# Mebendazole Induces Apoptosis via Bcl-2 Inactivation in Chemoresistant Melanoma Cells

Nicole Doudican,[1] Adrianna Rodriguez,[1] Iman Osman,[1,2,4] and Seth J. Orlow[1,3,4]

[1]The Ronald O. Perelman Department of Dermatology, [2]Department of Medicine, and [3]Department of Cell Biology, New York University School of Medicine; and [4]New York University Cancer Institute, New York, New York

## Abstract

**Most metastatic melanoma patients fail to respond to available therapy, underscoring the need for novel approaches to identify new effective treatments. In this study, we screened 2,000 compounds from the Spectrum Library at a concentration of 1 μmol/L using two chemoresistant melanoma cell lines (M-14 and SK-Mel-19) and a spontaneously immortalized, nontumorigenic melanocyte cell line (melan-a). We identified 10 compounds that inhibited the growth of the melanoma cells yet were largely nontoxic to melanocytes. Strikingly, 4 of the 10 compounds (mebendazole, albendazole, fenbendazole, and oxybendazole) are benzimidazoles, a class of structurally related, tubulin-disrupting drugs. Mebendazole was prioritized to further characterize its mechanism of melanoma growth inhibition based on its favorable pharmacokinetic profile. Our data reveal that mebendazole inhibits melanoma growth with an average IC$_{50}$ of 0.32 μmol/L and preferentially induces apoptosis in melanoma cells compared with melanocytes. The intrinsic apoptotic response is mediated through phosphorylation of Bcl-2, which occurs rapidly after treatment with mebendazole in melanoma cells but not in melanocytes. Phosphorylation of Bcl-2 in melanoma cells prevents its interaction with proapoptotic Bax, thereby promoting apoptosis. We further show that mebendazole-resistant melanocytes can be sensitized through reduction of Bcl-2 protein levels, showing the essential role of Bcl-2 in the cellular response to mebendazole-mediated tubulin disruption. Our results suggest that this screening approach is useful for identifying agents that show promise in the treatment of even chemoresistant melanoma and identifies mebendazole as a potent, melanoma-specific cytotoxic agent.(Mol Cancer Res 2008;6(8):1308–15)**

## Introduction

The lack of response of metastatic melanoma to a wide range of antineoplastic treatments suggests the presence of complex drug resistance mechanisms. Although most cancers selectively acquire drug resistance following drug treatment, drug resistance in melanoma is thought to be largely due to intrinsic survival factors (1). In this regard, several lines of evidence suggest that defects in apoptotic signaling cascades may represent a critical event in melanoma progression (2). Consistent with this notion are reports of modified expression of proapoptotic and antiapoptotic factors such as Bcl-2, Bcl-XL, and Mcl-1 in melanoma (3, 4).

In this study, we screened a library of 2,000 small molecules to identify new antimelanoma agents. The major advantage of this library is its collection of well-characterized drugs, many of which are already approved for human use in the United States, allowing a reduction in the time and effort required to advance promising candidates in a clinical setting.

Our study describes the therapeutic utility and mechanism of action of mebendazole, one of the compounds identified from this screen as an antimelanoma agent. Antitumor activity of mebendazole has been previously described in lung and adrenocortical carcinomas (5, 6). Furthermore, mebendazole has been shown to induce depolymerization of tubulin in a variety of cancer models (6, 7). However, to date, the effect of this well-tolerated drug in melanoma cells has not been examined.

The data presented in this report show that mebendazole selectively inhibits growth and induces apoptosis in melanoma cells at clinically achievable doses that are largely nontoxic to melanocytes. Our data provide evidence that the antineoplastic effects of mebendazole in human melanoma cells result from differential Bcl-2–mediated cellular responses to mebendazole-induced tubulin disruption.

## Results

*Library Screening Identifies Mebendazole as an Inhibitor of Melanoma Cell Proliferation*

To discover new antineoplastic agents useful in the treatment of melanoma, we did a screen of 2,000 small molecules in chemoresistant melanoma cell lines. M-14 and SK-Mel-19 melanoma cell lines display resistance to a variety of traditional chemotherapies, including temozolomide, cisplatin, and arsenic trioxide (8-10).[5] Additionally, these cell lines were chosen based on their differential p53 status—M-14 expresses mutant

[5] Unpublished results.

p53 (11) and SK-Mel-19 expresses wild-type p53 (12)—to eliminate any dependence on the tumor suppressor. From a screen of the Spectrum Library (MicroSource Discovery Systems, Inc.), we identified 10 compounds that differentially inhibit cellular proliferation in melanoma cell lines (M-14 and SK-Mel-19) versus spontaneously immortalized melanocytes (melan-a; data not shown). Table 1 shows the $IC_{50}$ of four of the compounds identified, which belong to a class of structurally related drugs called the benzimidazoles. The $IC_{50}$ for each benzimidazole identified was 2- to 6-fold lower in melanoma cells compared with melanocytes. Of these compounds, mebendazole showed the most striking growth-inhibitory effects in melanoma cells. The $IC_{50}$s for mebendazole in SK-Mel-19 and M-14 were 0.32 and 0.3 μmol/L, respectively. By contrast, in melan-a cells, mebendazole displayed an $IC_{50}$ of 1.9 μmol/L.

