).

Previously, we showed that the combination of ABT-737 with a proteasome inhibitor (MG-132) synergistically killed melanoma cells *in vitro*. Recent studies have also shown that Bcl-2 overexpression mediates resistance to another proteasome inhibitor, Bortezomib, and that ABT-737 can overcome this resistance in lymphoid cells (Paoluzzi et al., 2008; Smith et al., 2011). Bortezomib (Velcade) is the first therapeutic proteasome inhibitor approved in the U.S. for treating cancers (Chen et al., 2011), and combining ABT-737 with an already approved drug would be a more efficient way of moving it to clinic. In this study, we explored whether ABT-737 is effective in killing melanoma cells either alone or in combination with Bortezomib *in vitro* and *in vivo*, and evaluated the mechanisms of action.

Materials and Methods

Cell lines and culture conditions

The following melanoma cell lines were obtained from ATCC (Manassas, VA): A375, 1205Lu, and HT-144. WM852c, WM115, and 451Lu were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Cells were maintained in RPMI1640 (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Gemini Bio-Products, Inc., West Sacramento, CA). All cell lines except WM852c harbor *BRAF*^{V600E} mutations and no common mutations in *NRAS* (exons 1 or 2), and WM852c exhibits an *NRAS*^{Q61R} mutation but no *BRAF* mutations (exons 11 or 15).

Reagents

Bortezomib for *in vitro* experiments was purchased from LC Laboratories (Woburn, MA). For mouse experiments, Bortezomib formulated as a mannitol boronate ester (Millennium Pharmaceuticals, Cambridge, MA) was purchased from the University of Colorado Hospital pharmacy. ABT-737 was kindly provided by Abbott Laboratories (Abbott Park, IL).

Cell Titer 96TM Aqueous One solution cell proliferation assay for quantification of cell viability (MTS assay)

Cells were seeded in a 96-well plate for 24 h, and then treated with indicated compounds for 48 h before being subjected to MTS assays. The assay was obtained from Promega Corp. (Madison, WI), and procedures were followed as previously described (Shellman et al., 2005). All control treatments used vehicle (DMSO) concentrations equal to that of the highest concentration of the drug treatment groups.

Measurement of apoptosis using Annexin V staining

Cells were seeded in 10 cm dishes for indicated treatments before being subjected to analyses. The Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA) was used 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.

Immunoblots

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). Blots were developed with HRP substrate (SuperSignal West Pico or Femto solutions, Pierce, Rockford, IL) for 5 min at room temperature, and analyzed using a Chemi-doc chemiluminescence detector (Bio-Rad, Hercules, CA). The following antibodies were used at suggested dilutions from the manufacturers: Bax, Bcl-X_L, PARP, and α/β Tubulin were from Cell Signaling Technology (Danvers, MA); Noxa was from EMD Biosciences, Inc. (San Diego, CA); Mcl-1 was from BD Biosciences (San Jose, CA); Bcl-2 was from Dako (Glostrup, Denmark); Bak was from Millipore Corp. (Billerica, MA); and HRP-conjugated goat anti-mouse and anti-rabbit antibodies were from Jackson Immuno-Research (West Grove, PA).

Densitometry measurements were obtained using the Quantity One[®] program (Bio-Rad, Hercules, CA), normalizing all samples to α/β Tubulin to account for loading variation.

RNA interference

ON-TARGETplus SMARTpools of siRNA were purchased from Dharmacon, Inc. (Lafayette, CO), targeting human Noxa (PMAIP1, NM_021127), human Mcl-1 (MCL1, NM_182763), human Bcl-2 (BCL2, NM_000657), human Bcl-X_L (BCL2L1, NM_001191), and the non-targeting control pool. siRNA was introduced into cells using an Amaxa Nucleofector II device (Amaxa Inc., Gaithersburg, MD) as described previously (Miller et al., 2009). Cells were nucleofected with the indicated siRNAs and cultured for indicated periods of time. Subsequently, all growth medium was removed and fresh medium containing indicated compounds was added for indicated time periods before the cells were harvested for analysis.

Mouse tumor model

Female NCRNU nude mice, aged 5–6 weeks, were purchased from Taconic (Hudson, NY). Each mouse was subcutaneously injected on each flank with 1 million 1205Lu cells in a 100 μ l volume consisting of 50% BD Matrigel Matrix (BD Biosciences) prepared according to the manufacturer's protocol. We performed *in vitro* experiments with 1205Lu cells to ensure that they responded to the drugs in a similar fashion as the other cell lines (supplementary material Fig. S3).

Drug treatments began after tumors reached approximately 100 mm³, at around 1 week. Mice were randomly divided into four treatment groups: (1) vehicle only (control, n=10 tumors); (2) ABT-737 only (n=10); (3) Bortezomib only (n=12); (4) Bortezomib plus ABT-737 (n=18). Bortezomib and ABT-737 were administered at 0.8 mg/kg and 80 mg/kg respectively. All mice received either the drug or vehicle for both drugs. ABT-737 was prepared fresh every 7 days by dissolving it in vehicle consisting of 65% D5W, 30% propylene glycol, 5% Tween-80, pH 1.0. The pH was raised to ~3.5 after the drug was fully dissolved. ABT-737 or vehicle was administered daily for 21 days via intraperitoneal (i.p.) injection. Bortezomib was diluted in normal saline (0.9%) each day prior to use and administered on days 1, 5, and 9 via intravenous (i.v.) tail vein injection. On days when both drugs were administered, Bortezomib was administered at least 5 h prior to ABT-737. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Statistical analysis

One-Way Analyses of Variance (ANOVA) were performed to compare group means, and Tukey's Post Hoc tests were used for subsequent pair-wise comparisons. Kaplan-Meier curve analysis was used to compare individual tumor doubling rates. Log-rank (Mantel-Cox) tests were used to compare Kaplan-Meier curves with the program GraphPad Prism 5 (GraphPad Software, San Diego, CA), and *p*-values of 0.05 and below were considered significant.

