Capsaicin Is a Novel Blocker of Constitutive and Interleukin-6–Inducible STAT3 Activation

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Abstract

Purpose: Capsaicin, a constituent of green and red peppers, has been linked with suppression of tumorigenesis through a mechanism that is not well understood. Because the transcription factor signal transducer and activator of transcription 3 (STAT3) has been closely linked with tumorigenesis, we investigated the effect of this vanilloid on the STAT3 pathway in human multiple myeloma cells.

Experimental Design: The effect of capsaicin on both constitutive and interleukin-6–induced STAT3 activation, associated protein kinases, and STAT3-regulated gene products involved in proliferation, survival and angiogenesis, cellular proliferation, and apoptosis in multiple myeloma cells was investigated.

Results: We found that capsaicin inhibited constitutive activation of STAT3 in multiple myeloma cells in a dose- and time-dependent manner, with minimum effect on STAT5. Capsaicin also inhibited the interleukin-6–induced STAT3 activation. The activation of Janus-activated kinase 1 and c-Src, implicated in STAT3 activation, was also inhibited by the vanilloid, with no effect on extracellular signal-regulated kinase 1/2 activation. Pervanadate reversed the capsaicin-induced down-regulation of STAT3, suggesting the involvement of a protein tyrosine phosphatase. Capsaicin down-regulated the expression of the STAT3-regulated gene products, such as cyclin D1, Bcl-2, Bcl-xL, survivin, and vascular endothelial growth factor. Finally, capsaicin induced the accumulation of cells in G_1 phase, inhibited proliferation, and induced apoptosis, as indicated by caspase activation. Capsaicin also significantly potentiated the apoptotic effects of Velcade and thalidomide in multiple myeloma cells. When administered i.p., capsaicin inhibited the growth of human multiple myeloma xenograft tumors in male athymic *nu/nu* mice.

Conclusion: Overall, these results suggest that capsaicin is a novel blocker of the STAT3 activation pathway, with a potential role in the prevention and treatment of multiple myeloma and other cancers.

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a principal pungent ingredient of hot red and chili peppers that belong to the plant genus *Capsicum* (Solanaceae). In addition to alleviating neuropathic pain and itching in humans (1–4), capsaicin has exhibited anticancer effects in animal models, suppressing carcinogenesis of the skin (5), colon (6), lung (7), tongue (8), and prostate (9, 10). The mechanism by which this vanilloid mediates its anticarcinogenic effects is not understood but has been shown to alter the metabolism of carcinogens such as aflatoxin B1 (11) and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (12, 13). In culture, capsaicin has been found to selectively suppress the growth of various human tumor cells (14, 15), including leukemic (16–18), gastric (19), hepatic (20), glioma (21), and prostate cells (9). How capsaicin mediates its anticancer effects is not fully understood, but the roles of NADH oxidase activity (14), proteasome (9), cyclooxygenases (22), c-Jun NH_2-terminal kinase (23), nuclear factor-κB (24), peroxisome proliferator-activated receptor γ (25, 26), peroxynitrite (21), and mitochondrial respiration (27) have been implicated. Its immunosuppressive effects have been linked to its ability to suppress nuclear factor-κB activation (28).

Members of the signal transducer and activator of transcription (STAT) family of transcription factors regulate the expression of gene products involved in cell survival, proliferation, chemoresistance, and angiogenesis (29, 30). The activation of STATs involves the phosphorylation of a critical tyrosine residue by Janus-activated kinases (JAK), or the Src family kinases, leading to dimerization of STAT monomers, nuclear

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translocation, and binding to specific DNA response elements in the promoters of target genes. Among the STATs, STAT3 is perhaps the most intimately linked to tumorigenesis (31). Although STAT3 is activated by interleukin-6 (IL-6), epidermal growth factor, and other growth factors, constitutive activation of STAT3 has been discovered in a wide variety of tumors (31–34).

Because of the critical role of STAT3 in tumor cell survival, proliferation, and angiogenesis and its expression in various tumor cells, we hypothesized that capsaicin must mediate its effects in part through the suppression of the STAT3 pathway. We found that capsaicin does indeed suppress both constitutive and inducible STAT3 activation. This correlated with down-regulation of expression of cell survival, proliferative, and angiogenic gene products, leading to suppression of proliferation, induction of apoptosis, and enhancement of apoptosis induced by Velcade and thalidomide.

Materials and Methods

Materials. Capsaicin (molecular weight, 305.42) was purchased from Tocris Cookson. A 50 mmol/L stock solution of capsaicin was prepared in DMSO and then further diluted in cell culture medium. Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide, Tris, glycine, NaCl, SDS, and bovine serum albumin were purchased from Sigma-Aldrich. RPMI 1640, fetal bovine serum, 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Life Technologies. Rabbit polyclonal antibodies to STAT3 and STAT5 and mouse monoclonal antibodies against phosphorylated STAT3 (Tyr^{705}) and phosphorylated STAT5, phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), ERK, Bcl-2, Bcl-xL, cyclin D1, survivin, procaspase-3, and poly(ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology. Goat anti-rabbit horseradish peroxidase conjugate was purchased from Bio-Rad. Anti–vascular endothelial growth factor (VEGF) was purchased from NeoMarkers. Antibodies to phospho-specific src (Tyr^{416}), src, phospho-specific JAK1 ($Tyr^{1022/1023}$), and JAK1 were purchased from Cell Signaling Technology. Goat anti-mouse horseradish peroxidase was purchased from Transduction Laboratories (Lexington, KY), and goat anti-rabbit Alexa 594 was purchased from Molecular Probes. Bacteria-derived recombinant human IL-6 was kindly provided by Novartis Pharmaceuticals. Velcade (PS-341) was obtained from Millennium. Thalidomide was obtained from Tocris Cookson.

Cell lines. The human multiple myeloma cell lines U266 and MM.1S were obtained from the American Type Culture Collection. Cell line U266 (ATCC TIB-196) is a plasmacytoma of B-cell origin and is known to produce monoclonal antibodies and IL-6 (35, 36). The MM.1S cell line, established from the peripheral blood cells of a patient with IgA myeloma, secretes light chain, is negative for the presence of the EBV genome, and expresses leukocyte antigen DR, plasma cell Ag-1, and T9 and T10 antigens (37). U266 and MM.1S cells were cultured in RPMI 1640 containing 1× antibiotic-antimycotic solution with 10% fetal bovine serum. Human myeloid leukemia (KBM-5) cells were obtained from the American Type Culture Collection and cultured in Iscove's modified Dulbecco's medium with 15% fetal bovine serum containing 1× antibiotic-antimycotic solution.

Western blot analysis. For detection of STAT proteins, capsaicin-treated whole-cell extracts were lysed in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8), 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 $NaVO_4$]. Lysates were then centrifuged at 14,000 rpm for 10 min to remove insoluble material and resolved on a 7.5% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-STAT antibodies (1:1,000) overnight at 4°C. The blot was washed, exposed to horseradish peroxidase–conjugated secondary antibodies for 1 h, and finally examined by enhanced chemiluminescence (Amersham Pharmacia Biotech).

To detect the expression of STAT3-regulated proteins and caspase-3, U266 cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin for the indicated times. The cells were then washed and extracted by incubation for 30 min on ice in 0.05 mL of buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 250 mmol/L NaCl, 0.1% NP40, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5 μg/mL benzamidine, 1 mmol/L DTT, and 1 mmol/L sodium vanadate. The lysate was centrifuged, and the supernatant was collected. Whole-cell protein extract (50 μg) was resolved on 12% SDS-PAGE; electrotransferred onto a nitrocellulose membrane; blotted with antibodies against survivin, Bcl-2, Bcl-xl, cyclin D1, VEGF, caspase-3; and then detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay for STAT3-DNA binding. STAT3-DNA binding was analyzed by electrophoretic mobility shift assay using a ^{32}P-labeled high-affinity sis-inducible element probe as previously described (38). Briefly, nuclear extracts were prepared from capsaicin treated cells and incubated with a high-affinity sis-inducible element probe (5′-CTTCATTTCCCGTAAATCCCTAAAGCT-3′ and 5′-AGCTT-TAGGGATTTACGGGAAATGA-3′). DNA-protein complex formed was separated from free oligonucleotide on 5% native polyacrylamide gels. The dried gels were visualized, and the radioactive bands were quantitated with a Storm 820 and Imagequant software (Amersham).