To test the therapeutic potential of mebendazole, we examined its effect on cellular proliferation in a genetically diverse panel of melanoma cells. Cells were treated with physiologic concentrations of mebendazole ranging from 0.01 to 1.5 μmol/L for 72 h. Figure 1 shows that mebendazole treatment strongly inhibits the growth of all melanoma cells examined in a dose-dependent manner. In contrast, the growth-inhibitory effects of mebendazole on melan-a cells and on human epidermal melanocytes (HEM) were not as pronounced. After treatment with 0.5 μmol/L mebendazole, 86% of melan-a cells and 83% of HEM cells remained proliferative. In contrast, only 26% of SK-Mel-19 cells maintained proliferative capacity. Given that similar growth-inhibitory effects at this concentration of mebendazole were noted in all of the melanoma cell lines examined, we chose to conduct our mechanistic studies in SK-Mel-19 and M-14.

### Mebendazole Selectively Induces Apoptosis in Melanoma Cells through the Intrinsic and Extrinsic Mitochondrial Pathways

Figure 2A shows that 24 h after cells were treated with 1 μmol/L mebendazole, 25% of M-14 cells and 31% of SK-Mel-19 were positive for Annexin V staining, indicating that these cells were undergoing apoptosis. However, only 5% of melan-a cells display Annexin V staining under the same conditions. These data suggest the differential cytotoxicity of mebendazole results from a preferential induction of apoptosis in melanoma cell lines compared with immortalized melanocytes. Figure 2B shows that cleavage of caspase-9, caspase-7,
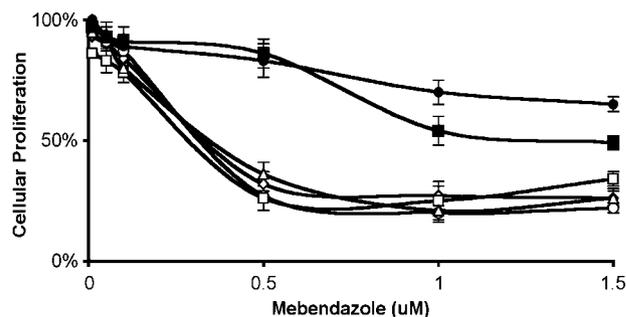


**FIGURE 1.** Differential growth inhibition of mebendazole in melanoma cell lines versus melanocytes. Melanoma cells (○, M-14; □, SK-Mel-19; ◇, Yusac-2; △, SK-Mel-173) and melanocytes (■, melan-a; ●, HEM) were treated with indicated concentrations of mebendazole for 72 h. Cellular proliferation was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay. Points, mean from a minimum of four independent experiments; bars, SE.

caspase-3, and poly(ADP-ribose) polymerase is evident 6 h after treatment with 0.5 μmol/L mebendazole in M-14 and SK-Mel-19 melanoma cells, indicating that treatment of melanoma cells with mebendazole induces rapid activation of the intrinsic, mitochondrial-mediated apoptotic pathway. Additionally, activation of the extrinsic pathway (caspase-8) is also evident in both cell lines after 18 h of mebendazole treatment.

### Mebendazole Alters Tubulin Structure in Both Melanocytes and Melanoma Cells

The effects of mebendazole on microtubule formation in melan-a, M-14, and SK-Mel-19 cells were directly assessed through immunofluorescence microscopy (Fig. 3). After treatment with 0.5 μmol/L mebendazole for 14 h, we observed overall microtubular network disarray in melan-a, M-14, and SK-Mel-19 cells, characterized by diffuse staining (Fig. 3B, D, and F). In contrast, Fig. 3A, C, and E shows that vehicle-treated melanocytes and melanoma cells in interphase display discrete networks of microtubule structure. Mitotic figures, when observed in mebendazole-treated cells, had an abnormal appearance with improperly aligned chromosomes (Fig. 3B and D, *arrowhead*).

### Mebendazole Induces a Differential Cellular Response to Microtubular Damage

Given that the antitubulin effects of mebendazole were evident in both melanocytes and melanoma cells, we hypothesized that the melanoma-specific effects of mebendazole might be due to differential cellular response to microtubular damage. We focused on the apoptosis-regulatory protein Bcl-2, as it is a key mediator of microtubule damage that is rapidly phosphorylated and inactivated in response to exposure of cells to microtubular-disrupting agents (13). Figure 4A reveals that mebendazole treatment results in Bcl-2 phosphorylation in M-14 and SK-Mel-19 melanoma cells but not in melan-a cells or HEM. Furthermore, phosphorylation of Bcl-2 in M-14 and SK-Mel-19 melanoma cells occurs at $Ser^{70}$ (Fig. 4B), a critical site for drug-induced Bcl-2 phosphorylation in cancer cells (14).

