

Combining Curcumin (Diferuloylmethane) and Heat Shock Protein Inhibition for Neurofibromatosis 2 Treatment: Analysis of Response and Resistance Pathways

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Abstract

Neurofibromatosis type 2 (NF2) is a genetic condition characterized by inactivation of the *NF2* tumor suppressor gene and the development of schwannomas. The *NF2* gene product, *merlin*, is activated (dephosphorylated) by contact inhibition and promotes growth suppression. We investigated the effect of curcumin (diferuloylmethane), a molecule with anti-inflammatory and antitumorogenic properties, on human schwannoma cell growth and the regulation of merlin by curcumin in both *NF2* cells and neuroblastoma (non-*NF2*) cells. Curcumin inhibited the growth of HEI-193 schwannoma cells *in vitro* and downregulated the phosphorylation of Akt and extracellular signal-regulated kinase 1/2. Curcumin also activated MYPT1-pp1 δ (a merlin phosphatase), which was associated with dephosphorylation of merlin on serine 518, an event that results in the folding of merlin to its active conformation. In addition, curcumin induced apoptosis and generated reactive oxygen species in HEI-193 cells. Consequently, hsp70 was upregulated at the mRNA and protein levels, possibly serving as a mechanism of escape from curcumin-induced apoptosis and growth inhibition. Endogenous merlin and hsp70 proteins interacted in HEI-193 schwannoma and SK-N-AS neuroblastoma cells. The combination of curcumin and an hsp inhibitor synergistically suppressed schwannoma cell growth. Our results provide a rationale for combining curcumin and KNK437 in the treatment of *NF2*. *Mol Cancer Ther*; 10(11); 2094–103. ©2011 AACR.

Introduction

Neurofibromatosis type 2 (*NF2*) is characterized by development of bilateral vestibular schwannomas and a subsequent loss of hearing. Schwannomas of other cranial nerves can accompany the disease, as can meningiomas, spinal schwannomas, and ependymomas. *NF2* occurs in 1 of 33,000 live births (1, 2). The mainstay of treatment of *NF2* has been surgery, though recently an anti-VEGF antibody, bevacizumab, showed encouraging results in a pilot trial (3). However, most patients suffer progressive debilitation, and new treatments and rationally developed clinical trials are needed (4).

The *NF2* gene is a tumor suppressor gene that fits the Knudson 2-hit hypothesis. The initial mutation of *NF2* occurs either in the germline, affecting all cells in the

body, or as an early zygotic constitutional mutation, affecting only a subpopulation of cells (5, 6). Each tumor is initiated by a second somatic event that inactivates the remaining functional allele. Several growth-suppressing functions have been ascribed to the *NF2* gene product, *merlin*, including regulation of cell adherence, contact growth inhibition, regulation of cyclins, and inhibition of phosphoinositide 3-kinase (PI3K).

Little is known about the regulation of merlin protein expression in schwannoma cells. Merlin exists in an "open" or inactive conformation when it is phosphorylated at (serine 518) ser518, abrogating N-terminal/C-terminal domain interaction and subsequent folding of merlin to its "closed" or active conformation. The phosphorylation of Merlin at ser518 can be induced by activation of p21-activated kinase (Pak1) or protein kinase A via 2 distinct mechanisms (7–10). Protein kinase B (Akt) also directly binds to and phosphorylates merlin on threonine 230 (thr230) and serine 315 (ser315; ref. 11). This phosphorylation abolishes merlin N-terminal/C-terminal domain interactions causing merlin to revert to its "open" or inactive conformation, resulting in loss of binding to PI3K enhancer-long (PIKE-L) and other binding partners (12). Furthermore, Akt-mediated phosphorylation leads to merlin degradation by ubiquitination (11).

We report increased programmed cell death and decreased proliferation of schwannoma cells following

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treatment with curcumin (diferuloylmethane), a yellow polyphenol extracted from the rhizome of turmeric (*Curcuma longa*), with known anti-inflammatory and anti-tumorigenic properties (13). Curcumin upregulates hsp70 in several cell lines, including HEI-193 schwannoma cells, which may serve as a means of escape from curcumin-induced apoptosis (14–16). In addition, the combination of curcumin and the hsp inhibitor KNK437 is strongly synergistic in its inhibition of HEI-193 cell growth and moderately synergistic in a curcumin-resistant variant (HEI-193 Resistant) that expresses a high baseline level of hsp70. Our results indicate that patients with *NF2* mutation-positive schwannomas may benefit from treatment with a combination of curcumin and hsp inhibitors.

