Combining the Endoplasmic Reticulum Stress–Inducing Agents Bortezomib and Fenretinide as a Novel Therapeutic Strategy for Metastatic Melanoma

David S. Hill,1 Shaun Martin,1 Jane L. Armstrong,2 Ross Flockhart,1 Joge J. Tonison,1 Dominic G. Simpson,1 Mark A. Birch-Machin,1 Christopher P.F. Redfern,2 and Penny E. Lovat1

Abstract

Purpose: Single-agent chemotherapy is largely the treatment of choice for systemic therapy of metastatic melanoma, but survival rates are low, and novel adjuvant and systemic therapies are urgently required. Endoplasmic reticulum (ER) stress is a potential therapeutic target, and two relatively new drugs, fenretinide and bortezomib (Velcade), each acting via different cellular mechanisms, induce ER stress leading to apoptosis in melanoma cells. The aim of this study was to test the hypothesis that apoptosis of melanoma cells may be increased by combining clinically achievable concentrations of fenretinide and bortezomib.

Experimental Design: Three human melanoma cell lines were used to assess changes in viability and the induction of apoptosis in response to fenretinide, bortezomib, or both drugs together. A s.c. xenograft model was used to test responses in vivo.

Results: Fenretinide and bortezomib synergistically decreased viability and increased apoptosis in all three melanoma lines at clinically achievable concentrations. This was also reflected by increased expression of GADD153, a marker of ER stress-induced apoptosis. In vivo, fenretinide in combination with bortezomib gave a marked reduction in xenograft tumor volume and an increase in apoptosis compared with fenretinide or bortezomib alone. The cell cycle stage of tumor cells in vivo were similar to that predicted from the effects of each drug or the combination in vitro.

Conclusions: These results suggest that fenretinide and bortezomib, both of which are available in clinical formulation, warrant clinical evaluation as a combination therapy for metastatic melanoma.

Cutaneous malignant melanoma, the most aggressive form of skin cancer, is one of the most difficult forms of human cancer to treat, with an increasing incidence in developed countries that has risen faster than any other malignancy over the past 40 years (1). Improvements in early detection and surgical management of early-stage disease have resulted in cure for most patients with primary melanoma (1, 2). However, advanced metastatic melanoma is highly invasive and has the capacity to develop resistance to drug-induced apoptosis and host immunologic defenses (1). Single-agent chemotherapy remains largely the treatment of choice for systemic therapy of metastatic melanoma (3). Dacarbazine, temozolomide, and fotemustine are widely used because of their low toxicity and simplicity of administration; more-toxic combination therapies do not improve survival (1). Other chemotherapies, including cisplatin and paclitaxel, have shown some activity, but despite clinical trials of differing combinations of chemotherapeutic agents, none have proved superior to single-agent dacarbazine. Because dacarbazine still only yields a response rate of 16%, which is rarely sustained beyond six months (1), other therapeutic approaches combining immunotherapy with either interleukin 2 or IFN-α with dacarbazine (or temozolomide and fotemustine) have been tried (1, 4). However, although initial response rates were slightly improved, these regimes have not been associated with better overall survival. Furthermore, administration of either interleukin 2 or IFN has multiple adverse side effects (1). Hence, overall, little progress has been made towards an effective treatment for metastatic melanoma, and with median survival rates rarely exceeding 12 months in clinical trials, novel adjuvant and systemic therapies are urgently required.

Melanoma cells are frequently resistant to apoptosis induced by conventional mechanisms of death receptor ligation (5) or mitochondrial-mediated apoptosis (6, 7). It has been suggested that the activation of endoplasmic reticulum (ER) stress is an important factor limiting melanoma progression (8), and recent studies have shown that the induction of apoptosis resulting from ER stress may offer novel therapeutic strategies.

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Translational Relevance

Single-agent chemotherapy is largely the treatment of choice for systemic therapy of metastatic melanoma, but survival rates are low, and novel adjuvant and systemic therapies are urgently required. Endoplasmic reticulum stress has emerged as a potential therapeutic target, and two relatively new drugs, fenretinide and bortezomib, each acting via different cellular mechanisms, induce endoplasmic reticulum stress leading to apoptosis in melanoma cells. The results of this study show that apoptosis of melanoma cells in vitro and in vivo may be increased by combining clinically achievable concentrations of fenretinide and bortezomib and that the effects of these drugs are synergistic when used together. Fenretinide and bortezomib are both available in clinical formulations, and this study suggests that clinical evaluation as a combination therapy for metastatic melanoma would be worthwhile.

