The Proteasome Inhibitor PS-341 Markedly Enhances Sensitivity of Multiple Myeloma Tumor Cells to Chemotherapeutic Agents[1]

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ABSTRACT

Increased nuclear factor κB (NF-κB) activity is associated with increased tumor cell survival in multiple myeloma. The function of NF-κB is inhibited through binding to its inhibitor, IκB. Release of activated NF-κB follows proteasome-mediated degradation of IκB resulting from phosphorylation of the inhibitor and, finally, conjugation with ubiquitin. We report that myeloma cells have enhanced IκBα phosphorylation and increased NF-κB activity compared with normal hematopoietic cells. The proteasome inhibitor PS-341 blocked nuclear translocation of NF-κB, blocked NF-κB DNA binding, and demonstrated consistent antitumor activity against chemoresistant and chemosensitive myeloma cells. The sensitivity of chemoresistant myeloma cells to chemotherapeutic agents was markedly increased (100,000–1,000,000-fold) when combined with a noncytotoxic dose of PS-341 without affecting normal hematopoietic cells. Similar effects were observed using a dominant negative super-repressor for IκBα. Thus, these results suggest that inhibition of NF-κB with PS-341 may overcome chemoresistance and allow doses of chemotherapeutic agents to be markedly reduced with antitumor effects without significant toxicity.

INTRODUCTION

MM[3] is a BM-based B-cell malignancy characterized by the aberrant monoclonal expansion of plasma cells. Although patients may initially respond to chemotherapy and/or steroids, they ultimately suffer from resistant disease (1, 2). Recent studies using high-dose chemotherapy followed by autologous BM transplantation demonstrated a survival benefit compared with conventional chemotherapy (3). However, MM remains incurable and is associated with significant morbidity. Clearly, more effective and less toxic treatment options are needed in the battle against MM.

Members of the NF-κB transcription factor family may play an important role in the pathogenesis of lymphoid malignancies (4–8). The formation of a heterodimer between subunits p50 and p65 is the most common NF-κB complex (9, 10). In unstimulated cells, NF-κB complexes are associated with a class of inhibitory proteins, called IκB. NF-κB is bound to IκB in the cytoplasm and the transcription factor is thereby rendered inactive. The family of inhibitor proteins includes IκBα, IκBβ, IκBγ, and the putative proto-oncogene product bcl-3 (11). NF-κB activation generally requires signal-induced degradation of IκBα, thus releasing the NF-κB transcription factor so that it can translocate to the nucleus (12–14). The IκB proteins contain multiple ankyrin repeats and physically associate with NF-κB proteins. Signal-induced activation of NF-κB is preceded by phosphorylation and degradation of IκBα (15–18) through the ubiquitin-proteasome pathway (19). NF-κB is involved in the control of various cellular processes, such as immune and inflammatory responses, cell growth, bone resorption, and apoptosis. Importantly, NF-κB activation increases the survival of tumor cells and confers their resistance to chemotherapy (20–23). Elevated levels of NF-κB activity were found in relapsing MM (24), suggesting that this transcription factor could be used as a prognostic marker as well as a target for therapy to prevent progression of the disease.

Newly developed proteasome inhibitors, such as PS-341 (Millennium Inc., Boston, MA), suppress NF-κB activity by inhibiting IκBα degradation (25–27). The inhibition of NF-κB activity by this proteasome inhibitor correlated with antitumor activity against human prostate cancer and Burkitt's lymphoma in murine models (26, 27). However, it would be an oversimplification to attribute all of the antitumor effects of PS-341 to the inhibition of NF-κB activity. PS-341 has been shown to affect many intracellular regulatory molecules such as p53 and

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[3] The abbreviations used are: MM, multiple myeloma; NF-κB, nuclear factor κB; BM, bone marrow; BMMC, bone marrow mononuclear cell; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GC, glucocorticosteroid.

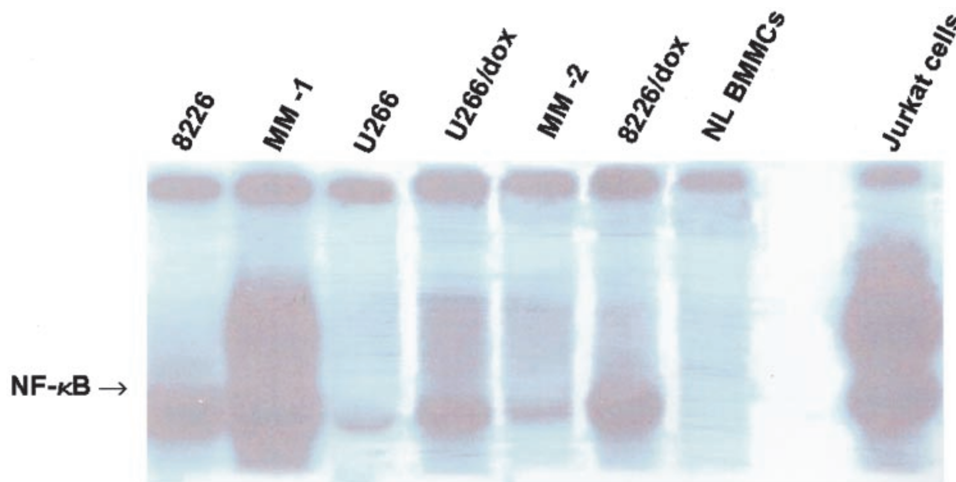

Fig. 1 NF-κB activity is elevated in MM cell lines. EMSA analysis of the activity of NF-κB in both chemoresistant (U266/dox and RPMI 8226/dox) and chemosensitive (U266 and RPMI8226) cell lines, two BMMCs (MM-1 and MM-2) from MM patients, and normal BMMCs is shown. Nuclear extracts derived from 12-*O*-tetradecanoylphorbol-13-acetate + calcium ionophore-treated Jurkat cells served as a positive control. The same amount of nuclear proteins was loaded for each sample.

