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Curcumin Circumvents Chemoresistance *in vitro* and Potentiates the Effect of Thalidomide and Bortezomib against Human Multiple Myeloma in Nude Mice Model

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Abstract

Curcumin (diferuloylmethane), a yellow pigment in turmeric, has been shown to inhibit the activation of NF-κB, a transcription factor closely linked to chemoresistance in multiple myeloma (MM) cells. Whether curcumin can overcome chemoresistance and enhance the activity of thalidomide and bortezomib, used to treat patients with MM, was investigated *in vitro* and in xenograft model in nude mice. Our results show that curcumin inhibited the proliferation of human MM cells regardless of their sensitivity to dexamethasone, doxorubicin, or melphalan. Curcumin also potentiated the apoptotic effects of thalidomide and bortezomib by downregulating the constitutive activation of NF-κB and Akt; and this correlated with the suppression of NF-κB-regulated gene products, including cyclin D1, Bcl-xL, Bcl-2, TRAF1, cIAP-1, XIAP, survivin, and VEGF. Furthermore, in a nude mice model, we found that curcumin potentiated the antitumor effects of bortezomib ($P < 0.001$, vehicle vs. bortezomib plus curcumin; $P < 0.001$, bortezomib vs. bortezomib plus curcumin), and this correlated with suppression of Ki-67 ($P < 0.001$ vs. control), CD31 ($P < 0.001$ vs. vehicle), and VEGF ($P < 0.001$ vs. vehicle) expression. Collectively, our results suggest that curcumin overcomes chemoresistance and sensitizes MM cells to thalidomide and bortezomib by downregulating NF-κB and NF-κB-regulated gene products.

Keywords

Curcumin; multiple myeloma; NF-κB; chemotherapy

Introduction

In the United States, 50,000 patients are affected by multiple myeloma (MM), 16,000 patients are newly diagnosed with MM, and 11,000 MM patients die of the disease each year (1). MM is a late-stage B-cell malignancy characterized by the infiltration of malignant plasma cells in bone marrow, and is associated with high monoclonal (M) protein in the blood. MM can occur

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de novo or it can evolve from benign monoclonal gammapathy of undetermined significance (MGUS) that involves in low levels of bone marrow plasmacytosis and M protein. Approximately 1% of patients with MGUS develop MM each year. The current standard treatments for MM are high-dose chemotherapy and stem cell transplantation. Although a wide range of new drugs, including bortezomib (Velcade), thalidomide (Thalomid), and lenalidomide (Revlimid), a thalidomide analogue, have been recently approved, MM remains an incurable disease (2). The majority of MM patients eventually experience a relapse, their disease becomes chemoresistant, and they die of the disease.

Why MM patients develop resistance to chemotherapy is not well understood, but numerous lines of evidence suggest that the transcription factor NF-κB may plays a major role in pathogenesis of MM. First, NF-κB has been shown to be constitutively active in MM cell lines (3) and in $CD138^+$ MM cells obtained from patients (4). Second, the NF-κB-regulated cyclin D1 gene is frequently dysregulated in MM patients (5). Third, IL-6, which is also regulated by NF-κB, can suppress apoptosis in MM cells (6). Fourth, the NF-κB-regulated antiapoptotic proteins, C-reactive protein (CRP), and cell adhesion molecules have been linked to chemoresistance in MM cells (7–9). Fifth, two recent reports used a whole genome-based screen approach and showed that cells from MM patients have alterations in numerous genes that control the constitutive activation of NF-κB (10,11). The mechanisms involved are the inactivation of *TRAF2*, *TRAF3*, *CYLD*, *cIAP-1*, and *cIAP-2* and the activation of *NF-κ B1*, *NF-κ B2*, *CD40*, *LTBR*, *TACI*, and *NIK*. Sixth, NF-κB signaling in stromal cells can lead to production of IL-6, BAFF, or APRIL, which can activate NF-κB and cause proliferation of MM cells (12). Based on these lines of evidence, agents that can inhibit NF-κB activation and are pharmacologically safe have the potential to overcome chemoresistance and potentiate the antitumor effects of existing chemotherapeutic agents, such as thalidomide and bortezomib.

Because of its ability to suppress NF-κB activation (13), we hypothesize that curcumin (diferuloylmethane), a pharmacologically safe agent, can reverse chemoresistance and potentiate effects of existing chemotherapy. First, curcumin can suppress NF-κB activation and inhibit the proliferation of MM cells (3,4). Additional support for this hypothesis include the following: curcumin can downregulate cyclin D1 expression (14); curcumin has been shown to suppress the expression of IL-6 (15); curcumin can inhibit cell survival, proliferation, invasion, and angiogenic gene products (16); curcumin can downregulate the activation of STAT3 (4,15), a transcription factor that has also been linked with chemoresistance in MM cells (17); curcumin binds directly to P-glycoprotein, inhibits drug transport (18). In addition, a clinical trial of curcumin showed that it is safe even when consumed at 12 g/day (19). Furthermore, the administration of curcumin at a dose of 2 g/day for 4 weeks to asymptomatic MM patients downregulated NF-κB activation in peripheral blood lymphocytes (20).

We tested our hypothesis by examining whether curcumin overcomes the resistance of MM cells to chemotherapy and potentiates the antitumor effects of thalidomide and bortezomib. Our results show that curcumin can overcome the chemoresistance of MM cells to conventional chemotherapeutic agents and potentiate the effects of bortezomib and thalidomide by inhibiting the NF-κB activation pathway, which leads to the downregulation of NF-κB-regulated antiapoptotic gene products.

Materials and Methods

Materials

Curcumin (77.5% curcumin, 18.27% demethoxycurcumin, 4.21% bisdemethoxycurcumin; also called C3 complex) was kindly supplied by Sabinsa Corp. (Piscataway, NJ). A 25-mM solution was prepared in dimethyl sulfoxide, stored as small aliquots at −20°C, and diluted as needed in cell culture medium. Penicillin, streptomycin, and RPMI 1640 medium were

obtained from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Bortezomib (PS-341) was obtained from Millennium (Cambridge, MA), and thalidomide was obtained from Tocris Cookson (St. Louis, MO). Dexamethasone, melphalan, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and anti-β-actin were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Antibodies against PARP, cIAP-1, Bcl-2, Bcl-xL, and TRAF1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-XIAP and survivin antibodies were obtained from BD Biosciences (San Jose, CA). Anti-VEGF and Ki-67 antibodies were obtained from NeoMakers (Fremont, CA). Polyclonal antibody against p65 was obtained from Abcam, Inc. (Cambridge, MA). γ^{32}P-ATP was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Phospho-specific anti-Akt (Ser 473) and anti-Akt antibodies were obtained from Cell Signaling (Beverly, MA).

Cell lines

Human MM cell lines U266, RPMI-8226, RPMI-8226-Dox-6 (a doxorubicin-resistant clone), RPMI-8226-LR-5 (a melphalan-resistant clone), MM.1 (also called MM.1S) and MM.1R (a dexamethasone-resistant variant of MM.1) cell lines were described previously (21). The U266, RPMI-8226, MM.1, and MM.1R cells as well as the Dox-6 and LR-5 variants were cultured in RPMI 1640 medium supplemented with 10% FBS and 1 × antibiotic/antimycotic.

Proliferation assay

The antiproliferative effects of curcumin on drug-sensitive and drug-resistant cells were determined by the MTT uptake method as described previously (21).

