

## The Bcl-2 Family Protein Inhibitor, ABT-737, Has Substantial Antimyeloma Activity and Shows Synergistic Effect with Dexamethasone and Melphalan

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**Abstract Purpose:** The aim of this study is to investigate the antimyeloma activity of a novel Bcl-2 family inhibitor, ABT-737, in preclinical treatment of multiple myeloma.

**Experimental Design:** The antimyeloma activity of ABT-737 was evaluated in cultured myeloma cell lines and patient myeloma samples, and in a xenograft mouse myeloma model. Drug combination therapy using ABT-737 with other commonly used myeloma drugs was also investigated.

**Results:** MY5 and JLN3 cell lines exhibited the most sensitivity to ABT-737 with an  $EC_{50}$  of 0.2 and 0.5  $\mu\text{mol/L}$ , respectively, with increased cell apoptosis and elevated activated caspase-3. We identified two distinct groups of myeloma patient samples that were either sensitive or resistant to the drug. Four of 15 patient bone marrow samples (27%) were highly sensitive to ABT-737 at doses of 0.25 and 0.5  $\mu\text{mol/L}$ , which eliminated 80% to 90% of myeloma cells as a result of cellular apoptosis 3 days after drug treatment. ABT-737 showed a synergistic effect when combined with dexamethasone or melphalan in inducing myeloma cell death. Furthermore, the dexamethasone-resistant MM1 (Dex)R myeloma cell line was highly sensitive to 0.2  $\mu\text{mol/L}$  ABT-737. As determined by colony assay, little or no detectable toxicity to patient hematologic progenitor cells was observed at 1  $\mu\text{mol/L}$  ABT-737. ABT-737 dose dependently suppressed tumor growth in a xenograft MY5 mouse model.

**Conclusions:** These studies show substantial antimyeloma activity of ABT-737 as a single agent or in combination with dexamethasone or melphalan and suggest a rationale for future clinical trials.

Multiple myeloma (MM) is a neoplasm of terminally differentiated, antibody-secreting plasma cells, characterized by accumulation of a monoclonal plasma cell population in the bone marrow, elevated plasma and/or urine immunoglobulin, and the presence of end-organ damage, such as osteolytic lesions and renal insufficiency. In MM pathogenesis, slow

proliferation of malignant plasma cells leads to their accumulation in the bone marrow. This feature suggests that resistance to apoptosis may play an important role in myeloma tumorigenesis, which is now supported by a wide spectrum of experimental data (1). There are two pathways that can lead to activation of caspases and cell apoptosis: the death receptor (or extrinsic) pathway and the mitochondrial (intrinsic) pathway. The mitochondrial pathway is regulated mainly by Bcl-2 family members with both proapoptotic and antiapoptotic proteins. The antiapoptotic members include Bcl-2, Bcl-X<sub>L</sub>, Bcl-w, Mcl-1, and Bfl-1/A1. These proteins are characterized by the presence of all four Bcl-2 homology domains (BH1-BH4) and function in preventing cell death by binding and sequestering proapoptotic proteins. The proapoptotic proteins include Bax, Bak, and Bok, which only contain three BH domains (BH1-BH3). When activated, they undergo conformational changes, leading to increased permeability of the outer mitochondrial membrane, and the release of cytochrome *c* and activation of caspase-9 (2). The third subgroup of Bcl-2 family members includes the proapoptotic ligands containing only the BH3 domain, which serve as ligands for the channel-forming Bcl-2 proteins by forming functional heterodimers to regulate apoptosis. Members of this subgroup include BAD, Bim, Bid, and Bik. Both the death receptor and the mitochondrial apoptosis pathways are reported to be involved in MM tumorigenesis (3, 4).

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Bcl-2, frequently expressed in follicular lymphomas bearing the t(14;18) chromosomal translocation, is also widely expressed in myeloma cell lines and in primary patient samples. In one report that compared the protein expression of 13 myeloma cell lines and 8 immunosorted myeloma patient samples, 12 of 13 and 8 of 8 express Bcl-2; 10 of 13 and 7 of 8 express Bcl-X<sub>L</sub>; and 12 of 13 and 7 of 8 overexpress Mcl-1 (5). Another independent study has linked the myeloma malignant phenotype to Mcl-1 overexpression and decreased Bax expression (6). Interleukin 6 (IL-6) is a major survival factor for MM cells and induces signal through the signal transducers and activators of transcription 3 pathway (7). Many myeloma cell lines produce IL-6 via an IL-6 autocrine loop (8). Those that do not produce IL-6 depend on the bone marrow stroma cells to secrete IL-6 in supporting the tumor cells in the bone marrow microenvironment. The IL-6 survival signal has been linked to up-regulation of Bcl-X<sub>L</sub> and Mcl-1 (5). Most chemotherapeutic agents, such as doxorubicin, induce apoptosis by a mechanism of cytochrome *c* release from the mitochondria and subsequent activation of caspases. This process can be blocked by overexpression of Bcl-2. Therefore, the Bcl-2 family proteins play a critical role in MM tumorigenesis and response to treatment.

A nuclear magnetic resonance-based high-throughput screen by Abbott Laboratories (Abbott Park, IL) has identified a small molecule that binds to the BH3-binding groove of Bcl-X<sub>L</sub>. After chemical modification of this molecule to reduce its side activity of binding to human serum albumin, the resultant compound, ABT-737, was generated with high affinity to Bcl-X<sub>L</sub>, Bcl-2, and Bcl-w but not to less homologous proteins such as Bcl-B, Mcl-1, and A1. In murine models, ABT-737 improved survival and induced regression of established tumors of lymphoma and small-cell lung cancer (9, 10). Aimed at exploring ABT-737 as an antimyeloma drug, we have done preclinical studies to investigate the efficacy of ABT-737 in treatment of MM. Here, we report that ABT-737 has substantial antimyeloma activity both *in vitro* and *in vivo*, with a synergistic antitumor efficacy when combined with dexamethasone and melphalan.

## Materials and Methods

**Chemical compounds, antibodies, Western blotting, and immunohistochemistry.** ABT-737 was kindly provided by Abbott Laboratories and prepared in solutions according to publication (9). Dexamethasone and melphalan were purchased from Sigma (Milwaukee, WI). Antibodies recognizing Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, Bak, Bax, and Bid were purchased from BD Biosciences (San Jose, CA). Antibody against Bim was purchased from Stressgen (Victoria, BC, Canada). Antibodies against Bak and caspase-3 were purchased from Cell Signaling Technology (Danvers, MA). Western blotting and immunohistochemistry were done as previously described (11).