Supplemental Materials and Methods

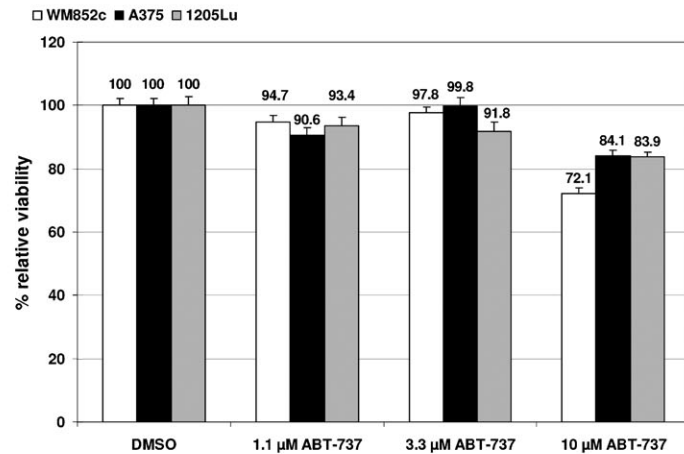
Calculation of combination indexes using Calcsyn.

The cytotoxic effects from MTS assays were entered into Calcsyn software (Biosoft, Ferguson, MO) to determine whether the treatment combinations had synergistic, additive, or antagonistic effects with 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.

Creation of shRNA 1205Lu cells.

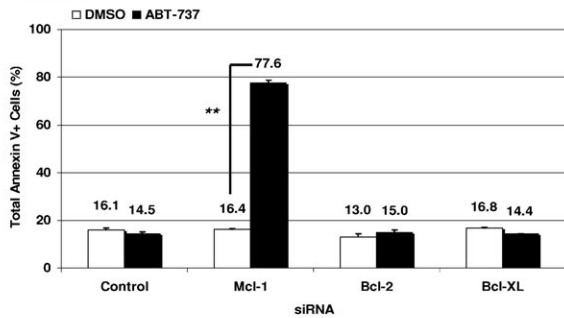
1205Lu cells expressing short hairpin RNA (shRNA) against Noxa, or scrambled control, were constructed using shRNA Lentiviral Particles from Santa Cruz Biotechnology (Santa Cruz, CA) according to the manufacturer's instructions with slight modification. Briefly, cells were seeded in 12-well plates for 24 h at concentrations sufficient to reach 50% confluency. The media was removed from each well and replaced with 1 ml of chilled polybrene working solution (5 μ g/ml in RPMI medium) and incubated at RT for 5 min. The solution was removed and replaced with 1 ml of chilled polybrene working solution with up to 20 μ l of viral particle and incubated at 37 °C. The solution was then removed 24 h later, cells were rinsed once with fresh media, and then 1 ml of fresh media was added. Cells were grown until sufficient numbers were available for selection. Transduced cells were selected by supplementing the media with puromycin (1–4 μ g/ml) for several days, replacing the medium with fresh puromycin-containing medium every 3–4 days until resistant

A. MTS



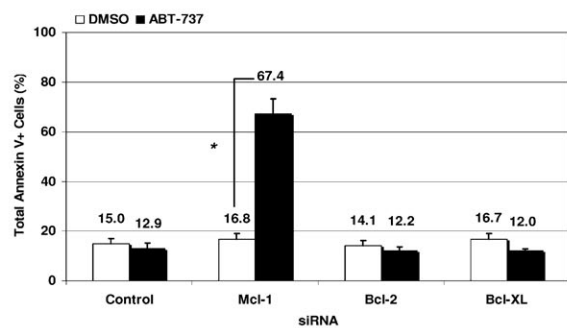
WM852c

B. Annexin V

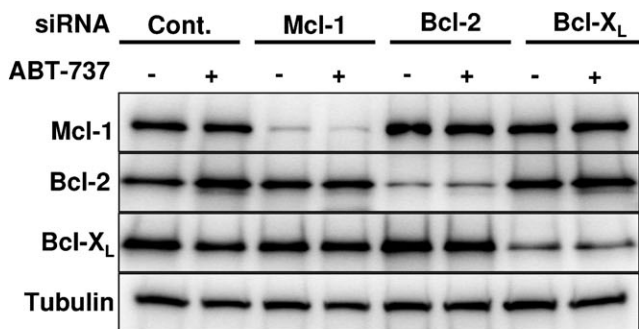


A375

D. Annexin V



C. Immunoblots



E. Immunoblots

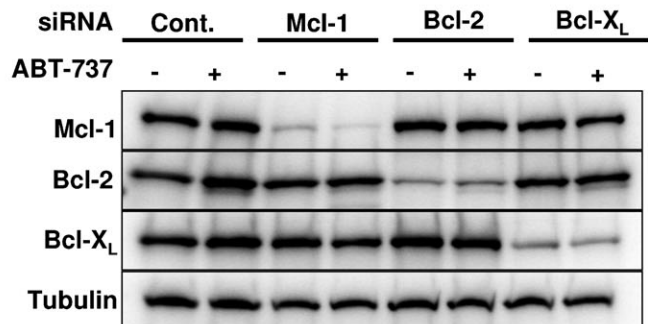


Fig. 1. ABT-737 alone has only a small killing effect on melanoma cells, and Mcl-1 is the main mediator of resistance. MTS assays (A) of WM852c, A375, and 1205Lu cells show only a small killing effect from ABT-737 after 48 h treatments. The control for each cell line was set to 100%. Annexin V Assays (B,D) and Immunoblots (C,E) were performed with melanoma cells transfected with indicated siRNAs. Cells were then treated for 24 h with DMSO control or 3.3 μM ABT-737, 24 h post-transfection. Total Annexin V-positive cells were averaged ± s.e.m. for WM852c cells (B) and A375 cells (D), representing two or three independent experiments, respectively. * One-Way ANOVA; $p=0.001$; ** $p=0.0004$. Remaining WM852c cells (C) and A375 cells (E) from Annexin V assays were lysed for immunoblot analyses.

colonies could be identified. Knockdown of Noxa was confirmed by immunoblotting of cell lysates (data not shown).

