

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 nuclear factor- κ B (NF- κ B), a transcription factor closely linked to chemoresistance in multiple myeloma cells. Whether curcumin can overcome chemoresistance and enhance the activity of thalidomide and bortezomib, used to treat patients with multiple myeloma, was investigated *in vitro* and in xenograft model in nude mice. Our results show that curcumin inhibited the proliferation of human multiple myeloma cells regardless of their sensitivity to dexamethasone, doxorubicin, or melphalan. Curcumin also potentiated the apoptotic effects of thalidomide and bortezomib by down-regulating the constitutive activation of NF- κ B and Akt, and this correlated with the suppression of NF- κ B-regulated gene products, including cyclin D1, Bcl-xL, Bcl-2, TRAF1, cIAP-1, XIAP, survivin, and vascular endothelial growth factor. Furthermore, in a nude mice model, we found that curcumin potentiated the antitumor effects of bortezomib ($P < 0.001$, vehicle versus bortezomib + curcumin; $P < 0.001$, bortezomib versus bortezomib + curcumin), and this correlated with suppression of Ki-67 ($P < 0.001$ versus control), CD31 ($P < 0.001$ versus vehicle), and vascular endothelial growth factor ($P < 0.001$ versus vehicle) expression. Collectively, our results

suggest that curcumin overcomes chemoresistance and sensitizes multiple myeloma cells to thalidomide and bortezomib by down-regulating NF- κ B and NF- κ B-regulated gene products. [Mol Cancer Ther 2009;8(4):959–70]

Introduction

In the United States, 50,000 patients are affected by multiple myeloma, 16,000 patients are newly diagnosed with multiple myeloma, and 11,000 multiple myeloma patients die of the disease each year (1). Multiple myeloma is a late-stage B-cell malignancy characterized by the infiltration of malignant plasma cells in bone marrow and is associated with high monoclonal protein in the blood. Multiple myeloma can occur *de novo* or can evolve from benign monoclonal gammopathy of undetermined significance that involves low levels of bone marrow plasmacytosis and monoclonal protein. Approximately 1% of patients with monoclonal gammopathy of undetermined significance develop multiple myeloma each year. The current standard treatments for multiple myeloma 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, multiple myeloma remains an incurable disease (2). The majority of multiple myeloma patients eventually experience a relapse, their disease becomes chemoresistant, and they die of the disease.

Why multiple myeloma patients develop resistance to chemotherapy is not well understood, but numerous lines of evidence suggest that the transcription factor nuclear factor- κ B (NF- κ B) may play a major role in pathogenesis of multiple myeloma. First, NF- κ B has been shown to be constitutively active in multiple myeloma cell lines (3) and CD138⁺ multiple myeloma cells obtained from patients (4). Second, the NF- κ B-regulated cyclin D1 gene is frequently dysregulated in multiple myeloma patients (5). Third, interleukin-6 (IL-6), which is also regulated by NF- κ B, can suppress apoptosis in multiple myeloma cells (6). Fourth, the NF- κ B-regulated antiapoptotic proteins, C-reactive protein, and cell adhesion molecules have been linked to chemoresistance in multiple myeloma cells (7–9). Fifth, two recent reports used a whole genome-based screen approach and showed that cells from multiple myeloma patients have alterations in numerous genes that control the constitutive activation of NF- κ B (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- κ B signaling in stromal cells can lead to production of IL-6, BAFF, or APRIL, which can activate NF- κ B and cause proliferation of multiple myeloma cells (12).

Received 9/18/08; revised 1/29/09; accepted 1/30/09.

Grant support: Clayton Foundation for Research (B.B. Aggarwal), NIH grants CA-16 672 and CA-124787-01A2, and Center for Targeted Therapy of The University of Texas M. D. Anderson Cancer Center.

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doi:10.1158/1535-7163.MCT-08-0905

Based on these lines of evidence, agents that can inhibit NF- κ B 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- κ B 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- κ B activation and inhibit the proliferation of multiple myeloma cells (3, 4). Additional support for this hypothesis include the following: curcumin can down-regulate 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 down-regulate the activation of STAT3 (4, 15), a transcription factor that has also been linked with chemoresistance in multiple myeloma cells (17); and curcumin binds directly to P-glycoprotein and inhibits drug transport (18). In addition, a clinical trial of curcumin showed that it is safe even when consumed at 12 g/d (19). Furthermore, the administration of curcumin at a dose of 2 g/d for 4 weeks to asymptomatic multiple myeloma patients down-regulated NF- κ B activation in peripheral blood lymphocytes (20).

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

Materials and Methods

Materials

Curcumin (77.5% curcumin, 18.27% demethoxycurcumin, and 4.21% bisdemethoxycurcumin; also called C3 complex) was kindly supplied by Sabinsa. A 25 mmol/L solution was prepared in DMSO, stored as small aliquots at -20°C , and diluted as needed in cell culture medium. Penicillin, streptomycin, and RPMI 1640 were obtained from Invitrogen. Fetal bovine serum was obtained from Atlanta Biologicals. Bortezomib (PS-341) was obtained from Millennium, and thalidomide was obtained from Tocris Cookson. Dexamethasone, melphalan, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, and anti- β -actin were obtained from Sigma-Aldrich. Antibodies against poly (ADP-ribose) polymerase, cIAP-1, Bcl-2, Bcl-xL, and TRAF1 were obtained from Santa Cruz Biotechnology. Anti-XIAP and survivin antibodies were obtained from BD Biosciences. Anti-vascular endothelial growth factor (VEGF) and Ki-67 antibodies were obtained from NeoMarkers. Polyclonal antibody against p65 was obtained from Abcam. γ ³²P-ATP was obtained from ICN Pharmaceuticals. Phosphospecific

anti-Akt (Ser⁴⁷³) and anti-Akt antibodies were obtained from Cell Signaling.