Immunocytochemistry for STAT3 localization. Capsaicin-treated multiple myeloma cells were plated on a glass slide by centrifugation using a Cytospin 4 (Thermoshendon), air-dried for 1 h at room temperature, and fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human STAT3 antibody (dilution, 1:100). After overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1:100) for 1 h and counterstained for nuclei with Hoechst (50 ng/mL) for 5 min. Stained slides were mounted with mounting medium (Sigma-Aldrich) and analyzed under an epifluorescence microscope (Labophot-2, Nikon). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).

Antiproliferative assay. The antiproliferative effects of capsaicin against U266 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide dye uptake method as described earlier (39).

Flow cytometric analysis. To determine the effect of capsaicin on the cell cycle, U266 cells were first synchronized by serum starvation and then exposed to capsaicin for the indicated time intervals. Thereafter cells were washed, fixed with 70% ethanol, and incubated for 30 min at 37°C with 0.1% RNase A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 μg/mL propidium iodide for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a FACSCalibur flow cytometer (Becton Dickinson).

Immunoblot analysis of PARP degradation. Ursolic acid–induced apoptosis was examined by proteolytic cleavage of PARP. Briefly, cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin for indicated time points at 37°C. The cells were then washed and extracted by incubation for 30 min on ice in 0.05 mL buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 250 mmol/L NaCl, 0.1% NP40, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5 μg/mL benzamidine, 1 mmol/L DTT, and 1 mmol/L sodium vanadate. The lysate was centrifuged, and the supernatant was collected. Cell extract protein (50 μg) was resolved on 7.5% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with anti-PARP antibody, and then detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Live/Dead assay. Apoptosis of cells was also determined by the Live/Dead assay (Molecular Probes), which measures intracellular esterase activity and plasma membrane integrity, as described previously (40). Briefly, 1×10^6 cells were incubated with capsaicin/Velcade/thalidomide alone or in combination for 24 h at 37°C. Cells were stained with the Live/Dead reagent (5 μmol/L ethidium homodimer, 5 μmol/L calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2).

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 in the standard mice plexiglass cages (five per cage) in a room maintained at constant temperature and humidity under 12-h light and darkness cycle and fed with regular autoclave chow diet 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 harvested from subconfluent cultures were washed once in serum-free medium and resuspended in PBS. Cell suspensions consisting of single cells, with >90% viability, were used for the injections. Mice were anesthetized with ketamine-xylazine solution, and 2×10^6 cells in 100 μL (sterile PBS and Matrigel, 1:1) were injected into the left flank of mice, s.c. using a 25-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe Co.). 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 a vernier caliper. The mice were randomized into the following treatment groups ($n = 5$) based on the initial tumor volume: (A) untreated control (DMSO, 100 μL) and (B) capsaicin (1 mg/kg in 100 μL). The control group was injected with DMSO and treated group with capsaicin thrice per week, i.p. The treatment was continued up to 3 weeks from the date of randomization. The tumor volume was measured once a week using the formula $V = 0.5236 \times d_1^2 \times d_2$, where d_1 is the shortest diameter, and d_2 is the longest diameter. The mice were sacrificed 25 days after the start of treatment. The tumors were carefully excised, and the diameters were measured for final tumor volume calculation. The final tumor volume was measured using the formula $V = 4/3\pi r^3$, where r is the mean of the three dimensions (length, width, and depth). The tumor volumes were compared among two groups using Student's t test, with $P < 0.05$ considered as significant.

Results

To further define the antitumor effects of capsaicin, we investigated the effect of capsaicin on both constitutively active and IL-6–inducible STAT3 phosphorylation in multiple myeloma cells in the present report. We also evaluated the effect of capsaicin on various mediators of cellular proliferation,