**Cell lines, tissue culture, and patient samples.** All human MM cell lines (KMS11, LP1, JLN3, U266, ANBL6, MY5, 8226, H929, and OPM1) were maintained in Iscove's modified Dulbecco's medium supplemented with 5% FCS, 100 µg/mL penicillin, and 100 µg/mL streptomycin (Hyclone, Logan, UT). Bone marrow stromal cells were obtained from bone marrow samples of MM patient and prepared as previously described (11). Briefly, mononuclear cells were isolated from patient bone marrow samples and cultured in 10 mL Iscove's modified Dulbecco's medium supplemented with 10% FCS. After the 1st week, the medium and suspension cells were removed and replenished every

3 days. When an adherent cell monolayer developed, the adherent cells were trypsinized and harvested, which represent bone marrow stromal cells. For the purposes of viability assays, bone marrow stromal cells were irradiated with 20 Gy after plating on 96-well plates. Bone marrow aspirates were obtained with consent under a protocol approved by the Research Ethic Board of University Health Network, Toronto, Canada.

**Cell viability assay.** Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Cells were seeded in 96-well plates at a density of 20,000 per well for MM cell lines. Cells were incubated with various concentrations of ABT-737, with or without IL-6 (10 ng/mL), bone marrow stromal cells, dexamethasone, or melphalan in 96-well plates for 48 or 72 h. Cell viability was determined using MTT assay. Each experimental condition was done in triplicate and repeated at least once.

**Cell apoptosis analysis.** For studies of apoptosis in MM cell lines, cells were seeded at a density of  $2 \times 10^5$ /mL to  $4 \times 10^5$ /mL in medium supplemented with DMSO or 1 µmol/L ABT-737 and cultured for 48 h. Apoptosis was determined by Annexin V staining (Boehringer Mannheim, Indianapolis, IN) or by immunostaining with antibody against cleaved caspase-3 and analyzed by flow cytometry. For cell apoptosis analyses in primary samples, mononuclear cells were purified from bone marrow samples and plated at a cell density of  $5 \times 10^5$ /mL in Iscove's modified Dulbecco's medium plus 15% FCS in the presence of diluted DMSO, and 0.25 or 0.5 µmol/L ABT-737 for 3 days. Cells were then double stained with anti-CD138-phycoerythrin, and FITC-conjugated Annexin V as previously described (11). Samples were analyzed by FACScan analysis using CellQuest software.

**Drug synergism analysis.** We used the Chou-Talalay method (12) to determine the compound combination synergism as described by Catley et al. (13). Briefly, the Chou-Talalay method uses the following equation: combination index (CI) =  $(D)_1/(Dx)_1 + (D)_2/(Dx)_2$ , where  $(D)_1$  and  $(D)_2$  are the doses of drugs 1 and 2 that have *x* effect when used in combination; and  $(Dx)_1$  and  $(Dx)_2$  are the doses of drugs 1 and 2 that have the same *x* effect when used alone. The combination is additive when CI equals 1.0, synergistic when CI is <1.0, and antagonistic when CI is >1.0.

**Cell colony formation assay.** For colony assay,  $2 \times 10^4$  mononuclear cells from bone marrow are plated in 1 mL METHOCULT GF H4434 (StemCell Technologies, Inc., Vancouver, BC, Canada) and cultured for 6 to 7 days with DMSO, and 0.5 or 1 µmol/L ABT-737. Total colony numbers [including granulocyte-erythrocyte-monocyte-megakaryocyte colony forming units, granulocyte-macrophage colony-forming unit, and blast-forming unit (erythroid)] in ABT-737-treated or nontreated groups were counted under microscope.

**Xenograft mouse model.** Method for establishing xenograft mouse myeloma model was described previously (11). Briefly, 6- to 8-week-old female BNX mice purchased from Frederick Cancer Research and Development Centre (Frederick, MD) were inoculated s.c. into the right flank with  $2 \times 10^7$  MY5 cells in 100 µL Iscove's modified Dulbecco's medium, together with 100 µL Matrigel basement membrane matrix (Becton Dickinson, Bedford, MA). Mice were divided into four groups (eight mice per group). Treatment of ABT-737 was initiated when tumors reached volumes of ~150 mm<sup>3</sup> (group IV) or ~380 mm<sup>3</sup> (groups II and III). The first group (group I) was injected with diluent only and used as a control. Caliper measurements were done every 5 days to estimate tumor volume, using the following formula:  $V = Lx(W^2/2; \text{ref. } 9)$ .

## Results

**Efficacy of ABT-737 in inducing apoptosis of myeloma cell lines.** We first investigated the expression of antiapoptotic proteins (Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1), proapoptotic proteins (Bak and Bax), and the BH3-only proapoptotic ligands (Bim and

Bid) in nine myeloma cell lines by Western blot analyses. For antiapoptotic proteins, all cell lines ubiquitously expressed Bcl-2 and Mcl-1. However, Bcl-X<sub>L</sub> was expressed at variable levels with the highest levels detected in U266, ANBL6, KMS11, and 8226 cells, and lowest levels in MY5 and 8226 cells (Fig. 1A). For proapoptotic proteins, Bax is expressed ubiquitously at similar levels in all the cell lines tested. Variable expression levels of Bak were detected with the highest levels in ANBL6, MY5, and H929 (Fig. 1B). For the proapoptotic ligands, Bim (extra large, large, and small) is expressed in all cell lines except LP1; Bid is expressed strongly in ANBL-6, H929, and MY5 but weakly in other cell lines (Fig. 1C).