**TABLE 1.** $IC_{50}$ **of Benzimidazole Compounds That Display Melanoma-Specific (M-14 and SK-Mel-19) Growth Inhibition as Compared with Melanocytes (Melan-a)**

|  | Melan-a | SK-Mel-19 | M-14 |
|---|---|---|---|
| Mebendazole | 1.9 μmol/L (0.05) | 0.3 μmol/L (0.1) | 0.32 μmol/L (0.2) |
| Albendazole | 3.8 μmol/L (0.3) | 0.7 μmol/L (0.07) | 1.2 μmol/L (0.1) |
| Fenbendazole | 3.4 μmol/L (0.2) | 1.2 μmol/L (0.3) | 1.4 μmol/L (0.4) |
| Oxybendazole | 3.5 μmol/L (0.1) | 0.8 μmol/L (0.05) | 1.1 μmol/L (0.2) |

NOTE: Cellular proliferation was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay. Each data point is derived from a minimum of four independent experiments. Error bars represent SE.
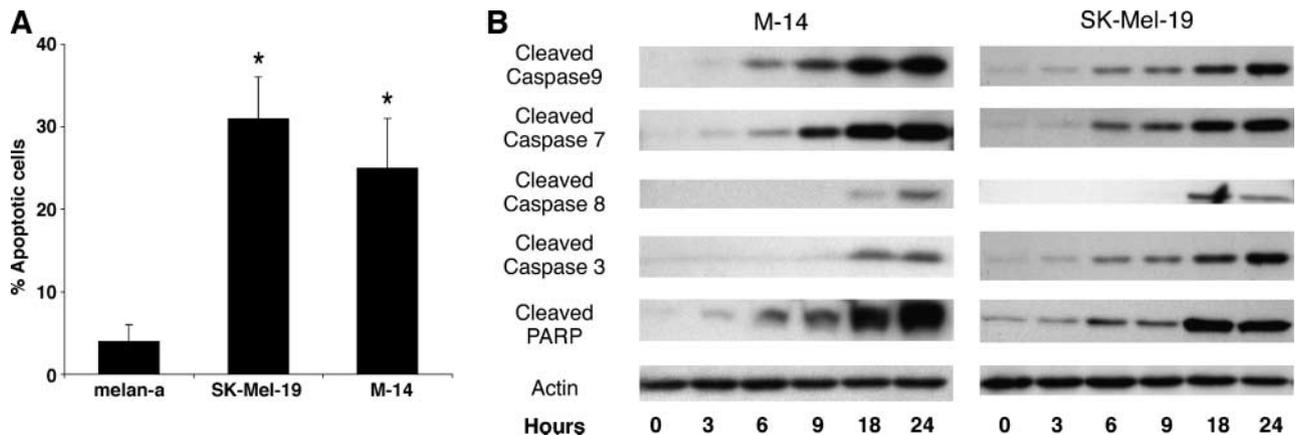
**FIGURE 2.** Mebendazole treatment induces apoptosis through the intrinsic and extrinsic pathways in melanoma cells but not in melanocytes. **A.** Cells were treated with 1 μmol/L mebendazole for 24 h. The percentage of cells positive for Annexin V binding (indicated as apoptotic cells) was quantified by flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$, statistically different from untreated control. **B.** M-14 and SK-Mel-19 cells were treated with 0.5 μmol/L mebendazole for indicated times. Proteins were extracted from the cells for analysis by immunoblotting. Experiments were repeated thrice, with representative blots displayed.

## Phosphorylated Bcl-2 Promotes Melanoma Apoptosis

We next examined whether Bcl-2 phosphorylation could promote apoptosis by altering its interactions with the proapoptotic protein Bax. Bcl-2 phosphorylation has been shown to prevent formation of Bax-Bcl-2 heterodimers. Instead, unbound Bax is available to form homodimers, a situation that promotes apoptosis (15). Coimmunoprecipitation studies depicted in Fig. 4A show that mebendazole treatment reduces the interaction between Bax and Bcl-2 in M-14 and SK-Mel-19 melanoma cells by 46% and 64%, respectively. In contrast, inhibition of Bcl-2 and Bax interaction was not observed in melan-a cells treated with mebendazole (Fig. 4A). Mebendazole treatment did not alter total levels of Bcl-2 or Bax protein in any of the cell lines (Fig. 4A).