Results

Targeting Mcl-1, but not Bcl-2 or Bcl-X_L, sensitizes melanoma cells to ABT-737

ABT-737 treatment alone was only moderately effective at killing melanoma cells at 10 μM for 48 h, and ineffective at lower doses (Fig. 1A). We thus examined how the anti-apoptotic proteins Bcl-2, Bcl-X_L, and Mcl-1 modulate melanoma's sensitivity to

ABT-737 using RNA interference techniques. Fig. 1 shows that simply knocking-down protein expression of these anti-apoptotic Bcl-2 family members individually did not induce cell death in melanoma cells. This suggests that melanoma cells have multiple redundant anti-apoptotic defenses. In contrast, knocking-down Mcl-1 (but not Bcl-2 or Bcl-X_L) protein expression sensitized these cells to ABT-737 treatment dramatically (Fig. 1B,D), from 16.4% to 77.6% Annexin V+ cells in WM852c cells (Fig. 1B), and from 16.8% to 67.4% Annexin V+ cells in A375 cells (Fig. 1D) (note that transfection of siRNA increased background cell death by

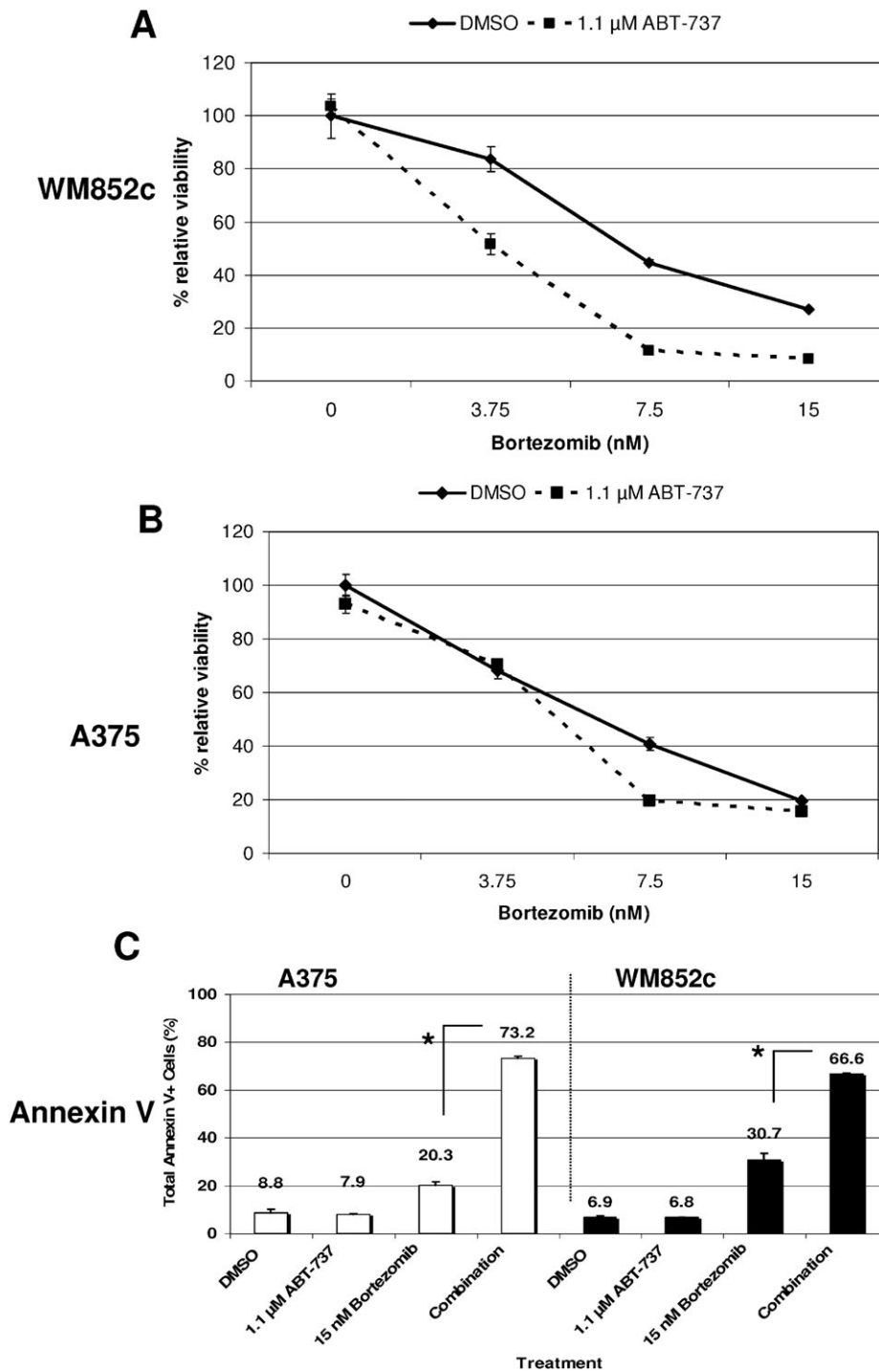


Fig. 2. ABT-737 combined with Bortezomib synergistically kills melanoma cells. For MTS viability assays, WM852c (A) and A375 (B) melanoma cells were treated with indicated compounds for 48 h. Results represent at least three independent experiments. For Annexin V assays (C), A375 and WM852c cells were seeded 24 h prior to treatment with indicated compounds for another 24 h. Total Annexin V-positive cells were averaged \pm s.e.m., representing three independent experiments. *One-Way ANOVA; Tukey's post-hoc test; $p < 0.001$ (individual Bortezomib treatments compared to combination treatment with ABT-737).

5–10% as compared to data in Fig. 2C). These changes were highly significant statistically, and demonstrate that Mcl-1 is the critical factor for melanoma resistance to ABT-737. Immunoblot analyses (Fig. 1C,E) and densitometry measurements confirmed 50–90% inhibition of target proteins in these experiments.

ABT-737 synergistically kills human melanoma cells when combined with Bortezomib

We examined the effects of ABT-737 alone or in combination with Bortezomib for treating A375 and WM852c human

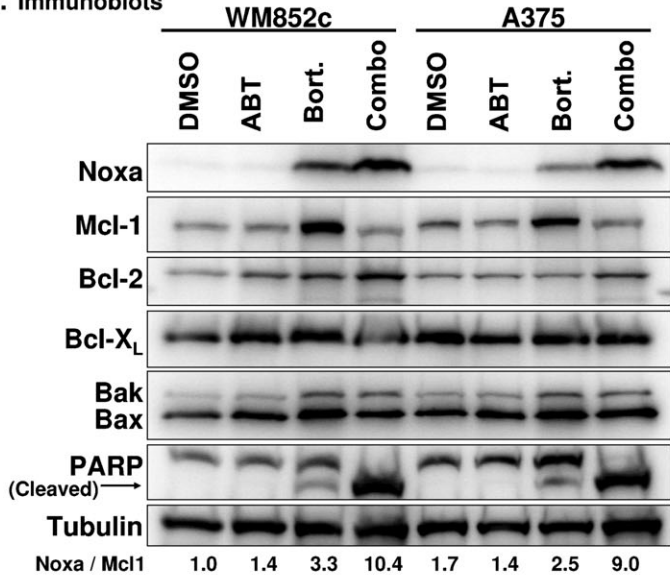
melanoma cells using MTS assays to measure relative cell viability. We found that ABT-737 alone at 1.1 μM caused no cytotoxicity, consistent with results shown in Fig. 1A, but ABT-737 increased the effect of Bortezomib on relative cell viability (Fig. 2A,B). To quantify whether these drug combinations exhibited synergistic, additive, or antagonistic effects, we used CalcuSyn software to calculate the combination index (CI) value for each experimental combination (supplementary material Fig. S1). CI values less than, equal to, or greater than one indicate synergy, additivity, or antagonism, respectively. Most of

the CI values were less than 0.7 (indicating synergy), and many CI values were less than 0.3 (indicating strong synergy). These results show that a majority of the combination conditions of ABT-737 with Bortezomib displayed strong synergistic killing effects for these melanoma cell lines (supplementary material

