XIAP downregulation accompanies mebendazole growth inhibition in melanoma xenografts

Nicole A Doudican, Sara A Byron, Pamela M Pollock, Seth J Orlow

The Ronald O. Perelman Department of Dermatology (N.A.D., S.J.O.) and the Department of Cell Biology (S.J.O.), New York University School of Medicine, New York, NY, USA; Cancer and Cell Biology Division (S.A.B., P.M.P), Translational Genomics Research Institute, Phoenix, AZ, USA

Dr Pollock’s current address is Institute of Health and Biomedical Innovation, QUT, Brisbane Australia.

Running title: Mebendazole downregulates XIAP

Corresponding author:
Seth J Orlow, MD, PhD
NYU School of Medicine
550 First Avenue, H-100
New York, NY 10016 USA
Phone: 212-263-5245
Fax: 212-263-8752
seth.orlow@nyumc.org

Request for reprints can be made to Seth J Orlow.

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Abbreviations
MBZ: mebendazole
siRNA: small interfering ribonucleic acid
TMZ: temozolomide
IC50: half maximal inhibitory concentration
FCS: fetal calf serum
XIAP: X-linked inhibitor of apoptosis
SMAC/DIABLO: second mitochondria-derived activator of caspases/ direct IAP binding protein with low pl
BCL2: B-cell lymphoma 2
BAX: BCL2–associated X protein
BRAF: v-Raf murine sarcoma viral oncogene homolog B1
PARP: Poly (ADP-ribose) polymerase
Abstract
Mebendazole (MBZ) was identified as a promising therapeutic based upon its ability to induce apoptosis in melanoma cell lines via a BCL2 dependent mechanism. We now show that in a human xenograft melanoma model, oral MBZ is as effective as the current standard of care temozolomide (TMZ) in reducing tumor growth. Inhibition of melanoma growth in vivo is accompanied by phosphorylation of BCL2 and decreased levels of XIAP. Reduced expression of XIAP upon treatment with MBZ is partially mediated by its proteasomal degradation. Furthermore, exposure of melanoma cells to MBZ promotes the interaction of SMAC/DIABLO with XIAP, thereby alleviating XIAP’s inhibition on apoptosis. XIAP expression upon exposure to MBZ is indicative of sensitivity to MBZ as MBZ resistant cells do not display reduced levels of XIAP after treatment. Resistance to MBZ can be partially reversed by siRNA knockdown of cellular levels of XIAP. Our data suggest that MBZ is a promising anti-melanoma agent based upon its effects on key antiapoptotic proteins.

Key Words: Melanoma, apoptosis, XIAP, mebendazole, xenograft, drug repositioning
**Introduction**

Melanoma is the most aggressive form of skin cancer, with a high propensity to metastasize. Based on National Cancer Institute estimates, the lifetime risk of a person in the USA developing melanoma has doubled in the last 20 years [1]. Because the risk of developing melanoma may be correlated with UV exposure and recent societal trends have resulted in increased sun exposure, the increasing trend is expected to continue its trajectory [2]. Despite recent advances in the treatment of melanoma using targeted therapies, patients still face a dismal prognosis as most responses are transient, with relapse and resistance occurring in most cases [3]. Temozolomide, the standard of care for advanced non-mutant BRAF melanoma and often administered in vemurafenib-resistant melanoma, is associated with response rates of less than 20% [4].

Drug repositioning is the application of known drugs to new indications. Given the successes for a number of repositioned drugs including duloxetine, sildenafil, and thalidomide, the process has become recognized as an effective alternative to the development of novel chemical entities [5,6]. Because repositioned drugs have often been through several stages of clinical development, and in many cases are already marketed, the time and cost required to take a drug from bench to bedside is dramatically reduced. With regard to the immediate clinical need for effective melanoma therapies, drug repositioning holds the promise of a rapid solution to a pressing clinical problem. Here, we present data demonstrating that mebendazole (MBZ), currently marketed in the US as an oral anti-parasitic drug with a remarkable safety profile, also exhibits promising anti-melanoma growth properties *in vivo*.