## Modulation of Bcl-2 Protein Levels Sensitizes Melan-a Cells to Mebendazole

Given that melan-a cells do not undergo apoptosis in response to mebendazole treatment nor do they show Bcl-2 phosphorylation and inactivation in response to mebendazole (Fig. 4A), we hypothesized that if Bcl-2 was critical to mebendazole-induced apoptosis, mebendazole-insensitive melanocytes should be sensitized to mebendazole if Bcl-2 was "inactivated" through down-regulation of protein levels. Figure 5A shows that electroporation of Bcl-2 small interfering RNA (siRNA) into both melan-a and M-14 cells resulted in a significant reduction in the levels of Bcl-2 protein, whereas the control scrambled siRNA had no effect on Bcl-2 protein levels. Reduction of Bcl-2 protein levels in melan-a cells rendered them significantly more sensitive to the growth-inhibitory effects of mebendazole compared with cells treated with a scrambled siRNA (Fig. 5B). The IC$_{50}$ for mebendazole in Bcl-2 siRNA-treated cells was 26-fold less compared with control scrambled siRNA–treated cells (0.05 and 1.3 μmol/L, respectively). Reduction of Bcl-2 protein levels also enhanced mebendazole-mediated growth inhibition in mebendazole-sensitive M-14 melanoma cells, although these effects were not as dramatic (Fig. 5C). These data are consistent with the

notion that Bcl-2 is a critical mediator of mebendazole-induced apoptosis in melanoma cells.

## Discussion

We have identified mebendazole as a potentially effective agent in the treatment of melanoma. We showed that mebendazole has selective cytotoxicity against a panel of melanoma cell lines, whereas it has a minimal effect on the
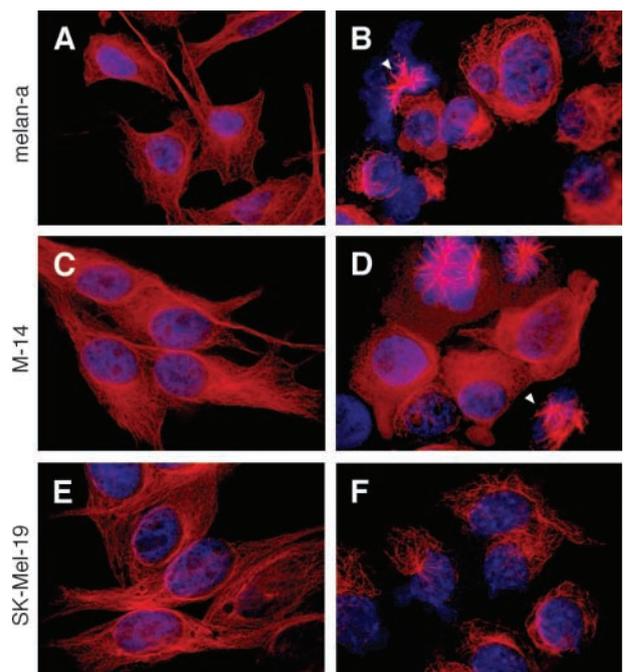


**FIGURE 3.** Mebendazole-induced microtubule disruption in melan-a, M-14, and SK-Mel-19 cells. Melan-a, M-14, and SK-Mel-19 cells were treated with vehicle alone (**A, C**, and **E**) or 0.5 μmol/L mebendazole for 14 h (**B, D**, and **F**). After permeabilization and fixation, cells were stained with anti-α-tubulin antibody (*red*) and 4′,6-diamidino-2-phenylindole (*blue*). Arrowheads, mitotic figures. Experiment was repeated thrice, with representative images shown.

**FIGURE 4.** Mebendazole treatment results in Bcl-2 phosphorylation and prevents Bcl-2-Bax interaction in melanoma cells but not in melanocytes. **A.** Cells were treated with vehicle alone (−) or 0.5 μmol/L mebendazole for 18 h (+). Equal amounts of cell lysates were resolved by SDS-PAGE gel followed by immunoblotting with indicated antibodies. For immunoprecipitation studies, cell lysates equivalent to 150 μg of total protein were immunoprecipitated (*IP*) with anti-Bax IgG, resolved by SDS-PAGE gel, and immunoblotted with anti-Bcl-2 IgG or anti-Bax IgG as indicated. Densitometric values are reported for Bax protein levels immunoprecipitated with antibodies to Bcl-2. **B.** Melanoma cells were treated with 0.5 μmol/L mebendazole for indicated times. Proteins were extracted from the cells for analysis by immunoblotting using anti-phospho-Bcl-2 (Ser[70]) IgG.
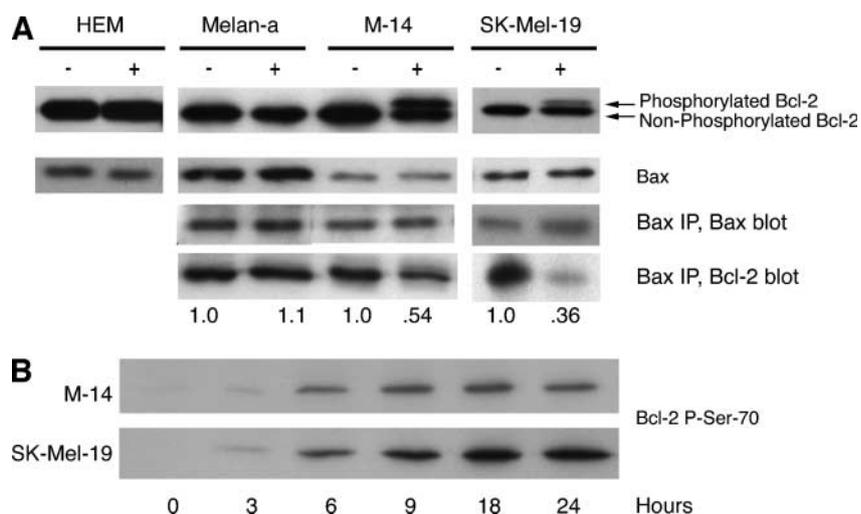