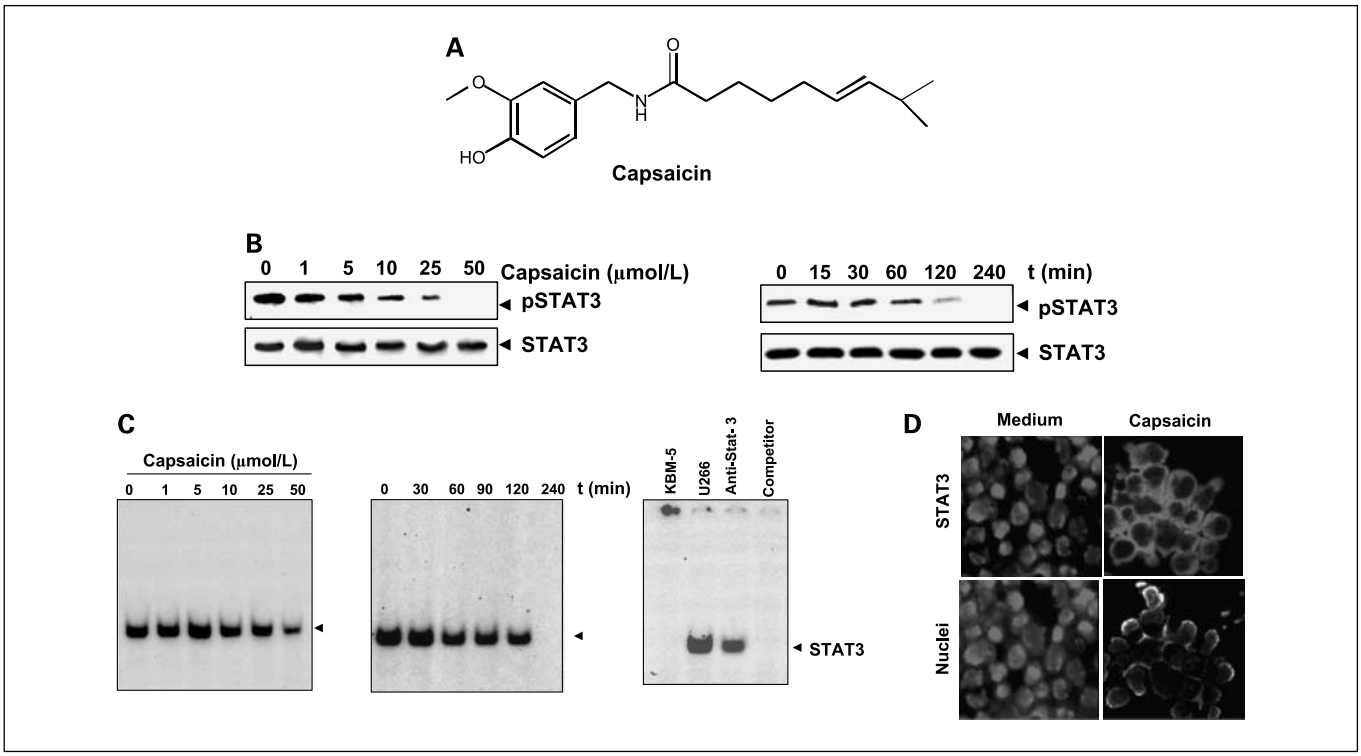

Fig. 1. Capsaicin inhibits constitutively active STAT3 in U266 cells. *A,* structure of capsaicin. *B, left,* capsaicin suppresses phosphorylated STAT3 (*pSTAT3*) levels in a dose-dependent manner. U266 cells (2×10^6 per mL) were treated with the indicated concentrations of capsaicin for 4 h. Whole-cell extracts were then prepared, and 30 μg of protein was resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phosphorylated STAT3. *B, right,* capsaicin suppresses phosphorylated STAT3 levels in a time-dependent manner. U266 cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin for the indicated times, after which Western blotting was done as described for left panel. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. *C, left,* capsaicin suppresses STAT3 DNA binding in a dose-dependent manner. U266 cells (2×10^6 per mL) were treated with the indicated concentrations of capsaicin for 4 h and analyzed for nuclear STAT3 levels by electrophoretic mobility shift assay. *C, middle,* U266 cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin for the indicated times and analyzed for nuclear STAT3 levels by EMSA. *C, right,* nuclear extracts from U266 cells were incubated with STAT3 antibody and an unlabeled STAT3 oligo probe. Nuclear extracts from untreated myeloid leukemia (KBM-5) cells were taken alone. They were then assayed for STAT3 DNA binding by electrophoretic mobility shift assay. *D*, capsaicin causes inhibition of translocation of STAT3 to the nucleus. U266 cells (1×10^5 per mL) were incubated with or without 50 μmol/L capsaicin for 4 h and then analyzed for the intracellular distribution of STAT3 by immunocytochemistry.

cell survival, and apoptosis. The structure of capsaicin is shown in Fig. 1A. The dose and duration of capsaicin used for the STAT3 experiments had no effect on cell viability (data not shown).

Capsaicin inhibits constitutive STAT3 phosphorylation in multiple myeloma cells. There are reports from our laboratory and others that multiple myeloma U266 cells express constitutively active STAT3 (31, 33, 34). Whether capsaicin can modulate the constitutive STAT3 activation in these cells was investigated. Western blot analysis with specific antibodies to phosphorylated STAT3 indicated that capsaicin inhibited constitutively active STAT3 in U-266 cells in a dose-dependent (Fig. 1B1) and time-dependent (Fig. 1B2) manner. This inhibition appeared as early as 1 h, with complete abrogation at 4 h after 50 μmol/L capsaicin treatment. Capsaicin treatment did not alter the overall expression of STAT3 protein.

We also investigated whether capsaicin affects the activation of other STAT proteins in U266 cells. We found that besides STAT3, U266 cells expressed constitutively active STAT5 and capsaicin altered neither the levels of constitutively phosphorylated STAT5 nor the expression of STAT5 proteins (data not shown).

Capsaicin inhibits DNA binding of STAT3 in multiple myeloma cells. The translocation of STAT3 to the nucleus leads to a specific DNA binding, which in turn regulates gene transcription (38). We examined whether capsaicin suppresses the DNA-binding activity of STAT3. Electrophoretic mobility shift assay analysis of nuclear extracts prepared from U266 cells showed that capsaicin decreased STAT3 DNA-binding activity in a dose-dependent (Fig. 1C1) and time-dependent (Fig. 1C2) manner. Supershift analysis indicated that the binding of STAT3 to the DNA was blocked by anti-Stat3 antibody and by cold competitor oligonucleotide, thus confirming that the protein/DNA complex observed actually contained STAT3. No constitutive activation of STAT3 could be detected in human myeloid KBM-5 cells (Fig. 1C3). These results show that capsaicin abrogates the DNA-binding ability of STAT3.

Capsaicin depletes the nuclear pool of STAT3 in multiple myeloma cells. Under resting conditions and in the non-phosphorylated state, STAT3 is retained in the cytoplasm. It translocates to the nucleus when phosphorylated (29, 30). Phosphorylation induces STAT3 dimerization, thus permitting its translocation into the nucleus. To determine whether capsaicin affects nuclear pools of STAT3, capsaicin-treated and untreated cells were plated on a glass slide, immunostained with antibody to STAT3, and then visualized by using the Alexa 594–conjugated second antibody. Figure 1D clearly shows that capsaicin depleted the nuclear pool of STAT3 in U266 cells.

Capsaicin inhibits inducible STAT3 phosphorylation in human multiple myeloma cells. Because IL-6 is a growth factor for multiple myeloma cells and induces STAT3 phosphorylation (35, 41), we determined whether capsaicin could inhibit IL-6–induced STAT3 phosphorylation. MM.1S cells were pretreated with capsaicin for different time intervals and then stimulated with IL-6 for 15 min. As shown in Fig. 2A, IL-6–induced STAT3 phosphorylation was blocked by capsaicin in a time-dependent manner. Exposure of the cells to capsaicin for 4 h was sufficient to completely suppress IL-6–induced STAT3 phosphorylation.

Capsaicin-induced inhibition of STAT3 phosphorylation is reversible in human multiple myeloma cells. We further examined whether capsaicin-induced inhibition of STAT3 phosphorylation is reversible. U266 cells were first treated for 60 min with capsaicin, and then the cells were washed twice with PBS to remove capsaicin. The cells were then cultured in fresh medium for various durations, and the levels of phosphorylated STAT3 were measured. Capsaicin induced the suppression of STAT3 phosphorylation (Fig. 2B), and the removal of capsaicin resulted in a gradual increase in phosphorylated STAT3 (Fig. 2C). The reversal was complete by 24 h and did not involve changes in STAT3 protein levels (Fig. 2C, *bottom*).

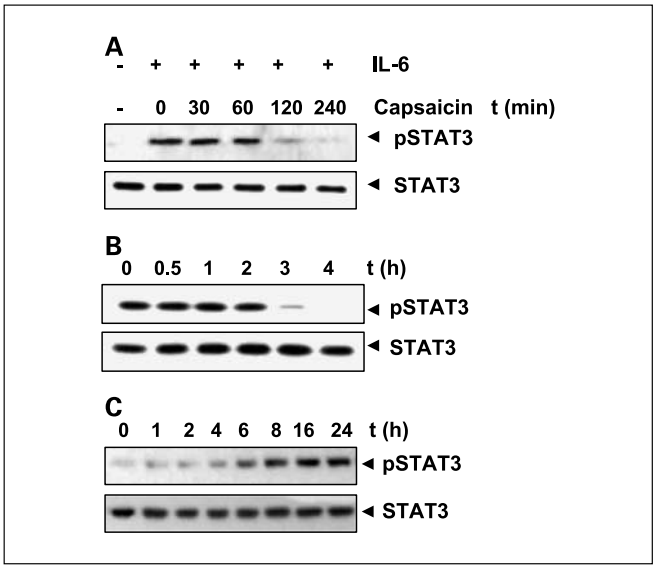

Fig. 2. Capsaicin down-regulates IL-6–induced phosphorylated STAT3. *A,* MM1.S cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin for the indicated times and then stimulated with IL-6 (10 ng/mL) for 15 min. Whole-cell extracts were then prepared and analyzed for phosphorylated STAT3 by Western blotting. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. Representative results of three independent experiments. *B,* capsaicin-induced inhibition of STAT phosphorylation is reversible. U266 cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin for the indicated times or treated for 1 h and washed with PBS twice to remove capsaicin before resuspension in fresh medium. *C,* cells were removed at the indicated times and lysed to prepare the whole-cell extract. Thirty micrograms of whole-cell extracts were resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, probed for the phosphorylated STAT3, and stripped and reprobed for STAT3 antibodies. Representative results of three independent experiments.

Capsaicin suppresses constitutive activation of JAK1. STAT3 has been reported to be activated by soluble tyrosine kinases of the Janus family (JAK; ref. 30). We examined whether capsaicin affects constitutive activation of JAK1 in U266 cells. We found that capsaicin suppressed the constitutive phosphorylation of JAK1 in a time-dependent manner (Fig. 3A). The levels of non-phosphorylated JAK1 remained unchanged under the same conditions (Fig. 3A, *bottom*).

