

Curcumin-Induced Antiproliferative and Proapoptotic Effects in Melanoma Cells Are Associated with Suppression of I κ B Kinase and Nuclear Factor κ B Activity and Are Independent of the B-Raf/Mitogen-Activated/Extracellular Signal-Regulated Protein Kinase Pathway and the Akt Pathway

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The authors thank Karen Ramirez for her flow cytometric work in the annexin V-propidium iodide cell staining experiments.

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Received October 1, 2004; revision received March 7, 2005; accepted April 14, 2005.

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DOI 10.1002/cncr.21216

Published online 11 July 2005 in Wiley InterScience (www.interscience.wiley.com).

BACKGROUND. Nuclear factor- κ B (NF- κ B) plays a central role in cell survival and proliferation in human melanoma; therefore, the authors explored the possibility of exploiting NF- κ B for melanoma treatment by using curcumin, an agent with known, potent, NF- κ B-inhibitory activity and little toxicity in humans.

METHODS. Three melanoma cell lines (C32, G-361, and WM 266-4), all of which had B-*raf* mutations, were treated with curcumin, and the authors assessed its effects on viability ((3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide assay) and apoptosis (flow-cytometric analysis of annexin V/propidium iodide-stained cells). Curcumin-treated cells also were examined for NF- κ B binding activity (electrophoretic mobility shift assay) and for the activity of its upstream regulator, I κ B kinase (IKK) (immune complex kinase assay). In addition, relevant signaling, as reflected by B-Raf kinase activity (kinase cascade assay), and steady-state levels of activated, downstream effectors, as reflected by mitogen-activated signal-regulated protein kinase (MEK), extracellular signal-regulated protein kinase (ERK), and Akt phosphorylation levels (immunoblots), were assessed.

RESULTS. Curcumin treatment decreased cell viability of all 3 cell lines in a dose-dependent manner (50% inhibitory concentration = 6.1–7.7 μ M) and induced apoptosis. NF- κ B and IKK were active constitutively in all melanoma cell lines examined, and curcumin, under apoptosis-inducing conditions, down-regulated NF- κ B and IKK activities. However, curcumin did not inhibit the activities of B-Raf, MEK, or ERK, and Akt phosphorylation was enhanced. Furthermore, in the presence of curcumin, the Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-methyl-3-*O*-octadecylcarbonate] no longer suppressed Akt phosphorylation.

CONCLUSIONS. Curcumin has potent antiproliferative and proapoptotic effects in melanoma cells. These effects were associated with the suppression of NF- κ B and IKK activities but were independent of the B-Raf/MEK/ERK and Akt pathways. *Cancer* 2005;104:879–90. © 2005 American Cancer Society.

KEYWORDS: curcumin, nuclear factor κ B, melanoma, signaling pathways.

Melanoma is a cutaneous tumor characterized by abnormal proliferation of melanocytes that invade the basement membrane. The majority of metastatic melanomas are resistant to diverse chemotherapeutic agents (for reviews, see Sonetas and Lowe¹ and Molife and Hancock²), and long-term survival for patients with melanoma who have metastatic disease is dismal.^{3,4} Consequently, the search for novel antimelanoma agents continues.

The natural compound curcumin (diferuoylmethane) has antiproliferative and proapoptotic effects against a variety of tumors *in vitro* (for review, see Aggarwal et al.⁵). These effects correlate with the inhibition of signaling proteins, including transcription factors (such as nuclear factor κ B [NF- κ B], activator protein-1, and early growth response-1), as well as cyclooxygenase 2, nitric oxide synthase, matrix metalloproteinase-9, urinary plasminogen activator, tumor necrosis factor, chemokines, cell surface adhesion molecules, cyclin D1, growth factor receptors (such as epidermal growth factor receptor [EGF] and human EGF receptor 2), other protein tyrosine kinases, and serine/threonine kinases.⁵ Despite its broad range of activity *in vitro*, curcumin is nontoxic *in vivo*; no dose-limiting toxicity was observed in individuals who ingested 8 g per day for 3 months.⁶

In the current study, we tested curcumin against melanoma cell lines to examine its effects on cell viability, apoptosis, and cell signaling molecules associated with melanoma pathogenicity. It has been shown previously that several signaling molecules are critical to the proliferation of melanoma *in vitro* or *in vivo*, including B-Raf (through mutation⁷), mitogen-activated protein kinase (MAPK),⁸ Akt,⁹ and NF- κ B.¹⁰ We show that curcumin exposure at micromolar concentrations both decreased viability and induced apoptosis in a dose-dependant manner in the melanoma cell lines. Furthermore, curcumin treatment, under apoptosis-inducing conditions, attenuated NF- κ B binding activity without suppressing the B-Raf kinase pathway or Akt phosphorylation in these cell lines. These results suggest that curcumin induces apoptosis in melanoma cells by inhibiting NF- κ B in a B-Raf-independent and Akt-independent manner.

MATERIALS AND METHODS

Cell Lines

The human melanoma cell lines C32, G-361, and WM 266-4 were purchased from American Type Cell Culture (Manassas, VA). All cells were grown in medium supplemented with 10% fetal bovine serum; G-361 cells were grown in McCoy 5A medium; WM 266-4 cells were grown in minimum essential medium (MEM); and C32 cells were grown in MEM supplemented with nonessential amino acids and sodium pyruvate. All media were purchased from Invitrogen Corporation (Carlsbad, CA).

The 3-[4,5-Dimethylthiazol-2-yl]2,5-Diphenyltetrazolium Bromide Cell-Viability Assay

To determine the effect of curcumin on cell viability, the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay was used. Cells were seeded $3.0\text{--}5.0 \times 10^3$ cells per well, depending on the

cell line, in a 96-well plate and incubated overnight. Cells were treated in sextuplicate with 0 μ M, 5 μ M, 10 μ M, 20 μ M, or 40 μ M of curcumin (Sigma-Aldrich Company, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO) and were added to their respective medium for a final DMSO concentration of 0.1%. Cells were protected from light for the duration of curcumin treatment and were incubated for 96–120 hours, depending on the cell line. Medium was aspirated from the wells; the cells were rinsed with phosphate buffered saline (PBS), and 200 μ L of the MTT solution (Sigma-Aldrich Company; final concentration, 0.5 mg/mL) were added to the cells. (MTT solution was prepared by adding 1 part of stock 5-mg/mL MTT dissolved in PBS to 9 parts of serum-supplemented medium.) Cells were incubated for 4–7 hours before the medium was aspirated again, and the precipitated formazan was dissolved by adding 200 μ L of DMSO and placing it on a shaker for 10–20 minutes. The samples were read at 560 nm (minus the 650-nm wavelength reference) on a plate reader (Molecular Devices Corp., Sunnyvale, CA). The mean and standard error for each treatment was determined and then was converted to the percent relative to control, with 0.1% DMSO only = 100%. The concentration at which cell growth was inhibited by 50% (the 50% inhibitory concentration [IC₅₀]) was determined by linear interpolation [(50% – low percentage)/(high percentage – low percentage)] \times (high concentration – low concentration) + low concentration]. The IC₉₀ was determined similarly, with 90% substituting for 50% in the equation.