We next studied the efficacy of ABT-737 in inducing apoptosis in myeloma cell lines. All nine cell lines were treated in culture with ABT-737 at doses of 0.5, 1, 2, 4, 8, and 16  $\mu\text{mol/L}$  for 48 h. By MTT assay, all cell lines were responsive to ABT-737 (Fig. 2A) with EC<sub>50</sub> values ranging from 0.2 to 15  $\mu\text{mol/L}$  (Fig. 2B). MY5 and JIN3 were the most sensitive cell lines with EC<sub>50</sub> of 0.2 and 0.5  $\mu\text{mol/L}$ , respectively (Fig. 2B). MY5 had the lowest level of Bcl-X<sub>L</sub> expression in all the cell lines tested (Fig. 1A). Relatively resistant cell lines included LP1, U266, H929, and OPM1 (EC<sub>50</sub> > 6  $\mu\text{mol/L}$ ). LP1 lacked Bim

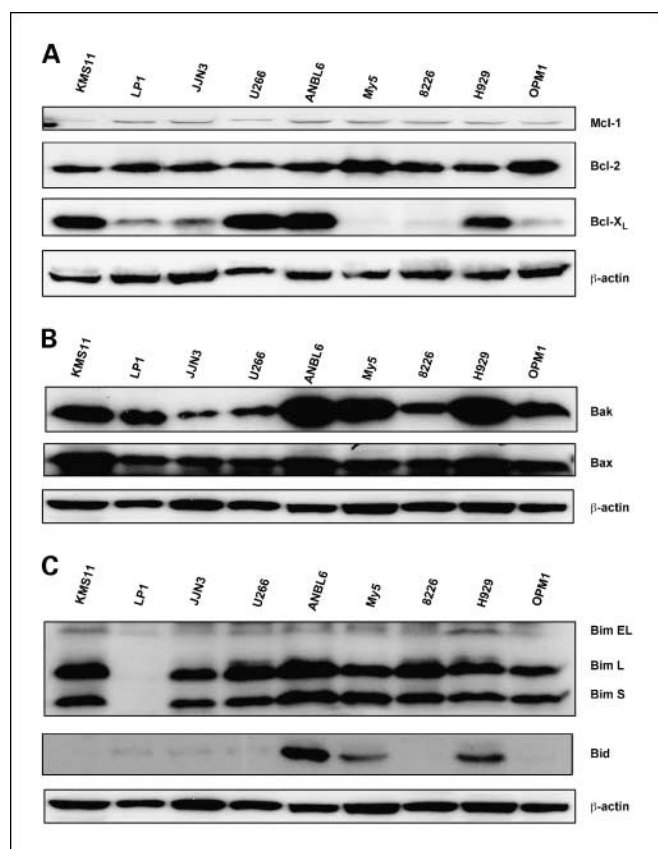
expression, whereas U266 and H929 had highest levels of Bcl-X<sub>L</sub> expression. OPM1 had the highest level of Bcl-2 expression (Fig. 1). Annexin V staining of MY5 and JIN3 cells following treatment with 1  $\mu\text{mol/L}$  ABT-737 for 48 h showed 94% and 43% cell apoptosis, respectively (Fig. 2C). This correlated with a 9- and 3-fold increase in the levels of activated caspase-3 in MY5 and JIN3 cells, respectively (Fig. 2D).

**Protective effects of IL-6 and stromal cells on survival of ABT-737-treated myeloma cells.** As IL-6 is an important survival factor for MM cells and stromal cells are important sources of IL-6 in the bone marrow, we next investigated the protective effect of these elements on myeloma cell survival in ABT-737-treated cells. All nine MM cell lines were treated with ABT-737 at the concentration of their EC<sub>50</sub> values for 48 h in the presence of 10 ng/mL recombinant human IL-6. As determined by MTT assay, coculture of IL-6 with ABT-737 had a protective effect in most of the MM cell lines (Fig. 3A). Complete or near-complete protection was observed in KMS11, U266, ANBL-6, and MY5 cells compared with the control of ABT-737-treated cells alone (Fig. 3A). IL-6 provided little protection for LP1, H929, and OPM1 cells, and no protection for JIN3 and 8228 cells. Similarly, cocultured stromal cells from pooled patients exerted strong protective effects in KMS11, 8226, and H929 cells; little protection in MY5 and OPM1 cells; and no protection in JIN3 and U266 (Fig. 3B). ABT-737 had no effects on the survival of bone marrow stromal cells (data not shown).