Capsaicin suppresses constitutive activation of c-Src. STAT3 has also been reported to be activated by soluble tyrosine kinases of the Src kinase families (34). Hence, we determined the effect of capsaicin on constitutive activation of Src kinase in U266 cells. We found that capsaicin suppressed the constitutive phosphorylation of c-Src kinase (Fig. 3B). The levels of non-phosphorylated c-Src kinase remained unchanged under the same conditions.

Capsaicin does not affect constitutive activation of ERK. Apart from tyrosine phosphorylation, STAT3 also undergoes phosphorylation at serine residues (30) through the mitogen-activated protein kinase/RAS pathway (42). We therefore

investigated whether capsaicin modulates constitutive activation of ERK1/2 kinases in U266 cells. We found that capsaicin did not affect the constitutive phosphorylation of ERK1/2 kinase in U266 cells (Fig. 3C). The levels of non-phosphorylated ERK1/2 remained unchanged under the same conditions.

Tyrosine phosphatases are involved in capsaicin-induced inhibition of STAT3 activation. Because protein tyrosine phosphatases (PTP) have also been implicated in STAT3 activation (43), we determined whether capsaicin-induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a protein tyrosine phosphatase (PTPase). Treatment of U266 cells with the broad-acting tyrosine phosphatase inhibitor sodium pervanadate reversed the capsaicin-induced inhibition of STAT3 activation (Fig. 3D). This suggests that tyrosine phosphatases are involved in capsaicin-induced inhibition of STAT3 activation.

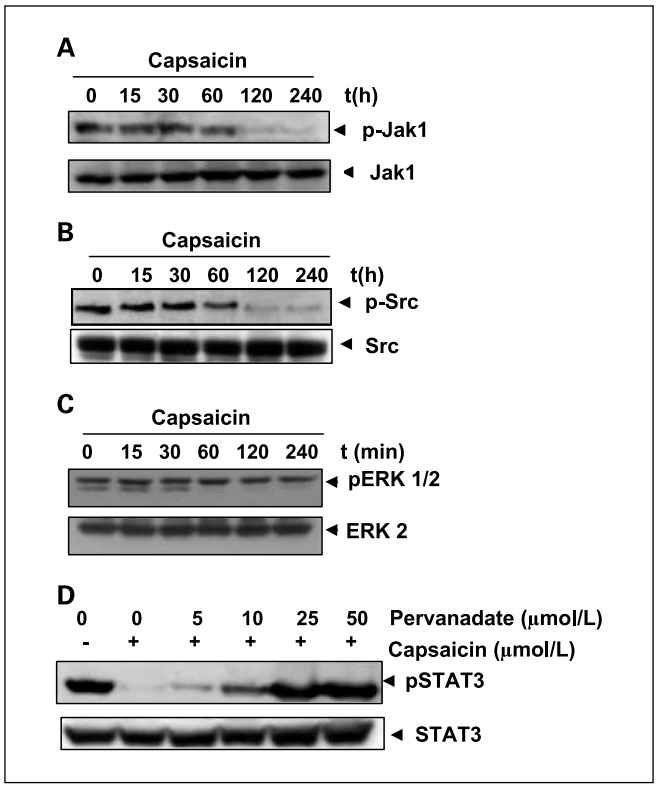

Fig. 3. Capsaicin suppresses phosphorylated Jak1 (*p-Jak1*) levels in a time-dependent manner. *A,* U266 cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin, after which whole-cell extracts were prepared, and 30-μg portions of those extracts were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phosphorylated JAK1 antibody. The same blot was stripped and reprobed with JAK1 antibody to verify equal protein loading. *B,* U266 cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin, after which whole-cell extracts were prepared, and 30-μg aliquots of those extracts were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phosphorylated src (*p-Src*) antibody. The same blot was stripped and reprobed with src antibody to verify equal protein loading. *C,* U266 cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin, after which whole-cell extracts were prepared, and 30-μg portions of those extracts were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phosphorylated ERK1/2 (*pERK 1/2*) antibody. The same blot was stripped and reprobed with ERK1/2 antibody to verify equal protein loading. *D,* pervanadate reverses the phosphorylated STAT3 inhibitory effect of capsaicin. U266 cells (2×10^6 per mL) were treated with the indicated concentrations of pervanadate and 50 μmol/L capsaicin for 4 h, after which whole-cell extracts were prepared, and 30-μg portions of those extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phosphorylated STAT3 and STAT3.

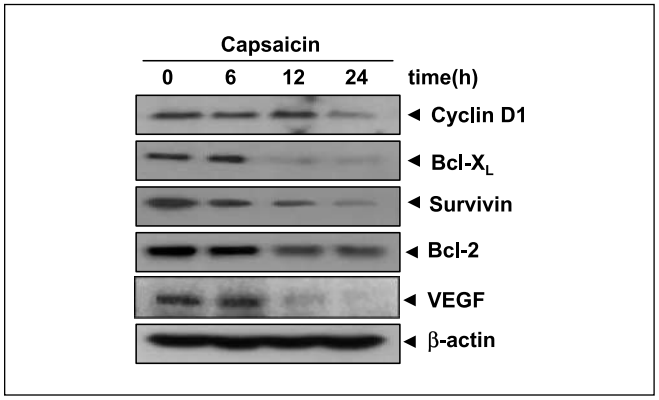

Fig. 4. Capsaicin suppresses STAT3-regulated gene products in U266 cells. U266 cells (2×10^6 per mL) were treated with 50 μmol/L capsaicin, after which whole-cell extracts were prepared, and 30-μg portions of those extracts were resolved on 10% SDS-PAGE gel, membrane sliced according to molecular weight, and probed against cyclin D1, Bcl-2, Bcl-XL, survivin, and VEGF antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading.

Capsaicin down-regulates the expression of cyclin D1, Bcl-2, Bcl-xL, survivin, and VEGF. STAT3 activation has been shown to regulate the expression of various gene products involved in cell survival, proliferation, and angiogenesis (34). We investigated whether the expression of the cell cycle regulator protein cyclin D1; the antiapoptotic proteins Bcl-2, Bcl-xL, and survivin; and the angiogenic gene product VEGF, all reported to be regulated by STAT3 (29–31, 44), was modulated by capsaicin treatment. Capsaicin treatment down-regulated expression of these proteins in a time-dependent manner, with maximum suppression observed at ~24 h (Fig. 4).

Capsaicin inhibits the proliferation of multiple myeloma cells. Because capsaicin suppressed the activation of STAT3 and STAT3-regulated gene products, we examined whether it modulates the proliferation of cells. We found that capsaicin suppressed the increase in proliferation of U266 cells in a dose-dependent manner (Fig. 5A).

Capsaicin causes the accumulation of the cells in G_1 phase. Because D-type cyclins are required for the progression of cells from the G_1 phase of the cell cycle to S phase (45), and because we observed a rapid decline in levels of cyclin D1 in the capsaicin-treated cells, we determined the effect of capsaicin on cell cycle phase distribution. We found that treatment with capsaicin caused significant accumulation of the cell population in G_1 phase (Fig. 5B).

Capsaicin activates caspase-3 and causes PARP cleavage. Whether suppression of constitutively active STAT3 in U266 cells by capsaicin leads to apoptosis was also investigated. Cell extracts prepared from capsaicin-treated cells showed a time-dependent activation of caspase-3 (Fig. 5C). Activation of downstream caspases led to the cleavage of a 116-kDa PARP protein into an 87-kDa fragment (Fig. 5D). These results clearly suggest that capsaicin induces caspase-3–dependent apoptosis in U266 cells.