Cell Recovery Assay

To determine whether the effects on viability were reversible, the following cell recovery assay was performed. Cells were seeded 5.0×10^5 cells per 100-mm plate and incubated overnight. Cells were treated with 0 μ M, 10 μ M, 20 μ M, or 40 μ M curcumin dissolved in DMSO, added to medium (final DMSO concentration, 0.1%), and incubated for 96 hours. Cells were trypsinized and counted; the same number of cells for each treatment (5.0×10^3 cells per well) were seeded in sextuplicate into 96-well plates with curcumin-free medium and incubated for 96 hours. The degree of cell recovery was determined with the MTT assay.

Annexin V-Propidium Iodide Staining for Apoptotic Cells

To determine the effect of curcumin on apoptosis, cells were stained with annexin V/propidium iodide and assessed by fluorescence-activated cell sorting analysis. Cells were seeded 5.0×10^5 cells per 100-mm plate and incubated overnight. Cells were treated with 0 μ M, 10 μ M, 20 μ M, or 40 μ M curcumin in DMSO (final DMSO concentration, 0.1%) and incubated for

72 hours. Then, cells were harvested by quick (< 5 minutes) trypsinization to minimize potentially high annexin V background levels in adherent cells, and they were washed and stained with fluorescein 5(6)-isothiocyanate (FITC)-annexin V-propidium iodide, as directed on the Annexin V-FLUOS staining kit (Roche Diagnostics Corp., Indianapolis, IN). Stained cells were placed on ice and protected from light until they were read by flow cytometry. The cells were analyzed on an Epics XL-MCL flow cytometer using the System II version 3.0 software (both hardware and software from Beckman Coulter, Inc., Miami, FL) with the laser excitation wavelength at 488 nm. The green signal from FITC-annexin V was measured at 525 nm, and the red signal from propidium iodide was measured at 620 nm.

Electrophoretic Mobility Shift Assay for NF- κ B Binding

Because melanoma cells often have constitutively activated NF- κ B,^{10–12} the effect of curcumin on NF- κ B binding was determined using electrophoretic mobility shift assay (EMSA). Cells seeded at $5.0\text{--}12.0 \times 10^5$ cells per 100-mm plate (depending on the cell line) and incubated overnight were treated with 0 μ M or 10 μ M curcumin (final DMSO concentration, 0.1%) and incubated for 72–96 hours, depending on the cell line. Cells were harvested by trypsinizing and washing twice in cold PBS, and they were stored at -80°C and used within 2 weeks.

Cells were thawed on ice and induced to swell by adding cold cytoplasmic extraction buffer (CEB) (10 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulphate [HEPES], pH 7.9; 10 mM KCl, 0.1 mM ethylenediamine tetraacetic acid [EDTA]; 0.1 mM ethylene guanine tetraacetic acid [EGTA], 1.0 mM dithiothreitol [DTT], 0.5 mM phenylmethanesulfonyl fluoride [PMSF]; 2 μ g/mL Leupeptin; 2 μ g/mL Aprotinin; and 0.5 mg/mL benzamidine) followed by a 15-minute incubation on ice. The cytoplasmic cell fraction was lysed by adding 3.13 μ L/100 μ L CEB of 10% Igepal (Sigma-Aldrich Company) and vortexing for 20 seconds. The cell suspension was centrifuged for 5 minutes, the cytosolic supernatant was discarded, and cold nuclear extraction buffer (20 mM HEPES, pH 7.9; 400 mM NaCl; 1.0 mM EDTA; 1.0 mM EGTA; 1.0 mM DTT; 0.5 mM PMSF; 2 μ g/mL Leupeptin; 2 μ g/mL Aprotinin; and 0.5 mg/mL benzamidine) was added to the pellet. Nuclear suspensions were incubated for 30 minutes on ice (vortexing every 10 minutes), then centrifuged for 10 minutes. Nuclear extracts from the supernatant were collected and stored at -80°C and were used within 4 days. Protein concentration was determined by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). The binding reaction was initiated by adding 20–30 μ g of nuclear extract to

binding buffer (100 mM HEPES, pH 7.9; 50 mM EDTA; 100 mM DTT; 10% glycerol); 2 μ g poly dI:dC (Amersham Biosciences, Piscataway, NJ); 3.0×10^5 cpm of ^{32}P -labeled, NF- κ B, double-stranded oligonucleotide; and 10% Igepal (total volume, 20 μ L), and incubating the mixture for 15 minutes at 37°C . The reaction was terminated by adding 4 μ L of $6 \times$ DNA loading dye and placing samples on ice. Samples were loaded on a prerun 5.5% polyacrylamide gel and electrophoresed under nondenaturing conditions. The gel was dried and placed on film for autoradiography.

For supershifts, 2 μ g of anti-p50 or p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the nuclear extract and incubated at room temperature for 15 minutes prior to its addition in the binding reaction. Quantitation of autoradiographs was performed on the Fluorchem[®] 8900 Imaging System using AlphaEase[®] FC software (Alpha Innotech, San Leandro, CA).

Oligonucleotides

The consensus sequence wild-type and mutant, blunt-end, double-stranded oligonucleotides used for the NF- κ B electrophoretic mobility shifts assays were purchased from Santa Cruz Biotechnology, Inc. The NF- κ B wild-type sense strand sequence was 5'-AGT-TGAGGGGACTTTCCCAGGC-3', and the mutant sequence was 5'-AGTTGAGGCGACTTTCCCAGGC-3'.¹³ Oligonucleotides were γ - ^{32}P -end-labeled with T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA), purified with Quick Spin G-50 Sephadex columns (Roche Diagnostics Corp.), and stored at -20°C .

IKK Assay

A kinase assay using phosphorylation of substrate was used to examine IKK activity. Cells were seeded and treated with curcumin under the conditions described above for the NF- κ B EMSA. Cells were lysed in IKK lysis buffer (20 mM HEPES, pH 7.9; 50 mM NaCl; 1% Igepal; 2 mM EDTA; 0.5 mM EGTA; 2 μ g/mL Aprotinin, 2 μ g/mL Leupeptin; 0.5 mM PMSF; 2 mM Na_3VO_4), and IKK complexes were precipitated from whole cell extracts with 1 μ g each of anti-IKK α and IKK β antibodies (Imgenex Corporation, San Diego, CA) overnight at 4°C followed by 20 μ L of protein A/G-conjugated Sepharose beads (Pierce, Rockford, IL). After 2 hours, the beads were washed with IKK lysis buffer; the kinase assay was initiated upon addition of 15 μ L kinase assay mixture (50 mM HEPES, pH 7.4; 20 mM MgCl_2 ; 2 mM DTT; 20 μ Ci [γ - ^{32}P] adenosine triphosphate [ATP]; 10 μ M unlabeled ATP) containing 2 μ g of the substrate glutathione-S-transferase (GST)-I κ B α (residue 1–54 of I κ B α conjugated to GST). After incubation at 30°C for 30 minutes, the reaction

was terminated by the addition of $5 \times$ sodium dodecyl sulfate (SDS) sample buffer and boiling for 5 minutes. Proteins were resolved on a 10% polyacrylamide gel under reducing conditions, the gel was dried, and the radiolabeled bands were visualized using a Phosphor-Imager.