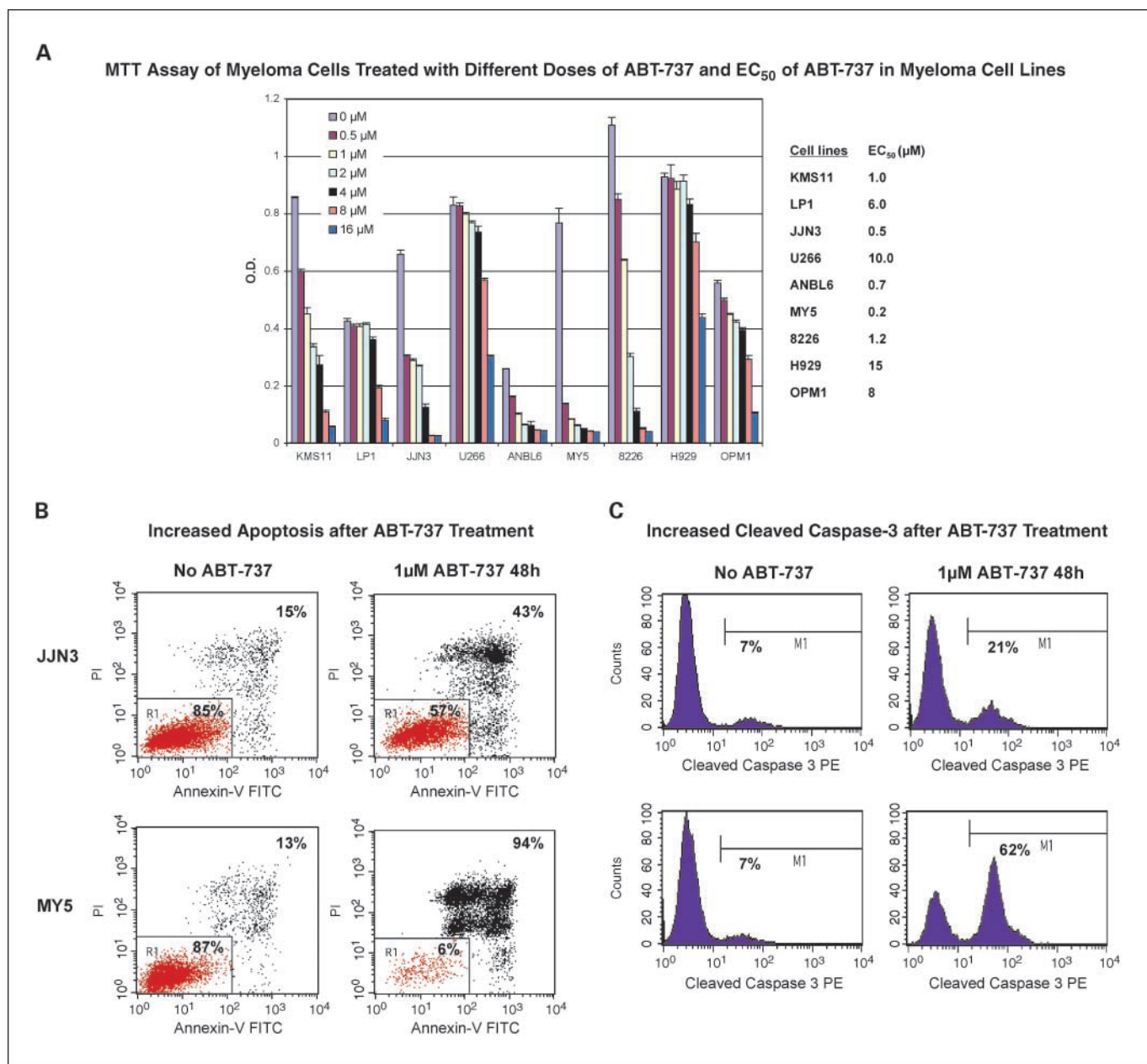
**Increased apoptosis of cultured primary myeloma cells after ABT-737 treatment.** We next studied the efficacy of ABT-737 in inducing myeloma cell apoptosis in bone marrow samples of patients. Bone marrows from newly diagnosed myeloma patients were collected, and mononuclear cells were isolated and cultured with ABT-737 at doses of 0.25 and 0.5  $\mu\text{mol/L}$  for 3 days. Vehicle-treated samples were cultured as controls. After ABT-737 treatment, the cells were harvested and stained with CD138 antibody for viable plasma cell population plus Annexin V staining for cellular apoptosis (Fig. 4A). It is interesting to note that the 15 patient samples studied fall into two distinct groups: responsive or nonresponsive (Fig. 4B). Four of 15 patient samples (27%) were highly sensitive to ABT-737 at doses of 0.25 and 0.5  $\mu\text{mol/L}$ . Normalization of the data reveals that the samples had decreased viable plasma cell populations to 23% ( $\pm 12\%$ ) and 11% ( $\pm 11\%$ ) of the untreated controls, respectively (Fig. 4C). Samples from the remaining 11 patients were not sensitive to ABT-737, with viable plasma cell population of 105% ( $\pm 19\%$ ) and 89% ( $\pm 16\%$ ) compared with controls after 3 days of treatment with 0.25 and 0.5  $\mu\text{mol/L}$  ABT-737, respectively (Fig. 4C).

**Synergistic effect of ABT-737 with dexamethasone and melphalan in inducing myeloma cell death.** To study whether ABT-737 is active against chemoresistant myeloma, we compared the drug efficacy of ABT-737 on dexamethasone-sensitive and dexamethasone-resistant cell lines MM1(Dex)S and MM1(Dex)R, respectively. Both cell lines were cultured at 0, 0.1, 0.2, and 0.4  $\mu\text{mol/L}$  ABT-737 for 3 days. The MTT assay indicated that dexamethasone-resistant MM1(Dex)R cells were highly sensitive to ABT-737 with an EC<sub>50</sub> of 0.16  $\mu\text{mol/L}$ , compared with an EC<sub>50</sub> of 0.30  $\mu\text{mol/L}$  for MM1(Dex)S cells (Fig. 5A).

We next studied the drug combination effect of ABT-737 with dexamethasone. As shown in Fig. 5B, various concentrations of



**Fig. 1.** Expression of Bcl-2 family proteins in human myeloma cell lines. Nine human myeloma cell lines (KMS11, LP1, JIN3, U266, ANBL6, MY5, 8226, H929, and OPM1) were investigated by Western Blot analyses. *A*, expression of antiapoptotic proteins: Bcl-2 and Mcl-1 were ubiquitously expressed in all the cell lines whereas Bcl-X<sub>L</sub> was expressed strongly in KMS11, U266, ANBL6, and H929. *B*, expression of proapoptotic proteins: Bax is expressed ubiquitously in cell lines whereas Bak is expressed at variable levels with the highest levels in ANBL-6, MY5, and H929. *C*, expression of BH3-only proteins: Bim is expressed in all cell lines except LP1. Bid is expressed strongly in ANBL-6, H929, and MY5.



**Fig. 2.** Efficacy of ABT-737 in inducing apoptosis of cultured myeloma cell lines. Nine myeloma cell lines were treated with various doses of ABT-737 for 48 h in culture. (A) MTT assay was done to determine cell viability (left), and the EC<sub>50</sub> values of ABT-737 in all the nine cell lines were calculated (right). MY5 and JLN3 were most sensitive to ABT-737 associated with increased cell apoptosis as determined by Annexin V staining (B) and increased activated caspase-3 (C). PE, phycoerythrin.

ABT-737 (50-500 nmol/L) and dexamethasone (4-1,024 nmol/L) were tested alone or in combination for drug-induced cell death of MM1(Dex)S cells by MTT assay. The CI values were calculated by using the following formula:  $CI = (D)1/(Dx)1 + (D)2/(Dx)2$  (see Materials and Methods). In the combinations of 4 nmol/L dexamethasone + 50 nmol/L ABT-737, 8 nmol/L dexamethasone + 100 nmol/L ABT-737, and 16 nmol/L dexamethasone + 150 nmol/L ABT-737, the CI values are 0.56, 0.38, and 0.32 respectively, suggesting a synergistic combination effect at these concentrations.

Similarly, we also investigated the combination effect of ABT-737 with another commonly used antimyeloma drug, melphalan. Various concentrations of ABT-737 (0.2-4 μmol/L) and

melphalan (5-60 μmol/L) were tested alone or in combination on 8226 cells. In the combinations of 5 μmol/L melphalan + 0.2 μmol/L ABT-737, 10 μmol/L melphalan + 0.4 μmol/L ABT-737, 20 μmol/L melphalan + 0.6 μmol/L ABT-737, and 30 μmol/L melphalan + 0.8 μmol/L ABT-737, the CI values are 0.75, 0.70, 0.77, and 0.87, respectively, indicating a synergistic combination effect at these concentrations (Fig. 5C).