cyclin-dependent kinase inhibitors p21 and p27 (25). PS-341 can also block the antiapoptotic effects of Bcl-2, even though the antiapoptotic effects of Bcl-2 are independent of NF-κB activity. PS-341 induced apoptosis of human IgAκ (ARP-1) myeloma cells that overexpressed Bcl-2 protein in a dose-dependent fashion (28). PS-341 can also decrease the binding of MM cells to BM stromal cells, and we know that the interaction between MM cells and BM stromal cells through extracellular molecules plays an important role in the survival of MM cells (29). PS-341 has shown growth-inhibitory effects in Lewis lung carcinoma treated with doxorubicin, 5-fluorouracil, cisplatin, and paclitaxel (27). This drug also demonstrated antiangiogenic effects in an orthotopic pancreatic cancer model (32). PS-341 has shown anti-MM activity *in vitro* (29). Importantly, a Phase I clinical trial evaluating single-agent PS-341 for patients with chemoresistant MM showed antitumor responses (31). In addition, early results from an ongoing Phase II clinical trial show evidence of antitumor activity in relapsing MM patients (32). We investigated the relationship between the level of NF-κB activity and the sensitivity of MM cells to cytotoxic agents and whether the proteasome inhibitor PS-341 would suppress the growth of MM cells and sensitize them to treatment with cytotoxic agents.

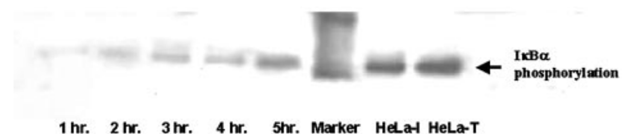

Fig. 2 Phosphorylation of IκBα is increased in MM cells exposed to doxorubicin. The MM cell line U266 was treated with doxorubicin (4×10^{-9} M) for up to 5 h. Phosphorylation of IκBα was determined using a Western blot assay. Cytosolic protein extracts were collected hourly after these cells were exposed to doxorubicin. Positive controls were HeLa cells 12 min after TNF-α (*HeLa-T*) or IL-1 (*HeLa-I*) treatment. Equivalent amounts of cytosol protein were loaded for each sample.

MATERIALS AND METHODS

Cell Lines and Fresh BM/PB Samples. Myeloma cell lines U266, RPMI8226, NCI-H929, WAD-1, and ARH77 were considered to be chemosensitive. Various chemoresistant MM cell lines were developed by Dr. William Dalton (University of South Florida, Tampa, FL) through selection for resistance to specific drugs, such as doxorubicin (U266/dox4 and 8226/dox4), melphalan (U266/LR7 and 8226/LR5), and mitoxantrone (8226/MR20). These drug-resistant lines were maintained in the medium with a certain concentration of chemotherapeutic agents. Five other cell lines including three leukemia cell lines (NB-4, U937, and molt-4), the primary effusion lymphoma cell line KS-1, and the kidney tumor cell line 293 were kindly supplied by Dr. Phillip Koeffler (Cedars-Sinai Medical Center, Los Angeles, CA). BM aspirates were collected from MM patients with high tumor burden and healthy individuals. PB samples were obtained from healthy individuals. Samples were obtained after informed consent was obtained in accordance with the Human Subjects Protection Committee. Informed consent was obtained from patients. Human experimentation guidelines of the United States Department of Health and Human Services and those of the authors' institutions were followed in the conduct of clinical research.

BM aspirates and PB specimens were collected in heparinized tubes and then diluted 1:1 with Leibovitz L-15 medium (Life Technologies, Inc., Grand Island, NY) supplemented with fetal bovine serum (Life Technologies, Inc.). The mononuclear cell fraction was enriched by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient sedimentation at $600 \times g$ for 20 min and washed twice in L-15/fetal bovine serum media. The percentage of tumor cells in fresh BM samples was determined by microscopic examination along with immunohistochemistry showing light chain restriction.

CD34-expressing cells were selected from healthy individuals' (donors for patients undergoing allogeneic transplantation) BMMCs using the Miltenyi column (Miltenyi Biotec, Auburn, CA). BMMCs were placed on the Miltenyi column and washed with Miltenyi washing buffer. The CD34-expressing cells were harvested by washing the column with Miltenyi elution buffer, and the harvested cells contained 80–95% CD34-expressing cells.