Cell Lines

Human multiple myeloma 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 supplemented with 10% fetal bovine serum and $1\times$ antibiotic/antimycotic.

Proliferation Assay

The antiproliferative effects of curcumin on drug-sensitive and drug-resistant cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide uptake method as described previously (21).

Activation of NF- κ B in Multiple Myeloma Cells and Tumor Samples

To assess NF- κ B activation, we isolated nuclei from multiple myeloma cell lines and tumor samples and carried out electrophoretic mobility shift assays (EMSA) essentially as described previously (22). In brief, nuclear extracts prepared from multiple myeloma cancer cells ($1 \times 10^6/\text{mL}$) and tumor samples were incubated with ³²P-end-labeled 45-mer double-stranded NF- κ B oligonucleotide from the HIV long terminal repeat (5'-TTGTTACAAGG-**GACTTTCGCTGGGGACTTTCAGGGAGGCGTGG-3'**; boldface indicates NF- κ B binding sites) for 15 min 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 nuclear extracts with 16 fmol ³²P-end-labeled with the octamer-binding protein (Oct-1) consensus oligonucleotide 5'-TGTCGAATGCAAATCACTAGAA-3' (boldface indicates Oct-1 binding site) for 30 min at 37°C and then analyzed using 5% native polyacrylamide gel. The dried gels were visualized, and radioactive bands were quantitated with a PhosphorImager (Molecular Dynamics) using ImageQuant software.

Western Blot Analysis

To determine the expression levels of the cIAP-1, XIAP, survivin, Bcl-2, Bcl-xL, TRAF1, VEGF, and poly(ADP-ribose) polymerase 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 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, and 4 mmol/L 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 h, and finally examined using an enhanced chemiluminescence reagent (Amersham).

Apoptosis Assay

To determine whether curcumin potentiates the apoptotic effects of bortezomib and thalidomide in multiple myeloma cells, we used a Live/Dead assay kit (Molecular Probes), 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 per well) were incubated in 12-well plates, pretreated with curcumin for 4 h, and treated with either thalidomide or bortezomib for 24 h. Cells were then stained with the assay reagents for 30 min 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 done. 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 done 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 per cage) in the standard mice Plexiglass cages in a room maintained at constant temperature and humidity under 12 h 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.

Implantation of U266 Cells

U266 cells were injected s.c. 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 per 100 μ L PBS/Matrigel (1:1)] were injected s.c. into the left flank of the mice using a 25-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe). To prevent leakage,

a cotton swab was held cautiously for 1 min 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 μ L, orally, daily) and saline (100 μ L, orally, weekly), group II was treated with curcumin alone (1 g/kg, orally, daily), group III was treated with bortezomib alone (0.25 mg/kg, 100 μ L, i.p., weekly), and group IV was treated with a combination of curcumin (1 g/kg, orally, daily) and bortezomib (0.25 mg/kg, 100 μ L, weekly). 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 three 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

Multiple myeloma tumor tissues (75-100 mg/mouse) from control and treated mice were minced and incubated on ice for 30 min in 0.5 mL ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L DTT, 0.1% Igepal CA-630, and 0.5 mmol/L phenylmethylsulfonyl fluoride]. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 \times g at 4°C for 10 min. The resulting nuclear pellet was suspended in 0.2 mL buffer B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 2 μ g/mL leupeptin] and incubated on ice for 2 h with intermittent mixing. The suspension was then centrifuged at 16,000 \times g at 4°C for 30 min. The supernatant (nuclear extract) was collected and stored at -70°C until use (25).

Immunolocalization of NF- κ B 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, multiple myeloma tumor samples were embedded in paraffin and fixed with paraformaldehyde. After being washed in PBS, the slides were blocked with protein block solution (DakoCytomation) for 20 min 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.