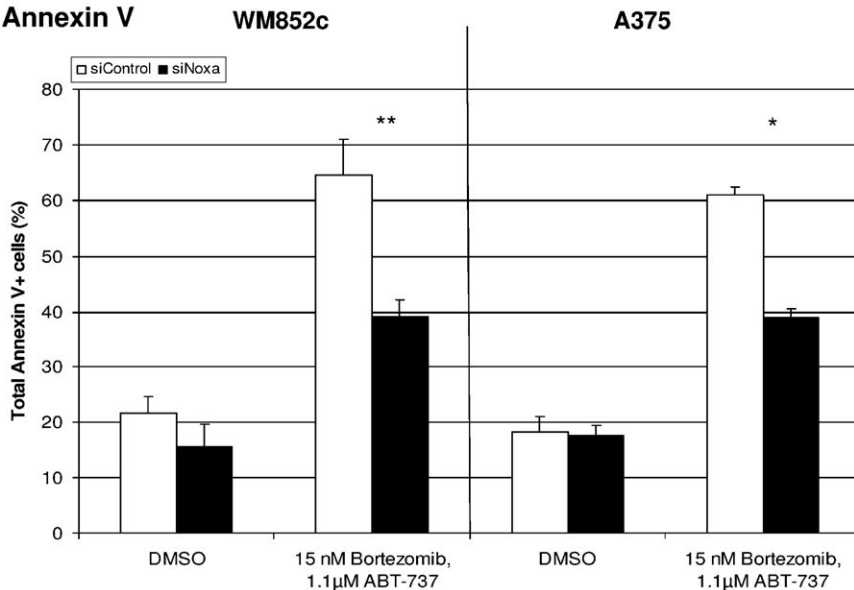
Fig. S1). We have observed similar results in treating several additional melanoma cell lines (supplementary material Figs. S2, S3 and data not shown).

We then performed Annexin V/PI apoptosis assays to determine if the combination treatments induced killing synergistically in

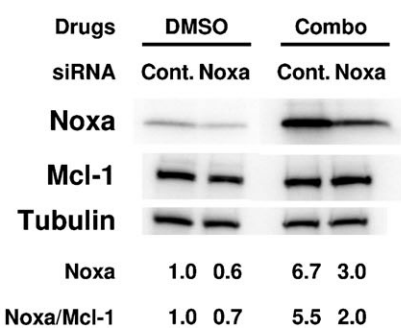
A. Immunoblots



B. Annexin V



C. WM852c immunoblots



D. A375 immunoblots

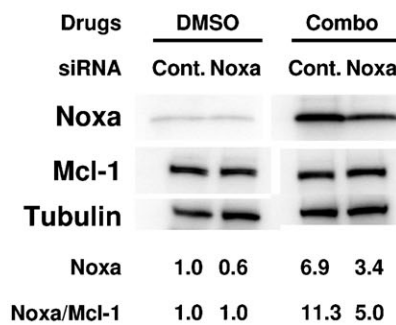


Fig. 3. Combination of Bortezomib with ABT-737 increases the Noxa/Mcl-1 protein ratio and induces Noxa-dependent apoptosis. Immunoblots (A) show changes in Bcl-2 family member proteins upon treatments. Numbers at the bottom indicate relative Noxa/Mcl-1 ratios. Cells were treated with vehicle control ('DMSO'), 1.1 μM ABT-737 ('ABT'), 15 nM Bortezomib ('Bort.'), or a combination of both drugs ('Combo') for 24 h before being lysed. Annexin V Assays (B) and Immunoblots (C,D) were performed with A375 and WM852c cells transfected with indicated siRNAs. 24 h post-transfection, cells were then treated for 18 h with the indicated compounds before being harvested for analyses. Total Annexin V-positive WM852c or A375 cells were averaged ± s.e.m., representing three independent experiments. One-Way ANOVA; * $p < 0.05$; ** $p < 0.01$.

A375 and WM852c melanoma cells (Fig. 2C). Consistent with our MTS results, ABT-737 single treatments did not significantly increase the percentage of Annexin V+ cells compared to the DMSO control. Similarly, Bortezomib single treatments only induced a small increase in the Annexin V+ cell populations. However, the ABT-737 combination treatments significantly increased the percentage of Annexin V+ cells compared to Bortezomib single treatments for both cell lines (Fig. 2C). The killing effect of the combination treatment was significantly higher than the sum effects of the individual treatments. Thus, MTS and Annexin V assays both indicate that combining ABT-737 with Bortezomib induces synergistic killing of melanoma cells.

Combination of ABT-737 and Bortezomib induces expression of the pro-apoptotic protein Noxa and degradation of the anti-apoptotic protein Mcl-1

To investigate the mechanism involved in synergy, we examined the effects of the treatments on Bcl-2 family members. Only Noxa and Mcl-1 protein levels exhibited consistent and dramatic changes induced by either the Bortezomib single treatment or the combination of Bortezomib and ABT-737 (Fig. 3A and data not shown). The ABT-737 single treatment had little effect on most proteins examined, while the Bortezomib single treatment increased both Noxa and Mcl-1 levels substantially (Fig. 3A). The combination treatment of Bortezomib and ABT-737 increased Noxa protein expression to levels similar to the Bortezomib single treatment. However, Mcl-1 protein levels in cells treated with the drug combination were reduced to similar levels as those treated with vehicle control or ABT-737 alone (Fig. 3A). This is very similar to what we observed for the treatment of ABT-737 combined with MG-132 (Miller et al., 2009). Thus, these data suggest that the combination treatment of ABT-737 and Bortezomib induces protein expression of pro-apoptotic Noxa and degradation of anti-apoptotic Mcl-1.