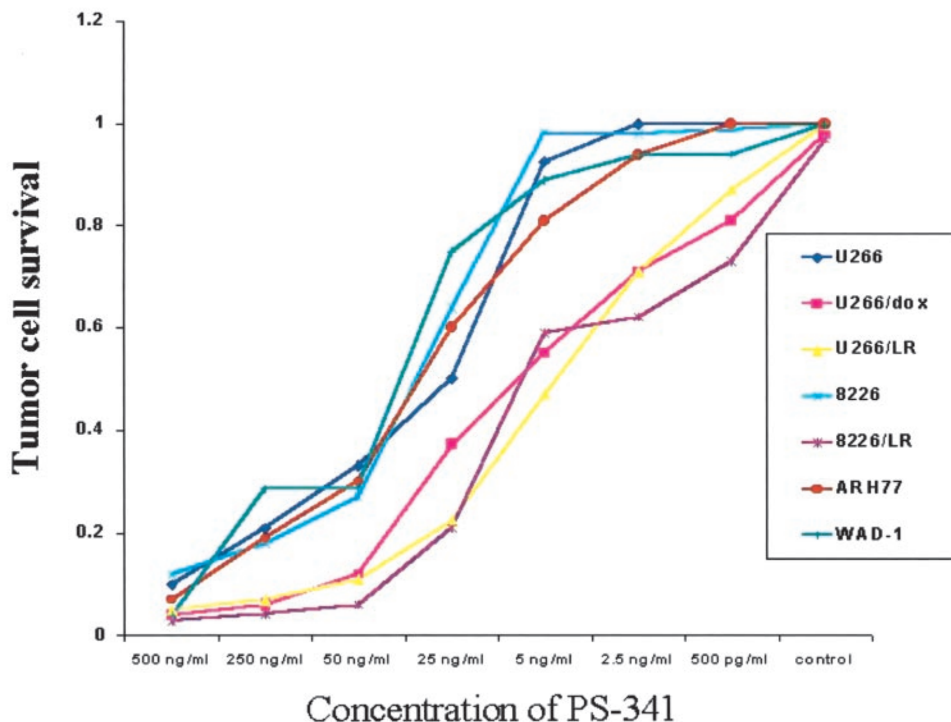

Fig. 3 PS-341 inhibits the growth of MM cells. The proliferation test (MTT assay) on chemosensitive (U266, ARH77, RPMI8226, and WAD-1) and chemoresistant (U266/LR7, U266/dox4, and RPMI8226/LR5) MM cell lines were treated with PS-341 (500 pg/ml to 500 ng/ml).

BMMCs, PBMCs, and MM cell lines were suspended in 10 ml of RPMI medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS (Gemini Bio-products, Calabasas, CA), penicillin (100 units/ml; Irvine Scientific), and streptomycin (100 mg/ml; Irvine Scientific) in 75-cm flasks (Costar, Cambridge, MA). Cells were incubated at 37°C in a CO_2 incubator.

Nuclear and Cytosolic Extract Preparation. Nuclear and cytosolic extracts were prepared according to the method of Schreiber *et al.* (33), with modifications. Briefly, cell pellets were suspended in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1× Complete (Boehringer Mannheim), and 0.5% NP40 (Fluka, Milwaukee, WI)] and incubated on ice for 15 min followed by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant that contained cytosolic protein was separated and stored at −80°C. The pellet was resuspended with buffer B [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, and 1× Complete], kept on ice for 30 min, and spun down at 12,000 rpm for 5 min at 4°C. The supernatants containing nuclear protein were stored at −80°C. The protein concentrations were determined with the Bradford method (Bio-Rad Laboratories).

EMSA. The sequence (5′-3′) of the oligonucleotide is derived from the NF-κB binding sequence from the promoter region of TNF-α. The oligonucleotide was end-labeled with [^{32}P]CTP and purified through G-25 columns. Ten μg of nuclear extracts from MM cell lines and fresh BM aspirates from either MM patients or healthy subjects were mixed with the radiolabeled NF-κB oligonucleotide (5–20 × 10^4 cpm) in binding buffer containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 10 mM Tris-HCl at pH 7.5. The reaction mixtures were incubated for 20 min at room temperature followed by electrophoresis on a 4% nondenaturing polyacrylamide gel.

Fluorescence Assay for Intracellular Presence of NF-κB. The relative amounts of nuclear and cytoplasmic NF-κB were evaluated using the NF-κB HitKit (Cellomics, Inc., Pittsburgh, PA). Using a 96-well plate, 5000 cells/well were incubated for at least 4 h. Next, culture medium was aspirated and replaced with 200 μl/well prewarmed fixation solution. The cells were incubated at room temperature for 10 min, and the fixation solution was replaced with permeabilization buffer. After washing the plate with 200 μl/well washing buffer, 50 μl of primary antibody solution were added into each well, followed by incubation for 1 h at room temperature. Next, 50 μl of staining solution were added to each well and also incubated for 1 h. The cells were incubated with 200 μl of detergent buffer for 10 min, which was replaced with 200 μl of washing buffer. The cells were evaluated using an UV light microscope.

Western Blot Analysis. Phosphorylation of IκBα was analyzed using the PhosphoPlus IκBα kit (Cell Signaling, Beverly, MA). Cytosol proteins were run on a 10% polyacrylamide gel and then transferred to polyvinylidene difluoride membrane and hybridized with anti-IκBα Ser-32 and anti-IκB antibodies. Western blots were developed using an electrochemiluminescence reagent kit (Kirkegard & Perry Laboratories). The intensity of protein bands was measured by autoradiography.