To determine the total amounts of IKK α and IKK β in each sample, immunoblotting was performed. Proteins (30 μ g) from whole cell extract were resolved on a 7.5% acrylamide gel then electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk for 1 hour then incubated with a 1:1000 dilution of either anti-IKK α or anti-IKK β antibody (Imgenex Corporation) for 1 hour. The membrane was washed and treated with horseradish peroxidase-conjugated, secondary antimouse immunoglobulin G antibody; then, proteins were detected by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Interleukin 8 Enzyme-Linked Immunosorbent Assay

The cytokine interleukin-8 (IL-8) has a proliferative role in melanomas,¹⁴ and its expression is regulated in part by NF- κ B.¹⁵⁻¹⁷ Therefore, the effect of curcumin on soluble, extracellular IL-8 levels was determined using enzyme-linked immunosorbent assays (ELISAs). Cells were seeded and treated with curcumin under conditions described above for the NF- κ B EMSA. After treatment, media from cells that were exposed to curcumin or to DMSO only were harvested and centrifuged to remove cellular debris. The supernatant was aliquoted and stored at -80°C . Samples were thawed, and the Quantikine[®] Human IL-8 Immunoassay kit (R&D Systems, Inc., Minneapolis, MN) was used to determine IL-8 concentration, according to the manufacturer's instructions, except that samples were read (on the Molecular Devices plate reader) at 490 nm with a wavelength correction at 560 nm. Results were calculated by generating a 4-parameter, logistic curve fit in the SOFTmax Pro version 2.6 software (Molecular Devices Corp., Sunnyvale, CA).

B-Raf Kinase Assay

Because melanoma cells often have activating B-*raf* mutations (see Davies et al.²¹), the effect of curcumin on B-Raf kinase activity was determined using the B-Raf Kinase Cascade Assay Kit (Upstate Cell Signaling Solutions, Charlottesville, VA). This kit examines Raf kinase activity by sequential phosphorylation of 0.4 μ g purified mitogen-activated signal-regulated protein kinase 1 (MEK1) (the Raf substrate) and 1.0 μ g extracellular signal-regulated protein kinase 2 (ERK2) (the MEK1 substrate) with nonradiolabeled ATP; 10% of this mixture is removed, and 50 μ g of myelin basic protein (the ERK2 substrate) along with radioisotope-

labeled ATP ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$) are added. The extent of myelin basic protein phosphorylation is determined by liquid scintillation counting. This method, due to increasing amounts of downstream components, enables the detection of Raf activity with great sensitivity. The specificity of B-Raf kinase activity is enabled by immunoprecipitation of B-Raf in cell lysates prior to the initiation of the kinase cascade assay using anti-B-Raf antibodies (Santa Cruz Biotechnology, Inc.).

Cells were seeded and treated with curcumin under the conditions described for the NF- κ B EMSA. Cells were harvested and stored at -80°C , and they were used within 2 weeks of harvest. Upon thawing, cells were lysed with lysis buffer (137 mM NaCl; 25 mM N-Tris[hydroxymethyl]methyl-2-animoethanesulfonic acid [TES], pH 7.4; 10% glycerol; 1% Triton X-100; 0.1% Igepal; 0.01% sodium deoxycholate) supplemented with 10% protease inhibitor cocktail (Sigma-Aldrich Company), 10 mM NaF, and 0.5 mM Na₃VO₄, and they were passed ≈ 8 times through a 21-gauge needle. Cell lysates were centrifuged in a microcentrifuge at $> 13,000$ revolutions per minute for 10 minutes at 4°C . Supernatants were collected, and the total protein concentration was determined; 1000 μ g of total protein (with sample volumes equalized to 500 μ L) were aliquoted for each treatment.

B-Raf was immunoprecipitated by adding 2 μ g of the rabbit anti-B-Raf antibody, incubating on ice for 1.5 hours, then adding 20 μ L of protein A/G-conjugated agarose beads (Oncogene Research Products, San Diego, CA), and incubating at 4°C on a rocker for 30 minutes. For negative controls, 2 μ g of rabbit antimouse antibody (ICN Pharmaceuticals, Inc., Aurora, OH) were used in place of the anti-B-Raf antibody. Immunoprecipitates were washed twice with lysis buffer supplemented with 10 mM NaF and 0.5 mM Na₃VO₄ and washed once with kinase assay buffer (25 mM TES, pH 7.4; 5 mM MgCl₂; 5 mM MnCl₂) supplemented with 10 mM NaF and 0.5 mM Na₃VO₄. B-Raf kinase activity was determined by the B-Raf Kinase Cascade Assay Kit (Upstate Group, Inc., Lake Placid, NY), as described above, according to manufacturer's instructions.

Immunoblots for MEK, ERK, and Akt Phosphorylation

The effects of curcumin on B-Raf/MEK/ERK signaling and on Akt activity were assessed using Western blots to examine phosphorylation of MEK, ERK, and Akt. Cells were seeded and treated with curcumin as described for the NF- κ B EMSA. Cells were thawed and lysed by resuspending in B-Raf kinase assay lysis buffer plus inhibitors (10% protease inhibitor cocktail, 10 mM NaF, 0.5 mM Na₃VO₄) and incubating on ice for 30 minutes with vortexing every 10 minutes. Cells

were then centrifuged, and the protein concentration of the supernatant was determined. Samples (40 μg of protein with sample volumes equalized to 20 μL) were aliquoted and prepared for SDS electrophoresis by adding 3 \times sample buffer and boiling.

After SDS-polyacrylamide gel electrophoresis, proteins were electrotransferred from gel to nitrocellulose membrane: The membrane was blocked using 20 mM TES, pH 7.6; 137 mM NaCl; and 0.5% Tween 20 (TBS-T) plus 5% (weight/volume) nonfat dry milk (Bio-Rad Laboratories) for 30 minutes at room temperature. The membrane was rinsed, and the primary antibody diluted in TBS-T plus 1% milk was added and incubated either overnight at 4 $^{\circ}\text{C}$ or for 2–3 hours at room temperature. The primary antibodies used were antiphosphorylated (antiphospho)-MEK1/2 (dilution, 1:3000), antiphospho-ERK1/2 (dilution, 1:3000), antiphospho-Akt (dilution, 1:1000), or anti-MEK1/2 (dilution, 1:3000), anti-ERK1/2 (1:3000), or anti-Akt (dilution, 1:3000). All antibodies were from Cell Signaling TechnologyTM (Beverly, MA). The membrane was rinsed 3 times with TBS-T, and peroxidase-conjugated antirabbit secondary antibody diluted 1:1000–1:2000 in TBS-T and 2.5% milk was added; and the membrane was incubated at room temperature for 2.0–2.5 hours. Excess antibody was washed from the membrane, and proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Akt Inhibition

To determine whether an Akt inhibitor would suppress Akt activity in the presence of curcumin, cells were treated with 30 μM , 45 μM , or 60 μM of Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-methyl-3-*O*-octadecylcarbonate], Calbiochem, San Diego, CA) with and without 10 μM curcumin and were incubated for 72 hours before harvesting, followed by analysis of Akt phosphorylation levels by anti-phospho-Akt Western blots.