Noxa protein knockdown protects cells from killing induced by the combination of ABT-737 with Bortezomib

The major function of Noxa in regulating apoptosis is to antagonize Mcl-1, since pro-apoptotic Noxa mainly binds to the anti-apoptotic protein Mcl-1, but binds to Bcl-2 and Bcl-X_L with 20- and 70-fold less affinity, respectively, and is not known to bind to Bcl-w (Chen et al., 2005; Willis et al., 2005; Smith et al., 2011). To further investigate the roles of Noxa expression in cell death induced by the combination drug treatments, we used RNAi techniques in WM852c and A375 cells (Fig. 3B,C,D). When cells were treated with combinations of ABT-737 and Bortezomib, blocking Noxa expression by siRNA significantly decreased Annexin V+ cells in both cell lines (Fig. 3B) (note the higher than usual positivity, compared to Fig. 2C, in the control cells due to siRNA transfection). Further, immunoblot analyses indicate that our siRNA inhibited Noxa protein induction by ~50% under the combination treatment conditions (Fig. 3C,D). Similar results were also observed in 1205Lu cells (supplementary material Fig. S3). These results suggest that Noxa is the major mediator of apoptosis induced by the combination drug treatment of ABT-737 and Bortezomib.

ABT-737 and Bortezomib reduce melanoma tumor growth in a mouse model

We tested the ability of ABT-737, Bortezomib, or a combination of the drugs to affect melanoma tumors *in vivo*. While ABT-737

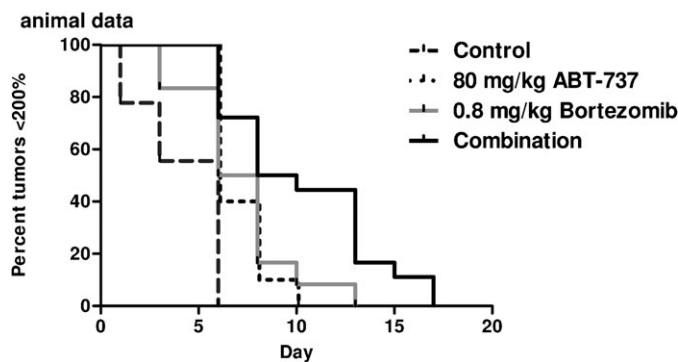


Fig. 4. ABT-737 and Bortezomib reduce tumor growth in a mouse model. Tumors were grown in nude mice by injecting 1205Lu cells subcutaneously. Mice were divided into four groups: (1) vehicle only, (2) ABT-737, (3) Bortezomib, (4) both drugs. Kaplan-Meier curves are based on tumor doubling times over the course of the experiment. Both ABT-737 and Bortezomib treatment groups were significantly different from the control ($p=0.005$ and 0.014 , respectively). The combination group was significantly different from the control ($p<0.0001$), ABT-737 ($p=0.008$) and Bortezomib groups ($p=0.0173$).

alone caused no observable adverse effects, Bortezomib treatment caused weight loss, lethargy, and the appearance of flaky skin. These effects were more pronounced in mice treated with both drugs, limiting the dose sizes. Although Bortezomib at doses of 1 mg/kg and above have been used in other mouse studies (Adams and Kauffman, 2004), we found that doses above 0.8 mg/kg, when combined with ABT-737 doses above 80 mg/kg, had a profound effect on tumor growth but also caused unacceptable weight loss (data not shown), requiring premature euthanasia of the mice. Therefore, we used relatively low drug doses (0.8 mg/kg Bortezomib, 80 mg/kg ABT-737). Since larger tumors had a tendency to ulcerate, requiring mice to be removed prior to the end of the study period, we constructed Kaplan-Meier curves (Fig. 4) to compare the rates at which individual tumors doubled their original size, which allowed the inclusion of every tumor. Both ABT-737 and Bortezomib treated mice showed significantly decreased tumor doubling rates compared to vehicle treated mice ($p=0.005$ and 0.011 , respectively). Mice treated with both drugs showed decreased tumor doubling rates significantly compared to the control ($p<0.0001$) and to mice treated individually with ABT-737 ($p=0.008$) or Bortezomib ($p=0.0173$). This indicates that ABT-737 and Bortezomib can effectively reduce melanoma tumor growth *in vivo*, and that combining them at these doses has a small but significant additive effect.

Discussion

The Bcl-2 family plays an important role in regulating apoptosis (see review (Thomadaki et al., 2006)), and targeting anti-apoptotic Bcl-2 family members combats the drug resistance exhibited by many cancer types (see review (Kang and Reynolds, 2009)). In this study, we investigated the effects and mechanisms of treating melanoma cells with a BH3 mimetic Bcl-2 inhibitor, ABT-737, as a single treatment or combined with a chemotherapeutic drug already used in clinic, Bortezomib.

We found that knocking-down protein expression of the anti-apoptotic proteins Bcl-2, Bcl-X_L, or Mcl-1 individually with siRNAs is not sufficient to induce cell death in melanoma cells. We determined that ABT-737 single treatment induced moderate

cytotoxicity at 10 μM , but little or no cytotoxicity at lower doses (Fig. 1A). However, knocking-down Mcl-1, but not Bcl-2 or Bcl-X_L, dramatically sensitized these melanoma cells to ABT-737 (Fig. 1B,D), demonstrating that Mcl-1 is the critical mediator of melanoma's resistance to ABT-737 treatment. Results here also indicate that melanoma cells have multiple anti-apoptotic defenses, and that neutralizing multiple anti-apoptotic Bcl-2 members at the same time is necessary to induce optimal killing effects for melanoma cells.

The Bcl-2 family can be divided into 3 groups (see review (Thomadaki et al., 2006)): (1) Anti-apoptotic proteins, including the homologous proteins Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1; (2) Multi-domain pro-apoptotic proteins Bax and Bak (and the less-studied Bok), which are critical downstream mediators of apoptosis; (3) Pro-apoptotic BH3-only proteins, including Noxa, Bad, Bim, Bid, Puma, Bmf, BNIP3, BNIP3L, Hrk, and Bik, which antagonize the function of anti-apoptotic Bcl-2 family members.