Materials and Methods

Cell culture, drug treatment, and analysis of cell viability and apoptosis in vitro. Human metastatic melanoma cell lines CHL1, A375, and WM266-4 were from the American Type Culture Collection and were cultured as previously described (10). Fenretinide (Janssen-Cilag Ltd), temozolomide (OSI Pharmaceuticals), bortezomib (Velcade; Janssen-Cilag Ltd), or thapsigargin (Sigma Chemical Co.) were added in ethanol to the media (17). After washing 3 times with PBS, sections were fixed in a solution of 10% formaldehyde for 10 min followed by further washes in distilled water and 1 mol/L sodium hydroxide (Sigma) for 10 min. Sections were then dehydrated for 1 to 2 min in 100% ethanol, cleared with xylene, and mounted. For the analysis of responses to combined treatment of melanoma cells in vitro, the Calcusyn program was used to derive parameter estimates for the median-effect equation.

Western blotting. Total protein was extracted from cell pellets, separated by electrophoresis through 4 to 20% SDS-PAGE gels (20 µg per track), and blotted onto polyvinylidene difluoride membranes as previously described (9). Blots were probed with an antibody to GADPH153 (Santa Cruz Biotechnology, B-3; Autogen Bioclear) diluted 1:500 and, as a loading control, with a β-actin antibody (Sigma) diluted 1:5,000. The binding of primary antibodies was detected with secondary peroxidase-conjugated antibodies (Upstate Biotechnology) diluted 1:2,000 and visualized with the use of the ECL system (Amersham Biosciences).

Xenograft mouse model. Female CD1 nude mice, 6 to 8 wk old (Charles River), were inoculated s.c. into the right flank with 7.5 × 10^6 A375 cells in 100 µL DMEM containing 4.5 g/L L-Glucose (BioWhittaker). On establishment of tumors 125 mm³ in volume, mice were randomized into 4 treatment groups (6 mice per group) and treated subsequently by daily i.v injection (tail) for 10 d with 1.45 mg/kg fenretinide or 0.1 mg/kg bortezomib, or the combination of both agents. The control group was treated with 100 µL of diluent only (DMEM containing 4.5 g/L glucose). Caliper measurements of tumor length (L) and width (W) were taken every day, and tumor volume was determined through the formula V = [(L * W) / 2].

Immunohistochemistry. Analysis of tumors from animals treated with fenretinide, bortezomib, or the combination of both agents was done by immunohistochemistry of frozen tissue sections (16). Frozen sections (6 µm) prepared on 3-Aminopropyltriethoxysilane–coated glass slides were fixed in ice-cold acetone for 10 min before staining with a Ki67 antibody or 4% paraformaldehyde before staining by terminal deoxyribonucleotide transferase–mediated dUTP-X nick-end labeling (TUNEL). After permeabilization for 10 min with 0.2% Triton X (Sigma) in PBS and two washes with PBS containing 0.05% Tween 20 (PBS/T; Sigma), sections were immersed in 10% goat serum for 1 h at room temperature, rinsed 3 times in PBS/T containing 0.05% Tween 20, and blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Nuclei for 15 min with 1:5,000-diluted primary antibody (1:5,000). The binding of primary antibodies was detected with secondary peroxidase-conjugated antibodies (Upstate Biotechnology) diluted 1:5,000 and, as a loading control, with a rabbit anti-Ki67 antibody (Novocastra Laboratories Ltd) diluted 1:200 in PBS/T for 1 h at room temperature. Ki67 staining was detected with a secondary FITC-conjugated goat anti-rabbit secondary antibody (Invitrogen) diluted 1:200 in PBS/T containing 2% bovine serum albumin (Sigma) for 1 h at room temperature before counterstaining nuclei with 15 min with 1:10 dilution of TOTO-3 iodide (Invitrogen) diluted 1:7,000 in PBS/T containing 2% bovine serum albumin. Stained sections were mounted in Vector Shield (Vector Laboratories) and analyzed by confocal microscopy with the use of a Leica TCS SP II laser-scanning confocal microscope with images captured and processed with the LCS Lite 2.61 software (Leica Microsystems). Tumors were processed for TUNEL staining in the same way and were detected with the use of a commercial kit (Roche Diagnostics) according to the manufacturer’s specifications.