**ABT-737 regressed tumors in a myeloma xenograft mouse model.** We next studied the antitumor efficacy of ABT-737 in a xenograft mouse myeloma model. MY5 cells ( $2 \times 10^7$ ) were injected s.c. in the right flank of BNX nude mice. Mice were divided into four groups: the first group (I) served as the control group and was injected i.p. with drug diluent daily; the second

and third groups (II and III) were treated with ABT-737 when tumors reached volumes of ~380 mm<sup>3</sup> (40 days after inoculation), at doses of 50 and 100 mg/kg/d, respectively; the fourth group of mice (IV) was treated with 100 mg/kg/d of ABT-737 when tumors grew to volumes of ~150 mm<sup>3</sup> (26 days after inoculation). Data from groups II and III showed that ABT-737 inhibited tumor growth in a dose-dependent manner (Fig. 6A). When the treatment was administered at an earlier stage (group IV), ABT-737 completely regressed the myeloma cell-derived solid tumors (Fig. 6A). Five days after drug treatment, tumor samples from groups I and III were collected and immunostained with antibody against cleaved caspase-3. The ABT-737-treated tumors exhibited massive immunostaining (Fig. 6B, b and d), indicating activated cellular caspase-3 and apoptosis.

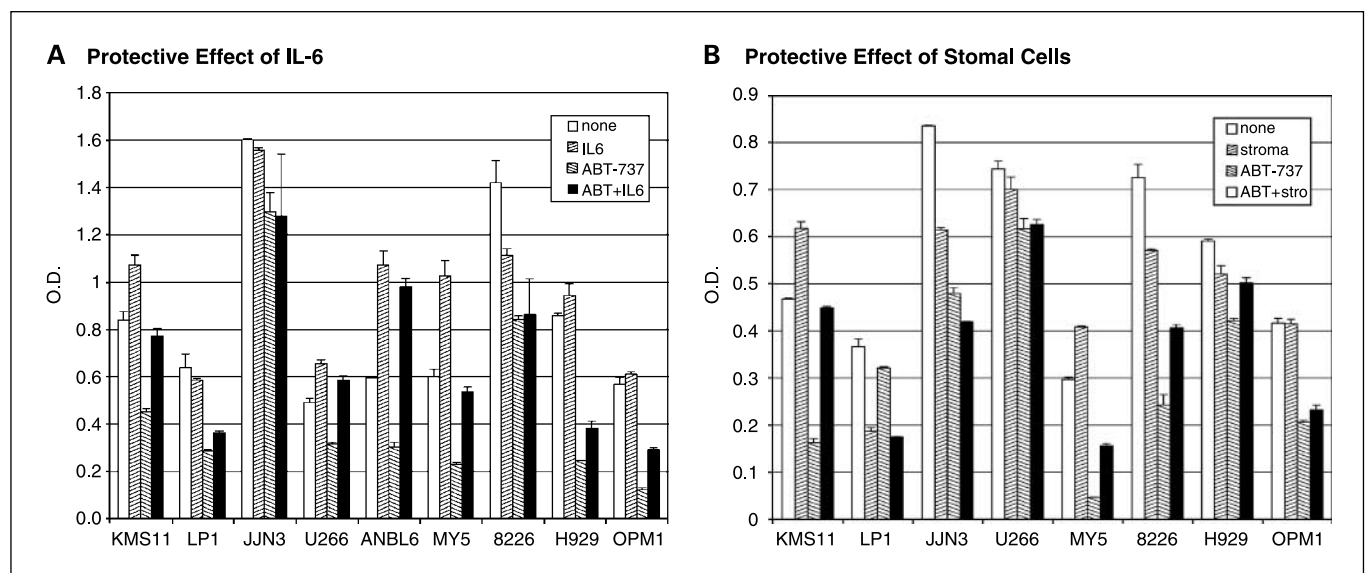
**ABT-737 is not cytotoxic to patient hematopoietic progenitor cells.** As ABT-737 seems to have clinical potential, we next investigated whether ABT-737 has cytotoxicity on hematopoiesis by performing a bone marrow soft agar colony assay. Mononuclear cells (2 × 10<sup>4</sup>) from bone marrow were plated in 1 mL METHOCULT GF H4434 mixed with 0.5 or 1.0 μmol/L ABT-737 and cultured for 6 to 7 days. Total colony numbers, including granulocyte-erythrocyte-monocyte-megakaryocyte colony forming units, granulocyte-macrophage colony-forming unit, and blast-forming unit (erythroid) were counted. The percentages of colony numbers compared with untreated controls were calculated (Table 1). At doses of 0.5 and 1.0 μmol/L, ABT-737 showed very little cytotoxicity against patient hematologic progenitor cells (Table 1).