Capsaicin potentiates the apoptotic effect of thalidomide and Velcade in multiple myeloma cells. Velcade, an inhibitor of proteasome, and thalidomide, which inhibits tumor necrosis factor expression, have been approved for the treatment of patients with multiple myeloma (46, 47). However, prolonged exposure to these agents is associated with toxicity and

development of chemoresistance. One of the mechanisms by which these agents confer resistance is due to the up-regulation of different antiapoptotic proteins (48–50). The ability of capsaicin to down-regulate the expression of antiapoptotic proteins, such as Bcl-2, Bcl-xL, and survivin, as described above prompted us to determine whether capsaicin can also lower the apoptotic threshold for these cytotoxic drugs. U266 cells were treated with capsaicin together with either thalidomide or Velcade and then examined the cells for apoptosis using the Live/Dead assay, which determines plasma membrane stability using esterase staining. As shown in Fig. 6A and B, capsaicin significantly enhanced the apoptotic effect of thalidomide from 15% to 60% and that of Velcade from 20% to 80%.

Thalidomide and Velcade enhance the effect of capsaicin in suppressing STAT3 activation. Whether potentiation of the apoptotic effects of capsaicin by thalidomide and Velcade was due to down-regulation of STAT3 activation was examined. To investigate this, U266 cells were exposed to sub-optimal doses of capsaicin, thalidomide, and Velcade and then examined for STAT3 phosphorylation. As shown in Fig. 6, thalidomide (C1) and Velcade (C2) potentiated the effect of capsaicin in down-regulating constitutive STAT3 activation.

***Capsaicin inhibits the growth of human multiple myeloma* in vivo.** Our *in vitro* experiments have shown that capsaicin is a potent inhibitor of STAT3 and antiapoptotic proteins in multiple myeloma cells. Next, we analyzed whether capsaicin can inhibit the growth of human multiple myeloma tumor in nude mice. Male nude mice were injected s.c. with U266 cells and treated with capsaicin administered i.p. for 3 weeks (thrice per week). The results showed that capsaicin significantly suppressed the tumor growth *in vivo* (Fig. 6D). At the end of treatment, we also noticed that two of five mice in the treatment group had barely detectable tumors. Our composite results show that capsaicin is an effective antitumor agent for multiple myeloma cells both under *in vitro* and *in vivo* conditions.

Discussion

The goal of this study was to determine whether the vanilloid capsaicin exerts its anticancer effects through the abrogation of the STAT3 signaling pathway in multiple myeloma cells. We found that capsaicin inhibited the constitutive and IL-6–inducible STAT3 activation in human multiple myeloma cells in parallel with the suppression of JAK1 and c-Src activation. However, capsaicin had no effect on the activation of Erk1/2. We also provided evidence for the role of PTP. Capsaicin also down-regulated the expression of STAT3-regulated gene products, including cyclin D1, survivin, Bcl-2, Bcl-xL, and VEGF. It induced the inhibition of proliferation, accumulation of cells in G_1-G_0 phase, and apoptosis, and it significantly potentiated the apoptotic effects of Velcade and thalidomide in multiple myeloma cells. Capsaicin also inhibited the growth of human multiple myeloma *in vivo*.

This is the first report to identify capsaicin as a novel blocker of the STAT3 pathway. We found that capsaicin could suppress both constitutive and inducible STAT3 activation, and that these effects were specific to STAT3, as capsaicin had no effect on STAT5 phosphorylation. We also observed that capsaicin suppressed nuclear translocation and DNA-binding activity of STAT3. Unlike curcumin, which has been shown to decrease STAT3 expression after 24 h of treatment (51), capsaicin had no effect on the total levels of STAT3. Besides multiple myeloma

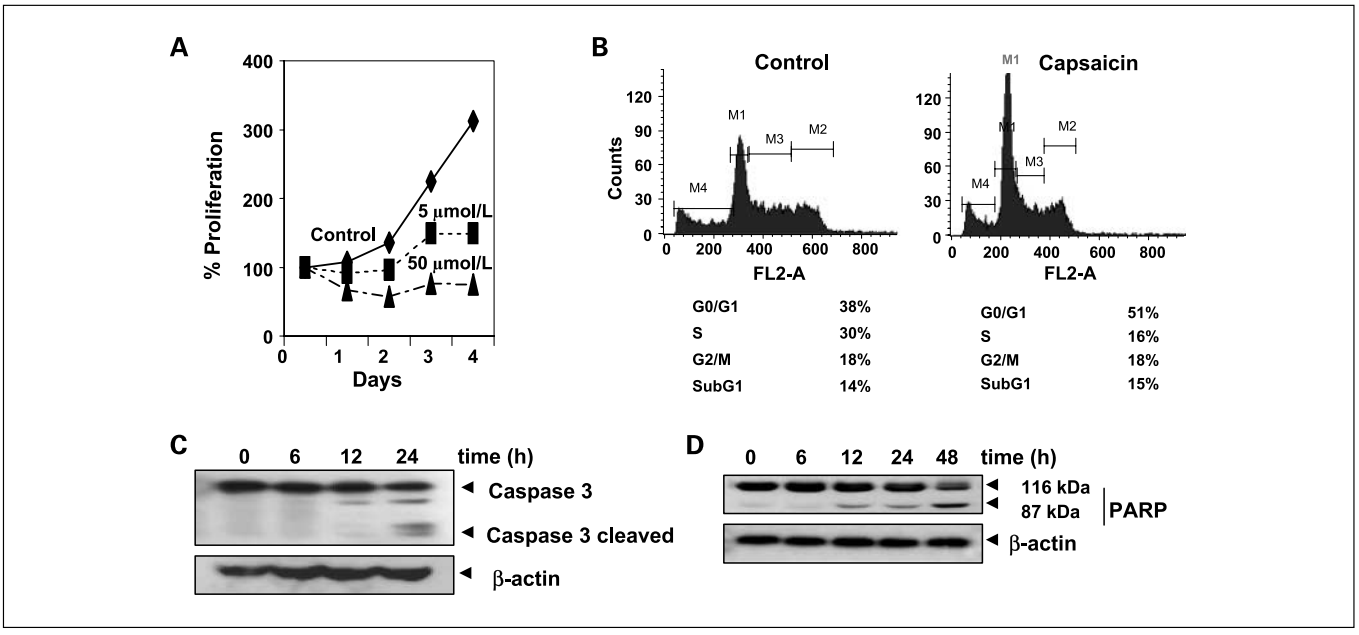

Fig. 5. Capsaicin suppresses proliferation, causes accumulation of cells in G_0-G_1 phase, and activates caspase-3. *A,* U266 cells were plated in triplicate, treated th 5 and 50 μmol/L capsaicin, and then subjected to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide assay on days 2 and 4 to analyze proliferation of cells. *B,* U266 cells (2×10^6 per mL) were synchronized by incubation overnight in the absence of serum and then treated with 50 μmol/L capsaicin for the indicated times, after which the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. *C,* U266 cells were treated with 50 μmol/L capsaicin for the indicated times. Whole-cell extracts were then prepared, separated on SDS-PAGE, and subjected to Western blotting against caspase-3 antibody. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. *D,* U266 cells were treated with 50 μmol/L capsaicin for the indicated times, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot against PARP antibody. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. Representative results of three independent experiments.

cells, other forms of cancer, including head and neck cancers (52), hepatocellular carcinoma (53), lymphomas, and leukemia (54), also express constitutively active STAT3. The suppression of constitutively active STAT3 in multiple myeloma cells raises the possibility that this novel STAT3 inhibitor might also inhibit constitutively activated STAT3 in other types of cancer cells.

The effects of capsaicin on STAT3 phosphorylation correlated with the suppression of upstream protein tyrosine kinases JAK1 and c-Src. It has been reported that all Src-transformed cell lines have persistently activated STAT3, and that dominant-negative STAT3 blocks transformation (55, 56). Interestingly, capsaicin had no affect on the activation of ERK1/2, suggesting that this kinase is not directly involved. The lack of effect of capsaicin on ERK1/2 in the current study agrees with the findings of Macho et al. (57), who reported that capsaicin and related vanilloid compounds did not affect ERK activity.