To assess changes in cell cycle parameters in vivo, tumor sections (prepared as above) were stained with methylene blue (17) to measure nuclear area. After a 5-min wash in distilled water and 30-s rinse in 1 mol/L hydrochloric acid, sections were incubated at 60°C in 1 mol/L HCl for 10 min followed by further washes in distilled water and 1 mol/L HCl each for 30 s before incubation in Schiff’s reagent for 1 h (Sigma). After washing 3 x with sulphite rinse (50 mmol/L HCl containing 6% aqueous sodium metabisulfite) for 2 min each, sections were stained with 0.5% methylene blue (BDH) for 10 s at room temperature, dehydrated through 100% ethanol, cleared in Histo-Clear II solution (Raymond Lamb Ltd.), and mounted in DPX (Raymond Lamb). Staining was visualized by light microscopy, and images were captured and analyzed with the Leica QWin software (Leica Microsystems).

Statistical analysis. For the analysis of responses to combined treatment of melanoma cells in vitro, the Calcusyn program was used to derive parameter estimates for the median-effect equation.
with single-drug treatments (fenretinide or bortezomib) and combination indices for combined treatments: combination index values around 1 indicate additivity; <1, synergy; and >1, inhibition (18). For cell cycle data, G0+G1/G2+M ratios, referred to here as G1/G2 ratio, were analyzed after log transformation to equalize the variances [Log10(1+G1/G2)] by SPSS Release 15 (SPSS Inc.); transformed values were analyzed by drug treatment separately (fenretinide, bortezomib, or the combination) with the use of two-way ANOVA (cells, drug dose), one-way ANOVA with Dunnett's post hoc test to compare with control treatments, and by general linear models on drug-treated cells with cell type as factor and drug dose as a covariate. In the xenograft experiment, tumor volumes were expressed as relative to day 1. For analysis of final relative tumor volumes, the data were log-transformed to equalize variances and were tested for normality with the use of a one-sample Kolmogorov-Smirnov (Lilliefors) test (P > 0.05); these data were analyzed conservatively by one-way ANOVA with Bonferroni correction for post hoc pair-wise comparisons. For analysis of Ki67 and TUNEL staining, image analysis was done with the use of Velocity version 4.3.1 (Improvision Ltd). Nuclei were defined with the use of the fluorescent intensity of the nuclear marker dye (TOTO-3) and by applying appropriate thresholding and size exclusion filters. For each nucleus, the fluorescence value and mean fluorescent intensity for TUNEL or Ki67 from each image was recorded. Percentage change in positivity was determined by setting a threshold value on the negative control for each analysis (kept constant between analyses). The same threshold value was set on drug-treated samples so that the change in nuclear positivity was normalized to the untreated control. For each tumor (6 animals per treatment group), values were reported as the mean percentage of positive nuclei from three independent staining analyses (a total of ≥12 images and ≥2,100 cells per treatment). For analysis of methylene blue staining, the mean nuclear area from 200 nuclei was derived with the use of Leica QWin. Values were reported as the means from four independent staining analyses for each treatment group (n = 6 per group). Results from all immunocytochemical analyses were compared by one-way ANOVA with Dunnett's post hoc test with the use of SPSS Release 15.