Cell Proliferation Test (MTT Assay). Cells were cultured in T25 flasks at 37°C. After starvation overnight, cells were placed in 96-well plates in fresh medium with cytotoxic agents (Becton Dickinson Labware, Franklin Lakes, NJ). MTT reagent (Sigma, St. Louis, MO) was added to each well after

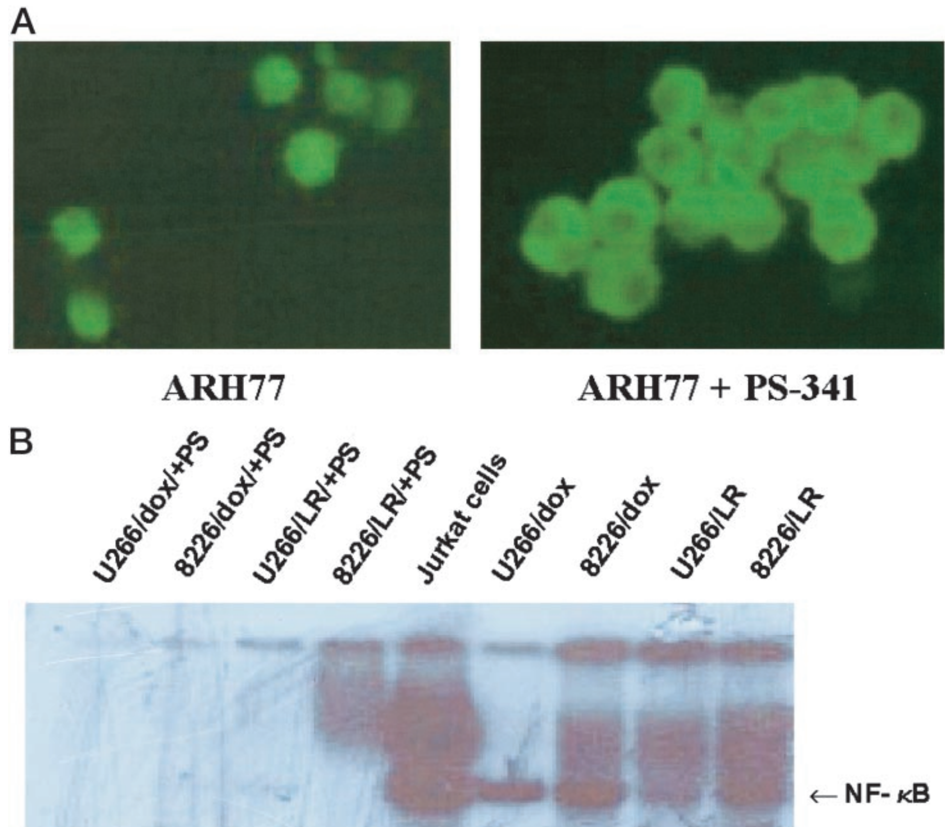

Fig. 4 PS-341 reduces NF-κB activity in MM cell lines. *A,* ARH77 cells before and after exposure to PS-341 (10 ng/ml) for 16 h. The NF-κB localization was detected using a fluorescence-labeled anti-NF-κB antibody. *B,* nuclear extracts were obtained from MM cell lines (U266/dox4, PMI8226/dox, U266/LR7, and RPMI8226/LR5) after treatment with PS-341 (10 ng/ml), and EMSA was performed. The nuclear extract derived from 12-*O*-tetradecanoylphorbol-13-acetate + calcium ionophore-treated Jurkat cells was a positive control.

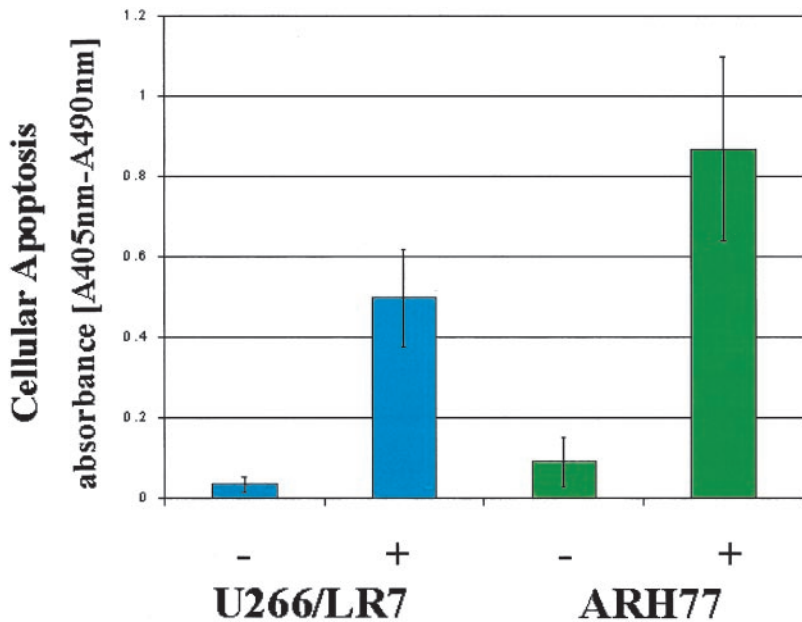

Fig. 5 Transfection with a dominant negative IκBα (dm IκBα) produced apoptosis in MM cells. MM cell lines (ARH77 and U266/LR7) were exposed to recombined adenovirus with the super-repressor dm IκBα and analyzed using photometric ELISA. ARH77 and U266/LR7 cell lines infected with the adenovirus control vector (without the IκBα gene) were used as negative controls. + indicates transduction with dm IκBα, and − indicates transduction with the control vector.

48 h. The absorbance of samples was determined using a microtiter plate (ELISA) reader at a wavelength of 575 nm. The reference wavelength was 650 nm.