Materials and Methods

Cell lines

HEI-193 schwannoma cells were obtained from the House Ear Institute (Los Angeles, CA, in 2005) and contain an LOH at chromosome 22. The remaining *NF2* allele contains a G to A point mutation at the exon 15 splice site acceptor, resulting in a splicing defect in the *NF2* transcript (17, 18). The cell line has been tested and authenticated in our laboratory by reverse transcriptase (RT)-PCR sequencing of this *NF2* mutation (2008). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM/Ham's F-12) supplemented with 15% fetal calf serum (FCS; Invitrogen, GIBCO/BRL). The SK-N-AS neuroblastoma cell line contains a wild-type *NF2* gene (19), and it was purchased from American Type Culture Collection and authenticated by short tandem repeat analyses. Cells were cultured in DMEM plus 10% FCS. HEI-193 Resistant cells were generated by culturing in the presence of 20 $\mu\text{mol/L}$ curcumin. All cells were maintained in a 37°C incubator with 5% CO₂.

Clonogenic assay

Cells were seeded at 1×10^5 cells per 60-mm dish (Nunc International) and grown overnight. Free or liposomal curcumin was added for 5 days. Cultures were washed, and fresh medium was added. Cultures were grown for 3 weeks, stained with crystal red in 0.5% methanol (Sigma-Aldrich), and colony numbers were counted. Statistical significance was assessed by the Fisher *t* test.

Preparation of liposomal curcumin

Preparation of liposomes was carried out as described (20). Curcumin (Sigma) was dissolved in dimethyl sulfoxide (DMSO). Liposomal curcumin was prepared by mixing liposomes and curcumin at a ratio of 10:1.

Annexin V apoptosis assay

Apoptosis was measured by staining cell-surface phosphatidylserine with fluorescein isothiocyanate (FITC)-conjugated Annexin V. Cells were seeded at

$1 \times 10^6/2.0$ mL per well of a 6-well plate, grown to 70% confluence, treated with various concentrations of curcumin for 24 hours, then harvested and stained with FITC-conjugated Annexin V and propidium iodide according to the manufacturer's instructions (BD Pharmingen). Cells were analyzed by fluorescence-activated cell-sorting analysis (Becton Dickinson).

Antibodies and reagents

Antibodies used for Western blotting included anti-N-terminal merlin (A19), anti-C-terminal merlin (amino acids 336–595), anti-p-Akt (ser473), anti-p-MYPT1 phosphatase (thr696), anti-MYPT1, anti-hsp70 (Santa Cruz Biotechnology, Inc.), anti-p-merlin (ser518; Rockland Immunochemicals for Research), anti-ERK 1/2 and anti-p-ERK 1/2 (Promega), and anti-Akt (Cell Signaling Technology). Anti- β -actin and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serve as loading controls (Santa Cruz Biotechnology). Free curcumin (Sigma) and KNK437 (EMD Biosciences) were dissolved in DMSO before use. *N*-Acetyl cysteine (NAC) was dissolved in water (Sigma).

Immunoprecipitation and Western blot analysis

For Western blots of phosphoproteins, cells were starved overnight in 0.5% FCS, treated with curcumin for the time indicated, and then stimulated with 20% FCS for 30 minutes. Cells were washed with PBS and lysed in solubilization buffer. A modified Lowry protein assay was conducted (Pierce Biotechnology), and equal amounts of protein were loaded onto 8% SDS-PAGE gels and then transferred to nitrocellulose membranes by electroblotting. Blots were blocked in 3.0% bovine serum albumin, incubated with primary antibody (22°C for 2 hours), washed, and then incubated with an appropriate secondary antibody conjugated to horseradish peroxidase (BioRad Laboratories) and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech Inc.). For immunoprecipitation experiments, 5×10^6 cells per lane were lysed with $1 \times$ NP-40 buffer. Lysates were precleared with an isotypic control (Santa Cruz Biotechnology; 4°C for 2 hours), then incubated with specific antibody (2–4 hours at 4°C), centrifuged, washed 4 times with cell lysis buffer, resuspended in $2 \times$ sample buffer (BioRad), and boiled before loading onto SDS-PAGE gels.