growth of melanocytes. Through an analysis of the tubulin-disrupting properties of mebendazole, we elucidated the mechanism underlying the differential cytotoxicity of mebendazole. Although the effects of mebendazole on microtubule structure were similar in both melanocytes and melanoma cells, we found that melanocytes and melanoma cells differ in their Bcl-2–mediated responses to mebendazole-induced microtubule disruption.

Our screen for antimelanoma agents consistently identified members of the benzimidazole family due to their selective, growth-inhibitory effects on melanoma cells compared with spontaneously immortalized, nontumorigenic melanocytes. Of the identified benzimidazoles, the favorable pharmacokinetic properties of mebendazole made it a preferred candidate for further characterization. It is important to note that the concentrations of mebendazole used in our studies are readily achievable by oral administration. The average reported peak serum concentration after a single dose of mebendazole used as an antiparasitic agent in the treatment of hydatid disease is 1.67 μmol/L (16), whereas we found striking effects using concentrations in the clinically relevant range of 0.5 to 1 μmol/L. Mebendazole has been extensively used to treat gastrointestinal parasitic infections in humans, and in this capacity, it has shown remarkable efficacy and safety (17, 18). Furthermore, clinical data from long-term mebendazole therapy in the treatment of alveolar echinococcosis suggest that mebendazole should also exhibit minimal toxicity when used as a cancer therapy (19, 20).

Benzimidazoles, specifically mebendazole and albendazole, have shown *in vivo* antitumor activity against a range of cancers, including hepatocellular, lung, and adrenocortical carcinoma (5, 6, 21). Mebendazole is more suited for clinical study than other benzimidazoles identified in our screen given its status as a marketed drug, its oral availability, and its favorable safety profile in long-term treatment regimens (19). In contrast, a pilot study of albendazole treatment in advanced cancer resulted in increased levels of neutropenia in treated subjects (22). Mebendazole and albendazole have different rates of absorption and hepatic metabolism, which may account for the differences in noted side effects (7).

Microtubules are widely recognized as effective targets for anticancer therapy. As key components of the cytoskeleton, microtubules are essential for cellular structure, intracellular transport, mitosis, and cell division (23). Microtubule-targeted drugs such as paclitaxel and *Vinca* alkaloids exert their inhibitory effect on cancer cell proliferation primarily by disrupting the mitotic spindle, causing cell cycle arrest at the metaphase-anaphase transition and inducing apoptosis (24, 25). In addition, some antimicrotubular drugs can act to depolymerize microtubules in newly formed vasculature and act as vascular-targeting agents to shut down the blood supply to tumors (26).

Our data suggest that mebendazole selectively inhibits melanoma cellular proliferation through a Bcl-2–mediated cellular response to microtubular damage. However, most metastatic melanomas fail to respond dramatically to "traditional" microtubule-targeted drugs such as paclitaxel and vinblastine (27). Mechanistically, mebendazole differs from these classic anticancer, microtubule-targeted drugs because it binds to the colchicine-binding site (28), whereas paclitaxel and vinblastine, respectively, bind to the Taxol- and vinblastine-binding sites (29). Additionally, benzimidazole compounds are structurally similar to nucleotides. This property allows for interaction with a variety of biomolecules, resulting in the diverse range of ascribed mechanisms of action, including reduction of fumarate, glucose uptake, and microtubule inhibition (30, 31). Consistent with this notion, numerous biological functions have been described for this class of compounds, including antihelminthic, antifungal, antimicrobial, antiviral, and antineoplastic activity (32-34). Given the variety of these activities, previous studies have suggested that inhibition of cellular proliferation by mebendazole may involve effects in addition to its microtubule disruption properties.