Apoptosis Assay. Apoptosis was determined by using the annexin V-EGFP kit (Clontech, Palo Alto, CA) or the cell death detection ELISA plus kit (Roche, Mannheim, Germany). Cells (1×10^5) were either treated with PS-341 or transduced by dominant negative IκBα. For the annexin V-EGFP kit, cells were incubated with 5 μl of annexin V and 10 μl of propidium iodide for 15 min and then evaluated by flow cytometry. For the

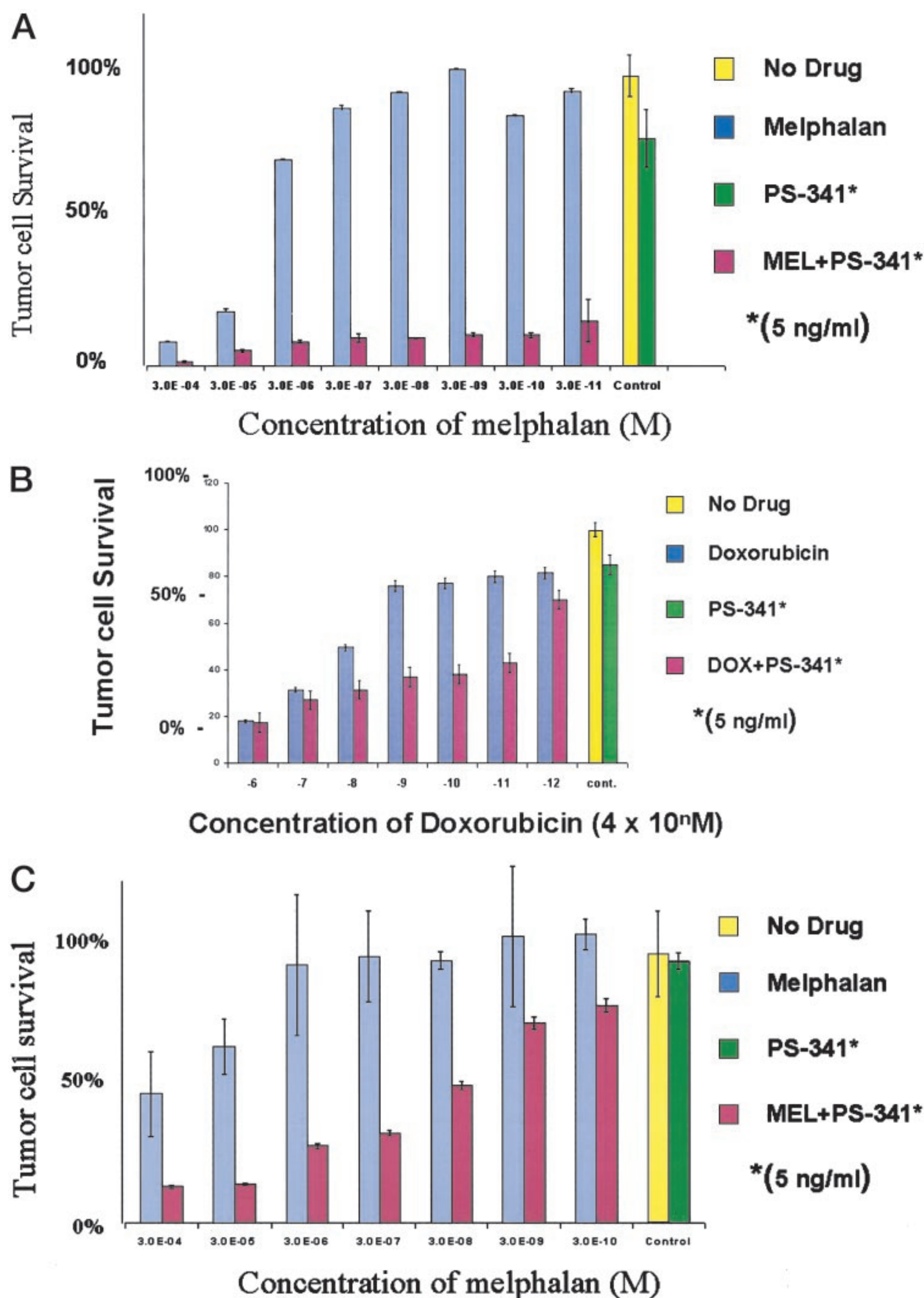

Fig. 6 Addition of PS-341 to chemotherapeutic agents enhances cytotoxicity of MM cells. *A* and *B,* MM cells (RPMI8228/LR and U266/dox4) treated for 24 h with melphalan (3×10^{-4} to 3×10^{-11} M) or doxorubicin (4×10^{-6} to 4×10^{-12} M) alone or melphalan with 5 mg/ml PS-341 (MTT assay). *C,* fresh MM BMMCs treated with melphalan (3×10^{-4} to 3×10^{-11} M) alone or melphalan with 5 mg/ml PS-341. *D,* analysis of apoptosis of the MM cell line RPMI8226/LR5 exposed to melphalan alone or melphalan with a relatively noncytotoxic dose of PS-341 (5 mg/ml). *E,* MTT assay on leukemia cell lines molt-4 and normal BMMCs and phytohemagglutinin-stimulated PBMCs treated with various concentrations of melphalan alone or melphalan with a relatively noncytotoxic dose of PS-341 (5 mg/ml).

cell death detection ELISA plus kit, 1×10^4 cells were lysed with 200 μl of lysis buffer. Cell lysates were placed on streptavidin-coated multiwell plates and incubated with immunoreagent for 2 h. Plates were read using a mQuant plate reader for photometric analysis.