Mebendazole has been used extensively to safely treat gastrointestinal parasitic infections in humans for over 50 years [7,8]. In the treatment of common roundworms, hookworms, and whipworms, adults and children 2 years of age and older are administered 100 mg two times a day, morning and evening, for three days. In addition, mebendazole has also demonstrated effectiveness in the management of cystic echinococcosis (also called “hydatid cysts”) and alveolar echinococcosis. For treatment of these conditions, mebendazole is administered at higher doses for prolonged periods of time. Mebendazole is typically used at a dosage of 40-50 mg/kg body weight either continuously or administered as 1–6 monthly cycles separated by 10–14 day intervals. [9]. Multiple studies have demonstrated that mebendazole is generally well tolerated for extended periods of time at this dose with rare reported adverse reactions including hepatotoxicity and gastrointestinal disturbances [10,11,12].

Melanoma's noted intrinsic resistance to therapy has been attributed to modulation of cellular survival pathways including apoptosis. We originally reported that MBZ, which acts as a microtubule-disrupting agent, is a potent inducer of phosphorylation of BCL2 and apoptosis in melanoma cells *in vitro*. Here, we show that in addition to its effects on BCL2, MBZ also modulates another key anti-apoptotic factor, X-linked inhibitor of apoptosis (XIAP), to promote melanoma apoptosis both *in vitro* and *in vivo*. XIAP is an important regulator of cellular apoptosis through direct binding to caspases and inhibition of their proteolytic activity [13]. Given its function, it is surmised that XIAP may play a critical role in melanoma chemoresistance. Indeed, overexpression of XIAP imparts resistance to various chemotherapies *in vitro* [14]. XIAP expression has also been shown to increase with progressive disease stage in melanoma [15].

Here we demonstrate that MBZ inhibits human melanoma xenograft growth and is accompanied by reduced XIAP expression. *In vitro*, MBZ mediated induction of apoptosis in melanoma cells involves enhanced interaction between XIAP and SMAC/DIABLO and subsequent proteasomal degradation of XIAP. This cellular response does not occur in cells resistant to MBZ and may serve as a potential biomarker of treatment response.
Methods
Reagents and cell lines: Human melanoma cell lines were cultured in DMEM supplemented with 10% FCS, 5 units/mL penicillin, and 5 μg/mL streptomycin. SK-Mel-19 was kindly provided by Alan Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY). M-14 cells were a generous gift from Gabriella Zupi (Instituto Regina Elena, Rome, Italy). A-375 was obtained from American Type Culture Collection (St. Louis, MO). The following cell lines were provided by Dr Pamela Pollock: MM622, MM540, D08, MM329, D17, and UACC1097 (UACC1097 was supplied by the Arizona Cancer Center Cell Culture Shared Resource, Tucson, AZ). All cells were maintained in an incubator with a humidified atmosphere of 5% CO₂ at 37°C. TMZ (NSC 362856) was kindly provided by the National Cancer Institute (Bethesda, MD). Mebendazole and MG132 were purchased from Sigma Aldrich (St. Louis, MO).

Xenograft studies: All animals were handled in strict accordance with good animal practice as defined by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. Animal protocols were approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. Athymic female nude mice aged 4-6 weeks (Taconic, Germantown, NY) were injected subcutaneously into the left flank with 2 × 10⁶ M-14 melanoma cells with 50% Matrigel (BD Biosciences, San Jose, CA) for tumor implantation. Mice were maintained in ventilated cages and fed/watered ad libitum with experiments carried out under an IACUC approved protocol as well as following institutional guidelines for the proper and humane use of animals in research. After development of a 3-5mm tumor (within 14 days), mice were randomly divided into one of four experimental groups: 1 mg MBZ administered by gavage every other day, 2 mg MBZ administered by gavage every other day, 100 mg/kg TMZ administered by intraperitoneal injection for 5 days, and DMSO administered by intraperitoneal injection for 5 days (mock-treated controls). Each experimental group consisted of 10 mice; the experiment was repeated three times. Treatment was administered for 5 weeks. During this time, mice were monitored for tumor growth through twice weekly caliper measurements of tumor diameter (determined by cross-sectional diameter) and tumor volume (using the formula a²×b/2, in which a is the short diameter of the tumor and b is the long diameter of the tumor). After the full course of treatment, mice were sacrificed by CO₂ euthanasia. Tumors were extracted and immediately frozen at −80°C.