Real-time PCR

HEI-193 cells were stimulated with 10 $\mu\text{mol/L}$ curcumin for 6 hours. Heat shock (43°C for 4 hours) and bortezomib (1 $\mu\text{mol/L}$) were used as positive inducers of hsp70 mRNA. Total RNA was extracted by the RNeasy Mini Kit from QIAGEN. mRNA levels for hsp70 and merlin were measured by the TaqMan RNA-to-C_T 1-Step Kit from Applied Biosystems according to the manufacturer's instructions. Primer and probe pairs for the reactions were as follows: hsp70, hs00271229_s1; GAPDH, GapDHhs99999905_m1; and *NF2*, Hs00738978_m1. Reactions were run on an ABI 7500 real-time PCR machine under the following

thermal cycling conditions: 48°C for 15 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Cytotoxicity assays

Survival of cells after treatment with curcumin and/or KNK437 was assessed by the MTT assay according to the manufacturer's instructions (Sigma). A total of 5×10^3 HEI-193 cells were seeded overnight followed by treatment with increasing doses of curcumin and KNK437 individually, or in combination, for 36 hours. The MTT assay was conducted as described (20, 21). Conversion of MTT was measured at 570 nm. Synergy calculations were conducted at a fixed 1:2 ratio of curcumin to KNK437 according to the method of Chou and Talalay (22, 23). A combination index (CI) was calculated with the CalcuSyn software (BioSoft), which uses a Monte Carlo method for determining 95% confidence intervals of the fractional affect versus CI plot (dashed lines).

Measurement of intracellular reactive oxygen species generation: 2',7'-dichlorofluorescein fluorescence

HEI-193 cells were seeded in a 96-well plate ($1 \times 10^4/0.1$ mL per well) and allowed to adhere for 48 hours. Cells were washed in Hank's balanced salt solution (HBSS) and incubated with 10 μ mol/L car-

boxy-H₂DCF-DA (Molecular Probes) in the loading medium (1% FCS in DMEM/Ham's F-12) for 45 minutes. Cells were washed to remove carboxy-H₂DCF-DA and replaced with HBSS containing 20 μ mol/L curcumin, 0.3%, H₂O₂, or HBSS alone. 2',7'-Dichlorofluorescein (DCF) fluorescence was measured by a fluorescence multi-well plate reader with excitation and emission wavelengths of 485 nm and 530 nm, respectively (24).

Results

Curcumin inhibits proliferation/survival of HEI-193 schwannoma cells *in vitro* and induces apoptosis

To determine whether human schwannoma cells are sensitive to the growth inhibitory effects of curcumin, HEI-193 cells were exposed to curcumin dissolved in DMSO (free curcumin), liposomal curcumin, or pegylated liposomal curcumin for 5 days (20, 25, 26), and the effect on proliferation was assessed by clonogenic assay. Cells treated with empty liposomes showed no significant decrease in colony formation, whereas cells treated with different forms of curcumin showed a dose-dependent decrease in colony formation (Fig. 1A). Treatment with 20 μ mol/L liposomal curcumin or 20 μ mol/L pegylated liposomal curcumin resulted in the most significant decrease in colony formation compared with control cells (***, $P < 0.001$ by the Fisher *t* test; Fig. 1A).