RESULTS

Curcumin Irreversibly Decreases Melanoma Cell Viability

Prior to treating all melanoma cell lines with varying doses of curcumin, time-course experiments in which a faster growing cell line (G-361 cells, doubling time \approx 24 hours) and a slower growing cell line (C32 cells, doubling time \approx 33 hours) were performed to determine optimal treatment time. These cells were treated with 0–40 μM of curcumin, and effects on cell viability, as assessed by the assay, were determined every 24–48 hours for a maximum of 144 hours after treatment. Maximum dose-dependent decreases in viability were observed at 96 hours and 120 hours for G-361

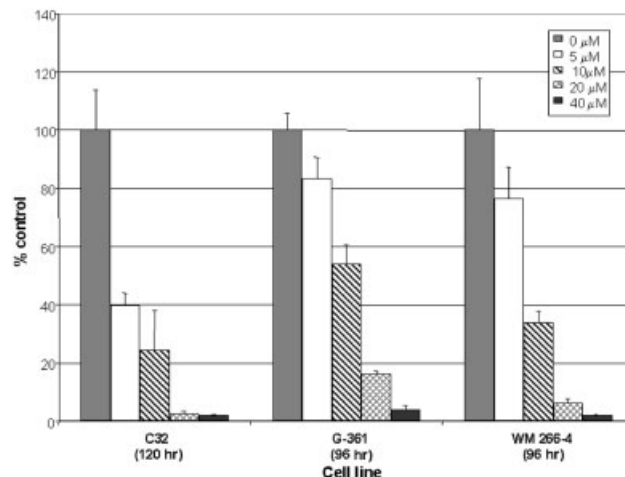


FIGURE 1. Curcumin inhibits melanoma cell viability. Cells plated in 96-well plates were treated for 96–120 hours with dimethyl sulfoxide (DMSO) only or with 5 μM , 10 μM , 20 μM , or 40 μM curcumin (final DMSO concentration, 0.1%) and were assayed using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide cell-proliferation assay. The mean \pm standard error values of six samples are shown. Optical density values were adjusted to % control values, with no curcumin (0.1% DMSO only) = 100%. This chart shows that curcumin inhibits melanoma cell viability in a concentration-dependent manner.

cells and C32 cells, respectively, corresponding to 3–4 cell doublings for each cell line (data not shown).

To further determine the effect of curcumin on cell viability, C32, G-361, and WM 266-4 cells (doubling time \approx 24 hours) were treated with 0–40 μM curcumin (final DMSO concentration of control and all curcumin concentrations, 0.1%), incubated for 96–120 hours (depending on the cell line), and assayed with MTT. Curcumin inhibited cell viability in all 3 melanoma cell lines in a dose-dependent manner (Fig. 1); the IC_{50} values in the cell lines ranged from 6.1 μM to 7.7 μM , whereas the IC_{90} values ranged from 15.9 μM to 21.4 μM .

To determine whether the effects of curcumin on viability were reversible, cells were treated with 0–40 μM curcumin for 96 hours, then replated with the same number of cells in curcumin-free medium, and allowed to grow for an additional 96 hours. Cell viability was then determined by MTT recovery assay. At 10 μM curcumin, C32 cells had dramatically reduced cell recovery, whereas recovery of WM 266-4 cells was comparable to that of controls (Fig. 2). At 20 μM , cell recovery was reduced greatly in both cell lines tested. These results indicate that curcumin induces irreversible effects on viability in melanoma cells, particularly at concentrations $\geq \text{IC}_{50}$.

To determine whether curcumin induced apoptosis in melanoma cells, the cells were treated with 0–40 μM curcumin and incubated for 72 hours; this was

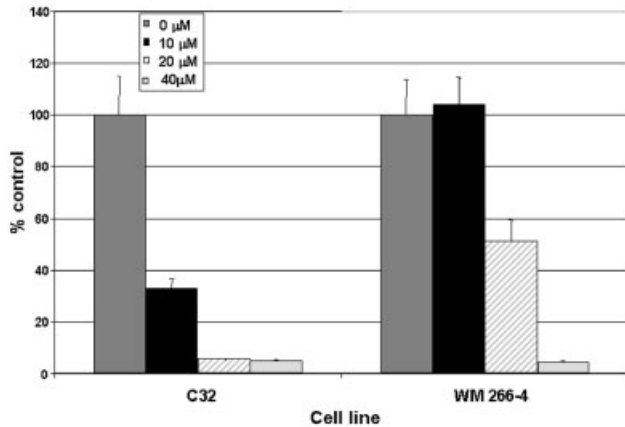


FIGURE 2. Curcumin induces irreversible loss of viability of melanoma cells. Cells were first treated with dimethyl sulfoxide (DMSO) only or with 10 μM , 20 μM , or 40 μM curcumin (final DMSO concentration, 0.1%) for 96 hours, then washed, counted, and plated in equal numbers in 96-well plates. Cells were incubated for an additional 96 hours in curcumin-free medium and then assayed with the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide viability assay. The mean \pm standard values of six samples are shown. Optical density values were adjusted to % control values, with no curcumin (0.1% DMSO only) = 100%. This chart shows that melanoma cells exposed to curcumin fail to recover their growth/survival capacity even after curcumin is removed from the media.

followed by FITC-annexin V-propidium iodide staining of lightly trypsinized cells. Flow cytometric analysis revealed an increase in the proportion of annexin V-positive cells with increasing concentrations of curcumin (Fig. 3A), indicating a dose-dependent increase in apoptotic cells upon curcumin treatment. Curcumin at 10 μM increased the proportion of apoptotic cells 1.9–2.8-fold, whereas curcumin at 20 μM increased the proportion of apoptotic cells 7.2–19.7-fold compared with controls, depending on the cell line (Fig. 3B).

Curcumin Inhibits NF- κ B Binding Activity in Melanoma Cells

Because the NF- κ B transcription factor has been implicated in the pathogenesis of diverse malignancies, including melanoma,¹⁰ we determined whether NF- κ B binding was decreased in curcumin-treated melanoma cells. Cells were treated with 0 μM or 10 μM curcumin for 72 hours and assayed for NF- κ B binding with an NF- κ B EMSA.

Comparison of bands from nuclear extracts that were obtained from untreated cells probed with wild-type or mutant NF- κ B oligonucleotides indicated that 1 band was NF- κ B-specific (Fig. 4A, lanes 1 and 2 for each cell line). Preincubation of nuclear extracts with anti-p50 or anti-p65 antibodies also reduced the in-

tensity of this band, confirming that the band consisted of the p50/p65 heterodimers.

NF- κ B was bound constitutively in the three cell lines tested. NF- κ B binding activity was decreased partially in all cell lines that were treated with curcumin compared with controls (Fig. 4A,B).