Bcl-2 is widely expressed in human melanoma and has been linked to melanoma chemoresistance through its antiapoptotic function (35-37). Oblimersen (Genasense) is an antisense compound that selectively targets Bcl-2 mRNA for degradation and decreases Bcl-2 protein production. Its use as a targeted therapy against malignant melanoma is currently being examined. Consistent with other studies (38), our data show

that Bcl-2 siRNA has a modest effect on cellular proliferation in melanoma cells and melanocytes (Fig. 5).

A recent phase III trial in patients with metastatic melanoma showed that oblimersen in combination with dacarbazine significantly improved multiple clinical outcomes, including response rate and progression-free survival. Although no difference in treatment-related mortality was observed between patients receiving dacarbazine alone or in combination with oblimersen, patients undergoing combination therapy reported a higher incidence of adverse events, including neutropenia, thrombocytopenia, and nausea (39). We have shown that mebendazole, like oblimersen, also acts through Bcl-2 to induce apoptosis in melanoma cells. However, oblimersen is difficult to administer and has a short half-life, necessitating prolonged s.c. infusion. In contrast, mebendazole is orally available and exhibits favorable pharmacokinetics, suggesting that it deserves further investigation as an antimelanoma agent given its potential for improved efficacy and notable safety profile.

We identified Bcl-2 as a critical mediator of the differential cellular response to mebendazole treatment in melanocytes versus melanoma cells. After mebendazole treatment, Bcl-2 is rapidly phosphorylated in melanoma cells but not in melanocytes. In the literature, the functional significance of Bcl-2 phosphorylation remains controversial. Some reports suggest that Bcl-2 phosphorylation renders the protein inactive and cells

more sensitive to induction of apoptosis (15), whereas other studies suggest that the antiapoptotic function of Bcl-2 is enhanced by phosphorylation (40). The differing consequences of Bcl-2 phosphorylation may be attributed to the agent used to induce phosphorylation in these studies. When antimitotic agents are used to induce Bcl-2 phosphorylation, this typically results in Bcl-2 inactivation. Consistent with this notion, our data suggest that tubulin-disrupting mebendazole causes Bcl-2 phosphorylation, which prevents its interaction with proapoptotic Bax, thereby promoting selective apoptosis in melanoma cells. Furthermore, we show that reduction of Bcl-2 protein levels through RNA interference sensitized initially mebendazole-resistant melan-a melanocytes to the growth-inhibitory properties of mebendazole. This result further supports the importance of Bcl-2 in the differential cellular responses between melanoma cells and melanocytes to mebendazole-mediated growth inhibition. In contrast, Sasaki et al. (7) reported that Bcl-2 phosphorylation was not a necessary event for mebendazole-induced apoptosis in non–small cell lung carcinoma cells based on the observation that Bcl-2 phosphorylation occurred in response to mebendazole treatment in H460 cells but not in A549 cells. However, it should be noted that A549 cells seem to express little or no Bcl-2 protein, whereas both the melan-a and M-14 cells used in our study express comparable levels of Bcl-2 protein, readily detectable by
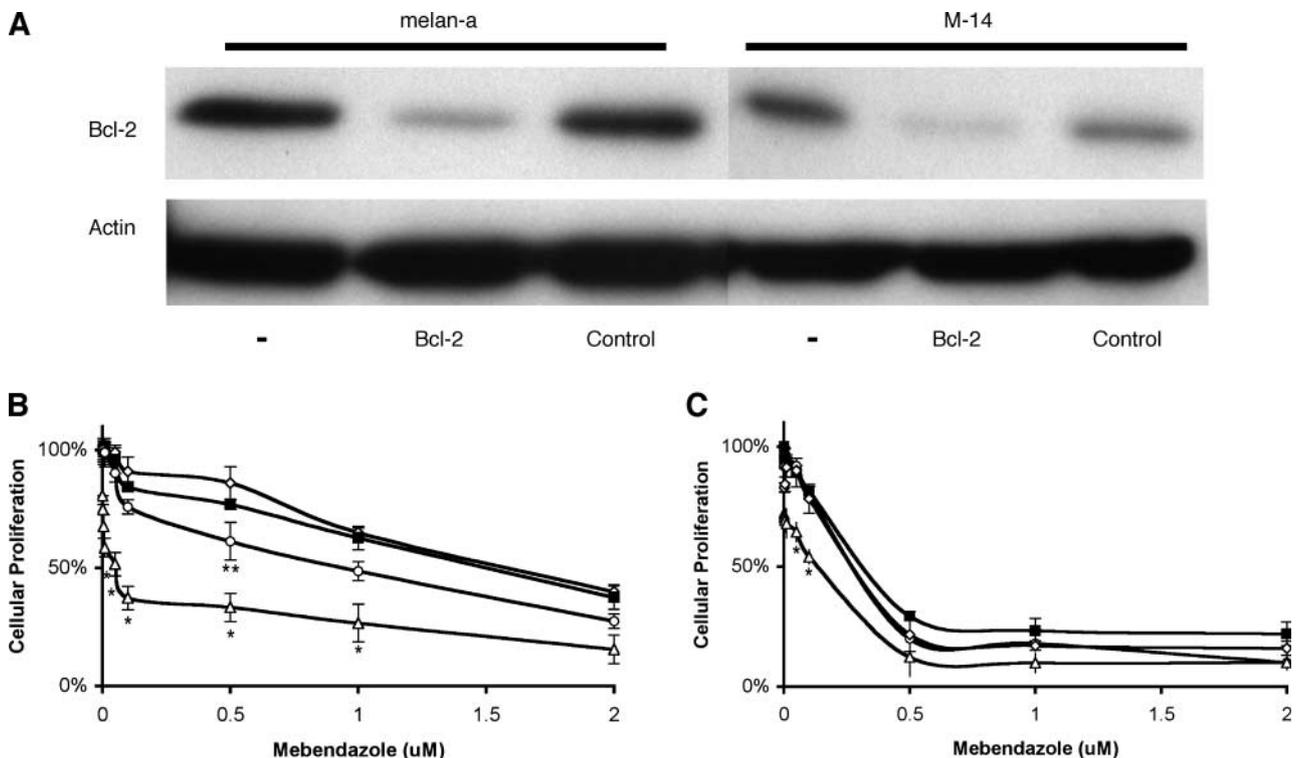