Results

Fenretinide and bortezomib inhibit cell viability and induce apoptosis of metastatic melanoma cells in vitro. Bortezomib and fenretinide induce ER stress and apoptosis when added as single agents to CHL1, A375, and WM266-4 melanoma cells in vitro (10). The viability dose-response curves for fenretinide gave half maximal inhibitory concentration (IC50) values of 14 [95% confidence interval (95% CI), 10-20], 24 (95% CI, 12-47), and 25 (95% CI, 13-51) μmol/L for CHL1, A375, and WM266-4 cells, respectively (Fig. 1); viability dose-response curves for bortezomib were shallower but with IC50 values of 0.1 (95% CI, 0.05-0.2), 0.4 (95% CI, 0.2-1), and 1.4 (95% CI, 0.7-2.6) μmol/L for the respective cell lines. Thus, CHL1 cells were more sensitive to these drugs than A375 or WM266-2 cells, with the latter showing greatest resistance to bortezomib. When fenretinide and bortezomib were added to melanoma cells in combination, there was a weak synergistic reduction in viability over fixed-ratio dose ranges of 2.5/0.05 to 20/0.4 μmol/L fenretinide/bortezomib (combination indices, 0.305-0.73; Fig. 1). The decrease in viability in response to fenretinide or bortezomib was mirrored by an increase in apoptosis and, for this measure of effect, the combination of the two drugs was weakly synergistic at fixed-ratio doses at concentrations of 2.5/0.05 to 5/0.1 μmol/L fenretinide/bortezomib (combination indices, 0.3-0.84) but additive or weakly inhibitory at higher doses of 15/0.3 to 20/0.4 μmol/L fenretinide/bortezomib (combination indices, 1-1.35). A375 cells showed the best apoptotic response to the drug combination in vitro, with combination indices of 0.3 to 0.5 over the 2.5/0.05 to 10/0.2 μmol/L dose range.

Because bortezomib has been reported to induce G2/M arrest (19), the ratio of G0+G1/G2+M (here referred to as G1/G2 ratio) in the cell lines was analyzed for all drug treatments. Although the G1/G2 ratio was substantially reduced in all three cell lines (increased proportion of cells in G2) in response to bortezomib treatment compared with control, there was no dose-dependent relationship between bortezomib and decreased G1/G2 ratio (Fig. 1). The greatest G2/M arrest in response to bortezomib was obtained in CHL1 cells, with the least effect obtained in A375 cells (Fig. 1). In contrast to bortezomib, there was no change in G1/G2 ratio in A375 cells in response to fenretinide (one-way ANOVA with Dunnett's test; P > 0.3), but there was an increase in G1/G2 ratio (greater proportion of cells in G1) in CHL1 and WM266-4 cells treated with fenretinide at 10 to 20 μmol/L (CHL1 cells) or 20 μmol/L (WM266-4 cells). In these two cell lines, the data suggest a dose-dependent effect of fenretinide (Fig. 1). For cells treated with bortezomib and fenretinide together at a constant dose ratio, there was a linear, dose-dependent increase in G1/G2 ratio and significant differences between cell lines (Fig. 1). These results suggest that, in the presence of both drugs, a bortezomib-induced G2/M arrest predominates at low fenretinide doses, but increased levels of apoptosis at higher fenretinide concentrations result in a relative decrease in cells in G2/M.

Expression of GADD153 is characteristic of the ER stress–mediated apoptosis pathway (20) and was therefore used as a marker of an increased ER stress response in melanoma cells in response to drug combinations. Fenretinide or bortezomib as single agents induced GADD153 in CHL1 cells, but induction was weaker or barely detectable in A375 and WM266-4 melanoma cells (Fig. 2). For these experiments, fenretinide and bortezomib were used at concentrations of 10 and 0.2 μmol/L, respectively; at these concentrations of each drug alone the decrease in viability or increase in apoptosis, relative to control cells, was small (Fig. 1), and this was also reflected by the low level of GADD153 induction at these doses. However, when fenretinide and bortezomib were used in combination at these doses, there was a marked induction of GADD153 (Fig. 2) in all three cell lines. In these experiments, thapsigargin, an ER stress inducer (9), and temozolomide, a DNA-damaging alkylating agent (21), were used as positive and negative controls, respectively. These results suggest that the synergistic induction of apoptosis and decrease in viability in response to fenretinide and bortezomib in combination is a result of synergy in the ER stress–apoptosis pathway.

Effective inhibition of melanoma growth in vivo by fenretinide and bortezomib in combination. Our observation that, in vitro, fenretinide and bortezomib were synergistic suggests that these drugs in combination may be effective in controlling melanoma growth in vivo. The previous data show that A375 cells were relatively resistant to fenretinide or bortezomib as single agents; because A375 cells also carry the BRAFV600E activating mutation (22), and activating mutations in BRAF are characteristic of >70% of human melanomas (23), this cell line was used to test the hypothesis that fenretinide and bortezomib will
be effective in controlling melanoma growth in vivo with the use of a s.c. xenograft model. Once tumors were established, daily treatment for 10 days with fenretinide, bortezomib, fenretinide and bortezomib, or control vehicle was initiated. Treatment with fenretinide and bortezomib together resulted in almost complete cessation of tumor growth. At the end of the experiment, relative tumor volumes in animals treated with fenretinide and bortezomib together were significantly lower than in control animals or in animals treated with fenretinide or bortezomib alone (Fig. 3; $F_{3,20} = 12.42; P < 0.0001$; fenretinide and bortezomib compared with other treatments: $P < 0.004$). Tumor volumes in animals treated with fenretinide...
or bortezomib alone were not significantly different from control ($P > 0.65$).