Binding affinities between different Bcl-2 family members are not equal and are important for their respective functions. For example, the BH3-only pro-apoptotic protein Bad binds tightly to the anti-apoptotic proteins Bcl-2, Bcl-X_L, and Bcl-w, but not to Mcl-1 or A1, whereas Noxa binds to Mcl-1 and A1 but far less strongly to Bcl-2 and Bcl-X_L, and is not known to bind Bcl-w (Chen et al., 2005; Willis et al., 2005; Smith et al., 2011).

ABT-737 is a mimetic of Bad, and as such, it binds tightly to Bcl-2, Bcl-X_L, and Bcl-w, antagonizing their anti-apoptotic function (Oltersdorf et al., 2005). However, ABT-737 does not bind to Mcl-1 or A1, just as Bad does not. Thus, Mcl-1 (or A1) may act as a barrier to ABT-737-induced cell death, and it has been shown that neutralizing Mcl-1 significantly potentiates the cytotoxicity induced by ABT-737 in treating multiple cancer types (Adams et al., 2005; Certo et al., 2006; Konopleva et al., 2006; Letai, 2006; Lin et al., 2006; van Delft et al., 2006; Chen et al., 2007; Tahir et al., 2007; Olberding et al., 2010; Harrison et al., 2011). Our results for treating melanomas are consistent with these studies, implying that combination treatments that antagonize multiple anti-apoptotic Bcl-2 family members are needed.

We attempted to determine if the anti-apoptotic Bcl-2 family member, A1, regulates melanoma's sensitivity to ABT-737. We found it was difficult to detect A1 protein expression in melanoma cells, probably due to low expression levels (data not shown). In addition, the dramatic increase in melanoma's sensitivity to ABT-737 with Mcl-1 protein knockdown indicates that Mcl-1 is the critical mediator of resistance to ABT-737 in melanoma cells. These results indicate that combination treatments of ABT-737 with drugs that neutralize the anti-apoptotic function of Mcl-1 are good treatment strategies. Moreover, resistance of melanoma cells to Bortezomib has been shown to be mediated by anti-apoptotic Bcl-2 family members (Wolter et al., 2007), making ABT-737 and Bortezomib a promising combination.

We therefore examined whether combining ABT-737 with the chemotherapeutic drug Bortezomib would synergistically kill melanoma cells. MTS and Annexin V assays showed that combining ABT-737 with Bortezomib synergistically killed melanoma cells (Fig. 2), and immunoblots of cleaved PARP corroborate the fact that the combination treatment induced apoptosis synergistically (Fig. 3A). Immunoblot analyses also demonstrated a significant increase in Noxa protein expression

and loss of Mcl-1 protein for the combination treatment, significantly increasing the Noxa/Mcl-1 protein ratio (Fig. 3A). Bortezomib primarily achieves this through drastic up-regulation of Noxa protein expression, though agents that promote Mcl-1 protein loss in the absence of Noxa up-regulation should have a similar effect.

Interestingly, Bortezomib treatment alone led to a significant increase of Mcl-1 levels at 24 h, most likely due to decreased proteolysis. While this effect has been observed previously in melanoma cells (Qin et al., 2006), it is in marked contrast to what is observed in multiple myeloma cells, where Mcl-1 undergoes caspase-dependent cleavage (Podar et al., 2008). Likewise, ABT-737 alone can decrease Mcl-1 in multiple myeloma (Kline et al., 2007), whereas we did not observe any Mcl-1 decrease with the concentrations used in the present study. These differences are likely due to the fact that apoptosis can induce caspase-dependent degradation of Mcl-1 (Podar et al., 2008; Miller et al., 2009), and that ABT-737 alone at 3.3 μM or less did not induce apoptosis in the present study, whereas similar concentrations induce significant cell death in multiple myeloma cell lines (Kline et al., 2007). Rather, in the present study, Mcl-1 degradation appears to have taken place only upon large-scale apoptosis induced by the combination treatment. These differences underscore the different sensitivities of various cancers to chemotherapeutic drugs, and show why, in the case of melanoma, targeting multiple cellular defenses is likely necessary.

To test whether Noxa up-regulation was responsible for the effects observed in the combination treatment of ABT-737 with Bortezomib, we inhibited Noxa expression via siRNA. This inhibition protected melanoma cells from cytotoxicity induced by the combination treatments (Fig. 3B). These results indicate that Bortezomib neutralizes Mcl-1's function specifically through Noxa.

Our *in vivo* mouse experiments showed that ABT-737 and Bortezomib treatments reduce the rate of tumor growth. Using relatively low doses of the drugs, we found reduced tumor growth for all treatment groups and significant decreases in the tumor doubling rate (Fig. 4), suggesting that each drug is promising as a candidate for treating melanoma. The combination treatment increased this time further still, and was statistically significant compared to both Bortezomib and ABT-737 individual treatments.

One critical limiting factor with the use of Bortezomib and ABT-737 in our mouse model was acute toxicity, particularly of Bortezomib. We therefore used relatively low doses that did not cause weight loss and found that the effect of the drug combination was still significant. However, without this dosing limitation, it is likely that we would observe a much more pronounced effect on tumor growth in the mice treated with the drug combination. This implies that newer proteasome inhibitors that are less toxic than Bortezomib, and can be used at higher doses, may work even better with ABT-737 and hold more promise for clinical trials.

The results from our *in vitro* Bortezomib treatments are consistent with our previous study on the combination treatment of ABT-737 with another proteasome inhibitor, MG-132 (Miller et al., 2009). In that study, we found that the combination treatment of MG-132 and ABT-737 antagonizes Mcl-1 through increased Noxa expression and caspase-dependent Mcl-1 cleavage (Miller et al., 2009). Noxa induction appears to be a consistent mechanism for cell death in melanoma cells treated with various proteasome inhibitors (Qin et al., 2005). These data

indicate that various proteasome inhibitors function through the same mechanisms when combined with ABT-737 in treating melanoma cells. Currently, more potent and less toxic, next-generation proteasome inhibitors are in development for clinical use (Orlowski and Kuhn, 2008; Kuhn et al., 2011). Our data suggest that combining these newer proteasome inhibitors with ABT-737 would be an effective means of treating melanomas, if they are less toxic, and that this is worthy of further investigation.

In conclusion, the data reported here indicate that drugs that neutralize Mcl-1's function are good candidates for combination therapy with ABT-737 for treating melanomas. This validates rational molecular approaches for targeting multiple anti-apoptotic Bcl-2 members in developing cancer treatments.