Protein extraction and Western blotting: Cells and/or homogenized tumors were harvested in extraction buffer containing 1% Triton X-100, 50 mmol/L Tris, 2 mmol/L EDTA, 150 mmol/L NaCl (pH 7.5), protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma Aldrich, St Louis, MO). The lysates were centrifuged at 10,000 × g, 4°C for 10 min in a microcentrifuge with protein concentration of supernatant determined with a protein assay kit (Bio-Rad, Hercules, CA). Proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Polyscreen, Perkin-Elmer, Waltham, MA). Estimation of protein molecular weight was determined through use of Precision Plus Kaleidoscope Protein Standard (BioRad, Hercules, CA). Antibodies against cleaved poly(ADP-ribose) polymerase (PARP, Cat. No. 9541), cleaved caspase 9 (Cat No. 9501), cleaved caspase 3 (Cat No. 9661) and XIAP (Cat No. 2042) were obtained from Cell Signaling Technology (Danvers, MA). BCL2 (Cat. No. sc-7382) and SMAC/DIABLO (Cat. No. sc-22766) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated forms of BCL2 protein appear as more slowly migrating species [16,17]. Anti-actin (Cat. No. A2103; Sigma Aldrich, St. Louis, MO) was used as a control. Immunoreactive bands were visualized using enhanced chemiluminescence detection reagent (Perkin-Elmer, Waltham, MA) and X-OMAT processing.

Immunoprecipitation: Cells were treated with MBZ for indicated times and then lysed in NP40 buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP40 (pH 8.0)) containing protease inhibitor cocktail and phosphatase inhibitor cocktail. Protein (150 μg) was combined with 10 μL anti-SMAC/DIABLO (Cat. No. sc-22766; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-XIAP
(Cat No. 9661; Cell Signaling Technology, Danvers, MA) antibodies and incubated at 4°C overnight. Immobilized protein A/G slurry (100 μL; Thermo Fischer Scientific, Rockford, IL) was added to the antigen-antibody complex and incubated at room temperature for 2 h with gentle mixing. After several washes with water, loading buffer was added to the slurry. The solution was incubated for 5 min at 95°C. From each sample, 30 μL of solution was used for evaluation by SDS-PAGE gel as described above.

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) assay: Following treatment with MBZ, cellular proliferation was assessed using a tetrazolium dye reduction assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI) performed according to the manufacturer’s instructions as previously described. Absorbance was recorded on a microplate reader at 495 nm. In Figure 5, cellular proliferation was expressed as percentage with vehicle-treated cells set at 100%.

Sulphorhodamine (SRB) assay: The growth inhibitory effect of MBZ was also measured using the SRB assay (Sigma Aldrich, St Louis, MO). Cells were plated in 96 well plates. The following day MBZ was added at half-log serial dilutions (10 µM to 1 nM). The SRB assay was performed 72 hours following drug. Data was analyzed and GI₅₀ values were generated by nonlinear regression analysis with variable slope using Prism software (GraphPad Software, La Jolla, CA).