Activation of NF-κB in MM cells and tumor samples

To assess NF-κB activation, we isolated nuclei from MM cell lines and tumor samples and carried out electrophoretic mobility shift assays (EMSAs) essentially as described previously (22). In brief, nuclear extracts prepared from MM cancer cells (1×10^6/mL) and tumor samples were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide from the HIV long terminal repeat (5′-TTGTTACAA**GGGACTTTC** CGCTG **GGGACTTTC** CAGGGA GGCGT GG-3′; boldface indicates NF-κB binding sites) for 15 minutes at 37°C. The resulting DNA-protein complex was separated from free oligonucleotides on 6.6% native polyacrylamide gels. To verify the equal loading of nuclear proteins, the binding of Oct-1 to DNA was determined by incubating 15 μg of nuclear extracts with 16 fmol of ^{32}P-end-labeled with the octamer-binding protein (Oct-1) consensus oligonucleotide 5′-TGTCGA**ATGCAAAT**CACTAGAA-3′ (boldface indicates Oct-1 binding site) for 30 min at 37°C, and then analysed using 5% native polyacrylamide gel. The dried gels were visualized, and radioactive bands were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

Western blot analysis

To determine the expression levels of the cIAP-1, XIAP, survivin, Bcl-2, Bcl-xL, TRAF1, VEGF, and PARP proteins in the curcumin-treated cells, we subjected the whole-cell extracts to Western blot analysis as described previously (23). In brief, the whole-cell extracts were prepared by lysing curcumin-treated cells with lysis buffer (20 mM Tris [pH 7.4], 250 mM NaCl, 2 mM EDTA [pH 8.0], 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mM PMSF, and 4 mM sodium orthovanadate) and loaded. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blocked with 5% nonfat milk, and probed with various antibodies overnight at 4°C. The blots were then washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 hour, and finally examined using an enhanced chemiluminescence (ECL) reagent (Amersham, Piscataway, NJ).

Apoptosis assay

To determine whether curcumin potentiates the apoptotic effects of bortezomib and thalidomide in MM cells, we used a Live/Dead assay kit (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic, green fluorescent dye that is retained within live cells, and a red fluorescent ethidium bromide homodimer dye that can enter cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membranes of live cells (23). In brief, cells (1×10^6 well) were incubated in 12 well plates, pretreated with curcumin for 4 hours, and treated with either thalidomide or bortezomib for 24 hours. Cells were then stained with the assay reagents for 30 minutes at ambient temperature. Cell viability was determined under a fluorescence microscope by counting live (green) and dead (red) cells. This experiment was repeated twice, and the statistical analysis was performed. The values were initially subjected to one-way ANOVA, which revealed significant differences between groups, and then compared among groups using an unpaired Student's *t*-test ($P < 0.05$ considered to be statistically significant), which revealed significant differences between the two sample means.

Annexin V assay

One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cell's cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of annexin V. This assay was performed as described previously (23).

Animals

Male athymic *nu/nu* mice (4 weeks old) were obtained from the breeding colony of the Department of Experimental Radiation Oncology at The University of Texas M. D. Anderson Cancer Center. The animals were housed (5 mice/cage) in the standard mice plexiglass cages in a room maintained at constant temperature and humidity under 12-hour light and dark cycle and fed with regular autoclaved mouse chow with water *ad libitum*. None of the mice exhibited any lesions and all were tested pathogen-free. Before initiating the experiment, we acclimatized all mice to a pulverized diet for 3 days. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at The University of Texas M. D. Anderson Cancer Center.

Subcutaneous implantation of U266 cells

U266 cells were injected subcutaneously into the mice as described previously (24). In brief, U266 cells were harvested from subconfluent cultures, washed once in serum-free medium, and resuspended in PBS. Only suspensions consisting of single cells, with >90% viability, were used for the injections. Mice were anesthetized with a ketamine-xylazine solution, and U266 cells [2×10^6 cells/100 μL PBS:Matrigel (1:1)] were injected subcutaneously into the left flank of the mice using a 25-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe Co., Reno, NV). To prevent leakage, a cotton swab was held cautiously for 1 minute over the site of injection.

Experimental protocol

After 1 week of implantation, tumor diameters were measured using Vernier calipers. The mice were then randomized into treatment groups (n = 5) based on the tumor volume. Group I (control) was treated with corn oil (100 μL; orally; daily) and saline (100 μL; orally; once a week); group II was treated with curcumin alone (1 g/kg; orally; daily); group III was treated with bortezomib alone (0.25 mg/kg; 100 μL; once in a week; i.p.); and group IV was treated with a combination of curcumin (1 g/kg; orally; daily) and bortezomib (0.25 mg/kg; 100 μL; once in a week). Treatment was continued for up to 20 days from the date of randomization

(Day 0). The tumor volume was measured at 5-day intervals. The mice were killed 25 days after randomization. The tumors were carefully excised and measured to calculate tumor volume. The tumor volume was derived using the formula $V = 2/3\ \pi r^3$, where r is the mean of the 3 dimensions (length, width, and depth). The tumor volumes were initially subjected to one-way ANOVA and then later compared among groups using an unpaired Student's *t*-test, with $P < 0.05$ considered to be significant.

Preparation of nuclear extract from tumor samples

MM tumor tissues (75–100 mg/mouse) from control and treated mice were minced and incubated on ice for 30 minutes in 0.5 mL of ice-cold buffer A (10 mM HEPES [pH 7.9], 1.5 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1% Igepal CA-630, and 0.5 mM PMSF). The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 × *g* at 4°C for 10 minutes. The resulting nuclear pellet was suspended in 0.2 mL of buffer B (20 mM HEPES [pH 7.9], 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and 2 μg/mL leupeptin) and incubated on ice for 2 hours with intermittent mixing. The suspension was then centrifuged at 16,000 × *g* at 4°C for 30 minutes. The supernatant (nuclear extract) was collected and stored at −70°C until use (25).

Immunolocalization of NF-κB p65 and VEGF in tumor samples

The nuclear localization of p65 and the expression of VEGF was examined using an immunohistochemical method described previously (25). In brief, MM tumor samples were embedded in paraffin and fixed with paraformaldehyde. After being washed in PBS, the slides were blocked with protein block solution (DakoCytomation, Carpinteria, CA) for 20 minutes and then incubated overnight with rabbit polyclonal anti-human p65 and mouse monoclonal anti-human VEGF antibodies (1:400 and 1:100, respectively). Slides were washed and then incubated with biotinylated link universal antiserum followed by horseradish peroxidase-streptavidin conjugate (LSAB+kit). The slides were rinsed, and color was developed using 3,3′-diaminobenzidine hydrochloride as a chromogen. Finally, sections were rinsed in distilled water, counterstained with Mayer's hematoxylin, and mounted with DPX mounting medium for evaluation. Pictures were captured with a Photometrics CoolSnap CF color camera (Nikon, Lewisville, TX) using MetaMorph software (v. 4.6.5; Universal Imaging, Downingtown, PA).

Ki-67 immunohistochemistry

Frozen sections (5 μm) were stained with anti-Ki-67 antibody as described previously (25). Results were expressed as percent of $Ki\text{-}67^+$ cells ± SE per x40 magnification. A total of ten x40 fields were examined and counted from 3 tumors of each treatment group. The values were initially subjected to one-way ANOVA and then later compared among groups using an unpaired Student's *t*-test, with $P < 0.05$ considered to be significant.