We also found evidence that the capsaicin-induced inhibition of STAT3 activation involves a PTP. Numerous PTPs have been implicated in STAT3 signaling, including SHP-1, SHP-2, TC-PTP, PTEN, PTP-1D, CD45, PTP-ϵ, and low molecular weight PTP (58–66). The type of PTP involved in down-regulation of STAT3 phosphorylation is not clear. Loss of SHP1 has been shown to enhance JAK3/STAT3 signaling in ALK-positive anaplastic large-cell lymphoma (43). It is possible that capsaicin mediates its effects through activation of SHP-1, although this PTP is ubiquitously expressed in both

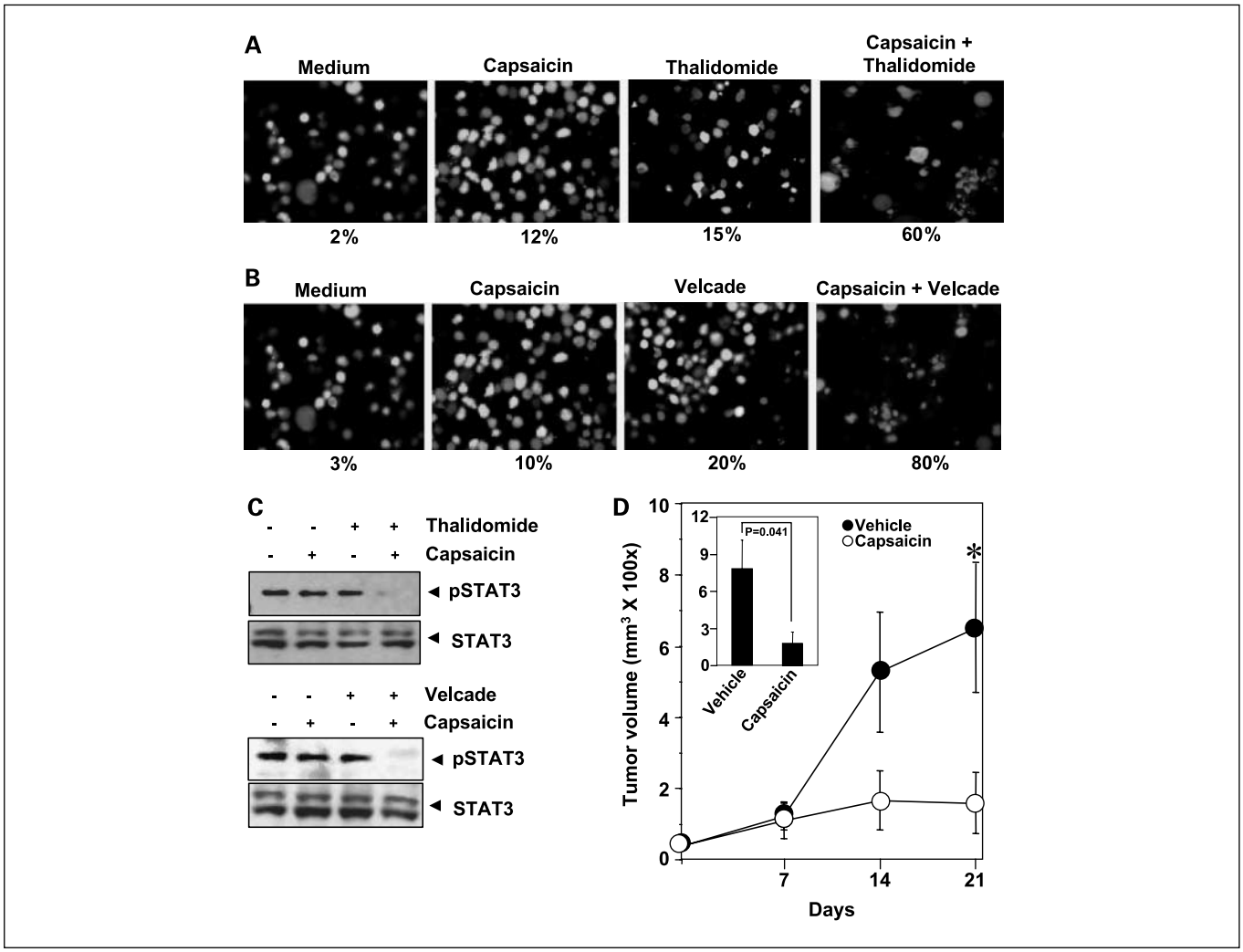

Fig. 6. Capsaicin potentiates the apoptotic effect of thalidomide and Velcade. *A* and *B,* U266 cells (1×10^6 per mL) were treated with 25 μmol/L capsaicin and 10 μg/mL thalidomide or 20 nmol/L Velcade alone or in combination for 24 h at 37°C. Cells were stained with the Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Materials and Methods. Representative results (% apoptosis) of three independent experiments. *C,* thalidomide and Velcade enhance the effect of capsaicin in suppressing STAT3 activation. *C, top,* U266 cells (2×10^6 per mL) were treated with 25 μmol/L capsaicin, or 10 μg/mL thalidomide alone or in combination for 4 h at 37°C. Whole-cell extracts were prepared, and 30-μg portions of those extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for antibody against phosphorylated STAT3. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. *C, bottom*, U266 cells (2×10^6 per mL) were treated with 25 μmol/L capsaicin or 20 nmol/L Velcade alone or in combination for 4 h at 37°C, after which Western blotting was done as described for top panel. Representative results of three independent experiments. *D,* capsaicin inhibits the growth of human multiple myeloma *in vivo*. U266 cells (2×10^6 per 100 μL) were injected into the left flank of mice, and the s.c. xenograft models were established as described in Materials and Methods. The diameters were measured once a week up to 3 wks using Vernier calipers, and the tumor volumes were calculated using the formula $V = 0.5236 \times d_1^2 \times d_2$, where d_1 is the shortest diameter, and d_2 is the longest diameter ($n = 5$). Points, mean tumor volume; bars, SE. *, $P = 0.038$ for control versus capsaicin on day 21. Inset, the mice were sacrificed on day 25 after injection of tumor cells; tumors were excised; and the final tumor volume was calculated using the formula $V = 4/3\pi r^3$, where r is the mean of the three dimensions (length, width, and depth). Points, mean tumor volume; bars, SE. *, $P = 0.041$ for control versus capsaicin on day 21.

hematopoietic and nonhematopoietic cells (58). However, capsaicin-induced apoptosis has been reported to be regulated by the protein phosphatase calcineurin (67).

STAT3 phosphorylation plays a critical role in the transformation and proliferation of tumor cells (32, 34). We found for the first time that capsaicin suppresses the expression of several STAT3-regulated proteins, including proliferative (cyclin D1), antiapoptotic (survivin, Bcl-2, and Bcl-xL), and angiogenic (VEGF) gene products. The down-regulation of cyclin D1 expression by capsaicin correlated with suppression of proliferation and accumulation of cells in G_1 phase of cell cycle. Expression of Bcl-xL is regulated by STAT3 and is overexpressed in multiple myeloma cells (68). Bcl-xL can also block cell death induced by a variety of chemotherapeutic agents, in parallel with an increase in chemoresistance (69).

The down-regulation of the expression of Bcl-2, Bcl-xL, and survivin is likely linked with the ability of capsaicin to induce apoptosis in multiple myeloma cells. Consistent with our observations, dominant-negative STAT3 has also been shown to induce apoptosis in cells with constitutively active STAT3 (70). Additionally, in view of the reported role of STAT3 in repressing p53 expression and function (71), inhibition of constitutive STAT3 activity by capsaicin could induce antitumor effects by enhancing p53 function as shown by a previous report (19). We also observed that capsaicin down-regulates the expression of VEGF in multiple myeloma cells. Our results differ from those of Patel et al. (72), who reported that capsaicin treatment induced VEGF expression by enhancing hypoxia-inducible factor-1α activity in human malignant melanoma cells. Whether this difference in results is due to cell type, dose, or time of exposure to capsaicin is unclear.