Statistics: Unless otherwise noted, experiments were performed in triplicate. All data are presented as the average ± standard error of the mean [18]. P values were determined by a two-sided Student’s t test with unequal variance, with P< 0.05 considered significant.
Results and Discussion

Mebendazole effectively inhibits melanoma xenograft growth
We originally identified MBZ as a potent inducer of Bcl2 phosphorylation and apoptosis in melanoma cell lines [17]. Based upon its promising in vitro activity, we wanted to assess the ability of MBZ to suppress melanoma tumor growth in vivo using a human melanoma xenograft model established from M-14 cells. We tested four experimental groups in these studies: 1 mg MBZ by gavage every other day, 2 mg MBZ by gavage every other day, 100 mg/kg TMZ by intraperitoneal (i.p.) injection for 5 days, and DMSO by intraperitoneal injection for 5 days (controls). MBZ concentrations are based upon upper and lower dosing equivalents used in long term treatment regimes for hydatid parasitic cysts [19]. TMZ treatment was used as a positive control, as it is a current standard of care for melanoma. The high dose of TMZ (100 mg/kg administered daily for 5 days) was chosen as it is commonly used for preclinical melanoma studies [20,21,22]. The results showed that MBZ inhibited M-14 melanoma xenograft tumor growth comparably at both doses tested (tumor growth inhibition = 83%, 77%, for 1 mg and 2 mg dosing, respectively). These data suggest that oral MBZ inhibits tumor growth as effectively as high dose i.p. TMZ (Figure 1A - B). The efficacy of MBZ as compared to TMZ was further demonstrated by a significant reduction in tumor volume as pictured in Figure 1C. No obvious toxicity was observed with MBZ treatment (Figure 1D).

Consistent with our previous in vitro studies, MBZ induced Bcl2 phosphorylation in vivo in response to both doses tested (Figure 2). Additionally, decreased levels of XIAP and enhanced cleavage of caspase 3 and 9 was also observed in mice receiving MBZ (Figure 2). In contrast, no effect on Bcl2 or XIAP was observed in tumors obtained from mice following treatment with TMZ or DMSO (control).

Mebendazole treatment causes XIAP downregulation
Given XIAP’s critical role in the regulation of apoptosis, we investigated its function in MBZ mediated apoptosis. As shown in Figure 3A, treatment of M-14 and A-375 melanoma cells with 0.5 μM MBZ results in a time dependent decrease in XIAP protein levels first evident at 24 hours following treatment. The decrease in XIAP levels in response to MBZ exposure inversely correlates with an increase in cleaved PARP (Figure 3A), a well-characterized marker of apoptosis. In addition to enhanced cleavage of PARP, we also observed enhanced cleavage of caspase 9 consistent with activation of the mitochondrial mediated apoptotic pathway (Figure 3A).

Upon conditions promoting apoptosis SMAC/DIABLO is capable of negatively regulating XIAP through selective binding to XIAP itself, thereby blocking inhibition of caspases by XIAP [23,24]. We therefore hypothesized that promotion of melanoma apoptosis by MBZ might be achieved through alterations of XIAP’s ability to interact with SMAC/DIABLO. To test this hypothesis, we immunoprecipitated XIAP from non-treated and MBZ-treated M-14 melanoma cell lines, and immunoblotted for SMAC/DIABLO. As shown in Figure 3B, a time dependent increase in SMAC/DIABLO association with XIAP is noted in MBZ treated cells as compared to untreated cells. This increase is initially detected 9 hours after treatment. Similar results were obtained when the opposite immunoprecipitation reaction was performed (i.e. SMAC/DIABLO immunoprecipitation, XIAP immunoblot). Altogether, these data suggest that MBZ treatment results in enhanced interaction of XIAP with SMAC/DIABLO, thereby alleviating XIAP’s inhibition on apoptosis.

Mebendazole-mediated XIAP degradation does not occur in MBZ-resistant cells
Given that XIAP has been linked to melanoma chemoresistance through its anti-apoptotic function, we wanted to determine whether XIAP expression could serve as a molecular marker for MBZ sensitivity in melanoma cell lines. To this end, we assessed XIAP levels in MBZ sensitive and resistant cell lines before and 24 hours following treatment with 1 μM MBZ. Interestingly, basal levels of XIAP in our panel of melanoma cell lines did not appear to be predictive of cellular
response to MBZ (Figure 4B). However, cells that were sensitive did show diminished levels of XIAP after treatment with MBZ whereas resistant cells (i.e. MBZ IC\textsubscript{50} \geq 10 \mu M) did not (Figure 4B). Reduced XIAP expression in response to MBZ treatment is partially reversible upon treatment with the proteasomal inhibitor MG132 (Figure 4A). These data suggest that the MBZ mediated decrease in XIAP levels results at least in part from its proteasomal degradation.