Tumors removed from the animals at sacrifice were processed for immunohistochemistry, and tumor sections were stained for proliferative activity (Ki67) and the presence of apoptotic cells (TUNEL). In addition, because bortezomib induced $G_2/M$ arrest in all three melanoma lines in vitro, sections were stained for DNA (methylene blue) to measure nuclear area as evidence of $G_2/M$ arrest in vivo. Proliferative activity, expressed as the percentage of Ki67-positive cells, was not significantly different between treatments (one-way ANOVA; $F_{3,20} = 1.773; P = 0.185$; Fig. 4). Conversely, the extent of apoptosis (percentage TUNEL-positive cells) differed significantly between treatments (one-way ANOVA; $F_{3,20} = 285.4; P < 0.001$), was higher than control in tumors from animals treated with bortezomib (Dunnett's; $P = 0.004$), and was substantially higher than control and the bortezomib-only treatments in tumors from animals treated with fenretinide and bortezomib together (Dunnett's; bortezomib versus fenretinide plus bortezomib contrast: $P < 0.001$; Fig. 4). The mean nuclear area of tumor cells differed significantly between treatment groups (one-way ANOVA; $F_{3,20} = 24.33; P < 0.001$) and, in tumors from animals treated with bortezomib alone (Dunnett's; $P < 0.001$), was $2 \times$ that in tumors from the other treatments, consistent with bortezomib-induced $G_2$ arrest. The mean nuclear areas in tumors from the fenretinide or the combined fenretinide and bortezomib treatments were not significantly different from control (Dunnett's; $P > 0.13$; Fig. 4). Therefore, with respect to the combination treatment, these in vivo results mirror the in vitro data in which the bortezomib-induced accumulation of cells in $G_2/M$ was counteracted by increased apoptosis in response to fenretinide or both drugs acting together.

**Discussion**

The results from this study show that fenretinide and bortezomib were synergistic at concentrations within the lower range of their dose-response curves for the induction of apoptosis and throughout the concentration range for decreased viability. The three cell lines used differed in their sensitivity to fenretinide or bortezomib, but showed similar levels of synergy with respect to decreased viability when the drugs were used in combination. As in studies on other cell types (24, 25), bortezomib induced $G_{2/M}$ arrest in all three melanoma cell lines even at the lowest doses used (0.05 $\mu$mol/L). There was no evidence for dose dependency in $G_{2/M}$ arrest, and the apoptotic dose-response curves for bortezomib were relatively shallow in all three cell lines. Because apoptosis was assessed by relative DNA content of sub-$G_1$ cells, cells entering apoptosis from $G_2$ would not be included, and the total apoptosis in response to bortezomib may be underestimated. However, viability dose-response curves for bortezomib were also relatively shallow, and it is likely that a $G_{2/M}$ arrest is the main outcome of bortezomib treatment over a short timescale in vitro.

In contrast to bortezomib, fenretinide gave steep dose-response curves for the induction of apoptosis in the range 10 to 15 $\mu$mol/L and produced a dose-dependent increase in the proportion of cells in $G_2$ in CHL1 and WM266-4 cells. The combination of drugs showed a markedly linear decrease in the proportion of cells in $G_{2/M}$ with increasing constant-ratio doses of fenretinide and bortezomib, and this may be an effect of increased fenretinide concentrations, perhaps preferentially inducing apoptosis in cells arrested in $G_{2/M}$. The different effects on cell cycle parameters shown by fenretinide or bortezomib may reflect their different modes of action: unlike bortezomib, fenretinide induces oxidative stress leading to ER stress in melanoma cells (9). Conversely, bortezomib is a proteasome inhibitor with effects on a range of cellular signaling pathways. Bortezomib-induced $G_{2/M}$ arrest is associated with altered levels of the cell cycle regulators $p21^{WAF1}$,
p27kip1, cyclin B1, CDK2, CDK4, and E2F4 in glioma cells (24), but the mechanism of bortezomib-mediated G2/M arrest is unknown. The proteasome is also a key component of the ER-associated degradation pathway that clears misfolded or excess proteins from the ER and is induced as part of the unfolded protein response (ref. 26). Inhibiting this process leads to ER stress (27, 28) and increases ER stress resulting from other ER stress inducers (28). This mechanism is likely to underlie the synergy between fenretinide and bortezomib in vitro and in vivo, and gives additional weight to studies suggesting that the most effective use of bortezomib will be in combination with drugs aimed at additional cellular targets (29).