**FIGURE 5.**   Reducing levels of Bcl-2 protein enhance mebendazole-mediated growth inhibition. **A.** Cells were electroporated with vehicle alone (−), Bcl-2 siRNA, or control scrambled siRNA. Twenty-four hours after electroporation, extracted proteins were analyzed by immunoblotting using anti-Bcl-2 IgG. **B** and **C.** Melan-a melanocytes (**B**) and M-14 melanoma cells (**C**) either without electroporation (■) or electroporated with vehicle alone (◇), Bcl-2 siRNA (△), or control scrambled siRNA (○). 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; bars, SE. *, $P < 0.05$, statistically different from control scrambled siRNA. There is no statistical difference between cells electroporated with vehicle alone or scrambled siRNA, with the exception of one data point indicated by **.

Western blotting (Fig. 4). In addition, Bcl-2 phosphorylation was observed in other melanoma cell lines (Yusac-2 and SK-Mel-19) treated with mebendazole (data not shown). These differences might explain the discrepancies between our work and the results reported by Sasaki et al.

Mebendazole shows potential as an effective antimelanoma therapy. However, further assessment of mebendazole using an *in vivo* model of chemoresistant melanoma is a prerequisite before embarking on human studies. In light of promising *in vivo* studies showing the remarkable tumor growth inhibition of mebendazole in lung and adrenocortical carcinoma, it is reasonable to suggest that mebendazole will also be effective against malignant melanoma (5, 6).

In conclusion, our data suggest that mebendazole-mediated microtubule disruption results in melanoma-specific phosphorylation of Bcl-2 and subsequent induction of apoptosis. Based on these results as well as its notable safety profile, mebendazole, a well-tolerated, orally available drug, deserves further investigation as a potentially effective treatment for advanced melanoma.

## Materials and Methods

### Cell Culture

Human melanoma cell lines were cultured in DMEM supplemented with 10% FCS, 5 units/mL penicillin, and 5 μg/mL streptomycin. SK-Mel-19 and SK-Mel-173 cells were kindly provided by Alan Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY). Yusac-2 and M-14 cells were a generous gift from Ruth Halaban (Yale University School of Medicine, New Haven, CT) and Gabriella Zupi (Instituto Regina Elena, Rome, Italy), respectively. Melan-a (kindly provided by Dorothy Bennett, St. George Hospital Medical School, London, United Kingdom) is an immortalized but nontumorigenic melanocyte line derived from C57BL/6J mice (41). Melan-a cells were grown in DMEM supplemented with 10% FCS, 5% sodium pyruvate, 5% glutamate, 5 units/mL penicillin, 5 μg/mL streptomycin, 1% nonessential amino acids, and 200 nmol/L 12-*O*-tetradecanoylphorbol-13-acetate as previously described (42). HEMs were purchased from ScienCell and cultured in melanocyte medium as recommended by ScienCell. All cells were maintained in an incubator with a humidified atmosphere of 5% $CO_2$ at 37°C. Mebendazole was purchased from Sigma.