Curcumin Inhibits I κ B Kinase Activity in Melanoma Cells

NF- κ B is activated by I κ B kinase (IKK), which phosphorylates and thereby initiates signals required for degradation of the negative NF- κ B regulator, I κ B α (for review, see Rothwarf and Karin¹⁸). Because curcumin modulated NF- κ B binding activity in melanoma cells, we examined IKK activity in curcumin-treated melanoma cells. An *in vitro* kinase assay using immunoprecipitated IKK from melanoma cells (either untreated or treated with 0.1% DMSO only or with 10 μM curcumin) and the GST-I κ B α as substrate showed that IKK was active constitutively in the cell lines examined. This activity was decreased after curcumin treatment (Fig. 5, top). Immunoblotting of the cell extracts of untreated, 0.1% DMSO only, or curcumin-treated cells showed no significant changes in the steady-state protein levels of the IKK subunits IKK α and IKK β in treated cells compared with cells that were untreated or exposed to vehicle only (Fig 5., middle and bottom, respectively).

IL-8 Secretion Is an Independent Factor in Curcumin-Treated Melanoma Cells

IL-8 is a cytokine in which increased serum levels are correlated with increased malignant phenotype and poor prognosis in patients with melanoma.¹⁹ IL-8 is regulated at least in part by NF- κ B.²⁰ To determine whether curcumin can modulate IL-8 secretion and whether these changes correlate with NF- κ B modulation, the media of cells exposed to 0 μM or 10 μM curcumin for 72–96 hours were examined for IL-8 levels using an ELISA. After curcumin exposure, IL-8 levels increased in 2 cell lines (G-361 and WM 266-4). Baseline levels in C32 cells were low and appeared to decrease after curcumin exposure (Fig. 6). There was no clear-cut correlation between IL-8 levels and growth inhibition or apoptosis, indicating that soluble, extracellular IL-8 is not a reliable marker of curcumin-induced melanoma cell growth inhibition or apoptosis. In addition, no precise correlation was observed between curcumin-induced NF- κ B inhibition and effect on IL-8, suggesting that IL-8 secretion is not dependent on NF- κ B binding activity in curcumin-treated melanoma cells.

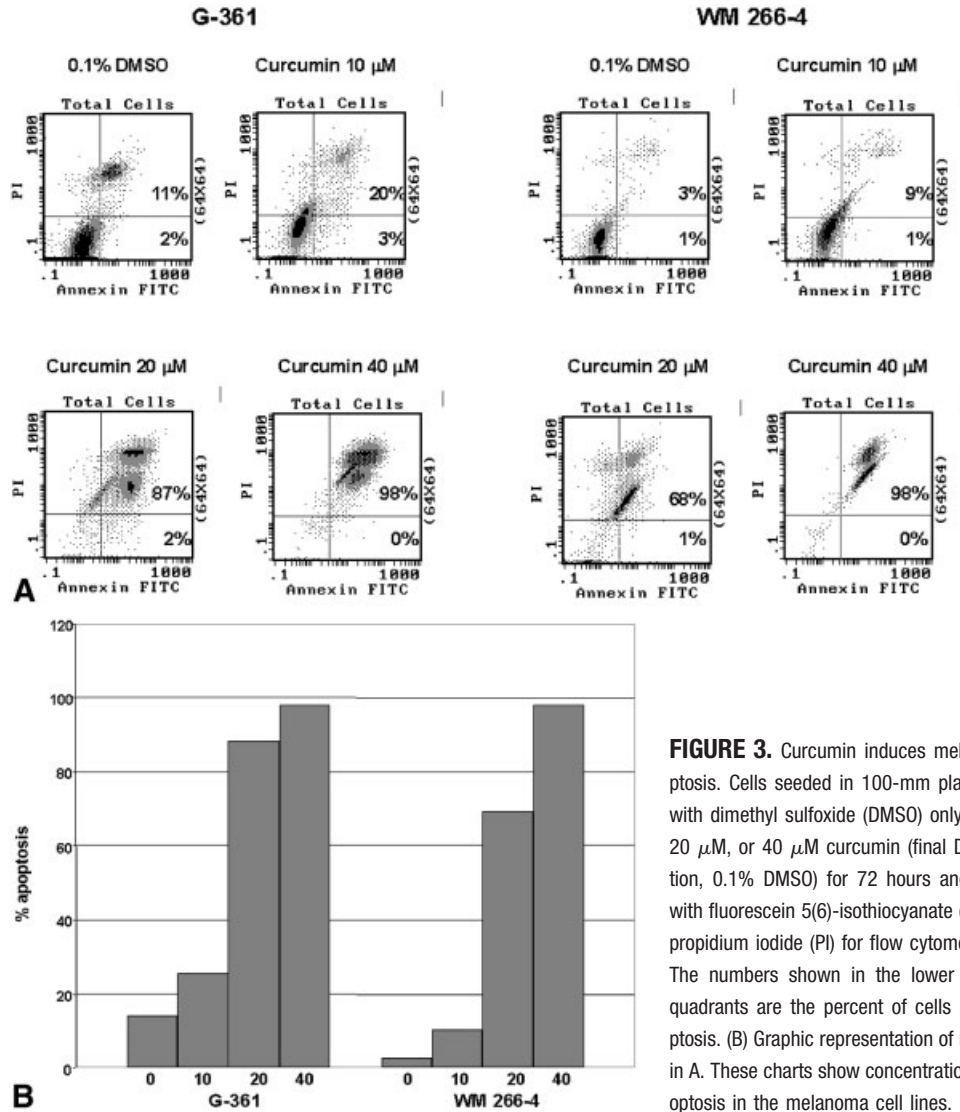


FIGURE 3. Curcumin induces melanoma cell apoptosis. Cells seeded in 100-mm plates were treated with dimethyl sulfoxide (DMSO) only or with 10 μ M, 20 μ M, or 40 μ M curcumin (final DMSO concentration, 0.1% DMSO) for 72 hours and doubly stained with fluorescein 5(6)-isothiocyanate (FITC)-annexin V-propidium iodide (PI) for flow cytometric analysis. (A) The numbers shown in the lower and upper right quadrants are the percent of cells staining for apoptosis. (B) Graphic representation of results presented in A. These charts show concentration-dependent apoptosis in the melanoma cell lines.

Curcumin Does Not Affect Raf, MEK, or ERK Activity in Melanoma Cells

Activating *B-raf* mutations are observed in the majority of melanomas.²¹ C32, G-361, and WM 266-4 cells have activating *B-raf* mutations,²¹ the gene product of which is activated by Ras. Activated forms of Raf reportedly activate NF- κ B,^{22,23} although NF- κ B is activated by other pathways as well; thus, we determined the effects of curcumin treatment on B-Raf kinase activity and on the phosphorylation of MEK (the downstream substrate of B-Raf) and ERK (the downstream substrate of MEK).

Melanoma cells treated with 0 μ M or 10 μ M curcumin for 72–96 hours were examined for B-Raf activity by immunoprecipitating B-Raf in these cells and using a Raf kinase cascade assay kit. This kit examines Raf kinase enzymatic activity by the sequential phosphorylation of increasing amounts of MEK1, ERK2,

and myelin basic protein. Myelin basic protein phosphorylation is detected by radioisotope labeling. All three cell lines had significant baseline levels of B-Raf kinase activity (probably due to activating *B-raf* mutations²¹). Changes in B-Raf kinase activity upon exposure to curcumin were insignificant (from + 3% to – 10%) (Fig. 7A).