**Discussion**

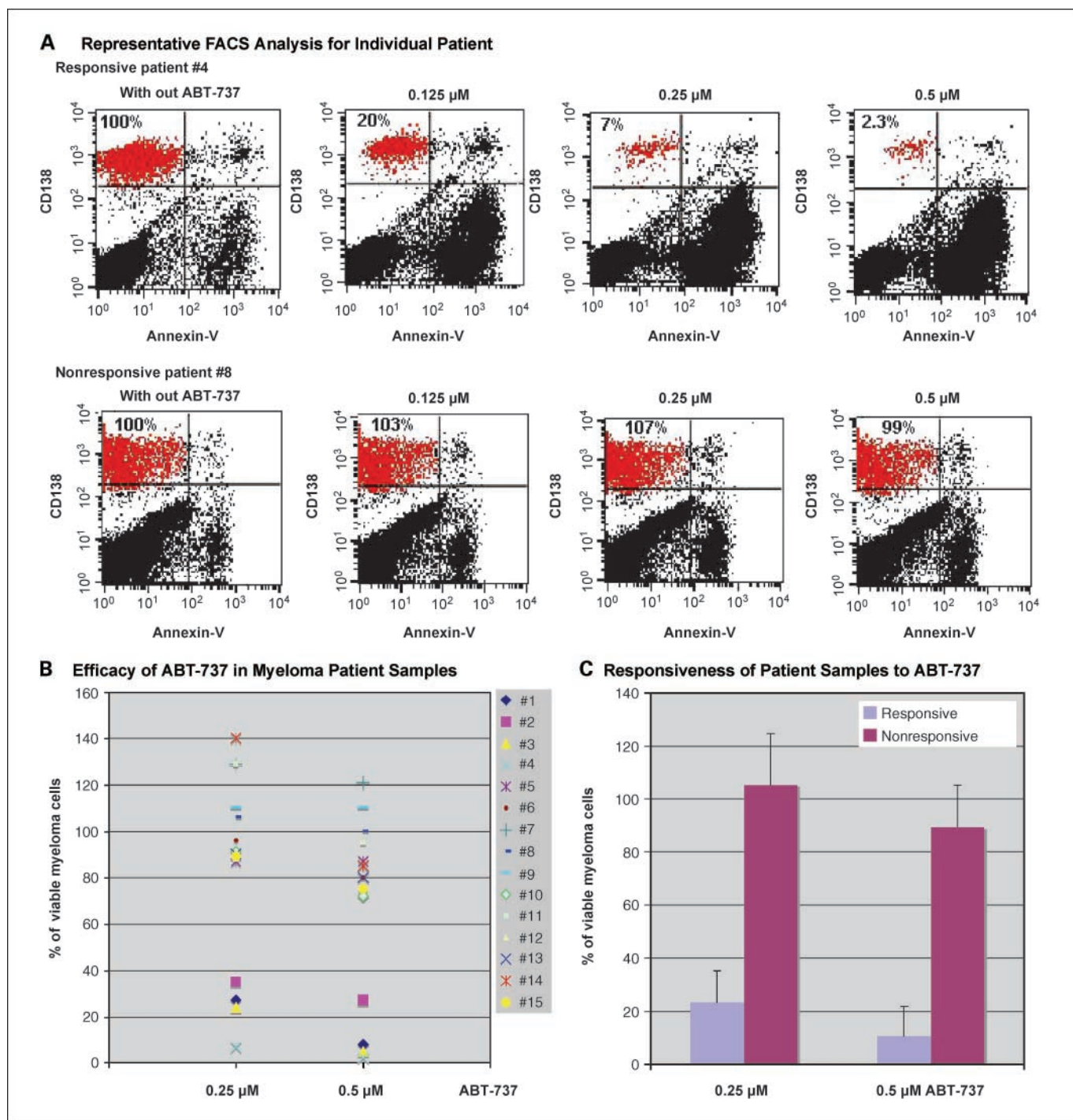
Unlike most tumors that result from proliferation of less-differentiated cell types, MM is a neoplasm of terminally differentiated cells with low-level proliferation. Numerous data have suggested that extended survival or resistance to apoptosis

is crucial in MM initiation, progression, and response to treatments. The mechanism for such resistance to apoptosis has been linked to the overexpression of the Bcl-2 family of antiapoptotic proteins (Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1) and to decreased expression proapoptotic proteins (1). High expression levels of Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1 are also linked to MM resistance to chemotherapy (14). As such, development of drugs that specifically target the Bcl-2 family members has recently emerged as a vital research field. Bcl-2 antisense therapy has been tested in various hematologic malignancies, including chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and MM (15). In myeloma patients G3139, an antisense oligonucleotide complementary to the first six codons of the Bcl-2 open reading frames has been shown to efficiently down-regulate Bcl-2 expression and significantly enhance sensitivity of tumor cells to chemotherapy (16, 17). However, clinical trials of this drug were ultimately disappointing. Imexon, a new antitumor agent with antimyeloma activity, was shown to activate caspase-9 and caspase-3 and promote translocation of Bcl-X<sub>L</sub> to the mitochondria in myeloma cells (18). Most recently, another small-molecule Bcl-2 family antagonist, GX15-070, has been tested in a preclinical setting for treatment of MM with proven antimyeloma activity (19, 20). In this study, we have investigated the drug efficacy of ABT-737, a specific inhibitor of Bcl-2, Bcl-X<sub>L</sub>, and Bcl-w, in treatment of myeloma. We show that ABT-737 has substantial antimyeloma activity both in cell culture and in a myeloma xenograft mouse model.

Among the nine genetically diverse myeloma cell lines tested, MY5 and JJN3 are the most sensitive cell lines to ABT-737 (EC<sub>50</sub> of 0.2 and 0.5 μmol/L, respectively) with increased cell apoptosis detected via activation of caspase-3. The relatively drug-resistant cell lines (EC<sub>50</sub> > 6 μmol/L) include LP1, U266, H929, and OPM1. Similarly, we have identified two distinct groups of patient samples, which are either responsive or resistant to ABT-737 treatment. Four of 15 (27%) myeloma



**Fig. 3.** Protective effect of IL-6 and stromal cells on survival of ABT-737-treated myeloma cells. Nine myeloma cell lines were treated with ABT-737 at the concentration of their EC<sub>50</sub> values for 48 h with or without 10 ng/mL IL-6 (A), or cocultured with bone marrow stromal cells (B). Both IL-6 and stromal cells have protective effects on the survival of the majority of ABT-737-treated myeloma cells.



**Fig. 4.** Responsiveness of primary myeloma cells to ABT-737 treatment. Mononuclear cells isolated from myeloma patient bone marrow samples were cultured and treated with ABT-737 for 3 d. The samples were labeled with Annexin V and CD138-phycoerythrin antibody. Viable CD138-positive plasma cells in drug-treated groups were normalized to vehicle-treated group (viable control plasma cell population was counted as 100%). Two distinct drug responses were identified, sensitive or resistant (A). Four of 15 (27%) patient samples were highly sensitive to ABT-737 at doses of 0.25 and 0.5  $\mu$ mol/L (B), which eliminated 80% to 90% myeloma cells as a result of apoptosis after the drug treatment in culture for 3 d (C).