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References

- Adams, J., and Kauffman, M. (2004). Development of the proteasome inhibitor Velcade (Bortezomib). *Cancer Invest.* **22**, 304-311.
- Adams, J. M., Huang, D. C., Strasser, A., Willis, S., Chen, L., Wei, A., van Delft, M., Fletcher, J. I., Puthalakath, H., Kuroda, J., et al. (2005). Subversion of the Bcl-2 life/death switch in cancer development and therapy. *Cold Spring Harb. Symp. Quant. Biol.* **70**, 469-477.
- Boisvert-Adamo, K., and Aplin, A. E. (2008). Mutant B-RAF mediates resistance to anoisiks via Bad and Bim. *Oncogene*. **27**, 3301-3312.
- Buzaid, A. C. (2004). Management of metastatic cutaneous melanoma. *Oncology (Williston Park)* **18**, 1443-1450; discussion 1457-1449.
- Cartledge, R. A., Thomas, G. R., Cagnol, S., Jong, K. A., Molton, S. A., Finch, A. J., and McMahon, M. (2008). Oncogenic BRAF(V600E) inhibits BIM expression to promote melanoma cell survival. *Pigment Cell Melanoma Res.* **21**, 534-544.
- Certo, M., Del Gaizo Moore, V., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S. A., and Letai, A. (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9**, 351-365.
- Chauhan, D., Velankar, M., Brahmandam, M., Hideshima, T., Podar, K., Richardson, P., Schlossman, R., Ghobrial, I., Raje, N., Munshi, N., et al. (2007). A novel Bcl-2/Bcl-X(L)/Bcl-w inhibitor ABT-737 as therapy in multiple myeloma. *Oncogene* **26**, 2374-2380.
- Chen, D., Frezza, M., Schmitt, S., Kanwar, J., and Dou, Q. P. (2011). Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Curr Cancer Drug Targets* **11**, 239-253.
- Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., Colman, P. M., Day, C. L., Adams, J. M., and Huang, D. C. (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol. Cell* **17**, 393-403.
- Chen, S., Dai, Y., Harada, H., Dent, P., and Grant, S. (2007). Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res.* **67**, 782-791.
- Chudnovsky, Y., Khavari, P. A., and Adams, A. E. (2005). Melanoma genetics and the development of rational therapeutics. *J Clin Invest* **115**, 813-824.
- Cummins, D. L., Cummins, J. M., Pantle, H., Silverman, M. A., Leonard, A. L., and Channugam, A. (2006). Cutaneous malignant melanoma. *Mayo Clin Proc* **81**, 500-507.
- Eggermont, A. M. (2010). Advances in systemic treatment of melanoma. *Ann Oncol* **21** Suppl 7, vii339-vii344.
- Gogas, H. J., Kirkwood, J. M., and Sondak, V. K. (2007). Chemotherapy for metastatic melanoma: time for a change? *Cancer* **109**, 455-464.
- Goldstein, N. B., Johannes, W. U., Gadeliya, A. V., Green, M. R., Fujita, M., Norris, D. A., and Shellman, Y. G. (2008). Active N-Ras and B-Raf Inhibit Anoikis by Downregulating Bim Expression in Melanocytic Cells. *J Invest Dermatol.* **129**, 432-437.
- Gray-Schopfer, V., Wellbrock, C., and Marais, R. (2007). Melanoma biology and new targeted therapy. *Nature* **445**, 851-857.
- Harrison, L. R., Micha, D., Brandenburg, M., Simpson, K. L., Morrow, C. J., Denny, O., Hodgkinson, C., Yunus, Z., Dempsey, C., Roberts, D., et al. (2011). Hypoxic human cancer cells are sensitized to BH-3 mimetic-induced apoptosis via downregulation of the Bcl-2 protein Mcl-1. *J Clin Invest.* **121**, 1075-1087.
- Hendrickson, A. W., Meng, X. W., and Kaufmann, S. H. (2008). Anticancer therapy: boosting the bang of Bim. *J Clin Invest* **118**, 3582-3584.
- Kang, M. H., and Reynolds, C. P. (2009). Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin Cancer Res* **15**, 1126-1132.
- Kang, M. H., Kang, Y. H., Szymanska, B., Wilczynska-Kalak, U., Sheard, M. A., Harned, T., Lock, R. B., and Reynolds, C. P. (2007). Activity of vincristine, L-ASP, and dexamethasone against acute lymphoblastic leukemia is enhanced by the BH3-mimetic ABT-737 *in vitro* and *in vivo*. *Blood*. **110**, 2057-2066.
- Kline, M. P., Rajkumar, S. V., Timm, M. M., Kimlinger, T. K., Haug, J. L., Lust, J. A., Greipp, P. R., and Kumar, S. (2007). ABT-737, an inhibitor of Bcl-2 family proteins, is a potent inducer of apoptosis in multiple myeloma cells. *Leukemia* **21**, 1549-1560.
- Kohl, T. M., Hellinger, C., Ahmed, F., Buske, C., Hiddemann, W., Bohlander, S. K., and Spiekermann, K. (2007). BH3 mimetic ABT-737 neutralizes resistance to FLT3 inhibitor treatment mediated by FLT3-independent expression of BCL2 in primary AML blasts. *Leukemia*. **21**, 1763-1772.
- Konopleva, M., Contractor, R., Tsao, T., Samudio, I., Ruvolo, P. P., Kitada, S., Deng, X., Zhai, D., Shi, Y. X., Sneed, T., et al. (2006). Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* **10**, 375-388.
- Kuhn, D. J., Orlowski, R. Z., and Bjorklund, C. C. (2011). Second Generation Proteasome Inhibitors: Carfilzomib and Immunoproteasome-Specific Inhibitors (IPSI). *Curr Cancer Drug Targets* **11**, 285-295.
- Labi, V., Grespi, F., Baumgartner, F., and Villunger, A. (2008). Targeting the Bcl-2-regulated apoptosis pathway by BH3 mimetics: a breakthrough in anticancer therapy? *Cell Death Differ* **15**, 977-987.
- Letai, A. (2005). BCL-2: found bound and drugged! *Trends Mol Med* **11**, 442-444.
- Letai, A. (2006). Restoring cancer's death sentence. *Cancer Cell* **10**, 343-345.
- Lin, X., Morgan-Lappe, S., Huang, X., Li, L., Zakula, D. M., Vernetti, L. A., Fesik, S. W., and Shen, Y. (2006). 'Seed' analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-X(L) inhibitor ABT-737. *Oncogene*. **26**, 3972-3979.
- Miller, A. J., and Mihm, M. C., Jr. (2006). Melanoma. *N Engl. J. Med.* **355**, 51-65.
- Miller, L. A., Goldstein, N. B., Johannes, W. U., Walton, C. H., Fujita, M., Norris, D. A., and Shellman, Y. G. (2009). BH3 mimetic ABT-737 and a proteasome inhibitor synergistically kill melanomas through Noxa-dependent apoptosis. *J Invest. Dermatol.* **129**, 964-971.
- Natarajan, N., Telang, S., Miller, D., and Chesney, J. (2011). Novel immunotherapeutic agents and small molecule antagonists of signalling kinases for the treatment of metastatic melanoma. *Drugs* **71**, 1233-1250.
- Olberding, K. E., Wang, X., Zhu, Y., Pan, J., Rai, S. N., and Li, C. (2010). Actinomycin D synergistically enhances the efficacy of the BH3 mimetic ABT-737 by downregulating Mcl-1 expression. *Cancer Biol. Ther.* **10**, 918-929.
- Oltsersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., et al. (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435**, 677-681.
- Orlowski, R. Z., and Kuhn, D. J. (2008). Proteasome inhibitors in cancer therapy: lessons from the first decade. *Clin. Cancer Res.* **14**, 1649-1657.
- Paoluzzi, L., Gonen, M., Bhagat, G., Furman, R. R., Gardner, J. R., Scotto, L., Gueorguiev, V. D., Heaney, M. L., Manova, K., and O'Connor, O. A. (2008). The BH3-only mimetic ABT-737 synergizes the antineoplastic activity of proteasome inhibitors in lymphoid malignancies. *Blood* **112**, 2906-2916.
- Podar, K., Gouill, S. L., Zhang, J., Opferman, J. T., Zorn, E., Tai, Y. T., Hideshima, T., Amiot, M., Chauhan, D., Harousseau, J. L., et al. (2008). A pivotal role for Mcl-1 in Bortezomib-induced apoptosis. *Oncogene* **27**, 721-731.
- Qin, J. Z., Xin, H., Sitailo, L. A., Denning, M. F., and Nickoloff, B. J. (2006). Enhanced killing of melanoma cells by simultaneously targeting Mcl-1 and NOXA. *Cancer Res.* **66**, 9636-9645.
- Qin, J. Z., Ziffra, J., Stennett, L., Bodner, B., Bonish, B. K., Chaturvedi, V., Bennett, F., Pollock, P. M., Trent, J. M., Hendrix, M. J., et al. (2005). Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. *Cancer Res.* **65**, 6282-6293.
- Reynoso, D., Nolden, L. K., Yang, D., Dumont, S. N., Conley, A. P., Dumont, A. G., Zhou, K., Duensing, A., and Trent, J. C. (2010). Synergistic induction of apoptosis by the Bcl-2 inhibitor ABT-737 and imatinib mesylate in gastrointestinal stromal tumor cells. *Mol. Oncol.* **5**, 93-104.
- Ruth, M. C., Xu, Y., Maxwell, I. H., Ahn, N. G., Norris, D. A., and Shellman, Y. G. (2006). RhoC promotes human melanoma invasion in a PI3K/Akt-dependent pathway. *J. Invest. Dermatol.* **126**, 862-868.
- Shellman, Y. G., Ribble, D., Miller, L., Gendall, J., Vanbuskirk, K., Kelly, D., Norris, D. A., and Dellavalle, R. P. (2005). Lovastatin-induced apoptosis in human melanoma cell lines. *Melanoma Res.* **15**, 83-89.
- Shoemaker, A. R., Oleksijew, A., Bauch, J., Belli, B. A., Borre, T., Bruncko, M., Deckwerth, T., Frost, D. J., Jarvis, K., Joseph, M. K., et al. (2006). A Small-Molecule Inhibitor of Bcl-XL Potentiates the Activity of Cytotoxic Drugs *in vitro* and *in vivo*. *Cancer Res.* **66**, 8731-8739.