Melanoma cells treated with 0 μ M or 10 μ M curcumin for 72 hours also were assayed for the extent of MEK phosphorylation using anti-phospho-MEK1/2 immunoblots. Endogenous MEK phosphorylation was detected readily in the melanoma cell lines (Fig. 7B, top), indicating that MEK is active constitutively in melanoma cells. However, no differences were observed between control and curcumin-treated melanoma cells in the cell lines examined.

ERK1/2 reportedly activates NF- κ B in melanoma cells.²⁴ Furthermore, ERK1/2 is a downstream effector

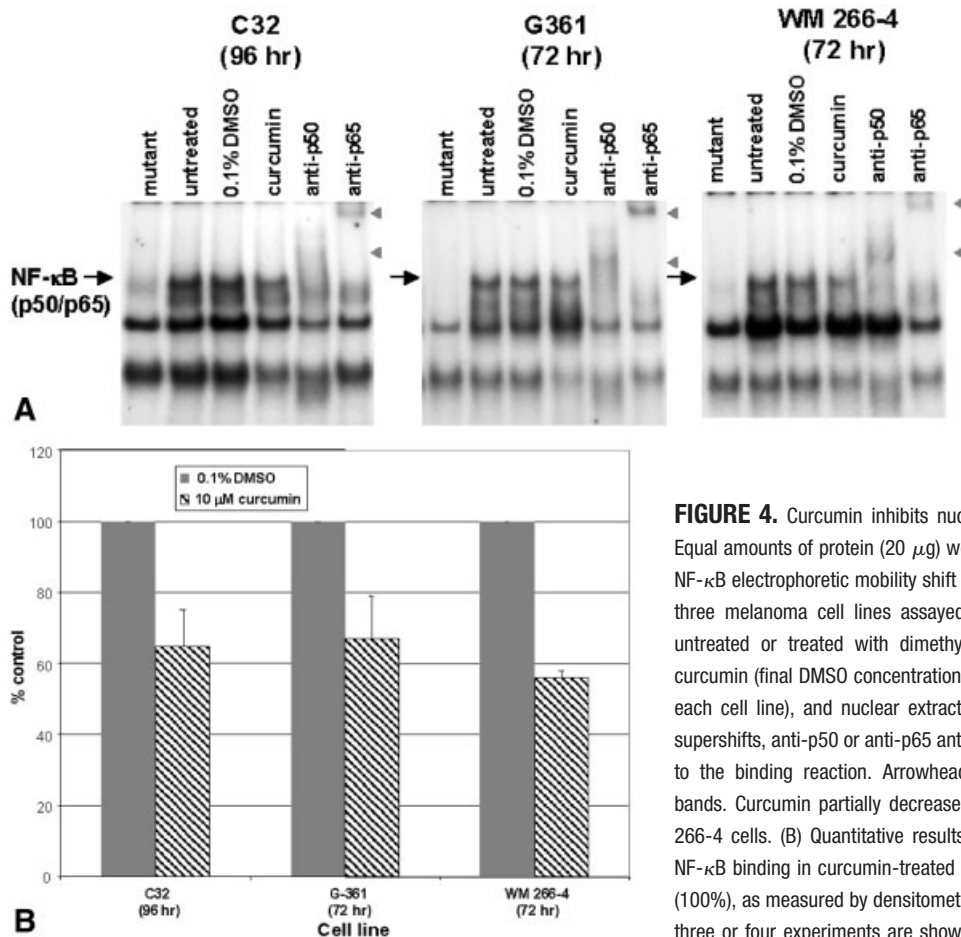


FIGURE 4. Curcumin inhibits nuclear factor κ B (NF- κ B) binding activity. Equal amounts of protein (20 μ g) were used in each lane. (A) Representative NF- κ B electrophoretic mobility shift assay (EMSA) results are shown from the three melanoma cell lines assayed. Cells plated in 100-mm dishes were untreated or treated with dimethyl sulfoxide (DMSO) only or in 10 μ M curcumin (final DMSO concentration, 0.1%) for 72–96 hours (as indicated for each cell line), and nuclear extracts were harvested for NF- κ B EMSA. For supershifts, anti-p50 or anti-p65 antibody was added to nuclear extracts prior to the binding reaction. Arrowheads indicated supershifted p50 and p65 bands. Curcumin partially decreased NF- κ B levels in C32, G-361, and WM 266-4 cells. (B) Quantitative results of EMSAs show a relative decrease in NF- κ B binding in curcumin-treated cells compared with DMSO-only controls (100%), as measured by densitometry. The mean \pm standard error values for three or four experiments are shown for each cell line.

in the Raf/MEK pathway.^{25–27} Therefore, we also determined whether ERK1/2 activity is affected by curcumin treatment using antiphospho-ERK1/2 immunoblot. With regard to MEK, endogenous levels of ERK1/2 phosphorylation were detected readily in the melanoma cell lines (Fig. 7B, middle), indicating that ERK1/2 also is active constitutively in melanoma cells. Curcumin exposure did not affect ERK1/2 phosphorylation. Taken together, these results indicate that, although the B-Raf/MEK/ERK pathway is active constitutively in B-*raf*-mutated melanoma cells, curcumin does not inhibit this pathway and that NF- κ B suppression is independent of this pathway.

Akt Activity Increased in Melanoma Cells with Exposure to Curcumin

The activation of Akt-mediated pathways induces NF- κ B activation in different cell types.^{28–31} In addition, several reviews have established that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway engages in crosstalk with the Ras/Raf/MEK/ERK pathway.^{32–34} To determine whether curcumin modulates Akt activity, melanoma cells treated with 0 μ M or 10 μ M of

curcumin for 72–96 hours were assessed for phospho-Akt levels using an antiphospho-Akt immunoblot. Low levels of phosphorylated Akt were detected in the cell lines (Fig. 7B, bottom). However, 3.9-fold and 1.7-fold increases in phospho-Akt levels, as determined by densitometry, were observed in G-361 and WM 266-4 cells, respectively, upon curcumin treatment. Total Akt levels remained unchanged in these cells.