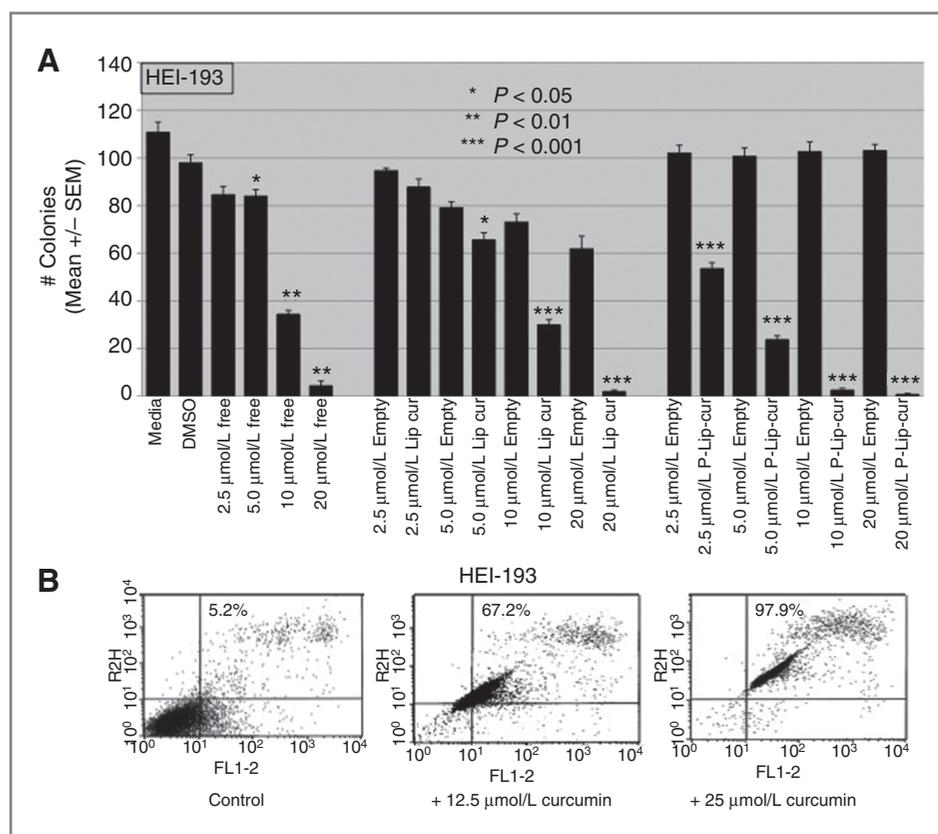


Figure 1. Curcumin inhibits HEI-193 schwannoma cell growth. A, HEI-193 cells were treated with increasing concentrations of free, liposomal, or pegylated liposomal curcumin or control for 5 days, and colonies were counted ($n = 5$). B, HEI-193 cells were treated for 24 hours with media alone (control), 12.5 μ mol/L, or 25 μ mol/L free curcumin. Apoptosis was assessed by staining with FITC-conjugated Annexin V and propidium iodide. Empty, empty vehicle; Lip cur, liposomal curcumin; P-Lip-cur, pegylated liposomal curcumin.

Curcumin also inhibited the proliferation of HEI-193 cells by MTT assay (data not shown). Because all 3 forms of curcumin inhibited HEI-193 cells in a comparable manner at concentrations greater than 10 $\mu\text{mol/L}$, free and/or liposomal curcumin were used interchangeably for the remainder of the study, with no significant difference between the different forms of curcumin.

Curcumin induces apoptosis in several different cell lines (27–29). To determine whether HEI-193 cells undergo apoptosis following curcumin treatment, cell-surface phosphatidylserine was measured with FITC-conjugated Annexin V as an indicator of apoptosis. Sixty-seven percent and 97.9% of HEI-193 cells underwent apoptosis with free curcumin concentrations of 12.5 and 25 $\mu\text{mol/L}$, respectively, after 24 hours of treatment (Fig. 1B). Hence, curcumin induces apoptosis in HEI-193 cells in a dose-dependent manner.

Curcumin inhibits phosphorylation of Akt and extracellular signal-regulated kinase 1/2 in HEI-193 cells

Curcumin downregulates several protein kinases and transcription factors necessary for survival pathways, including Akt and NF- κB (27, 30, 31). Akt is a prosurvival serine/threonine kinase that acts in a PI3K-dependent manner. Phosphorylated Akt (p-Akt) indicates that proliferative pathways are active (32). We investigated the effect of free or liposomal curcumin on p-Akt expression in HEI-193 cells. A significant decrease in p-Akt occurred with 5 $\mu\text{mol/L}$ of liposomal curcumin by 24 hours. Levels of total Akt protein remained unchanged (data not shown and Fig. 2A).