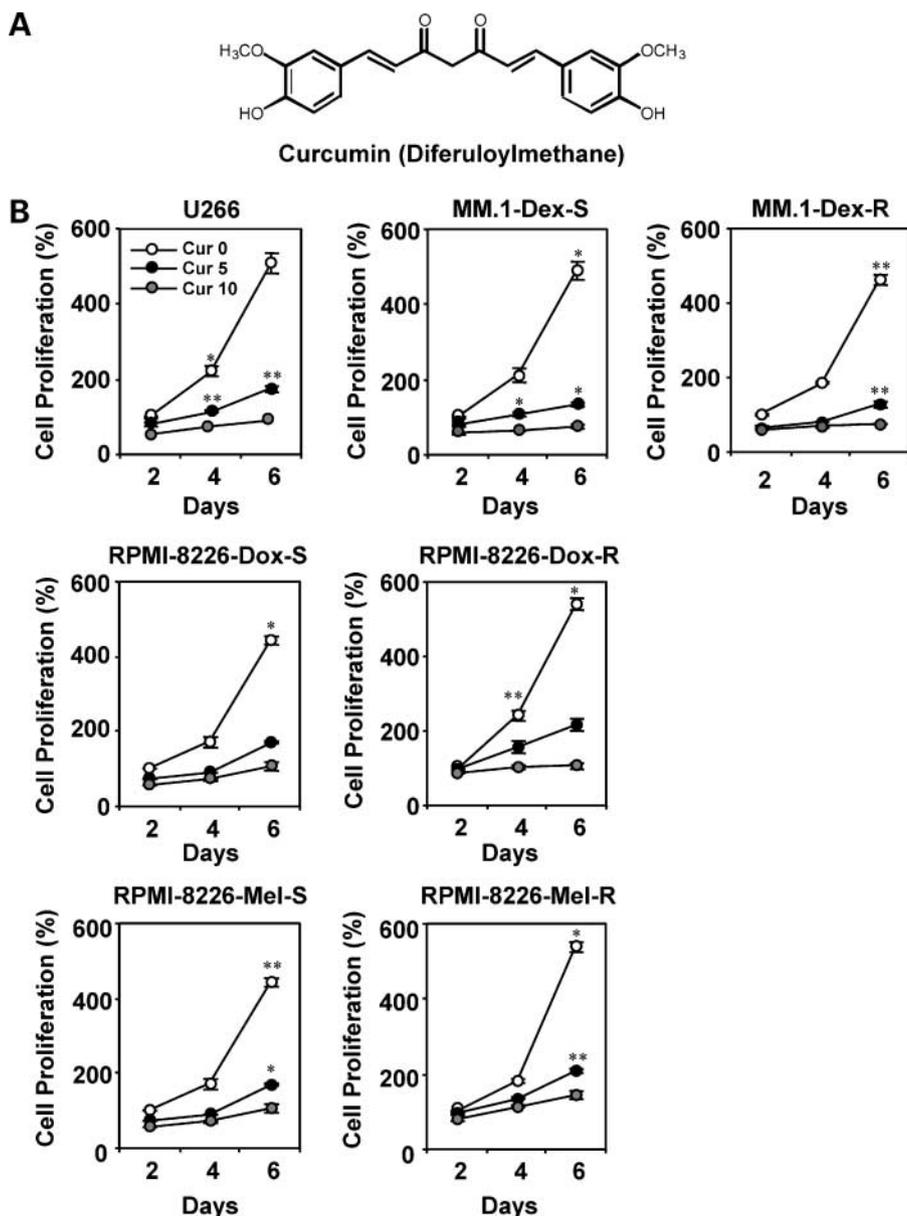


Figure 1. Curcumin suppresses the proliferation of drug-resistant multiple myeloma cells. **A**, structure of curcumin. **B**, U266 cells (5×10^3) treated with various concentrations of curcumin, dexamethasone-sensitive (*top middle*) and dexamethasone-resistant (*top right*) MM.1 cells (5×10^3), doxorubicin-sensitive (*middle left*) and doxorubicin-resistant (*middle right*) RPMI-8266 cells (5×10^3), and melphalan-resistant (*bottom right*) RPMI-8266 cells were plated in triplicate, treated with 5 and 10 $\mu\text{mol/L}$ curcumin, and then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay on days 2, 4, or 6 to analyze multiple myeloma cell proliferation. Mean \pm SD of three experiments. **, $P < 0.01$; *, $P < 0.05$ versus control.

Pictures were captured with a Photometrics CoolSnap CF color camera (Nikon) using MetaMorph software (version 4.6.5; Universal Imaging).

Ki-67 Immunohistochemistry

Frozen sections (5 μm) were stained with anti-Ki-67 antibody as described previously (25). Results were expressed as percentage \pm SE of Ki-67⁺ cells per $\times 40$ magnification. A total of ten $\times 40$ fields were examined and counted from three 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) as described pre-

viously (25). Areas of greatest vessel density were then examined under higher magnification ($\times 100$) and counted. Any distinct area of positive staining for CD31 was counted as a single vessel. Results were expressed as the mean \pm SE number of vessels per high-power field ($\times 100$). A total of 20 high-power fields were examined and counted from three 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 (a) to determine whether curcumin (Fig. 1A) sensitizes chemoresistant multiple myeloma cells that have developed resistance to conventional

chemotherapeutic agents, (b) to determine whether curcumin potentiates the antitumor effects of thalidomide and bortezomib, two chemotherapeutic agents used extensively to treat multiple myeloma patients, (c) to determine whether curcumin potentiates the effects of these chemotherapeutic agents *in vivo*, and (d) to determine the mechanism by which curcumin sensitizes multiple myeloma cells to

these drugs. We used multiple myeloma 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 Multiple Myeloma Cells

To determine whether curcumin suppresses the proliferation of drug-sensitive and drug-resistant human multiple