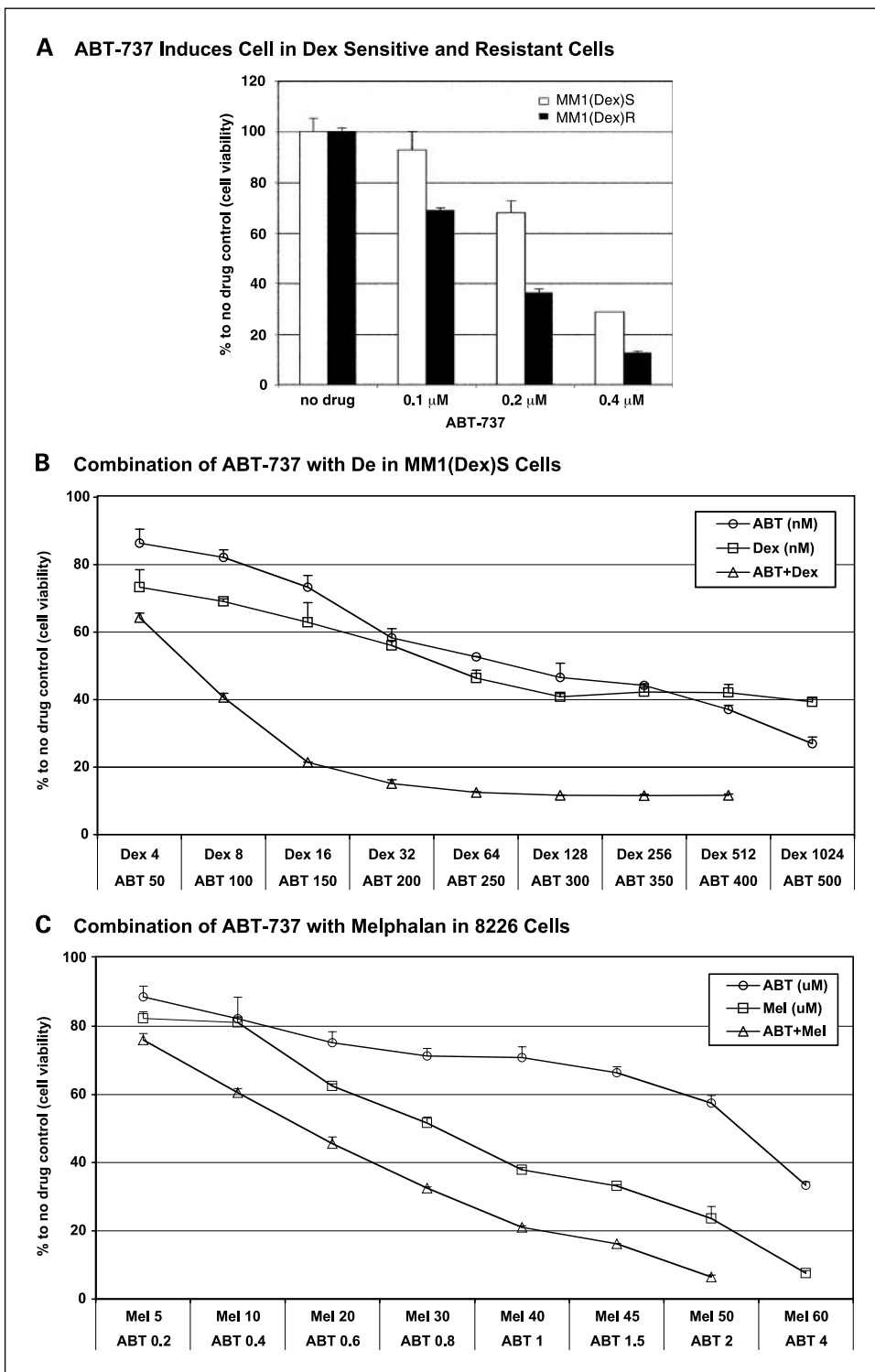
patient bone marrow samples are highly sensitive to 0.25 and 0.5  $\mu$ mol/L ABT-737 treatment in culture. Such distinct drug responsiveness may reflect their different cellular apoptosis regulatory mechanisms. However, the expression levels of individual antiapoptotic and proapoptotic proteins do not show a general correlation with differences in drug responsive-

ness as observed in our Western studies in myeloma cell lines. Since Letai et al. (21, 22) reported that BH3-only proteins Bim and Bid are important determinants of cytochrome *c* release and mitochondrial apoptosis, we further did Western blot analysis to investigate Bim and Bid protein expression in myeloma cell lines. However, their expression levels are not

correlating with antimyeloma efficacy of ABT-737. Therefore, we speculate that the ABT-737 drug efficacy could be attributed to balances in the levels of antiapoptotic and proapoptotic Bcl-2 family proteins, BH3-only proteins, as well as their interaction partners that deserves further investigation. It is also possible that the differences in drug response may have resulted from gene mutations of the Bcl-2 family members in the cell lines tested.

We have previously shown that overexpression of fibroblast growth factor receptor 3 in myeloma cells confers dexamethasone resistance with up-regulation of Bcl-X<sub>L</sub> (23). Here, we investigate the potential of using ABT-737 to treat dexamethasone-resistant myeloma. ABT-737 is able to induce cell death in dexamethasone-resistant MM1(Dex)R cells. It is also interesting to note that these dexamethasone-resistant cells are more sensitive to ABT-737 than their parental MM1(Dex)S