Constitutively active STAT3 has been implicated in the induction of resistance to apoptosis (70), possibly through the expression of Bcl-2 and cyclin D1 (73, 74). Recently, a proteasome inhibitor (PS341, also called Velcade) and a tumor necrosis factor inhibitor (thalidomide) were approved for the treatment of multiple myeloma (46, 47). We found for the first time that capsaicin potentiates the apoptotic effect of Velcade and thalidomide in multiple myeloma cells. How does capsaicin potentiates the effect of these drugs? This potentiation of apoptosis could be due to the down-regulation of antiapoptotic proteins, such as Bcl-2, Bcl-xL, and survivin, by capsaicin that contributes to resistance to these cytotoxic drugs in multiple myeloma patients. Additionally, we found that capsaicin when combined with thalidomide and Velcade was more effective in suppressing STAT3 activation than when used alone.

Whether these *in vitro* observations with capsaicin has any relevance to that *in vivo* was also investigated. Our results show for the first time that capsaicin significantly suppressed multiple myeloma tumor growth in nude mice. In summary, safety information from preclinical studies described and those reported previously (7–9); the ability of capsaicin to suppress constitutive and inducible STAT-3 activation; down-regulate the expression of cyclin D1, Bcl-2, Bcl-xL, survivin, and VEGF; inhibit cell proliferation; and potentiate the effects of Velcade and thalidomide provide a sound basis for pursuing the use of capsaicin further, either alone or in combination with other agents, to enhance treatment efficacy, reduce toxicity, and overcome the chemoresistance of relapsed or refractory multiple myeloma.

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References

1. Ellison N, Loprinzi CL, Kugler J, et al. Phase III placebo-controlled trial of capsaicin cream in the management of surgical neuropathic pain in cancer patients. J Clin Oncol 1997;15:2974–80.
2. Jancso G, Kiraly E, Such G, Joo F, Nagy A. Neurotoxic effect of capsaicin in mammals. Acta Physiol Hung 1987;69:295–313.
3. Dray A. Neuropharmacological mechanisms of capsaicin and related substances. Biochem Pharmacol 1992;44:611–5.
4. Jordt SE, Julius D. Molecular basis for species-specific sensitivity to "hot" chili peppers. Cell 2002; 108:421–30.
5. Park KK, Surh YJ. Effects of capsaicin on chemically-induced two-stage mouse skin carcinogenesis. Cancer Lett 1997;114:183–4.
6. Yoshitani SI, Tanaka T, Kohno H, Takashima S. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by dietary capsaicin and rotenone. Int J Oncol 2001;19:929–39.
7. Jang JJ, Kim SH, Yun TK. Inhibitory effect of capsaicin on mouse lung tumor development. In Vivo 1989;3: 49–53.
8. Tanaka T, Kohno H, Sakata K, et al. Modifying effects of dietary capsaicin and rotenone on 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. Carcinogenesis 2002;23:1361–7.
9. Mori A, Lehmann S, O'Kelly J, et al. Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. Cancer Res 2006;66:3222–9.
10. Sanchez AM, Sanchez MG, Malagarie-Cazenave S, Olea N, Diaz-Laviada I. Induction of apoptosis in prostate tumor PC-3 cells and inhibition of xenograft prostate tumor growth by the vanilloid capsaicin. Apoptosis 2006;11:89–99.
11. Teel RW. Effects of capsaicin on rat liver S9-mediated metabolism and DNA binding of aflatoxin. Nutr Cancer 1991;15:27–32.
12. Miller CH, Zhang Z, Hamilton SM, Teel RW. Effects of capsaicin on liver microsomal metabolism of the tobacco-specific nitrosamine NNK. Cancer Lett 1993; 75:45–52.
13. Zhang Z, Huynh H, Teel RW. Effects of orally administered capsaicin, the principal component of capsicum fruits, on the *in vitro* metabolism of the tobacco-specific nitrosamine NNK in hamster lung and liver microsomes. Anticancer Res 1997;17: 1093–8.
14. Morre DJ, Chueh PJ, Morre DM. Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. Proc Natl Acad Sci U S A 1995; 92:1831–5.
15. Morre DJ, Sun E, Geilen C, et al. Capsaicin inhibits plasma membrane NADH oxidase and growth of human and mouse melanoma lines. Eur J Cancer 1996; 32A:1995–2003.
16. Kang SN, Chung SW, Kim TS. Capsaicin potentiates 1,25-dihydoxyvitamin D3- and all-*trans* retinoic acid-induced differentiation of human promyelocytic leukemia HL-60 cells. Eur J Pharmacol 2001;420:83–90.
17. Zhang J, Nagasaki M, Tanaka Y, Morikawa S. Capsaicin inhibits growth of adult T-cell leukemia cells. Leuk Res 2003;27:275–83.
18. Ito K, Nakazato T, Yamato K, et al. Induction of apoptosis in leukemic cells by homovanillic acid derivative, capsaicin, through oxidative stress: implication of phosphorylation of p53 at Ser-15 residue by reactive oxygen species. Cancer Res 2004;64:1071–8.
19. Kim JD, Kim JM, Pyo JO, et al. Capsaicin can alter the expression of tumor forming-related genes which might be followed by induction of apoptosis of a Korean stomach cancer cell line, SNU-1. Cancer Lett 1997; 120:235–41.
20. Jung MY, Kang HJ, Moon A. Capsaicin-induced apoptosis in SK-Hep-1 hepatocarcinoma cells involves Bcl-2 downregulation and caspase-3 activation. Cancer Lett 2001;165:139–45.
21. Qiao S, Li W, Tsubouchi R, Haneda M, Murakami K, Yoshino M. Involvement of peroxynitrite in capsaicin-induced apoptosis of C6 glioma cells. Neurosci Res 2005;51:175–83.
22. Lee YS, Kwon EJ, Jin DQ, et al. Redox status-dependent regulation of cyclooxygenases mediates the capsaicin-induced apoptosis in human neuroblastoma cells. J Environ Pathol Toxicol Oncol 2002;21:113–20.
23. Kang HJ, Soh Y, Kim MS, et al. Roles of JNK-1 and p38 in selective induction of apoptosis by capsaicin in ras-transformed human breast epithelial cells. Int J Cancer 2003;103:475–82.
24. Singh S, Natarajan K, Aggarwal BB. Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is a potent inhibitor of nuclear transcription factor-kappa B activation by diverse agents. J Immunol 1996;157:4412–20.
25. Kim CS, Park WH, Park JY, et al. Capsaicin, a spicy component of hot pepper, induces apoptosis by activation of the peroxisome proliferator-activated receptor gamma in HT-29 human colon cancer cells. J Med Food 2004;7:267–73.

26. Park JY, Kawada T, Han IS, et al. Capsaicin inhibits the production of tumor necrosis factor alpha by LPS-stimulated murine macrophages, RAW 264.7: a PPARgamma ligand-like action as a novel mechanism. FEBS Lett 2004;572:266–70.

27. Hail N, Jr., Lotan R. Examining the role of mitochondrial respiration in vanilloid-induced apoptosis. J Natl Cancer Inst 2002;94:1281–92.

28. Sancho R, Lucena C, Macho A, et al. Immunosuppressive activity of capsaicinoids: capsiate derived from sweet peppers inhibits NF-kappaB activation and is a potent antiinflammatory compound *in vivo*. Eur J Immunol 2002;32:1753–63.

29. Darnell JE, Jr. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2002;2:740–9.

30. Ihle JN. STATs: signal transducers and activators of transcription. Cell 1996;84:331–4.

31. Aggarwal BB, Sethi G, Ahn KS, et al. Targeting signal-transducer-and-activator-of-transcription (STAT)-3 for prevention and therapy of cancer: modern target but ancient solution. Ann N Y Acad Sci 2006;1091:151–69.