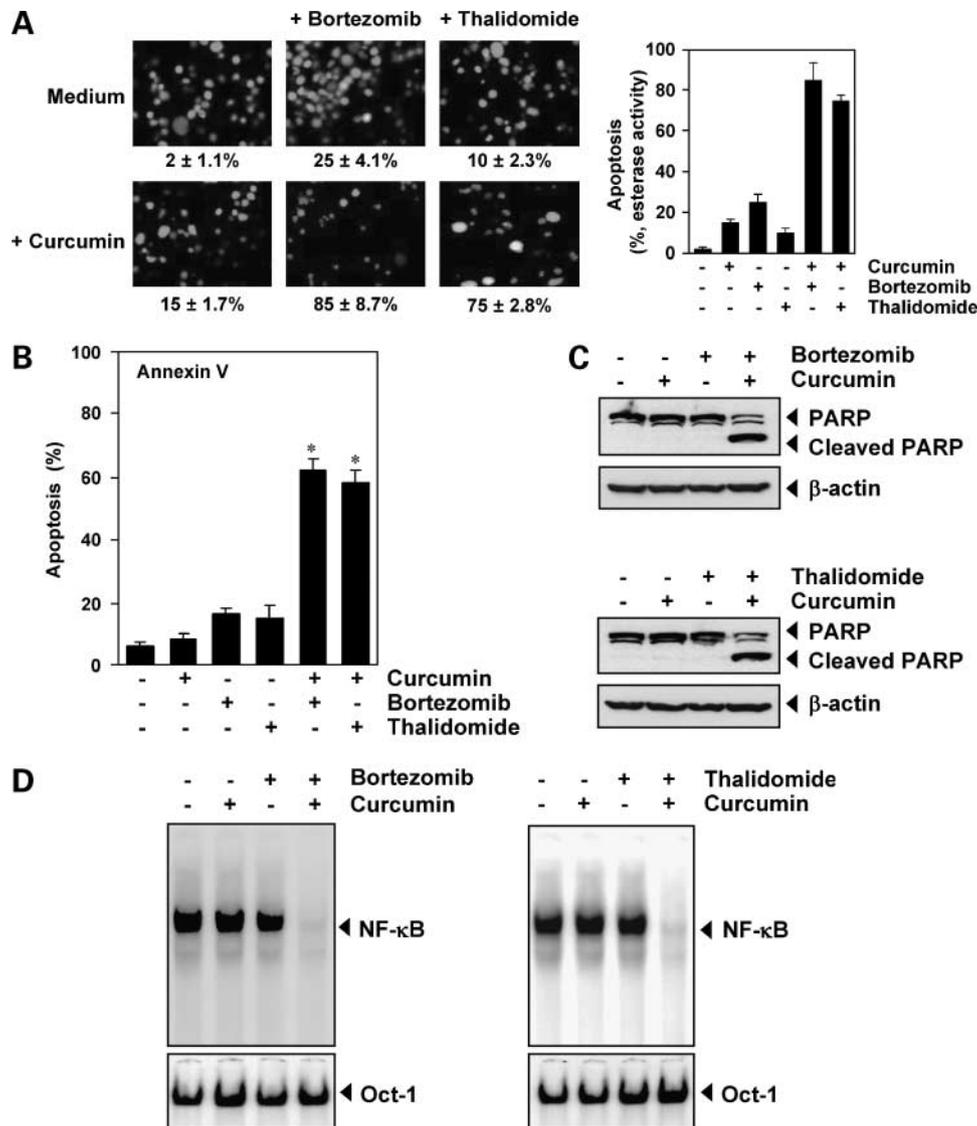


Figure 2. Curcumin potentiates the apoptotic effect of bortezomib and of thalidomide. **A**, *left*, U266 cells (1×10^6 /mL) were treated with 25 μ M/L curcumin, 20 nmol/L bortezomib, or 10 μ M/L thalidomide alone or a combination of these two agents with curcumin for 24 h at 37°C. Cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope. *Inset*, percentage of apoptosis. *Right*, graphic representation of the data with U266 cells. **B**, U266 cells (1×10^6 /mL) were treated with 25 μ M/L curcumin, 20 nmol/L bortezomib, or 10 μ M/L thalidomide alone or a combination of these agents with curcumin for 12 h at 37°C. Cells were incubated with anti-Annexin V antibody conjugated with FITC and then analyzed with a flow cytometry to detect early apoptotic effects. Mean \pm SD of three experiments. *, $P < 0.001$ versus control as well as single agent. **C**, U266 cells (1×10^6 /mL) were treated with 25 μ M/L curcumin, 20 nmol/L bortezomib, or 10 μ M/L thalidomide alone or a combination of these agents with curcumin for 12 h at 37°C. Whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis using antibody against poly(ADP-ribose) polymerase. The same blots were stripped and reprobed with β -actin antibody to show equal protein loading. Results of three independent experiments. **D**, U266 cells (1×10^6 /mL) were treated with 5 μ M/L curcumin or 10 nmol/L bortezomib alone or a curcumin + bortezomib combination for 12 h at 37°C and then tested for NF- κ B activation by EMSA (*left*). U266 cells (1×10^6 /mL) were treated with 5 μ M/L curcumin or 10 μ M/L thalidomide alone or a curcumin + thalidomide combination for 12 h at 37°C and then tested for NF- κ B activation by EMSA (*right*). Oct-1 EMSA served as a loading control. Results of three independent experiments.

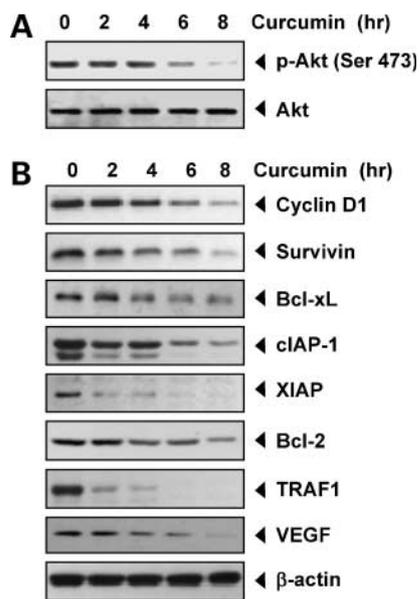


Figure 3. Curcumin suppresses the expression of antiapoptotic proteins in multiple myeloma cells. **A**, curcumin suppresses Akt activation in U266 cells. U266 cells (2×10^6 /mL) were treated with 50 μ mol/L curcumin for the indicated times. Whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis using the indicated proteins. The same blots were stripped and reprobbed 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 μ mol/L 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 reprobbed with β -actin antibody to show equal protein loading.

myeloma cells, we tested the cell proliferation using an (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay. Curcumin suppressed the proliferation of all multiple myeloma 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- and time-dependent manner (Fig. 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 Multiple Myeloma Cells

Bortezomib, a proteasome inhibitor, and thalidomide, a tumor necrosis factor inhibitor, have been approved for the conventional treatment of multiple myeloma 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% (Fig. 2A, *middle*) and thalidomide from 10% to 75% (Fig. 2A, *right*).