**Modulation of XIAP levels sensitizes MBZ-resistant cells to apoptosis**

Given that UACC1097 and D17 non-mutant BRAF melanoma cells are resistant to inhibition of growth by MBZ and do not display downregulation of XIAP upon treatment with MBZ (Figure 4B), we hypothesized that if XIAP was critical to MBZ induced apoptosis, MBZ-resistant cells should be rendered sensitive to MBZ if XIAP levels are reduced by siRNA. As shown in Figure 5, siRNA knockdown of XIAP results in a significant reduction in the levels of XIAP, whereas control, scrambled siRNA has no effect in both UACC1097 and D17 melanoma cells. Reduction of XIAP protein levels in UACC1097 and D17 melanoma cells rendered them more sensitive to the growth-inhibitory effects of MBZ compared with cells treated with a scrambled siRNA (Figure 5). These data are consistent with the notion that XIAP is a critical mediator of MBZ-induced apoptosis in melanoma cells.

We originally identified MBZ as a potent inducer of BCL2 phosphorylation and apoptosis in melanoma cell lines [17]. MBZ is a drug with known microtubule disrupting properties that has been used safely as an antihelminthic agent in the treatment of parasitic infections for over 50 years [7]. We found that phosphorylation of BCL2 in response to MBZ causes a decreased association with BAX, a condition that favors apoptosis [17]. BAX oligomerization promotes permeabilization of the outer mitochondrial membrane and subsequent release of apoptogenic proteins such as cytochrome C and SMAC/DIABLO [25].

Our data suggest that MBZ has the potential to be repositioned as an anti-melanoma agent. At concentrations achievable with oral administration, MBZ is able to effectively inhibit proliferation of melanoma xenografts in vivo (Figure 2) [17]. This growth inhibition may be attributable to MBZ’s ability to target cellular anti-apoptotic machinery and thereby promote apoptosis (Figure 2, 3). Of particular interest is the fact that mebendazole displays important pharmacokinetic properties (such as enhanced oral bioavailability and maximal plasma concentrations after oral administration that greatly exceed those necessary to induce apoptosis in melanoma cells) that make it an ideal candidate for further study in a clinical setting [26]. Additionally, several published studies demonstrate mebendazole’s ability to inhibit cellular proliferation in certain other cancer cells including lung, hepatocellular and adrenocortical carcinoma [27,28,29].

Shi et al [30] recently found that downregulation of XIAP was a predictor of cellular response to microtubule disrupting agents. In addition to rapid phosphorylation of Bcl2 and its enhanced dissociation from BAX [17], we have now shown that MBZ treatment of melanoma cells results in downregulation of XIAP as well as increased interaction of remaining XIAP with SMAC/DIABLO, which has been previously shown to relieve XIAP’s inhibitory effects on apoptosis (Figure 3) [23,24]. Phosphorylation of BCL2 is an early event, occurring at 3 to 6 hours post MBZ treatment [17]. Our data show enhanced interaction between XIAP and SMAC/DIABLO by 9 hours (although it is possible that this occurs at earlier time points not evaluated), followed by down-regulation of XIAP at 24 hours (Figure 1). Our data would suggest that this sequence of events is responsible for MBZ’s ability to promote apoptosis in melanoma cells. Additionally, in light of recent studies suggesting that renal cell cancer can be rendered sensitive to apoptosis through multiple targeting of XIAP and BCL2, a single agent like MBZ that impacts both proteins might prove valuable in overcoming apoptotic resistance to therapy in melanoma [31].