Microvessel density

Frozen sections (5 μm) were stained with rat anti-mouse CD31 monoclonal antibody (Pharmingen, San Diego, CA) as described previously (25). Areas of greatest vessel density were then examined under higher magnification (×100) and counted. Any distinct area of positive staining for CD31 was counted as a single vessel. Results were expressed as the mean number of vessels ± SE per high-power field (HPF, or ×100). A total of 20 HPFs were examined and counted from 3 tumors of each treatment group. The values were initially subjected to one-way ANOVA and then later compared among groups using an unpaired Student's *t*-test, with $P < 0.05$ considered to be significant.

Results

The goals of this study were, first, to determine whether curcumin (Figure 1A) can sensitizes chemoresistant multiple myeloma MM cells that have developed resistance to conventional chemotherapeutic agents; second, to determine whether curcumin can potentiates the antitumor effects of thalidomide and bortezomib, two chemotherapeutic agents used extensively to treat MM patients; third, to determine whether curcumin potentiates the effects of these chemotherapeutic agents *in vivo*; fourth, to determine the mechanism by which curcumin sensitizes MM cells to these drugs. We used MM cell lines that are sensitive and resistant to dexamethasone, doxorubicin, and melphalan in our studies.

Curcumin suppresses the proliferation of drug-sensitive and drug-resistant MM cells

To determine whether curcumin suppresses the proliferation of drug-sensitive and drug-resistant human MM cells, we tested the cell proliferation using an MTT assay. Curcumin suppressed the proliferation of all MM cell types tested, including U266 cells, MM.1R cells (resistant to dexamethasone), RPMI-8226-Dox-6 cells (resistant to doxorubicin), and RPMI-8226-LR-5 cells (resistant to melphalan) in a dose-dependent and time-dependent manner (Figure 1B). Whether a cell line is a sensitive or resistant to a conventional chemotherapeutic agent, no difference in the sensitivity to curcumin was observed.

Curcumin potentiates the apoptotic effects of bortezomib and thalidomide against MM cells

Bortezomib, a proteasome inhibitor, and thalidomide, a TNF inhibitor, have been approved for the conventional treatment of MM patients. To determine whether curcumin potentiates the apoptotic effect of these drugs, we treated U266 cells with curcumin combined with either bortezomib or thalidomide, and then examined these cells with a Live/Dead assay. Curcumin potentiated the apoptotic effect of bortezomib from 25% to 85% (Figure 2A, middle panel) and of thalidomide from 10% to 75% (Figure 2A, right panel).

To further confirm the potentiating effect of curcumin on bortezomib and thalidomide, we used the annexin V method, which detects an early stage of apoptosis. Again, curcumin enhanced the apoptotic effects of bortezomib from 16% to 60% and of thalidomide from 18% to 57% (Figure 2B).

When we examined the PARP cleavage, which indicates caspase-3 activation, a well-known characteristic of apoptosis, we found that curcumin potentiated the effect of bortezomib (Figure 2C, *top*) and of thalidomide (Figure 2C, *bottom*). The potentiating effect of curcumin was more pronounced on thalidomide than on bortezomib. This may be because bortezomib induces apoptosis by activating caspase-9, whereas thalidomide induces apoptosis by activating caspase-8 (26).

Curcumin potentiates the inhibitory effect of bortezomib and thalidomide on constitutive NF-κB activation in MM cells

Our results indicate that curcumin can potentiate the apoptotic effects of bortezomib and thalidomide. How the effects were potentiated, was investigated. The constitutive activation of NF-κB is associated with growth and survival of cancer cells, including MM cells (27,28). In addition, studies have shown that bortezomib and thalidomide have been shown to suppress NF-κB activation (29,30). To determine whether the apoptotic effects of the curcumin alone and of the combination of curcumin with these drugs, correlates with down-regulation of NF-κB activation was examined. To investigate this, we incubated U266 cells with suboptimal concentrations of curcumin, bortezomib, and thalidomide alone or a combination with curcumin and these drugs and then examined NF-κB activation by EMSA. Curcumin

potentiated the inhibitory effect of bortezomib and of thalidomide on NF-κB activation in MM cells (Figure 2D).

Curcumin suppresses Akt activation in MM cells

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is one of the signaling pathways whose dysregulation has been linked with chemoresistance in MM cells (31). We investigated whether curcumin suppressed the activation of Akt in MM cells. We found that Akt was constitutively active in U266 cells and that curcumin suppressed the levels of phosphorylated Akt in a time-dependent manner (Figure 3A), indicating that these reduced levels of activated Akt may contribute towards increasing the apoptosis in MM cells.

Curcumin inhibits the expression of NF-κB-regulated gene products in MM cells

Numerous proteins, including cyclin D1, survivin, Bcl-xL, cIAP-1, XIAP, Bcl-2, TRAF1, and VEGF, have been linked with chemoresistance; all regulated by NF-κB. To determine whether curcumin mediates its effects by downregulating the expression of NF-κB-regulated proteins, we examined the effect of curcumin on the expression of NF-κB-regulated gene products implicated in cell proliferation (cyclin D1), antiapoptosis (cIAP-1, XIAP, survivin, Bcl-2, Bcl-xL, and TRAF1), and angiogenesis (VEGF). Our results showed that curcumin downregulated the constitutive expression of cyclin D1, cIAP-1, XIAP, survivin, Bcl-2, Bcl-xL, and TRAF1 and VEGF in U266 cells (Figure 3B). The effect of curcumin on apoptosis showed the consistent to our previous report (3).

Curcumin potentiates the antitumor effects of bortezomib in human MM xenograft in nude mice

To determine whether curcumin enhances the antitumor effects of bortezomib against MM, we developed a human MM xenograft in nude mice using U266 cells. A week after implantation, the animals were randomized into 4 treatment groups based on tumor volume. Treatment was started 1 week after tumor cell implantation and was continued up to 20 days, in accordance with the experimental protocol (Figure 4A). The tumor diameters were measured at 5-day intervals. Animals were killed 32 days after tumor cell injection and 25 days after the treatment start date (Figure 4A), and the tumors were excised and the tumor diameters were measured. We found that the tumor volume increased rapidly in the control group compared with the other treatment groups (Figure 4B). Both curcumin alone and bortezomib alone significantly decreased the tumor volume; the tumor volume in the curcumin+bortezomib group was significantly lower than that in the bortezomib alone group and in the control group at Day 25 after treatment ($P < 0.001$, vehicle vs. curcumin+bortezomib; $P < 0.001$, bortezomib vs. curcumin+bortezomib) (Figure 4C). When examined for tumor volume on different days, we found that curcumin+bortezomib combination was much more effective in reducing the tumor volume compared to either agent alone ($P < 0.001$ vs. vehicle) (Figure 4D).

Curcumin inhibits NF-κB activation in human MM xenograft in nude mice

We evaluated whether the effects of curcumin on MM tumor growth was associated with the inhibition of NF-κB activation. The DNA binding assay for NF-κB in nuclear extracts from tumor samples showed that curcumin alone significantly suppressed NF-κB activation. The effect of bortezomib alone was less pronounced than curcumin in inhibiting NF-κB activation (Figure 5A). Interestingly, the curcumin+bortezomib combination was not more effective than curcumin alone ($P < 0.001$ vs. vehicle).