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 thalidomide from 18% to 57% (Fig. 2B).

When we examined the poly(ADP-ribose) polymerase cleavage, which indicates caspase-3 activation, a well-known characteristic of apoptosis, we found that curcumin potentiated the effect of bortezomib (Fig. 2C, *top*) and thalidomide (Fig. 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- κ B Activation in Multiple Myeloma 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- κ B is associated with growth and survival of cancer cells, including multiple myeloma cells (27, 28). In addition, studies have shown that bortezomib and thalidomide have been shown to suppress NF- κ B activation (29, 30). To determine whether the apoptotic effects of curcumin alone and the combination of curcumin with these drugs correlates with down-regulation of NF- κ B 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- κ B activation by EMSA. Curcumin potentiated the inhibitory effect of bortezomib and thalidomide on NF- κ B activation in multiple myeloma cells (Fig. 2D).

Curcumin Suppresses Akt Activation in Multiple Myeloma Cells

The phosphatidylinositol 3-kinase/Akt pathway is one of the signaling pathways whose dysregulation has been linked with chemoresistance in multiple myeloma cells (31). We investigated whether curcumin suppressed the activation of Akt in multiple myeloma 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 (Fig. 3A), indicating that these reduced levels of activated Akt may contribute toward increasing the apoptosis in multiple myeloma cells.

Curcumin Inhibits the Expression of NF- κ B-Regulated Gene Products in Multiple Myeloma 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- κ B. To determine whether curcumin mediates its effects by down-regulating the expression of NF- κ B-regulated proteins, we examined the effect of curcumin on the expression of NF- κ B-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 down-regulated the constitutive expression of cyclin D1, cIAP-1, XIAP, survivin, Bcl-2, Bcl-xL, and TRAF1 and VEGF in U266 cells

(Fig. 3B). The effect of curcumin on apoptosis showed the consistent to our previous report (3).

Curcumin Potentiates the Antitumor Effects of Bortezomib in Human Multiple Myeloma Xenograft in Nude Mice

To determine whether curcumin enhances the antitumor effects of bortezomib against multiple myeloma, we developed a human multiple myeloma xenograft in nude mice using U266 cells. A week after implantation, the animals were randomized into four 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 (Fig. 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 (Fig. 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 (Fig. 4B). Both curcumin alone and bortezomib alone significantly decreased the tumor volume; the tumor volume in the curcumin + borte-

zomib 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 versus curcumin + bortezomib; $P < 0.001$, bortezomib versus curcumin + bortezomib; Fig. 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 with either agent alone ($P < 0.001$ versus vehicle; Fig. 4D).

Curcumin Inhibits NF- κ B Activation in Human Multiple Myeloma Xenograft in Nude Mice