### Library Screening and Cell Proliferation Assay

The Spectrum Library (Microsource Discovery Systems) was used to screen SK-Mel-19, M-14, and melan-a cells. Library compounds were supplied in DMSO and tested at a final concentration of 1 μmol/L. Briefly, 5,000 cells per well were plated in 96-well culture plates. After overnight incubation, the cells were treated with 1 μmol/L of indicated compound. Following a 72-h incubation period, cellular proliferation was assessed using a tetrazolium dye reduction assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega) done according to the manufacturer's instructions. Absorbance was recorded on a microplate reader at 495 nm. Cellular proliferation was expressed as percentage with vehicle-treated cells set at 100%.

### Detection of Apoptosis by Annexin V Staining

An early marker for apoptosis is the translocation of the membrane phospholipid phosphatidyl serine to the outer leaflet of the plasma membrane where it becomes accessible to Annexin V binding (43). Annexin V staining was assessed using the Annexin V-FITC Apoptosis Detection kit (Medical and Biological Laboratories). Cells ($2 \times 10^5$) were treated with 1 μmol/L mebendazole for 24 h. After treatment, cells were harvested and stained with Annexin V-FITC according to the manufacturer's instructions. Annexin V-FITC binding was analyzed by flow cytometry with excitation set at 488 nm and emission at 530 nm. At least 10,000 cells were counted for each sample. Cells positive for Annexin V stain were considered as apoptotic and expressed as a percentage of total cell number.

### Protein Extraction and Western Blotting

Cells were harvested in extraction buffer [1% Triton X-100, 50 mmol/L Tris, 2 mmol/L EDTA, 150 mmol/L NaCl (pH 7.5)] containing protease inhibitor cocktail (Roche Applied Science) and phosphatase inhibitor cocktail (Sigma) after two washes with ice-cold PBS. The lysates were centrifuged at $10,000 \times g$, 4°C for 10 min in a microcentrifuge. The protein concentrations of supernatants were measured with a protein assay kit (Bio-Rad). Proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Polyscreen, Perkin-Elmer). For apoptotic studies, antibodies against full-length and cleaved forms of poly(ADP-ribose) polymerase, caspase-9, caspase-8, caspase-7, and caspase-3 were obtained from Cell Signaling Technology. For Bcl-2 phosphorylation and siRNA studies, mouse monoclonal anti-Bcl-2 IgG (Santa Cruz Biotechnology) and rabbit monoclonal anti-phospho-Bcl-2 (Ser[70]; Cell Signaling Technology) were used. Phosphorylated forms of Bcl-2 protein are detected as more slowly migrating species (40). Anti-actin (Sigma) was used as a control. Immunoreactive bands were visualized using enhanced chemiluminescence detection reagent (Perkin-Elmer) and X-OMAT processing.

### Immunofluorescence

Cells were seeded in 24-well plates containing glass coverslips at a density of 75,000/mL. After achieving 50% confluence, cells were treated with 0.5 μmol/L mebendazole for 14 h. Cells were fixed in 4% paraformaldehyde for 15 min and then permeabilized in 0.1% Triton X-100 solution for 10 min. Blocking was done with 2% bovine serum albumin for 1 h. Cells were incubated in mouse monoclonal anti-α-tubulin IgG (Sigma) for 2 h at room temperature before incubating with anti-mouse IgG labeled with Texas red (Invitrogen) for 1 h. Coverslips were washed in PBS and then incubated in 1 μg/mL 4′,6-diamidino-2-phenylindole for 1 min. Following additional washes in PBS, coverslips were dried and mounted. Cells were examined by microscopy using fluorescein filter sets (Zeiss Axiophot upright phase contrast).

### Immunoprecipitation

Cells were treated with indicated drugs for 18 h 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-Bax IgG (Santa Cruz Biotechnology) and incubated at 4°C overnight. Immobilized protein A/G slurry (100 μL; Pierce) 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 before evaluation by SDS-PAGE gel as described above. Densitometry values were calculated using ImageQuant TL software (GE Healthcare Biosciences). Densitometric values were normalized to vehicle-treated controls in each cell line.

### Down-Regulation of Bcl-2 Protein Levels by siRNA

Bcl-2 siRNA and control scrambled siRNAs were purchased from Santa Cruz Biotechnology. siRNA was delivered into cells by electroporation using the Amaxa Nucleofector System Kit L (Amaxa) according to the manufacturer's instructions. Briefly, $2 \times 10^6$ cells were resuspended in 100 μL nucleofector L solution plus 300 nmol/L of indicated siRNA. The suspension was transferred to a cuvette and nucleofected with an Amaxa nucleofector apparatus using the U-20 pulsing parameter. After electroporation, cells were immediately transferred into wells containing 37°C prewarmed culture medium in six-well plates (for protein analysis) or seeded into 96-well plates (for mebendazole dosing studies).

### Statistical Analysis

Mean activities were calculated from a minimum of three independent experiments done in triplicate, with SE reported. The Student's two-tailed $t$ test was used to determine statistically significant differences between treatment and control values. The significance level was set at $P < 0.05$.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

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