We also examined whether the EMSA results were consistent with the immunohistochemical analyses. We found that the nuclear translocation of p65 was significantly inhibited by

curcumin alone but not by bortezomib alone, which confirmed the EMSA results (Figure 5B, *left and right panel*).

Curcumin downregulates the expression of the cell proliferation marker Ki-67 in human MM xenograft in nude mice

To determine whether curcumin decreases MM tumor growth by inhibiting proliferation, we examined the expression of Ki-67$^+$ cells in MM tumors from mice. Our results showed that curcumin alone and bortezomib alone significantly decreased the expression of Ki-67 in tumor tissue (Figure 5C, *left and right panel*). The curcumin+bortezomib combination was more effective in reducing Ki-67 expression than either agent alone ($P < 0.001$ vs. vehicle).

Curcumin inhibits angiogenesis and downregulates the expression of VEGF in human MM xenograft in nude mice

To determine whether curcumin decreases MM tumor growth by inhibiting angiogenesis, we examined the expression of the microvessel density marker CD31+ in MM tumors from nude mice. Our results showed that curcumin alone and bortezomib alone significantly reduced microvessel density. The curcumin+bortezomib combination was more effective in reducing microvessel density than either agent alone (Figure 6A, *left and right panel*) ($P < 0.001$ vs. vehicle).

Because VEGF plays an important role in angiogenesis, we also examined its expression in MM tumors. We found that both curcumin alone and bortezomib alone effectively suppressed VEGF expression and that the curcumin+bortezomib combination had the highest inhibitory effect on VEGF expression in MM tumors (Figure 6B, *left and right panel*).

Discussion

Despite the fact that several treatments for MM are currently available, MM remains an incurable disease, with a median survival time of 3–5 years (32–34). The disease relapses in the majority of MM patients, regardless of the treatment regimen or their initial response to a given treatment. Recently, 3 new agents (bortezomib, thalidomide, and lenalidomide) were approved for the treatment of MM patients. Bortezomib (Velcade) was approved for the treatment of MM patients who have received at least one prior therapy; thalidomide (Thalomid) in combination with dexamethasone was approved for the treatment of newly diagnosed MM patients; and the thalidomide analogue lenalidomide (Revlimid) in combination with dexamethasone was recently approved for the treatment of MM patients who have received one prior therapy (35). All these three drugs mediate their antimyeloma activities through modulation of intracellular signaling pathways within tumor cells and microenvironments (36,37). Preclinical studies have shown that these drugs induce apoptosis in myeloma cells resistant to melphalan, doxorubicin, and dexamethasone and potentiate the antimyeloma activity of conventional therapies (38,39). However, these drugs exhibit several side effects, such as fatigue, anemia, and peripheral neuropathy. Furthermore, studies have shown that patients eventually develop resistance to these drugs. Thus, new treatment approaches that are more effective than conventional therapies against MM are required to improve the outcome and extend the survival of MM patients.

Because the transcription factor NF-κB has been closely linked with chemoresistance, cell survival, and proliferation, we investigated whether curcumin suppresses the proliferation of MM cells that have developed resistance to chemotherapeutic agents. We found that curcumin suppressed human MM cell proliferation regardless of the cells' sensitivity or resistance to melphalan, doxorubicin, or dexamethasone. Similar to our results these drug-resistant cells have been shown to be sensitive to bortezomib and thalidomide (38,40). We also found that

curcumin potentiated the antitumor effects of bortezomib and thalidomide. What is the mechanism of resistance of MM to melphalan, doxorubicin, or dexamethasone? First, cells that resistant to chemotherapeutic agents have been shown to express increased activation of NF-κB and suppression of this NF-κB can sensitize the cells to the drug (41). Second, multiple myeloma cells and mantel cell lymphoma are known to express constitutive active NF-κB that is resistant to bortezomib (42,43). Our results indicate that the mechanism of potentiation is the suppression of NF-κB activation. Why NF-κB is constitutively active in MM cells, is not fully understood. Recently, two studies found mutations on the genes encoding positive and negative regulators of NF-κB signaling in many MM cell lines and primary tumor cells; these mutations are thought to mediate the constitutive activation of NF-κB in MM cells (10,11). It is likely that the constitutive activation of NF-κB in MM cells leads to chemoresistance. For example, CRP, whose expression is regulated by NF-κB, has been shown to enhance the proliferation of myeloma cells and protect myeloma cells from chemotherapy-induced apoptosis both *in vitro* and *in vivo* (8). CRP has also been shown to bind to Fcgamma receptors and activate Akt, ERK, and NF-κB pathways. CRP also enhanced the secretion of IL-6 and synergized with IL-6 to protect myeloma cells from chemotherapy-induced apoptosis. In our study, we showed that curcumin downregulates Akt activation, another possible mechanism which curcumin sensitizes tumor cells to chemotherapeutic agents. Previously, we showed that curcumin also downregulates the expression of IL-6 (4), a major growth factor for MM cells. We also found that curcumin downregulated various proliferative and antiapoptotic proteins, including cyclin D1, cIAP-1, XIAP, survivin, Bcl-2, Bcl-xL, and TRAF1. As mentioned above, cyclin D1, Bcl-2, Bcl-xL and survivin have been linked with chemoresistance in MM cells (44–46). Thus, in addition to the suppression of NF-κB activation, these mechanisms may help explain the effects of curcumin.

Like curcumin, bortezomib inhibits NF-κB activation but through different mechanism. The pathway by which bortezomib prevents degradation of IκB, differs from that of curcumin. Bortezomib inhibits the proteasome, resulting in the accumulation of IκBα, whereas curcumin prevents IκB phosphorylation, thus blocking its subsequent ubiquitination and degradation through suppression of upstream kinase IKK. Thus, these different mechanisms of NF-κB suppression provide the rationale for combining these agents to effectively inhibit NF-κB activation. Furthermore, other mechanisms bortezomib and curcumin may support their combination to maximize their cytotoxicity against MM cells. For instance, curcumin is a potent blocker of STAT3 activation (15), a transcription factor that has been linked with chemoresistance (47). Moreover, bortezomib has been shown to overcome chemoresistance in MM cells both in the laboratory (41,48) and in the clinic (49). Indeed, bortezomib has been shown to exhibit considerable clinical efficacy against MM. Whether this activity is due to its anti-NF-κB activity is not clear (35); as its ability to suppress NF-κB in patients has not been demonstrated.

We next determined whether curcumin potentiates the effects of bortezomib on human MM xenografts in nude mice. Our results showed that curcumin alone and bortezomib alone inhibited the MM xenograft in nude mice. We found that the curcumin+bortezomib combination had a higher antimyeloma effect than either agent alone. When investigated the mechanism, we found that curcumin downregulated the expression of NF-κB, the cell proliferation marker Ki-67, and the microvessel density marker CD31, all which have been linked with chemoresistance. Since curcumin alone inhibited most of NF-κB activity whit partial anti-tumor effect, the downregulation of NF-κB by curcumin alone may not be sufficient to explain the effect of this agent *in vivo*. Moreover, when combined with bortezomib *in vivo* there is slight increase in NF-κB DNA binding activity even though this combination leads to the greatest effect on myeloma growth *in vivo*. Thus, it is possible that *in vivo* effect of curcumin are not entirely mediated though its ability to suppress NF-κB. Curcumin has been shown to modulate multiple targets (50) that may be involved in its *in vivo* antitumor action.