32. Gao SP, Bromberg JF. Touched and moved by STAT3. Sci STKE 2006;2006:pe30.

33. Bharti AC, Donato N, Aggarwal BB. Curcumin (diferuloylmethane) inhibits constitutive and IL-6-inducible STAT3 phosphorylation in human multiple myeloma cells. J Immunol 2003;171:3863–71.

34. Yu H, Jove R. The STATs of cancer: new molecular targets come of age. Nat Rev Cancer 2004;4:97–105.

35. Kawano M, Hirano T, Matsuda T, et al. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature 1988;332:83–5.

36. Nilsson K, Bennich H, Johansson SG, Ponten J. Established immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgE myeloma patient. Clin Exp Immunol 1970;7:477–89.

37. Goldman-Leikin RE, Salwen HR, Herst CV, et al. Characterization of a novel myeloma cell line, MM.1. J Lab Clin Med 1989;113:335–45.

38. Yu CL, Meyer DJ, Campbell GS, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. Science 1995;269:81–3.

39. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. J Biol Chem 1998;273:13245–54.

40. Takada Y, Gillenwater A, Ichikawa H, Aggarwal BB. Suberoylanilide hydroxamic acid potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis by suppressing nuclear factor-kappaB activation. J Biol Chem 2006;281:5612–22.

41. Klein B, Zhang XG, Lu ZY, Bataille R. Interleukin-6 in human multiple myeloma. Blood 1995;85:863–72.

42. Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol 1997;17:6508–16.

43. Han Y, Amin H, Franko B, Frantz C, Shi X, Lai R. Loss of SHP1 enhances JAK3/STAT3 signaling and decreases proteosome degradation of JAK3 and NPM-ALK in ALK-positive anaplastic large-cell lymphoma. Blood 2006;108:2796–803.

44. Aoki Y, Feldman GM, Tosato G. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. Blood 2003;101:1535–42.

45. Matsushime H, Roussel MF, Ashmun RA, Sherr CJ. Colony-stimulating factor 1 regulates novel cyclins during the G_1 phase of the cell cycle. Cell 1991;65:701–13.

46. Cavo M. Proteasome inhibitor bortezomib for the treatment of multiple myeloma. Leukemia 2006;20:1341–52.

47. Glasmacher A, Hahn C, Hoffmann F, et al. A systematic review of phase-II trials of thalidomide monotherapy in patients with relapsed or refractory multiple myeloma. Br J Haematol 2006;132:584–93.

48. Mitsiades N, Mitsiades CS, Poulaki V, et al. Molecular sequelae of proteasome inhibition in human multiple myeloma cells. Proc Natl Acad Sci U S A 2002;99:14374–9.

49. Chauhan D, Li G, Podar K, et al. The bortezomib/proteasome inhibitor PS-341 and triterpenoid CDDO-Im induce synergistic anti-multiple myeloma (MM) activity and overcome bortezomib resistance. Blood 2004;103:3158–66.

50. Chauhan D, Velankar M, Brahmandam M, et al. A novel Bcl-2/Bcl-X(L)/Bcl-w inhibitor ABT-737 as therapy in multiple myeloma. Oncogene 2007;26:2374–80.

51. Blasius R, Reuter S, Henry E, Dicato M, Diederich M. Curcumin regulates signal transducer and activator of transcription (STAT) expression in K562 cells. Biochem Pharmacol 2006;72:1547–54.

52. Song JI, Grandis JR. STAT signaling in head and neck cancer. Oncogene 2000;19:2489–95.

53. Yoshikawa H, Matsubara K, Qian GS, et al. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet 2001;28:29–35.

54. Zhang Q, Raghunath PN, Xue L, et al. Multilevel dysregulation of STAT3 activation in anaplastic lymphoma kinase-positive T/null-cell lymphoma. J Immunol 2002;168:466–74.

55. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474–88.

56. Brierley MM, Fish EN. Stats: multifaceted regulators of transcription. J Interferon Cytokine Res 2005;25:733–44.

57. Macho A, Blazquez MV, Navas P, Munoz E. Induction of apoptosis by vanilloid compounds does not require *de novo* gene transcription and activator protein 1 activity. Cell Growth Differ 1998;9:277–86.

58. Tenev T, Bohmer SA, Kaufmann R, et al. Perinuclear localization of the protein-tyrosine phosphatase SHP-1 and inhibition of epidermal growth factor-stimulated STAT1/3 activation in A431 cells. Eur J Cell Biol 2000;79:261–71.

59. Woetmann A, Nielsen M, Christensen ST, et al. Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and functional inhibition of STAT3. Proc Natl Acad Sci U S A 1999;96:10620–5.

60. Kim H, Baumann H. Dual signaling role of the protein tyrosine phosphatase SHP-2 in regulating expression of acute-phase plasma proteins by interleukin-6 cytokine receptors in hepatic cells. Mol Cell Biol 1999;19:5326–38.

61. Yamamoto T, Sekine Y, Kashima K, et al. The nuclear isoform of protein-tyrosine phosphatase TC-PTP regulates interleukin-6-mediated signaling pathway through STAT3 dephosphorylation. Biochem Biophys Res Commun 2002;297:811–7.

62. Sun S, Steinberg BM. PTEN is a negative regulator of STAT3 activation in human papillomavirus-infected cells. J Gen Virol 2002;83:1651–8.

63. Gunaje JJ, Bhat GJ. Involvement of tyrosine phosphatase PTP1D in the inhibition of interleukin-6-induced Stat3 signaling by alpha-thrombin. Biochem Biophys Res Commun 2001;288:252–7.

64. Irie-Sasaki J, Sasaki T, Matsumoto W, et al. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. Nature 2001;409:349–54.

65. Tanuma N, Nakamura K, Shima H, Kikuchi K. Protein-tyrosine phosphatase PTPepsilon C inhibits Jak-STAT signaling and differentiation induced by interleukin-6 and leukemia inhibitory factor in M1 leukemia cells. J Biol Chem 2000;275:28216–21.

66. Chiarugi P, Cirri P, Marra F, et al. The Src and signal transducers and activators of transcription pathways as specific targets for low molecular weight phosphotyrosine-protein phosphatase in platelet-derived growth factor signaling. J Biol Chem 1998;273:6776–85.

67. Wolvetang EJ, Larm JA, Moutsoulas P, Lawen A. Apoptosis induced by inhibitors of the plasma membrane NADH-oxidase involves Bcl-2 and calcineurin. Cell Growth Differ 1996;7:1315–25.

68. Tu Y, Renner S, Xu F, et al. BCL-X expression in multiple myeloma: possible indicator of chemoresistance. Cancer Res 1998;58:256–62.

69. Simonian PL, Grillot DA, Nunez G. Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. Blood 1997;90:1208–16.

70. Catlett-Falcone R, Landowski TH, Oshiro MM, et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 1999;10:105–15.

71. Niu G, Wright KL, Ma Y, et al. Role of Stat3 in regulating p53 expression and function. Mol Cell Biol 2005;25:7432–40.

72. Patel PS, Yang S, Li A, Varney ML, Singh RK. Capsaicin regulates vascular endothelial cell growth factor expression by modulation of hypoxia inducing factor-1alpha in human malignant melanoma cells. J Cancer Res Clin Oncol 2002;128:461–8.

73. Nielsen M, Kaestel CG, Eriksen KW, et al. Inhibition of constitutively activated Stat3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells. Leukemia 1999;13:735–8.

74. Sinibaldi D, Wharton W, Turkson J, Bowman T, Pledger WJ, Jove R. Induction of p21WAF1/CIP1 and cyclin D1 expression by the Src oncoprotein in mouse fibroblasts: role of activated STAT3 signaling. Oncogene 2000;19:5419–27.

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