Adenovirus Transfections with Dominant Negative IκBα. Recombinant replication-deficient adenoviruses containing dominant negative IκBα, which were kindly supplied by Dr. Richard Gaynor (University of Texas Southwestern Medical School, Dallas, TX), were used to infect 5×10^5 MM cells (ARH77 and U266/LR7) in serum-free medium at a ratio of 10,000 viral particles/cell for 72 h. The viral titers were determined based on the percentage of NIH3T3 cells infected after exposure to freshly collected virus. Adenovirus containing the transfection vector without the IκBα gene was used as negative control. After incubation, cells were washed with serum-free medium, followed by quantitative measurement of apoptosis by ELISA or annexin V assay.

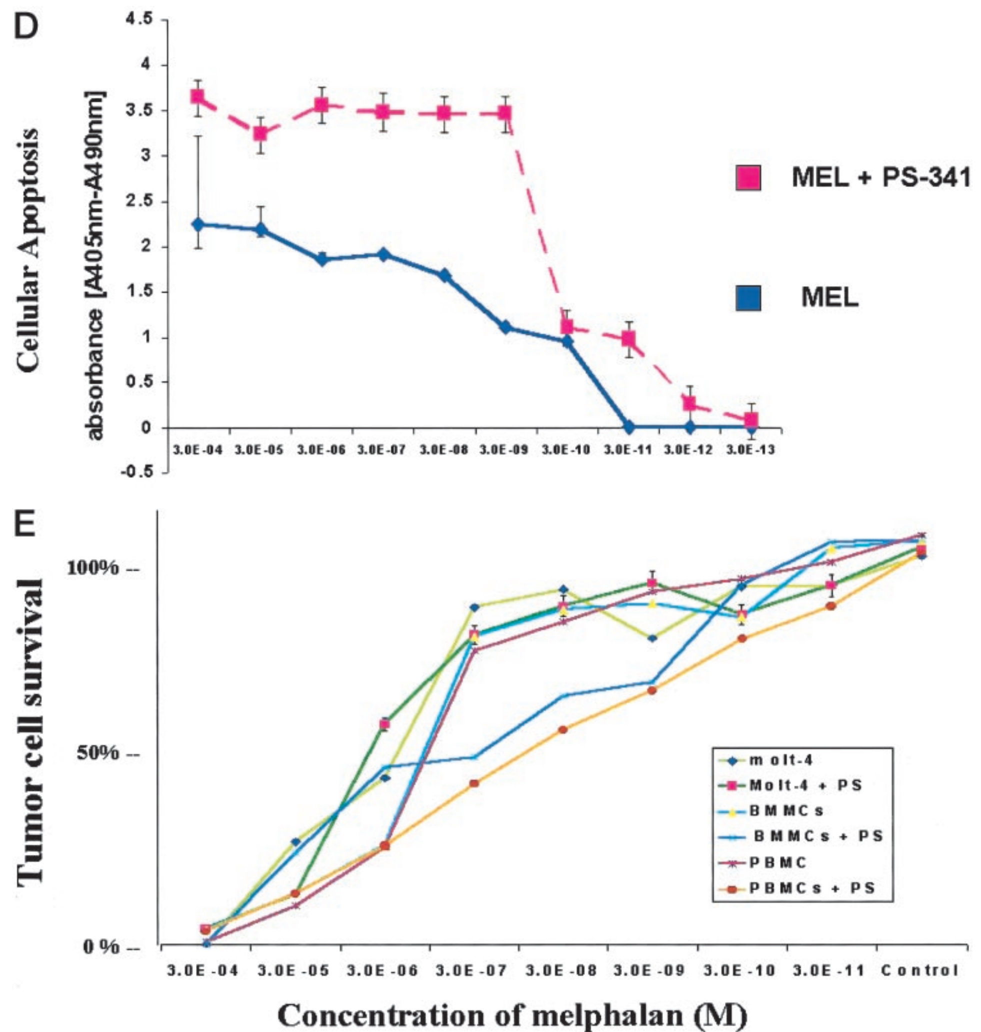

Fig. 6 Continued.

RESULTS

The Activity of NF-κB Is Elevated in MM Cells. To measure NF-κB activity, we obtained nuclear extracts from chemoresistant and chemosensitive MM cell lines and fresh BMMCs from MM patients ($n = 4$) with a high proportion of tumor cells (80–95%). These extracts were incubated with [^{32}P]CTP-labeled oligonucleotide probes containing a NF-κB-binding sequence derived from the promoter region of the TNF-α gene. We used the nuclear proteins from BMMCs from healthy individuals as controls. The activity of NF-κB was elevated in both chemoresistant and chemosensitive cell lines as well as in fresh BMMCs from MM patients when compared with normal BMMCs (Fig. 1). Moreover, among four MM cell lines, the density of the DNA-binding band showed that the NF-κB activity was approximately 3 times higher in the U266/dox chemoresistant MM cell line than in the U266 chemosensitive MM cell line. However, no difference in NF-κB activity was found between RPMI8226 and RPMI8226/dox4 MM cell lines.

Next, we determined whether the exposure of MM cells to chemotherapy decreases IκB protein levels. Cytosol protein extracts were obtained at 1, 2, 3, 4, and 5 h after doxorubicin treatment of the U266 cell line with low concentrations (4×10^{-9} M) of doxorubicin, which corresponds to approximately one-tenth of the serum concentration measured in patients who receive standard doses of this agent. First, the amount of IκBα phosphorylation, as determined by an antibody recognizing phosphorylated IκBα, increased after exposure to doxorubicin (Fig. 2). Second, as measured by a Western blot assay, the level of cytoplasmic IκBα protein decreased after exposure to this drug (data not shown).