Previous studies have suggested that other microtubule binding agents like paclitaxel cause a diminution of XIAP levels through proteasomal degradation [32]. Additionally, recent data suggest that XIAP can function as an E3 ubiquitin ligase [33]. Furthermore, XIAP can regulate itself through auto-ubiquitination [34]. Several studies have also shown that XIAP agonists like
SMAC/DIABLO can trigger the degradation of XIAP [35]. Our data using the proteasomal inhibitor MG132 suggests that the reduction in XIAP protein levels observed upon treatment with MBZ is likely mediated by proteasomal degradation (Figure 3).

We demonstrate that XIAP levels in response to treatment with MBZ correlate with sensitivity of melanoma cell lines to the anti-proliferative effects of MBZ (Figure 4). Interestingly, basal XIAP levels do not provide the same predictive value. This finding is also consistent with the report by Shi and coworkers who showed that sensitivity to paclitaxel and nocodazole does not correlate with pre-treatment levels of XIAP. Moreover, in keeping with the results reported here for MBZ, they found that sensitivity to microtubule targeting agents correlates with a loss of XIAP during the drug response [30].

As key components of the cytoskeleton, microtubules are essential for cell structure, intracellular transport, mitosis and cell division [36]. Through disruption of the mitotic spindle, classical microtubule-targeted drugs such as paclitaxel and *Vinca* alkaloids exert their inhibitory effect on cancer cell proliferation through inhibition of tubulin degradation and polymerization, respectively. This anti-tubulin mechanism results in arrest of the cell-cycle at the metaphase-anaphase transition and induction of apoptosis [37,38]. However, melanoma fails to respond sufficiently to “classical” microtubule targeted drugs like paclitaxel and vinblastine [39]. Like the *Vinca* alkaloids, MBZ inhibits tubulin polymerization. However, it should be noted that MBZ possesses different tubulin binding properties than the classical anti-cancer drugs. MBZ binds to the colchicine binding site [40], whereas paclitaxel and vinblastine bind to the paclitaxel and vinblastine binding sites, respectively [36]. Therefore, it is reasonable to suggest that MBZ has the potential to be an effective anti-melanoma agent despite the disappointing results with other microtubule disrupting agents.

*In vivo*, MBZ inhibits melanoma xenograft growth (Figure 1). Tumors of mice treated with MBZ exhibit activation of the apoptotic pathway as evidenced by caspase 3 and 9 cleavage (Figure 2). However, it should be noted that we did not observe regression in our *in vivo* model of human melanoma. While we showed that MBZ induces cell death *in vitro* and *in vivo* causes marked tumor growth inhibition, the question as to why we did not see tumor regression in the M14 melanoma xenografts still remains. It is possible that in the presence of MBZ, equilibrium exists between cellular proliferation and cellular death in these xenografts.

Previous *in vivo* studies have clearly demonstrated that MBZ is an effective anti-tumor agent in the treatment of lung and adrenocortical carcinomas at dosing concentrations of 1 and 2 mg [41,42]. Our decision to use similar MBZ doses in our study were based upon the efficacy of the aforementioned studies as well as our in vitro data demonstrating that MBZ potently inhibits melanoma cell growth and induces apoptosis at 0.5 µM in M-14 melanoma cells [17]. This concentration can be achieved in humans by giving 50 mg/kg orally per day as done in the treatment of cystic and alveolar echinococcosis and is equivalent to the 1 mg MBZ dose used in our xenograft studies [43]. Our data revealed that both 1 and 2 mg MBZ effectively inhibited melanoma xenograft growth, resulted in BCL2 phosphorylation and decreased XIAP expression, and was well tolerated (Figure 1 and 2). Because the effect of 1 mg MBZ appears to be strikingly similar to 2 mg MBZ and 1 mg MBZ dosing is clinically achievable using the dosing regimen established for the treatment of echinococcosis, we would conclude that this dose would be ideal for future clinical studies.