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

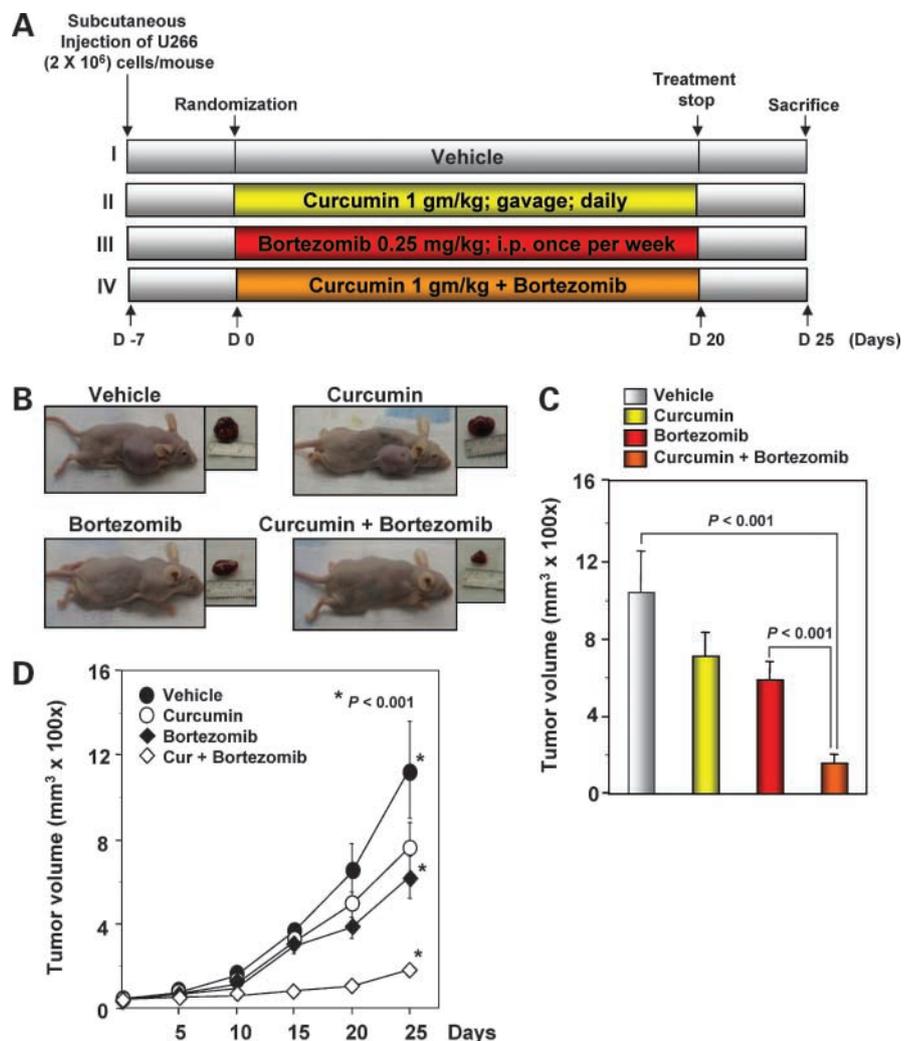


Figure 4. Curcumin potentiates the antitumor effects of bortezomib in myeloma tumor growth in nude mice induced by U266 cells. **A**, schematic representation of the experimental protocol as described in Materials and Methods. U266 cells (2×10^6 per mice) were injected s.c. into the left flank of the mice. The animals were randomized after 1 wk of tumor cell injection into four groups based on tumor volume. Group I was treated with corn oil (100 μ L, orally, daily) and saline (100 μ L, orally, weekly), group II was treated with curcumin alone (1 g/kg, orally, daily), group III was treated with bortezomib alone (0.25 mg/kg, orally, weekly), and group IV with a combination of curcumin (1 g/kg, orally, daily) and bortezomib (0.25 mg/kg, orally, weekly; $n = 6$). **B**, photographs of mice bearing xenotransplanted multiple myeloma. **C**, tumor volumes (mean \pm 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**, tumor diameters were measured at 5-d intervals with Vernier calipers, and the tumor volumes were calculated using the formula: $V = 2/3\pi r^3$ ($n = 5$).