The Proteasome Inhibitor PS-341 Reduces NF-κB DNA Binding and Causes Cytotoxicity of MM Cell Lines. Because the degradation of phosphorylated IκBα is mediated by the proteasome, inhibiting its function should increase IκBα levels, thus inhibiting NF-κB activity. We examined the effects of the proteasome inhibitor PS-341 (500 pg/ml to 500 ng/ml) on the growth of MM cell lines using the MTT assay. The growth of chemosensitive (U266, ARH77, RPMI8226, and WAD-1) and chemoresistant (U266/LR7, U266/dox4, and RPMI8226/

LR5) MM cell lines was substantially inhibited by PS-341 (Fig. 3) with an IC_{50} of 10–40 ng/ml. Interestingly, all chemoresistant cell lines demonstrated a right shift in their dose-response curves, indicating that these chemoresistant cell lines were more sensitive to treatment with PS-341 than chemosensitive lines, consistent with their enhanced NF-κB activity. We determined whether treatment with PS-341 decreased NF-κB DNA binding activity using the EMSA and fluorescence assay. After 10 h of treatment with 10 ng/ml PS-341 (a dose that is relatively equivalent to those used in the clinical patients), we performed EMSA on nuclear protein extracts from MM cell lines (U266/dox4, RPMI8226/dox4, U266/LR7, and RPMI8226/LR5), and we also tested the intracellular location of NF-κB in ARH77 cells using a fluorescence-based antibody assay. After treatment with PS-341, the vast majority of NF-κB was contained in the cytoplasm, where it cannot function as a transcription factor, whereas most of the protein complex is located in the nucleus in the absence of the proteasome inhibitor (Fig. 4*A*). We also evaluated the DNA binding capacity of NF-κB in MM cell lines after exposure to PS-341. PS-341 markedly decreased NF-κB DNA binding activity in a variety of chemosensitive and chemoresistant cell lines (Fig. 4*B*).

Transfection with a Dominant Negative IκBα Produced Apoptosis in MM Cells. To more fully establish the importance of inhibiting NF-κB activity as a potential therapeutic target in MM, we transduced two MM cell lines (ARH77 and the melphalan-resistant U266/LR7) with an adenovirus vector carrying an inhibitor of NF-κB activity, a dominant negative IκBα. This super-repressor of IκBα (dm IκBα) has alterations at amino acid positions 32 and 36 (from serine to alanine) and is resistant to phosphorylation, resulting in increased cellular levels of IκBα. With increased amounts of cellular IκBα bound to NF-κB, the activity of NF-κB will be suppressed. The cell lines were exposed to recombined adenovirus with the super-repressor form (dm IκBα) for 2 h and analyzed using both the annexin V apoptosis assay and photometric ELISA. ARH77 and U266/LR7 cell lines infected with the adenovirus control vector (without the IκBα gene) were used as negative controls. Compared with the negative controls, apoptosis was increased by approximately 13 and 8 fold in the U266/LR7 and ARH77 cells transfected with dm IκBα, respectively (Fig. 5). These results support the concept that increasing cellular levels of IκBα (which also results with PS-341 treatment) is a potential means of producing cytotoxicity in chemosensitive as well as chemoresistant MM.

Addition of PS-341 to Chemotherapeutic Agents Enhances Cytotoxicity of MM Cells. We determined whether there was a synergistic anti-MM effect between the proteasome inhibitor and a variety of chemotherapeutic agents. Chemosensitive MM cell lines (U266, ARH77, WAD-1, and RPMI 8226) and MM cell lines resistant to doxorubicin (U266/dox4 and RPMI8226/dox4), mitoxantrone (RPMI8226/MR20), and melphalan (U266/LR7 and RPMI8226/LR5) were evaluated using the drugs alone at various concentrations and then with the addition of a noncytotoxic dose of PS-341 (5 ng/ml). No significant growth inhibition of both of the melphalan-resistant MM cell lines was observed when using chemotherapeutic agents alone, unless their concentration was at high levels (3×10^{-5} M; Fig. 6*A*). In contrast, when the resistant MM cell lines were exposed to melphalan with PS-341, these cell lines became extremely sensitive to melphalan at a concentration of 3×10^{-11} M, which is 1,000,000-fold lower than the concentration necessary for melphalan alone to induce cytotoxicity. Similar effects were observed between PS-341 and doxorubicin or mitoxantrone, with PS-341 increasing the sensitivity of the doxorubicin-resistant and mitoxantrone-resistant MM cell lines by 100,000-fold each (Fig. 6*B*). Similar synergistic effects were also found in the treatment with the same combinations in fresh BM samples obtained from active patients with MM (Fig. 6*C*).

To further demonstrate the enhancement of chemotherapy-induced cytotoxicity by PS-341, we performed an ELISA-based apoptosis assay on RPMI 8226/LR5 cells with PS-341 (5 ng/ml) and varying concentrations of melphalan as outlined above (Fig. 6*D*). There was a marked increase in apoptosis induced by PS-341 and melphalan compared with melphalan alone, which correlated with the results of the MTT assay.

To determine the effects of PS-341 on other tumor types, we treated the T-cell leukemia cell line NB-4, human myeloid leukemia cell line U937, B-cell leukemia cell line molt-4, primary effusion lymphoma cell line KS-1, and kidney tumor cell line 293 with PS-341 alone or in combination with chemotherapeutic agents. Similar experiments were also performed on normal unstimulated and stimulated (with 20 nM phytohemagglutinin for 30 min) PBMCs and CD34-selected BMMCs obtained from healthy individuals. Suppression of proliferation in either these non-MM cell lines or normal hematopoietic cells was found with PS-341 treatment at higher concentrations (IC_{50}, 100–300 ng/ml) than in the MM cell lines (IC_{50}, 10^{-40} ng/ml; data not shown). When added to chemotherapy, PS-341 showed very little synergism in inhibiting the proliferation of these non-MM cell lines or normal CD34-selected BMMCs or PBMCs (Fig. 6*E*).