MM is usually diagnosed in elderly patients who are unable to tolerate highly toxic drugs, so curcumin, a pharmacologically safe compound, is a viable therapeutic agent for these MM patients. A recent phase I study of curcumin demonstrated that this agent can be administered safely at oral doses of up to 12 g/day (19). There was no dose-limiting toxicity; dosing was limited by the number of pills that patients could or would swallow daily. In addition, a recent phase II clinical trial with curcumin in pancreatic cancer patients showed that tumor progression was suppressed by 73% in some patients (51). Clinical trials with asymptomatic MM patients have shown that curcumin downregulates NF-κB activation in peripheral blood mononuclear cells even at dose of 2 g/day (20). Thus, curcumin can be used, first, to enhance the effects of current chemotherapeutic agents and, second, to overcome chemoresistance in MM to conventional therapy. Fatigue and peripheral neuropathy are two symptoms commonly observed in MM patients. Because these symptoms are mediated through the expression of proinflammatory cytokines, curcumin may be used to alleviate these symptoms. In fact, curcumin has been shown to reverse these symptoms (52–54), which further supports its use in MM patients.

In conclusion, the chemoresistance remains a major challenge in the treatment of patients with MM as well as other cancers. MM patients who have relapsed after conventional-dose chemotherapy or stem cell transplantation are typically treated with high-dose corticosteroids, thalidomide, or bortezomib. However, a large number of these patients do not respond to treatment with these agents. Moreover, prolonged exposure leads to the development of resistance and toxicity, nd progression-free and overall survival times for MM patients are short. The ability of curcumin to suppress NF-κB activation, downregulate the expression of cyclin D1 and Bcl-xL, inhibit cell proliferation, potentiate the effects of bortezomib and thalidomide, and overcome chemoresistance provides a sound basis for conducting clinical trials with curcumin, alone or in combination with other agents, to enhance treatment efficacy, reduce toxicity, and overcome chemoresistance of relapsed or refractory MM.

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Abbreviations

cIAP-1
: cellular inhibitor of apoptosis-1

CRP
: C-reactive protein

EMSA
: electrophoretic mobility shift assay

ERK
: extracellular signal-regulated kinase

IKK
: IκB kinase

IL-6
: interleukin-6

MM

multiple myeloma

NF-κB
nuclear factor-kappaB

PARP
poly (ADP-ribose) polymerase

PI3K
phosphatidylinositol 3-kinase

TRAF1
TNF receptor associated factor 1

VEGF
vascular endothelial growth factor

XIAP
X-linked IAP

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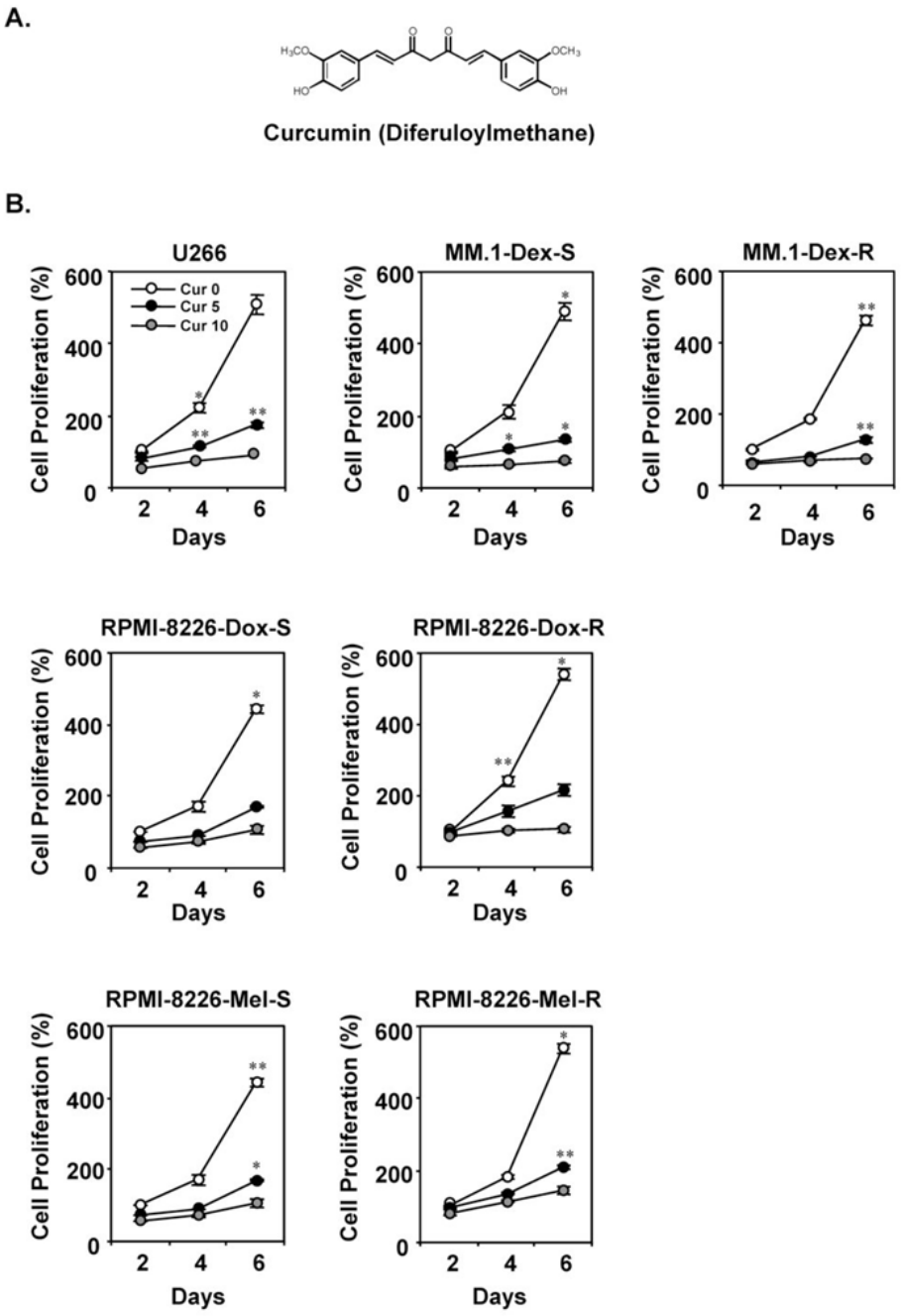

Figure 1. Curcumin suppresses the proliferation of drug-resistant MM cells

(A) Structure of curcumin. (B) U266 cells (5×10^3 cells) treated with various concentrations of curcumin, dexamethasone-sensitive (*top-middle panel*) and dexamethasone-resistant (*top-right panel*) MM.1 cells (5×10^3 cells), doxorubicin-sensitive (*middle-left panel*) and doxorubicin-resistant (*middle-right panel*) RPMI-8266 cells (5×10^3 cells), and melphalan-sensitive (*bottom-right panel*) and melphalan-resistant (*bottom-right panel*) RPMI-8266 cells were plated in triplicate, treated with 5 and 10 μM curcumin, and then subjected to MTT assay on days 2, 4, or 6 to analyze MM cell proliferation. Data are means ± SD for three experiments; **, $P < 0.01$; *, $P < 0.05$, vs. control.