Our data have clinical implications for the treatment of melanoma. Our *in vivo* data suggest that MBZ inhibits melanoma growth in an efficacious manner similar to TMZ (the current standard of care for non mutated BRAF melanoma) and is well tolerated (Figure 2). These studies lay the groundwork for further evaluation in a clinical trial of advanced melanoma.
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References

Braithwaite PA, Roberts MS, Allan RJ, Watson TR. Clinical pharmacokinetics of high
Figure Legends:

**Figure 1.** Mebendazole inhibits melanoma xenograft growth as effectively as TMZ. M-14 melanoma cells were implanted into athymic nude mice to allow for xenograft formation. Mice were treated with 100mg/kg TMZ administered by i.p. injection daily for 5 days, 1 mg or 2 mg MBZ delivered by gavage every other day, or DMSO administered by i.p. injection daily for 5 days (control). Tumor volume was measured twice weekly during the 5 weeks of treatment. (A) Average tumor volume or (B) scatter plot of actual tumor volumes with best fit trend line for each treatment group is plotted as follows DMSO (■), TMZ (◊), 2 mg MBZ (×), 1 mg MBZ (□). (C) Photograph of final tumor size after the course of treatment. Two representative tumors from each treatment group displayed. (D) Mean animal weight over the course of treatment for the following groups: DMSO (■), TMZ (◊), 2 mg MBZ (×), 1 mg MBZ (□). Error bars represent SEM.

**Figure 2.** Tumors from mice treated with mebendazole reveal phosphorylation of BCL2 and reduction in XIAP levels. Mouse tumor extracted following treatment regimen were homogenized and immunoblotted for BCL2, XIAP, cleaved caspase 9 (CC9), cleaved caspase 3 (C-C3) and actin (loading control). Expected protein sizes are as follows: Bcl2 = 26 KDa, XIAP = 53 KDa, CC9 = 37 KDa, CC3 = 17 KDa and actin = 42 KDa.

**Figure 3.** Mebendazole induced apoptotic response involves enhanced interaction of SMAC/DIABLO with XIAP. (A) M-14 and A-375 cells treated with 0.5 μM MBZ for 0, 18, 24 and 48 hours. Extracted proteins were immunoblotted for cleaved caspase 9 (C-C9), cleaved PARP (C-PARP), XIAP and actin (loading control). (B) M-14 and A-375 cells were treated with 0.5 μM MBZ for 9, 18, 24 hours. Cellular lysate was immunoprecipitated using antibodies to SMAC or XIAP, then immunoblotted for SMAC/DIABLO or XIAP (as input control). Expected protein sizes are as follows: CC9 = 37 KDa, CPARP = 89 KDa, XIAP = 53 KDa, SMAC = 21KDa and actin = 42 KDa.

**Figure 4.** Reduction of XIAP expression upon treatment with mebendazole only occurs in sensitive melanoma cells. (A) M-14 cells treated with 0.5 μM MBZ for 24 hours in the presence or absence of 10 μM MG 132. (B) Indicated melanoma cell lines were treated with 0.5 μM MBZ for 24 hours. Cellular lysates were immunoblotted for XIAP and actin (loading control). MBZ IC_{50} values for each cell line were determined through dosing experiment using the sulphorhodamine (SRB) assay. Expected protein sizes are as follows: XIAP = 53 KDa and actin = 42 KDa.

**Figure 5.** XIAP siRNA renders resistant melanoma cells sensitive to mebendazole. D17 (A) and UACC1097 (B) melanoma cells were electroporated with vehicle alone (NT), XIAP siRNA, or control scrambled siRNA. Twenty-four hours after electroporation, extracted proteins were analyzed by immunoblotting using anti-XIAP and actin (loading control). D17 and UACC1097 melanoma cells either electroporated with vehicle alone (☐), XIAP siRNA (○), or control scrambled siRNA (Δ). Expected protein sizes are as follows: XIAP = 53 KDa and actin = 42 KDa. Cells were treated with indicated concentrations of mebendazole for 72 h. Cellular proliferation was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay. Points, mean of three independent experiments.
Figure 4

A. MG132 - - +
   MBZ - + +
   50 KDa XIAP
   37 KDa Actin

B. 50 KDa XIAP
   37 KDa Actin

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