DISCUSSION

MM patients develop chemoresistance, and increasing the concentrations of cytotoxic drugs fails to significantly improve the therapeutic response. One hypothesis for the development of chemoresistance is related to acquired resistance of the tumor cells to apoptosis (34), allowing tumors to withstand high levels of chemotherapy. Tumor cells that are resistant to apoptosis also exhibit increased proliferative capacity. Wang *et al.* (35) reported that activation of NF-κB in response to chemotherapy is a principal mechanism of tumor chemoresistance. Inhibition of such inducible NF-κB activation enhances apoptosis. Increased activity of NF-κB proteins has been reported in solid tumors resistant to chemotherapy (36). In this report, we found that the activity of NF-κB was elevated in MM tumor cells. The activity of NF-κB appeared to be higher in chemoresistant MM cell lines compared with chemosensitive ones, although the difference was not always consistent between chemosensitive *versus* chemoresistant MM cells (Fig. 1). Further work is needed to clarify this. The level of IκBα phosphorylation was significantly higher, and amounts of this inhibitor were reduced in MM cells after exposure to chemotherapeutic agents. These results suggest that increases in NF-κB activity may help MM cells evade chemotherapy-induced cytotoxicity.

Phosphorylated IκBα becomes ubiquitinated and then becomes degraded through the proteasome pathway, resulting in release of activated NF-κB. The blockage of this degradation by proteasome inhibitors such as PS-341 increases the level of

IκBα, resulting in the inhibition of the function of NF-κB. In this report, PS-341 was shown to markedly reduce nuclear localization of this transcription factor and its DNA binding activity and markedly suppress growth of tumor cells from MM patients and cell lines. Among the MM cell lines analyzed, the chemoresistant lines were more sensitive to treatment with PS-341 than the chemosensitive lines.

The use of other combinations of treatment for cancer patients has been shown to be more efficacious than single-agent therapy (37–40). In MM patients, the addition of GCs to chemotherapy (melphalan) improves overall survival (41). Part of the efficacy of this combination may result from the effects of GCs on NF-κB activity. It has recently been shown that GCs interfere with the interaction of one of the NF-κB members, p65, with the basal transcription machinery (42). This inhibition of NF-κB function by GCs may lead to the sensitization of MM cells to the cytotoxic effects of chemotherapy, similar to what we have shown in this study with PS-341. By combining a noncytotoxic PS-341 dose with the chemotherapeutic agents melphalan, doxorubicin, or mitoxantrone, we overcame drug resistance in highly chemoresistant MM cell lines. These cell lines became sensitive to chemotherapeutic agents at markedly lower concentrations (100,000–1,000,000-fold) than were necessary to kill these cells without PS-341. Both increased apoptosis and a reduction in tumor cell accumulation result from exposure of MM cells to chemotherapeutic agents with the addition of PS-341. In contrast, we did not find any significant cytotoxic effect of the proteasome inhibitor alone or in combination with chemotherapy, except at very high doses in normal samples (CD34-selected BMMCs and PBMCs) that were obtained from healthy individuals and cell lines derived from other types of tumors. These results suggest that the antitumor effects of PS-341 alone and its enhancement of chemotherapy cytotoxicity have differential effects on MM cells than on normal hematopoietic stem and PB cells.

To provide further support for the importance of inhibiting NF-κB as an effective therapy for MM, we performed studies on MM cells that were infected with dominant negative IκBα, and we showed a similar apoptosis-enhancing effect. These data provide further support for inhibiting NF-κB activity as a new strategy for the treatment of MM. Although these results suggest that proteasome inhibitors and other agents that increase IκBα levels may overcome chemoresistance by inhibiting NF-κB activity in MM cells, it is still possible that other factors may contribute to this effect. In fact, recent studies show that another inhibitor of IκBα, Bay 11-7085, actually produces its antileukemic effects through a p38 mitogen-activated protein kinase-dependent but NF-κB-independent mechanism (43). In addition, drug resistance to camptothecins may result from proteasome-mediated degradation of topoisomerase I rather than IκB (44). It also remains to be determined what leads to the increased NF-κB activity in MM cells. Recent studies from our laboratory and others suggest that polymorphisms and/or mutations of the IκBα gene might reduce the function and/or levels of this inhibitor among patients with MM and other B-cell malignancies (45). Abnormalities in other parts of the signal transduction pathway may also be present in MM patients' tumor cells, such as alterations in the IκB kinase complex or tumor necrosis receptor-associated factors.

Our findings indicate that resistance to chemotherapy in MM may be related to overactivity of NF-κB. By using the proteasome inhibitor PS-341, which reduces NF-κB activity, chemoresistance in MM cells can be overcome without significant effects on normal hematopoietic cells. Combining PS-341 with chemotherapeutic reagents markedly reduces the concentrations of these drugs that are cytotoxic without affecting normal hematopoietic cells. Thus, these results should provide the basis for clinical trials using drug combinations involving much lower doses of chemotherapy with inhibitors of NF-κB activity that should produce maximal anti-MM activity with minimal toxicity.

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