Based on these results, it appears that the anti-proliferative/proapoptotic effect of curcumin was independent of Akt. However, it remains possible that retention and enhancement of Akt operates as a salvage pathway. Therefore, we used the Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate] and first determined its effect alone on cell proliferation/survival and Akt activity on G-361 cells. The IC₅₀ for cell proliferation/survival of the Akt inhibitor, as determined by the MTT assay, 61 μ M (data not shown) whereas Akt phosphorylation, as determined by Western blot analysis, was inhibited with an IC₅₀ of \approx 58 μ M. However, when 10 μ M of curcumin were added in combination with 60 μ M of the Akt inhib-

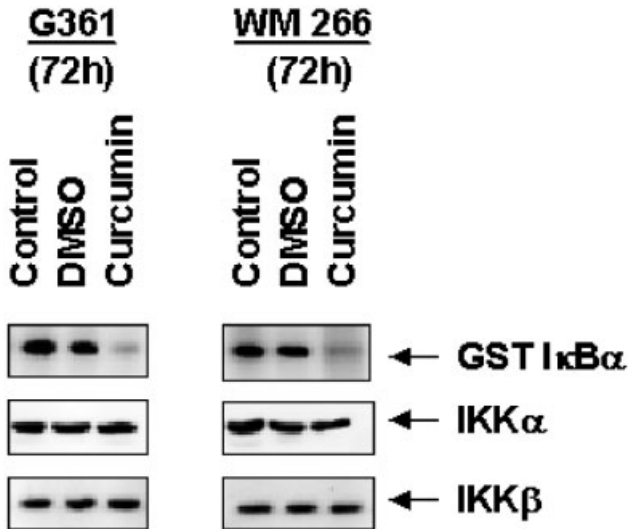


FIGURE 5. Curcumin inhibits $\text{I}\kappa\text{B}$ kinase (IKK) activity in melanoma cells. Cells plated in 100-mm dishes were either untreated or treated with DMSO only or 10 μM curcumin (0.1% DMSO final concentration) for 72 hours. Cells were harvested and lysed; equal amounts of protein from lysates were immunoprecipitated for IKK and assayed for IKK activity using an in vitro kinase assay with glutathione-S-transferase (GST)- $\text{I}\kappa\text{B}\alpha$ as a substrate. Melanoma cells have constitutively active IKK (top), and this enzymatic activity is down-regulated by curcumin (top). Steady-state $\text{IKK}\alpha$ levels (middle) and $\text{IKK}\beta$ levels (bottom), as determined by immunoblots, were unchanged by curcumin exposure.

itor, no decrease in Akt phosphorylation was observed (Fig. 8).

DISCUSSION

In the current study, we demonstrated that curcumin inhibited cell viability and induced apoptosis in a dose-dependent manner in melanoma cells (Figs. 1, 3). Furthermore, we showed that NF- κB activity, as measured by EMSA, was inhibited in part under conditions in which melanoma cells were induced to undergo apoptosis (72 hours of exposure to 10 μM of curcumin) (Fig. 4). Previous reports described NF- κB inhibition with exposure to higher concentrations of curcumin (60 μM) for shorter periods (6 hours) in melanoma cell lines.³⁵ Therefore, the NF- κB machinery is suppressed both by short exposures to high concentrations of curcumin and by longer exposures to lower concentrations of curcumin. In addition, we demonstrated that, under apoptosis-inducing conditions, IKK, the upstream regulator of NF- κB , is inhibited strongly by curcumin (Fig. 5). Partial inhibition of NF- κB but strong inhibition of IKK by curcumin suggests that, in melanoma cells, signaling molecules other than IKK can regulate NF- κB activity. Indeed, IKK-independent NF- κB activation has been demonstrated previously in ultraviolet-irradiated cells,^{36,37} in

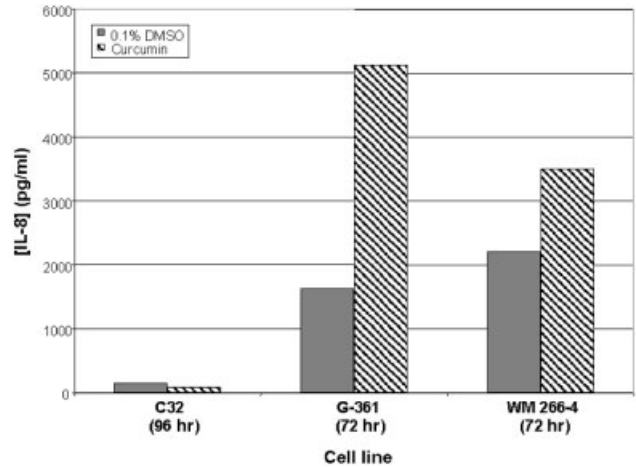
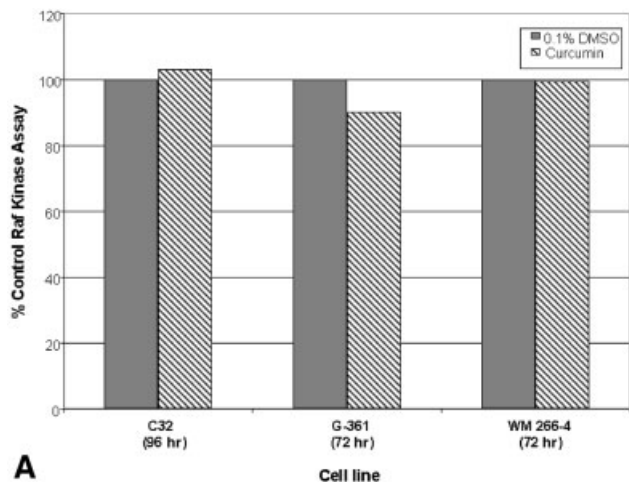


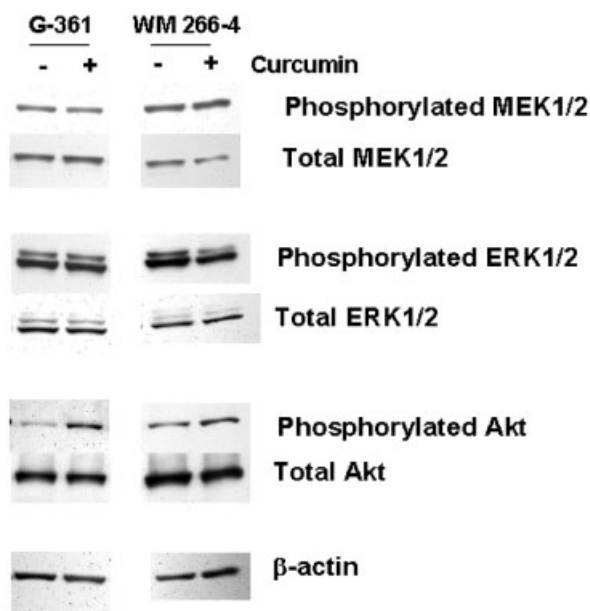
FIGURE 6. Interleukin 8 (IL-8) secretion does not correlate with melanoma cell growth inhibition, apoptosis, or neuronal factor κB (NF- κB) binding activity. Cells plated in 100-mm dishes were treated with dimethyl sulfoxide (DMSO) only or with 10 μM curcumin (final DMSO concentration, 0.1%) for 72–96 hours. Exposed medium was harvested and centrifuged to remove cellular debris. The IL-8 concentration was determined by an IL-8 enzyme-linked immunosorbent assay followed by interpolation based on a 4-parameter logistic curve fit.

nontypeable *Hemophilus influenzae*-infected cells,³⁸ and in mutant cultured human cells with constitutively activated NF- κB .³⁹ In melanoma cells, there is evidence that an ERK1/2-mediated pathway and an Akt-mediated pathway can contribute to NF- κB activation independent of IKK.^{24,40}

To determine whether other potential NF- κB regulators also are involved in curcumin-induced inhibition of proliferation or survival in melanoma cells, we examined the B-Raf/ERK kinase pathway as well as Akt activity. Both the Ras/ERK pathway, of which B-Raf is a signaling intermediate, and the PI3K/Akt pathway play a role in melanoma cell proliferation or survival^{41–43} in addition to regulating NF- κB activity.^{22,28,44–45} B-Raf is especially important in this regard, because activating B-*raf* mutations are a hallmark of melanomas.²¹ In the current investigation, we showed that, under apoptosis-inducing conditions, neither the B-Raf/ERK pathway nor the Akt pathway was inhibited upon exposure to curcumin. In fact, Akt phosphorylation was increased after curcumin treatment (Fig. 7B). These results are in contrast to those seen with curcumin-treated breast, prostate, and renal carcinoma cells, all of which showed decreased Akt activity upon exposure to curcumin.^{46–48} Therefore, it is possible that the induction of apoptosis in melanoma cells either does not require suppression of these pathways, or persistent activation of these signals eventually emerges as an escape pathway that could lead to chemoresistance in melanoma cells. The latter may be of concern.