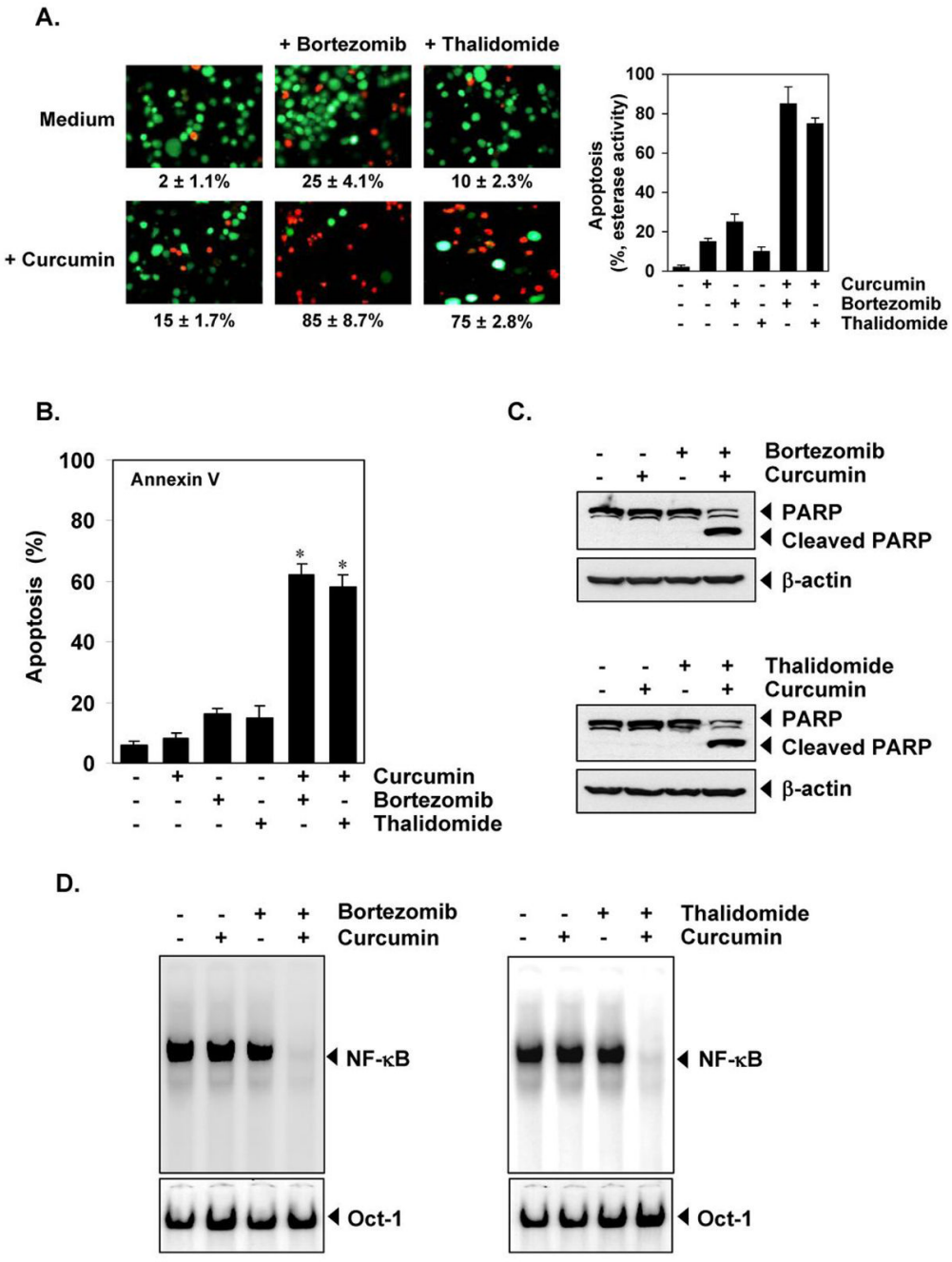

Figure 2. Curcumin potentiates the apoptotic effect of bortezomib and of thalidomide

(A) (*left*) U266 cells (1×10^6/mL) were treated with 25 μM curcumin, 20 nM bortezomib, or 10 μg/mL thalidomide alone or a combination of these two agents with curcumin for 24 hours at 37°C. Cells were stained with a Live/Dead assay reagent for 30 minutes and then analyzed under a fluorescence microscope. Percentage of apoptosis is indicated in the inset. (*right*) a graphic representation of the data with U266 cells. (B) U266 cells (1×10^6/mL) were treated with 25 μM curcumin, 20 nM bortezomib, or 10 μg/mL thalidomide alone or a combination of these agents with curcumin for 12 hours at 37°C. Cells were incubated with anti-annexin V antibody conjugated with fluorescein isothiocyanate and then analyzed with a flow cytometery to detect early apoptotic effects. Data are means ± SD for three experiments; *, $P < 0.001$, vs.

control as well as single agent. (C) U266 cells (1×10^6/mL) were treated with 25 μM curcumin, 20 nM bortezomib, or 10 μg/mL thalidomide alone or a combination of these agents with curcumin for 12 hours at 37°C. Whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis using antibody against PARP. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. The results shown represent 3 independent experiments. (D) U266 cells (1×10^6/mL) were treated with 5 μM curcumin or 10 nM bortezomib alone or a curcumin+bortezomib combination for 12 hours at 37°C and then tested for NF-κB activation by EMSA (*left panel*). U266 cells (1×10^6/mL) were treated with 5 μM curcumin or 10 μg/mL thalidomide alone or a curcumin+thalidomide combination for 12 hours at 37°C and then tested for NF-κB activation by EMSA (*right panel*). Oct-1 EMSA served as a loading control. The results shown represent 3 independent experiments.