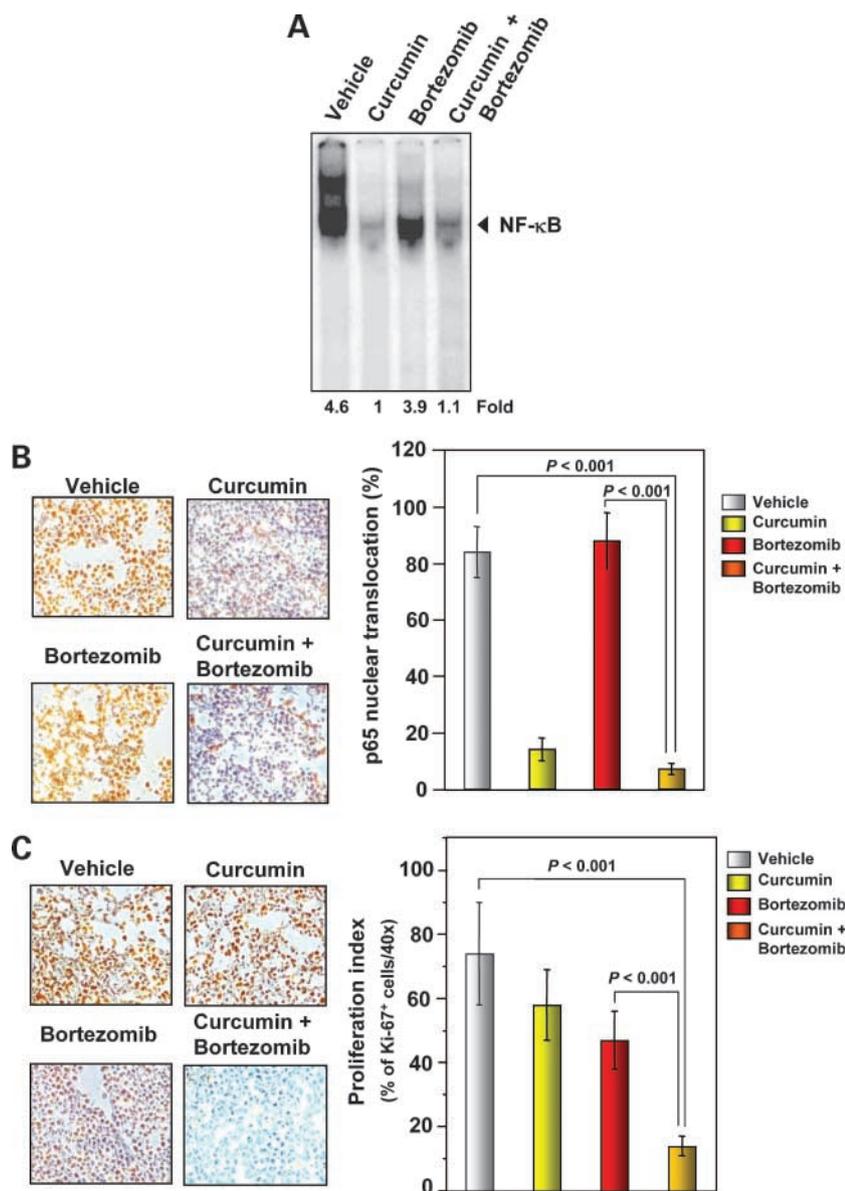


Figure 5. Curcumin enhances the inhibitory effect of bortezomib on the activation of NF- κ B and expression of the cell proliferation marker Ki-67 in multiple myeloma tumor samples. **A**, EMSA analysis revealed that curcumin inhibited NF- κ B activation in nuclear extracts from animal tissue. **B**, *left*, immunohistochemical analysis of nuclear p65 in tumor tissues showed that curcumin alone and in combination with bortezomib inhibited NF- κ B activation. **B**, *right*, quantification of NF- κ B expression in multiple myeloma tumor samples. Percentages indicate p65 nuclear positive cells. Samples from three animals in each treatment group were analyzed. Representative data. **C**, *left*, immunohistochemical analysis of Ki-67-positive cells in multiple myeloma indicated that curcumin alone and in combination with bortezomib suppressed cell proliferation. Samples from three animals in each treatment group were analyzed. Representative data. **C**, *right*, quantification of Ki-67 proliferation index as described in Materials and Methods. Mean \pm SE of triplicate.

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 (Fig. 5B, *left* and *right*).

Curcumin Down-Regulates the Expression of the Cell Proliferation Marker Ki-67 in Human Multiple Myeloma Xenograft in Nude Mice

To determine whether curcumin decreases multiple myeloma tumor growth by inhibiting proliferation, we examined the expression of Ki-67-positive cells in multiple myeloma tumors from mice. Our results showed that curcumin alone and bortezomib alone significantly decreased the expression of Ki-67 in tumor tissue (Fig. 5C, *left* and *right*). The curcumin + bortezomib combination was more effective

in reducing Ki-67 expression than either agent alone ($P < 0.001$ versus vehicle).

Curcumin Inhibits Angiogenesis and Down-Regulates the Expression of VEGF in Human Multiple Myeloma Xenograft in Nude Mice

To determine whether curcumin decreases multiple myeloma tumor growth by inhibiting angiogenesis, we examined the expression of the microvessel density marker CD31⁺ in multiple myeloma 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 (Fig. 6A, *left* and *right*; $P < 0.001$ versus vehicle).

Because VEGF plays an important role in angiogenesis, we also examined its expression in multiple myeloma

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 multiple myeloma tumors (Fig. 6B, left and right).

Discussion

Despite the fact that several treatments for multiple myeloma are currently available, multiple myeloma remains an incurable disease, with a median survival time of 3 to 5 years (32–34). The disease relapses in the majority of multiple myeloma patients regardless of the treatment regimen or their initial response to a given treatment. Recently, three new agents (bortezomib, thalidomide, and lenalidomide) were approved for the treatment of multiple myeloma patients. Bortezomib (Velcade) was approved for the treatment of multiple myeloma patients who have received at least one prior therapy, thalidomide (Thalomid) in combination with dexamethasone was approved for the treatment of newly diagnosed multiple myeloma patients, and the thalidomide analogue lenalidomide (Revlimid) in combination with dexamethasone was recently approved for the treatment of multiple myeloma 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 mel-

phalan, 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 multiple myeloma are required to improve the outcome and extend the survival of multiple myeloma patients.

Because the transcription factor NF- κ B has been closely linked with chemoresistance, cell survival, and proliferation, we investigated whether curcumin suppresses the proliferation of multiple myeloma cells that have developed resistance to chemotherapeutic agents. We found that curcumin suppressed human multiple myeloma 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 multiple myeloma to melphalan, doxorubicin, or dexamethasone? First, cells resistant to chemotherapeutic agents have been shown to express increased activation of NF- κ B and suppression of this NF- κ B can sensitize the cells to the drug (41). Second, multiple myeloma cells and mantle cell lymphoma are known to express constitutive active NF- κ B that is resistant to bortezomib (42, 43). Our results indicate that the mechanism of potentiation is the suppression of NF- κ B