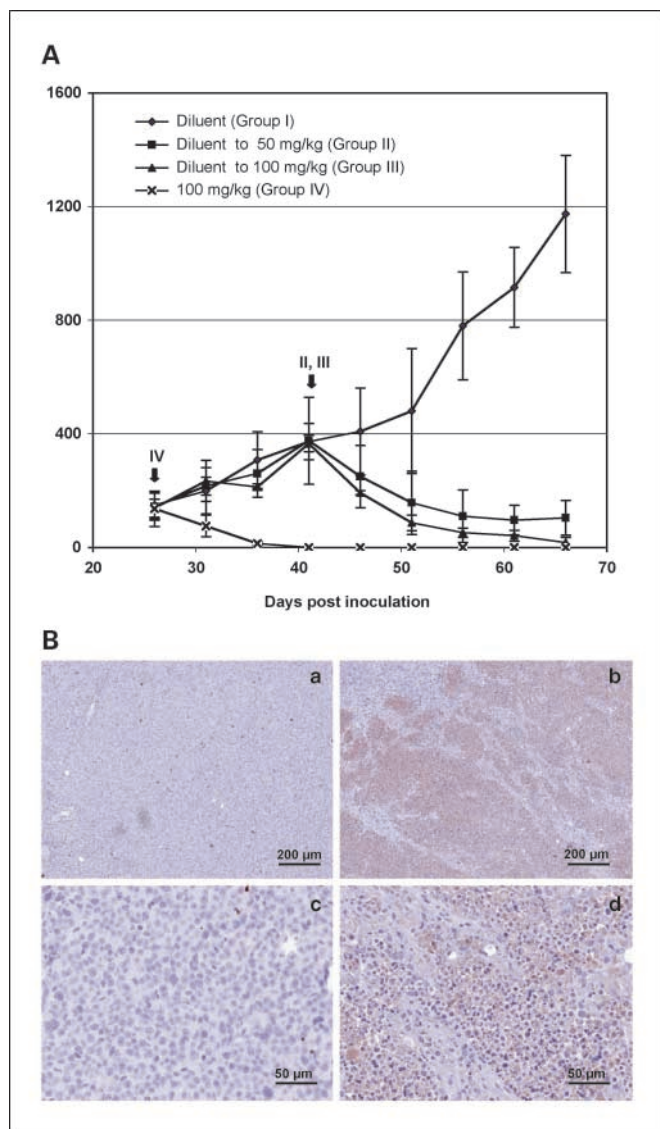
**Fig. 5.** ABT-737 is active against dexamethasone-resistant cells and has a synergistic effect with dexamethasone or melphalan. *A*, dexamethasone-sensitive, MM1 (Dex)S, or dexamethasone-resistant, MM1 (Dex)R, cells were cultured in various concentrations of ABT-737 for 3 d. MTT was done to determine cell viability with data normalized as percentage to untreated control. *B*, in similar cell culture conditions, MM1 (Dex)S cells were treated with ABT-737 in combination with dexamethasone (Dex) at various concentrations. In the combinations of 4 nmol/L dexamethasone + 50 nmol/L ABT-737, 8 nmol/L dexamethasone + 100 nmol/L ABT-737, and 16 nmol/L dexamethasone + 150 nmol/L ABT-737, the CI values were <1.0, indicating a synergistic combination effect. *C*, similarly, when tested on 8226 myeloma cells, combinations of melphalan (Mel) with ABT-737 (5 μmol/L melphalan + 0.2 μmol/L ABT-737; 10 μmol/L melphalan + 0.4 μmol/L ABT-737; 20 μmol/L melphalan + 0.6 μmol/L ABT-737; and 30 μmol/L melphalan + 0.8 μmol/L ABT-737) have a synergistic combination effect (CI <1.0).



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cells. One possible explanation for this phenomenon is that dexamethasone-resistant cells are more dependent on the mitochondrial pathway for survival and, thus, more accessible to ABT-737 inhibition. Indeed, there is experimental data to show that Bcl-2 is overexpressed in myeloma cells resistant to dexamethasone (24). These data strongly support a rationale for potential use of ABT-737 in treatment of chemoresistant myeloma.

Data from our drug combination studies of ABT-737 with dexamethasone and melphalan support a synergistic antimyeloma effect of ABT-737 with both agents. As dexamethasone and melphalan are drugs frequently used in myeloma clinics and chemoresistance to both are common, ABT-737 holds promise in drug combination therapy of myeloma. Although



**Fig. 6.** ABT-737 suppressed tumor growth in a xenograft mouse myeloma model. *A*,  $2 \times 10^7$  MY5 cells were injected s.c. into the BNX mice. ABT-737 was delivered i.p. when the tumors reached volumes of  $\sim 150 \text{ mm}^3$  (group IV) or  $\sim 380 \text{ mm}^3$  (groups II and III). Group I served as the untreated control. ABT-737 inhibited tumor growth in a dose-dependent manner (groups II and III) and completely regressed the tumors when the treatment was started 26 d after tumor cell inoculation (group IV). *B*, immunostaining with antibody against cleaved caspase-3 in tumor samples (groups I and III) from ABT-737-treated (*b* and *d*) and untreated (*a* and *c*) mice.

**Table 1.** ABT-737 is not cytotoxic to hematopoietic progenitors

Patient no.	Colony no. (%)		
	0 $\mu\text{mol/L}$	0.5 $\mu\text{mol/L}$	1 $\mu\text{mol/L}$
1	100	90	78
2	100	98	95
3	100	100	95
4	100	80	90
5	100	88	78
Average ( $\pm$ SE)	100%	91% ( $\pm 8\%$ )	87% ( $\pm 9\%$ )

dexamethasone has been used in myeloma treatment for over 30 years, the mechanism of dexamethasone-induced apoptosis is still not fully characterized. However, Bcl-X<sub>L</sub> seems to be down-regulated in MM1(Dex)S cells after 6 h of dexamethasone treatment (25), suggesting that ABT-737 and dexamethasone could target the same molecule in the mitochondrial apoptotic pathway. Melphalan-induced apoptosis of myeloma cells is associated with a cleavage of Mcl-1 and Bim and a decrease in the Mcl-1/Bim complex (26). Melphalan induces a drastic down-regulation of Mcl-1, Bcl-X<sub>L</sub>, and BimEL in melphalan-sensitive cells, whereas the most potent proapoptotic isoforms, BimL and BimS, are affected to a lesser extent. The down-regulation of Mcl-1 allows to release of Bim isoforms (L and S), which can exert their proapoptotic function leading to Bax activation and cytochrome *c* release (26).

As IL-6 is an important survival factor for myeloma cells, and stromal cells are important sources for IL-6 production in the bone marrow microenvironment, we investigated the protective effects of IL-6 and stromal cells on ABT-737-induced myeloma cell apoptosis. Both IL-6 and stromal cells had a protective effect in the majority of MM cell lines. Such a protective effect could potentially be associated with the balance of proapoptotic and antiapoptotic proteins in the Bcl-2 family. IL-6 is reported to up-regulate Bcl-X<sub>L</sub> and Mcl-1 (5) and down-regulate three major Bim isoforms (EL, L, and S; ref. 27). These data suggest that it may be important to combine Bcl-2 family inhibitors with agents targeting IL-6 or the bone marrow microenvironment. As a proteasome inhibitor with multiple antimyeloma activities, bortezomib (Velcade) is reported to abrogate IL-6-triggered signaling cascades and down-regulate gp130 in MM (28, 29). ABT-737 targeting of the Bcl-2 family, along with bortezomib targeting of IL-6 pathway, may prove to be beneficial in preclinical or clinical settings of myeloma treatment.

Our *in vivo* drug efficacy study using a xenograft mouse myeloma model s.c. injected with MY5 myeloma cells shows that ABT-737 inhibits tumor growth in a dose-dependent manner. At the dose generating drug response in primary patient samples, ABT-737 has no apparent cytotoxicity to patient hematologic progenitor cells. These results strongly support a rationale for clinical trials with ABT-737 for the treatment of MM.

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