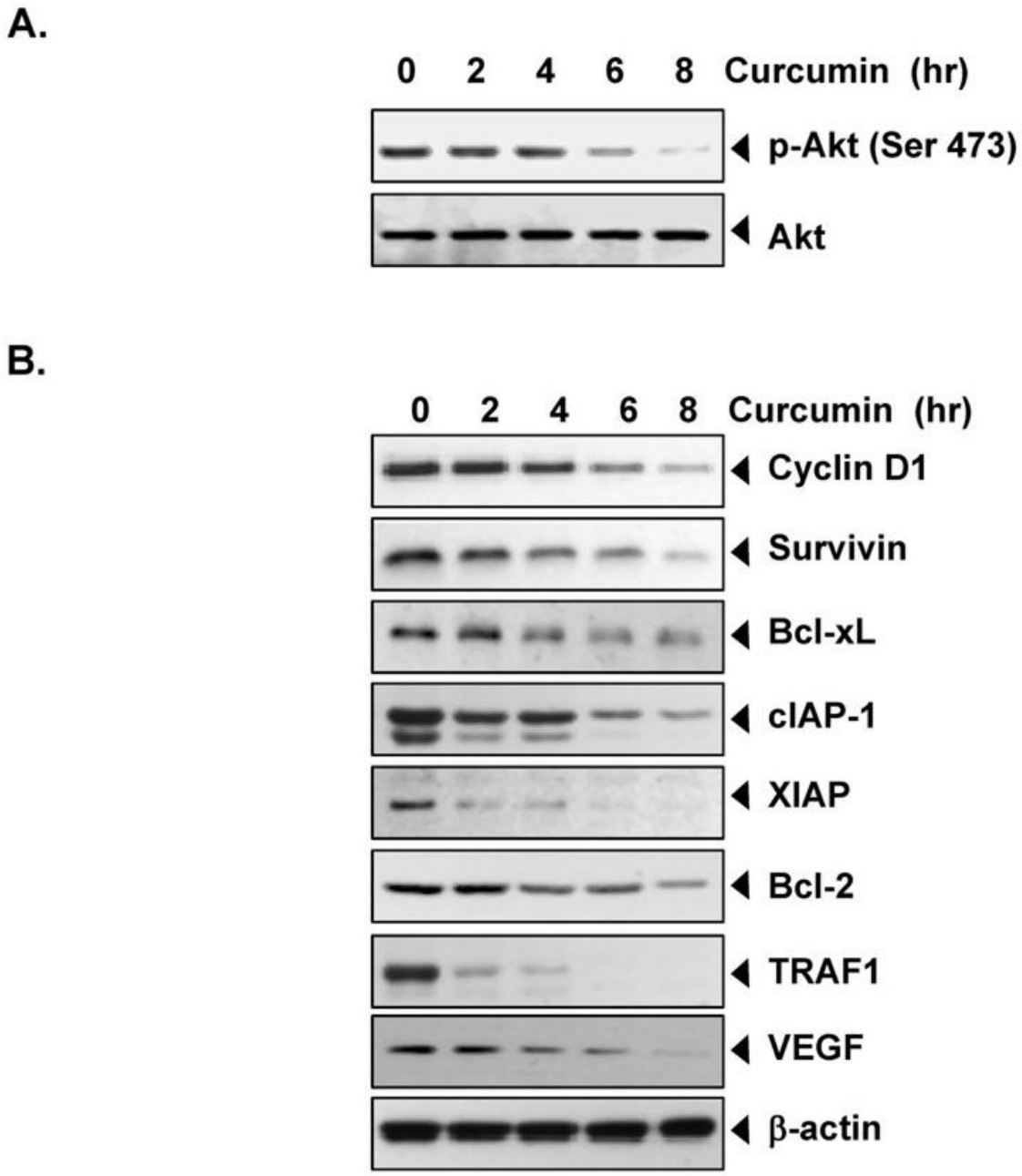

Figure 3. Curcumin suppresses the expression of antiapoptotic proteins in MM cells

(A) Curcumin suppresses Akt activation in U266 cells. U266 cells (2×10^6/mL) were treated with 50 μM curcumin for the indicated times. Whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis using the indicated proteins. The same blots were stripped and reprobed with Akt antibody to show equal protein loading. (B) Curcumin inhibits the expression of antiapoptotic gene products. U266 cells (2×10^6/mL) were treated with 50 μM curcumin for the indicated times. Whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis using the indicated proteins. The same blots were stripped and reprobed with Akt and β-actin antibody to show equal protein loading.

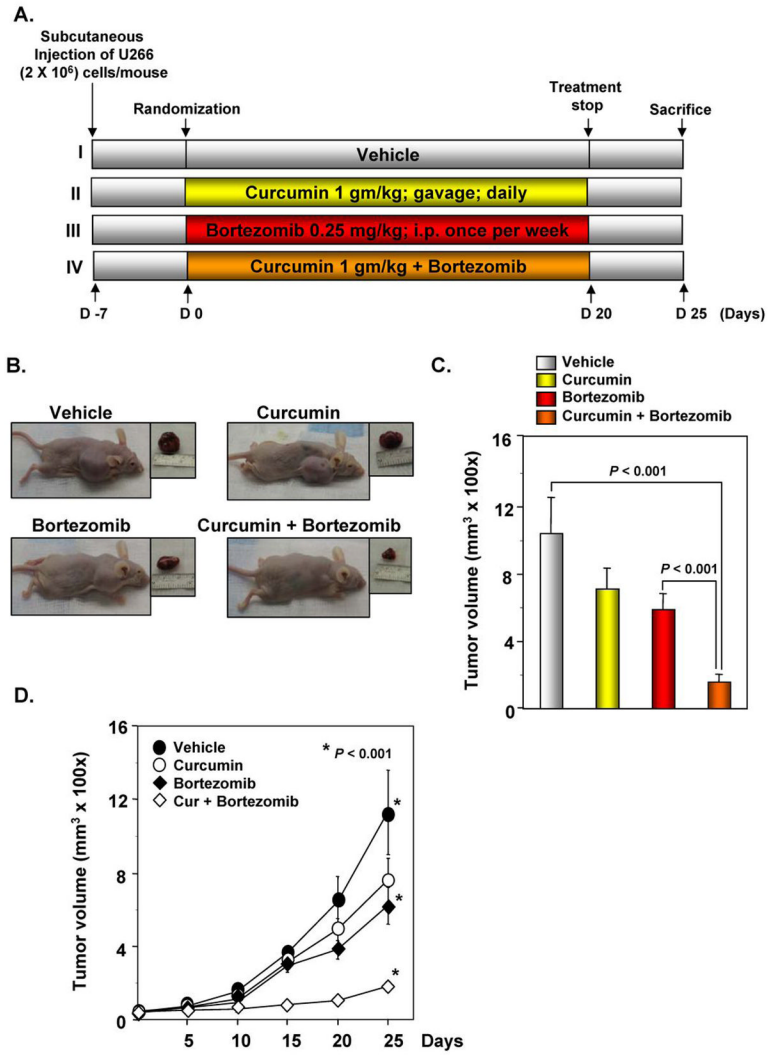

Figure 4. Curcumin potentiates the antitumor effects of bortezomib in myeloma tumor growth in nude mice induced by U266 cells

(A) Schematic representation of the experimental protocol as described in "Materials and Methods." U266 cells (2×10^6/mice) were injected subcutaneously into the left flank of the mice. The animals were randomized after 1 week of tumor cell injection into four groups based on tumor volume. Group I was treated with corn oil (100 μL; orally; daily) and saline (100 μL; orally; once a week), group II was treated with curcumin alone (1 g/kg daily; orally), group III was treated with bortezomib alone (0.25 mg/kg; orally; once a week), and group IV with a combination of curcumin (1 g/kg orally; daily) and bortezomib (0.25 mg/kg; orally; once a week) (n = 6). (B) The tumor diameters were measured at 5-day intervals with Vernier calipers, and the tumor volumes were calculated using the formula $V = 2/3\ \pi r^3$ (n = 5). (C, *left panel*) Necropsy photographs of mice bearing xenotransplanted MM. (C, right panel) Tumor volumes (mean ± SE) calculated using the formula $V = 2/3\ \pi r^3$ (n = 6) after tumor diameters were measured on the last day of the experiment at autopsy using Vernier calipers. (D) The tumor volume of mice at different time intervals.