Synergy between fenretinide and bortezomib was also apparent in vivo, as shown by the markedly reduced growth of s.c. A375 xenografts. Evidence that these drugs were working in vivo in a similar manner to that observed in vitro was provided by the histologic analyses of the xenograft tumors; these were in agreement with predictions from in vitro data with respect to the induction of apoptosis, the accumulation of cells in G2/M in response to treatment with bortezomib alone, and the abrogation of G2/M arrest by fenretinide and bortezomib used in combination. The synergy between fenretinide and bortezomib at the concentrations used in vitro and in vivo is important: pharmacokinetic studies of fenretinide and bortezomib in a variety of clinical settings define the plasma concentration of each drug achievable in patients. For fenretinide, low toxicity allows high doses to be ingested, and the amount of drug delivered orally is mainly limited by the high number of capsules that need to be taken; mean peak plasma concentrations up to 13 μmol/L are achieved in children and adult cancer patients (30, 31) with steady state levels in the range 0.9 to 10 μmol/L (30, 32). Peak plasma concentrations of bortezomib up to 0.5 μmol/L can be achieved at appropriate doses (33–35) with biological half-lives of around 12 h (34–36). The present study on melanoma cell lines in vitro show that, for cell viability and apoptosis, fenretinide and bortezomib were synergistic at concentrations clinically achievable in cancer patients: 2.5-10 μmol/L for fenretinide and 0.05-0.2 μmol/L bortezomib.

Clearly, although bortezomib has shown no evidence of clinical efficacy in metastatic melanoma on its own (11), on the basis of these results, combining bortezomib with fenretinide

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**Fig. 4.** Immunohistochemical analysis of A375 xenograft tumors treated with FenR and/or Bort. A, B, and C are micrographs of tumor sections stained with (A) Ki67, (B) TUNEL, or (C) methylene blue from animals treated for 10 d with 1.45 mg/kg FenR, 0.1 mg/kg Bort, or both agents together (FenR+Bort). In A and B: red, TOTO-3 iodide counterstain; green, staining for Ki67 or TUNEL; yellow, the signal from Ki67 or TUNEL staining on a cellular background. Bar graphs below micrographs, summary of data analysis from tumor sections. For Ki67 (A) and TUNEL staining (B): bar, mean % ± SE of positive-stained cells (from at least 2,100 cells from 3 independent staining runs) from 6 tumors in each treatment group. In graph for B: ***, significantly different from control (Dunnett’s; P < 0.001); ****, significantly different from control and Bort alone (Dunnett’s and ANOVA contrast; P < 0.0001). For methylene blue staining (C): bars, mean (6 tumors per treatment group) ± SE nuclear area (μm²) of 200 nuclei from 4 independent staining experiments, in all cases assessed by two independent observers; ***, significantly different from control (Dunnett’s; P < 0.0001); all other treatments were not significantly different from control. Bar, 100 μm for Ki67 and TUNEL staining (white) and 5 μm for methylene blue staining (red).
may be an effective therapeutic strategy. It is particularly pertinent that many tumor cells already have activated ER stress responses and display greater sensitivity to agents that increase ER stress still further, providing a mechanism to target tumor cells while minimizing the damage to normal cells. Furthermore, whereas combining bortezomib with an additional ER stress inducer, such as fenretinide, HDAC inhibitors (28), or inhibitors of ER stress recovery mechanisms (10) may be effective strategies, inhibiting downstream antiapoptotic elements of the Bcl2 family proteins also increases apoptosis of melanoma cells in response to bortezomib (37). This raises the possibility that drug cocktails of fenretinide and bortezomib with clinically available Bcl2-family inhibitors, such as (-)-gossypol (37) or ABT-737 (38), will be an exciting development for melanoma therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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