A



B

FIGURE 7. Curcumin does not affect the Raf/mitogen-activated/extracellular signal-regulated protein kinase (Raf/MEK/ERK) pathway and increases Akt activity in melanoma cells. Cells plated in 100 mm dishes were treated with dimethyl sulfoxide (DMSO) only or 10 μ M curcumin (final DMSO concentration, 0.1%) for 72 hours. (A) Cells were harvested and lysed; equal amounts of protein (500–1000 μ g) from lysates were immunoprecipitated for B-Raf and assayed for B-Raf activity using a Raf kinase cascade assay (Upstate Cell Signaling Solutions, Charlottesville, VA). This assay examines Raf kinase activity by sequential phosphorylation of MEK1 (the Raf substrate), ERK2 (the MEK1 substrate), and myelin basic protein (MBP) (the ERK2 substrate). The extent of MBP phosphorylation (the only substrate labeled by radioisotope) was determined by liquid scintillation counting. Resulting counts per minute were adjusted to percent control, with 0.1% DMSO only = 100%. (B) Harvested, lysed cells were assayed for MEK1/2, ERK1/2, or Akt activities using antiphospho-MEK1/2, phospho-ERK1/2, or phospho-Akt immunoblots. Both phosphorylated and unphosphorylated forms of MEK (top), ERK (middle), and Akt (bottom) are shown. Equal amounts of protein (40 μ g) were loaded on 10% sodium dodecyl sulfate-polyacrylamide gels. The figure demonstrates that melanoma cells have constitutive activity of the B-Raf kinase with phosphorylation of MEK and ERK1/2. The Raf kinase pathway is unaffected by curcumin, whereas Akt phosphorylation is increased.

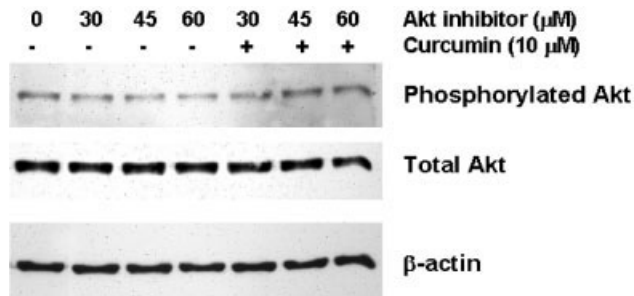


FIGURE 8. The Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-methyl-3-*O*-octadecylcarbonate] does not inhibit Akt phosphorylation in the presence of curcumin. Cells plated in 100-mm dishes were treated with 0.15% dimethyl sulfoxide (DMSO) only or with 30 μ M, 45 μ M, or 60 μ M Akt inhibitor with or without 10 μ M curcumin (final DMSO concentration, 0.15%) and incubated for 72 hours. Cells were harvested and lysed; equal amounts (40 μ g) of total protein from lysates were loaded on 10% sodium dodecyl sulfate-polyacrylamide gels. Akt activity was assessed by antiphospho-Akt immunoblots. Both phosphorylated and unphosphorylated forms of Akt are shown. The results demonstrate that the Akt inhibitor decreased Akt phosphorylation in a concentration-dependent manner but that, in the presence of curcumin, this suppression did not occur.

because we demonstrated that the Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-methyl-3-*O*-octadecylcarbonate] was unable to suppress Akt phosphorylation in the presence of curcumin (Fig. 8). Still, the implications of these experiments for the antitumor potential of curcumin is unclear, because the role of Akt in preventing apoptosis in melanoma cells remains controversial, with some experiments reporting that Akt inhibition induces apoptosis⁴⁹ and with others suggesting that Akt inhibition induces cell cycle arrest, but not programmed cell death.⁹ In addition, curcumin retains its antiproliferative effects in our melanoma cells despite the enhancement of Akt phosphorylation (Fig. 8). With regard to ERK, or MAPK, Smalley and Eisen reported that the inhibition of MAPK did not result in apoptosis of melanoma cells,⁹ and the current results suggesting that apoptosis is independent of MAPK suppression are in agreement with their report. Finally, based on the ability of ERK1/2 or Akt to activate NF- κ B independent of IKK,^{24,40} the lack of inhibition of ERK1/2 and Akt in our melanoma cells after curcumin treatment may explain the differences in inhibition observed between IKK activity and NF- κ B activity.

IL-8 stimulates melanoma cell proliferation *in vitro*,¹⁴ and increased levels of this cytokine correlate with a poor prognosis in patients with melanoma.¹⁹ The promoter region of IL-8 contains a recognition site for the NF- κ B transcription factor.²⁰ In this report, we also explored the consequence of NF- κ B inactivation by assessing IL-8 secretion levels. It was some-

what surprising that IL-8 levels increased after curcumin exposure in the high IL-8-secreting melanoma cell lines (Fig. 6). Therefore, it appears that IL-8 secretion is independent of curcumin-induced NF- κ B inhibition. This may be because IL-8 expression also is regulated transcriptionally by AP-1 and C/EBP as well as NF- κ B (reviewed by Hoffmann et al.⁵⁰). AP-1 activity is regulated by the ERK and Akt pathways, as reported in several reviews.^{51–54} Whereas the ERK pathway is preserved after curcumin exposure, it is possible that AP-1, through activation of the Akt pathway, induces IL-8 transcription in melanoma cells in the presence of curcumin-induced NF- κ B suppression.

Based on our studies, we conclude that curcumin is a potent suppressor of cell viability and inducer of apoptosis in melanoma cell lines. This activity is associated with inhibition of the IKK/NF- κ B transcriptional machinery, but not the B-Raf/ERK or Akt pathways. Indeed, curcumin enhances Akt activity and results in resistance to Akt suppression by the Akt inhibitor 1L-6-hydroxymethyl-*chiro*-inositol 2-[(R)-2-*O*-methyl-3-*O*-octadecylcarbonate]. These results imply that suppression of the viability of melanoma cells can occur despite the continued activation of the B-Raf/MEK/ERK and Akt pathways. Future investigations to determine the effects of curcumin in animal models of melanoma and clinical trials are planned.

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