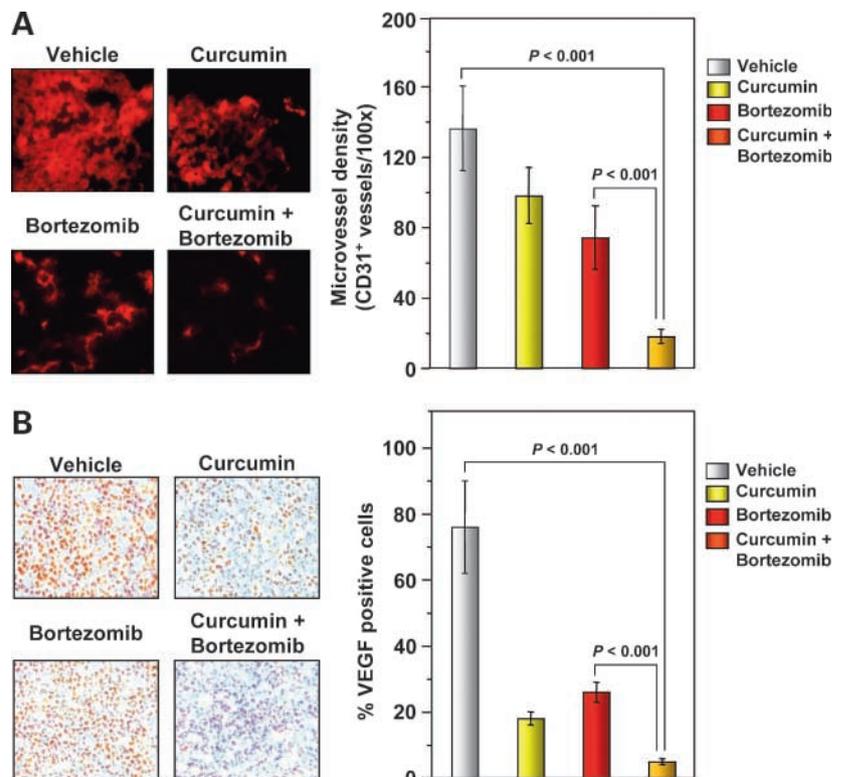


Figure 6. Curcumin enhances effects of bortezomib to inhibit angiogenesis in multiple myeloma tumor samples. **A**, left, immunohistochemical analysis of the microvessel density marker CD31 in multiple myeloma tumor samples. Samples from three animals in each treatment group were analyzed. Representative data. **A**, right, quantification of CD31⁺ microvessel density as described in Materials and Methods. Mean \pm SE of triplicate. **B**, left, immunohistochemical analysis of VEGF in multiple myeloma tumor samples revealed that curcumin alone and in combination with bortezomib suppresses angiogenesis. Samples from three animals in each treatment group were analyzed. Representative data. **B**, right, quantification of VEGF as described in Materials and Methods. Mean \pm SE of triplicate.

activation. Why NF- κ B is constitutively active in multiple myeloma cells is not fully understood. Recently, two studies found mutations on the genes encoding positive and negative regulators of NF- κ B signaling in many multiple myeloma cell lines and primary tumor cells; these mutations are thought to mediate the constitutive activation of NF- κ B in multiple myeloma cells (10, 11). It is likely that the constitutive activation of NF- κ B in multiple myeloma cells leads to chemoresistance. For example, C-reactive protein, whose expression is regulated by NF- κ B, 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). C-reactive protein has also been shown to bind to Fc γ receptors and activate Akt, extracellular signal-regulated kinase, and NF- κ B pathways. C-reactive protein 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 down-regulates Akt activation, another possible mechanism that curcumin sensitizes tumor cells to chemotherapeutic agents. Previously, we showed that curcumin also down-regulates the expression of IL-6 (4), a major growth factor for multiple myeloma cells. We also found that curcumin down-regulated 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 multiple myeloma cells (44–46). Thus, in addition to the suppression of NF- κ B activation, these mechanisms may help explain the effects of curcumin.

Like curcumin, bortezomib inhibits NF- κ B activation but through different mechanisms. The pathway by which bortezomib prevents degradation of I κ B differs from that of curcumin. Bortezomib inhibits the proteasome, resulting in the accumulation of I κ B α , whereas curcumin prevents I κ B phosphorylation, thus blocking its subsequent ubiquitination and degradation through suppression of upstream kinase IKK. Thus, these different mechanisms of NF- κ B suppression provide the rationale for combining these agents to effectively inhibit NF- κ B activation. Furthermore, other mechanisms of bortezomib and curcumin may support their combination to maximize their cytotoxicity against multiple myeloma 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 multiple myeloma cells both in the laboratory (41, 48) and in the clinic (49). Indeed, bortezomib has been shown to exhibit considerable clinical efficacy against multiple myeloma. Whether this activity is due to its anti-NF- κ B activity is not clear (35), as its ability to suppress NF- κ B in patients has not been shown.

We next determined whether curcumin potentiates the effects of bortezomib on human multiple myeloma xenografts in nude mice. Our results showed that curcumin alone and bortezomib alone inhibited the multiple myeloma xenograft in nude mice. We found that the curcumin + bortezomib combination had a higher antimyeloma

effect than either agent alone. When we investigated the mechanism, we found that curcumin down-regulated the expression of NF- κ B, the cell proliferation marker Ki-67, and the microvessel density marker CD31, all have been linked with chemoresistance. Because curcumin alone inhibited most of NF- κ B activity with partial antitumor effect, the down-regulation of NF- κ B 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- κ B DNA-binding activity, although this combination leads to the greatest effect on myeloma growth *in vivo*. Thus, it is possible that the *in vivo* effect of curcumin is not entirely mediated by its ability to suppress NF- κ B. Curcumin has been shown to modulate multiple targets (50) that may be involved in its *in vivo* antitumor action.

Multiple myeloma 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 multiple myeloma patients. A recent phase I study of curcumin showed that this agent can be administered safely at oral doses of up to 12 g/d (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 multiple myeloma patients have shown that curcumin down-regulates NF- κ B activation in peripheral blood mononuclear cells even at dose of 2 g/d (20). Thus, curcumin can be used (a) to enhance the effects of current chemotherapeutic agents and (b) to overcome chemoresistance in multiple myeloma to conventional therapy. Fatigue and peripheral neuropathy are two symptoms commonly observed in multiple myeloma 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 multiple myeloma patients.

In conclusion, the chemoresistance remains a major challenge in the treatment of patients with multiple myeloma as well as other cancers. Multiple myeloma 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, and progression-free and overall survival times for multiple myeloma patients are short. The ability of curcumin to suppress NF- κ B activation, down-regulate 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 multiple myeloma.

Disclosure of Potential Conflicts of Interest

Curcumin is commonly available from numerous health food stores and various companies, including Indogen which was set up by spouse of one of the authors (B. B. Aggarwal). No potential conflicts of interest were disclosed.

Acknowledgments

We thank Lionel Santibañez for carefully editing this article.

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