- Smith, A. J., Dai, H., Correia, C., Takahashi, R., Lee, S. H., Schmitz, I., and Kaufmann, S. H. (2011). Noxa/Bcl-2 protein interactions contribute to bortezomib resistance in human lymphoid cells. *J. Biol. Chem.* **286**, 17682-17692.
- Song, J. H., Kandasamy, K., Zemskova, M., Lin, Y. W., and Kraft, A. S. (2010). The BH3 mimetic ABT-737 induces cancer cell senescence. *Cancer Res.* **71**, 506-515.
- Tahir, S. K., Yang, X., Anderson, M. G., Morgan-Lappe, S. E., Sarthy, A. V., Chen, J., Warner, R. B., Ng, S. C., Fesik, S. W., Elmore, S. W., et al. (2007). Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. *Cancer Res.* **67**, 1176-1183.
- Thomadaki, H., Scorilas, A., and Hindmarsh, J. T. (2006). BCL2 family of apoptosis-related genes: functions and clinical implications in cancer. *Crit. Rev. Clin. Lab. Sci.* **43**, 1-67.
- van Delft, M. F., Wei, A. H., Mason, K. D., Vandenberg, C. J., Chen, L., Czabotar, P. E., Willis, S. N., Scott, C. L., Day, C. L., Cory, S., et al. (2006). The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* **10**, 389-399.
- Wang, Y. F., Jiang, C. C., Kiejda, K. A., Gillespie, S., Zhang, X. D., and Hersey, P. (2007). Apoptosis induction in human melanoma cells by inhibition of MEK is caspase-independent and mediated by the Bcl-2 family members PUMA, Bim, and Mcl-1. *Clin. Cancer Res.* **13**, 4934-4942.
- Weinstein, I. B., and Joe, A. K. (2006). Mechanisms of disease: Oncogene addiction—a rationale for molecular targeting in cancer therapy. *Nat. Clin. Pract. Oncol.* **3**, 448-457.
- Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., and Huang, D. C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev.* **19**, 1294-1305.
- Wolter, K. G., Verhaegen, M., Fernandez, Y., Nikolovska-Coleska, Z., Riblett, M., de la Vega, C. M., Wang, S., and Soengas, M. S. (2007). Therapeutic window for melanoma treatment provided by selective effects of the proteasome on Bcl-2 proteins. *Cell Death Differ.* **14**, 1605-1616.