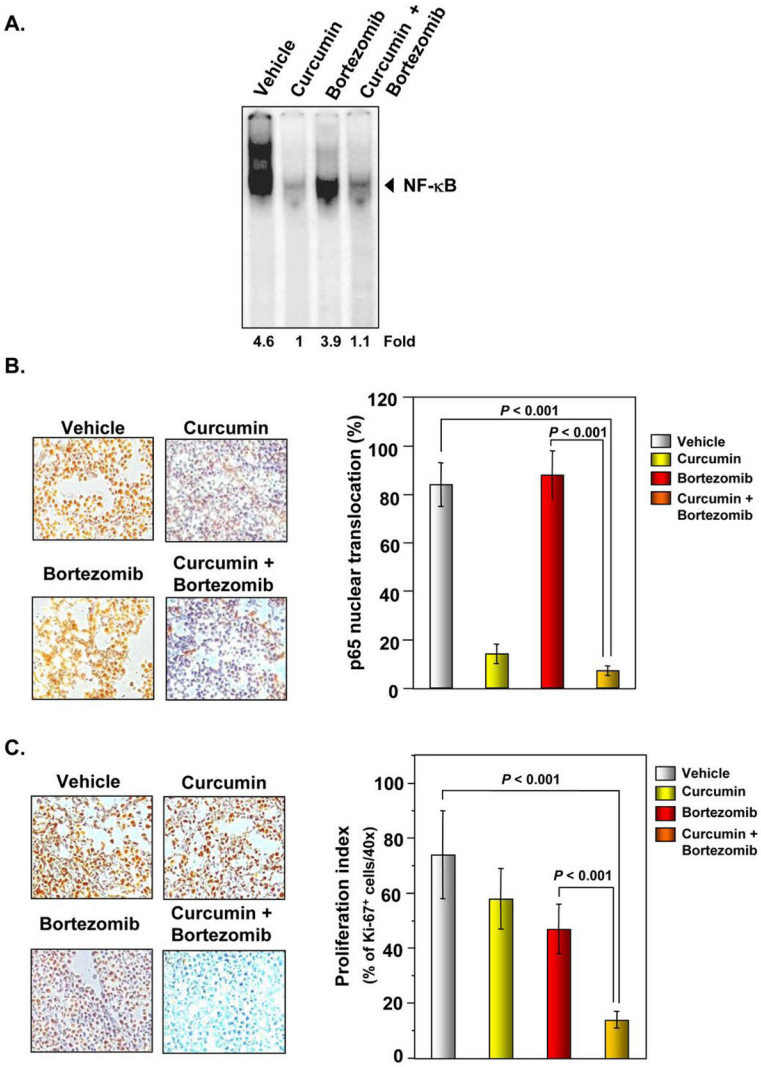

Figure 5. Curcumin enhances the inhibitory effect of bortezomib on the activation of NF-κB and expression of the cell proliferation marker Ki-67 in MM tumor samples

(A) EMSA analysis revealed that curcumin inhibited NF-κB activation in nuclear extracts from animal tissue. (B, *left panel*) Immunohistochemical analysis of nuclear p65 in tumor tissues showed that curcumin alone and in combination with bortezomib inhibited NF-κB activation. (B, *right panel*) Quantification of NF-κB expression in MM tumor samples. Percentages indicate p65 nuclear positive cells. Samples from 3 animals in each treatment group were analyzed, and representative data are shown. (C, *left panel*) Immunohistochemical analysis of Ki-67^{+} cells in MM indicated that curcumin alone and in combination with bortezomib suppressed cell proliferation. Samples from 3 animals in each treatment group were analyzed, and representative data are shown. (C, *right panel*) Quantification of Ki-67 proliferation index as described in "Materials and Methods." Values are represented as mean ± SE of triplicate.

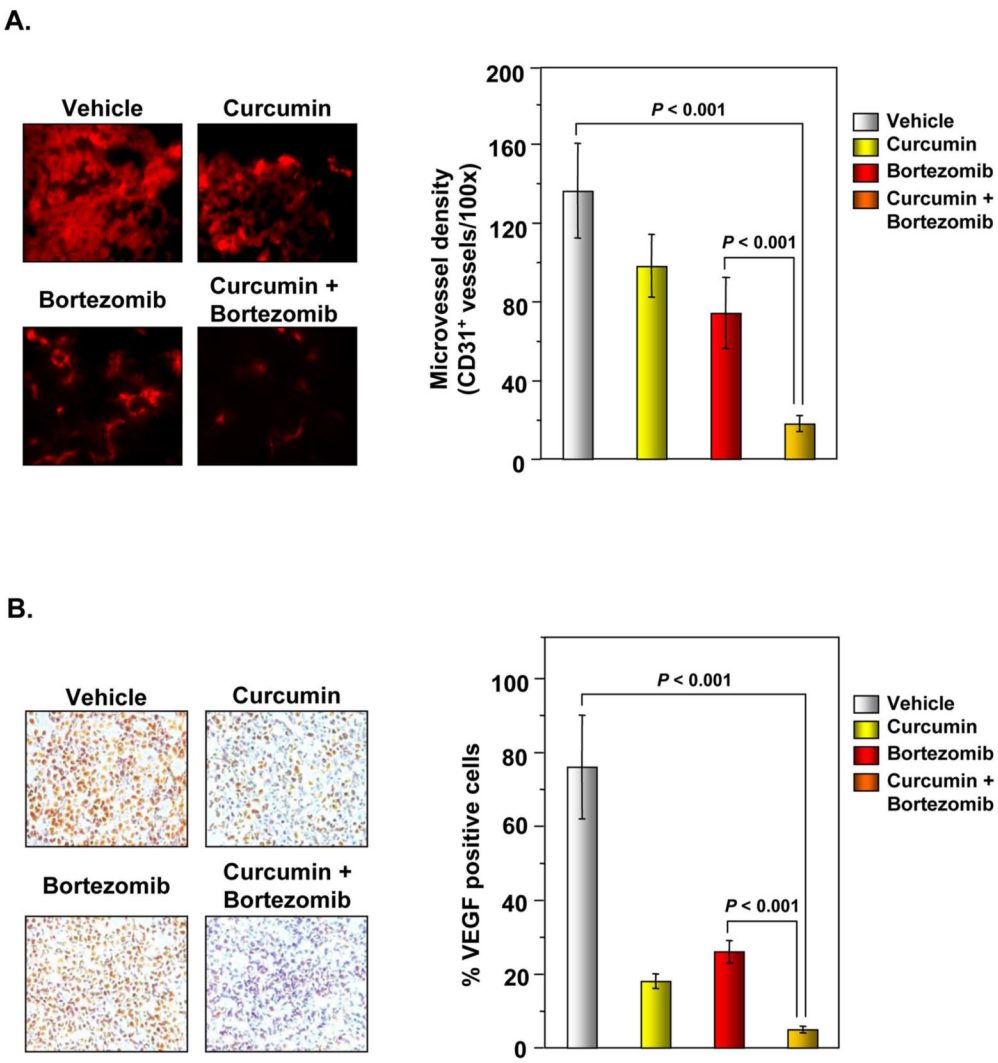

Figure 6. Curcumin enhances effects of bortezomib to inhibit angiogenesis in MM tumor samples

(A, *left panel*) Immunohistochemical analysis of the microvessel density marker CD31 in MM tumor samples. Samples from 3 animals in each treatment group were analyzed, and representative data are shown. (A, *right panel*) Quantification of $CD31^+$ microvessel density as described in "Materials and Methods." Values are represented as mean ± SE of triplicate. (B, *left panel*) Immunohistochemical analysis of VEGF in MM tumor samples revealed that curcumin alone and in combination with bortezomib suppresses angiogenesis. Samples from 3 animals in each treatment group were analyzed, and representative data are shown. (B, *right panel*) Quantification of VEGF as described in "Materials and Methods." Values are represented as mean ± SE of triplicate.