The effect of curcumin on extracellular signal-regulated kinase 1/2 (ERK 1/2), an important component of the mitogen-activated protein kinase (MAPK) pathways, was also examined. Total levels of ERK 1/2 kinase remained fairly stable, whereas phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK 1/2) decreased in a dose-dependent manner (Fig. 2A). Dephosphorylation of ERK and Akt did not occur in HEI-193 or SK-N-AS cells until at least 24 hours after curcumin treatment at concentrations up to 20 $\mu\text{mol/L}$ (data not shown).

Curcumin dephosphorylates (activates) merlin via activation of MYPT1-pp1 δ , a merlin phosphatase

Merlin becomes active after dephosphorylation of ser518, which allows N-terminal/C-terminal domain interaction and folding of the protein (7). Phosphorylation of merlin on ser518 was decreased by curcumin in a dose-dependent manner in both HEI-193 schwannoma cells and SK-N-AS neuroblastoma cells, indicating that merlin may become active following curcumin treatment (Fig. 2B). Dephosphorylation of merlin occurred as early as 8 hours after curcumin treatment (Fig. 2B, left), indicating that it precedes the dephosphorylation of ERK and Akt. These experiments were repeated at least 3 times with doses of curcumin ranging from 5 to 30 $\mu\text{mol/L}$. Time points included 8, 24, 48, and 72 hours. Densitometric analysis of 3 representative Western blots shows a significant decrease in phosphorylated merlin (p-merlin) starting at 10 $\mu\text{mol/L}$ curcumin ($P < 0.05$; Fig. 2C).

Levels of total merlin remained fairly constant after curcumin treatment in both cell lines (Fig. 2D). The predominant form of merlin expressed in HEI-193 cells

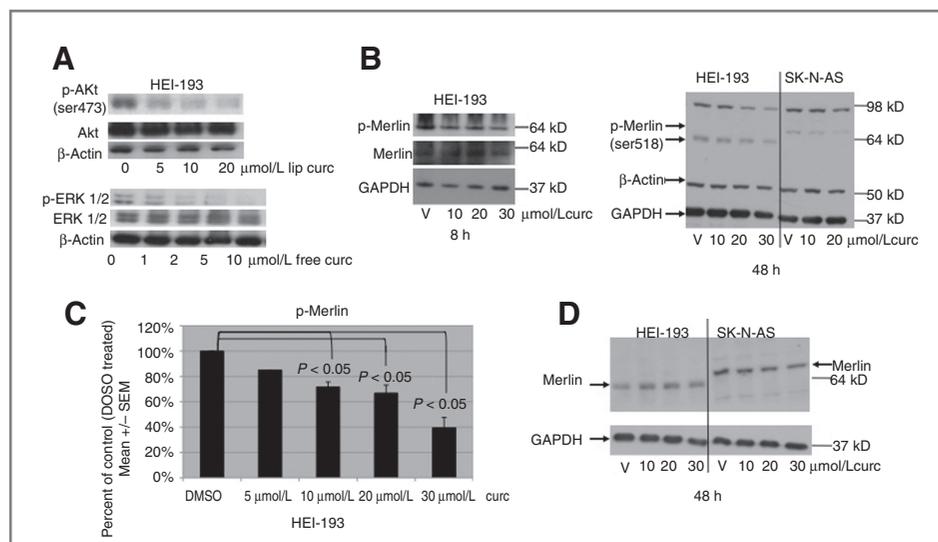


Figure 2. A, concentration-dependent inhibition of p-Akt and p-ERK 1/2 expression in HEI-193 cells treated with liposomal or free curcumin for 72 hours. Protein expression was examined by Western blotting. B, concentration-dependent inhibition of p-merlin in HEI-193 and SK-N-AS cells following 8- or 48-hour curcumin treatment. Merlin isoform III runs at approximately 60 kD in HEI-193; isoform I runs at approximately 70 kD in SK-N-AS. C, densitometry of p-merlin bands from 3 experiments (HEI-193). Only one experiment was available for analysis at 5 $\mu\text{mol/L}$. The Student *t* test was used for statistical analysis. D, levels of total merlin remain fairly constant in both cell lines (48-hour curcumin treatment). Lip curc, liposomal curcumin; curc, curcumin.

is a truncated protein, identical to the normally occurring merlin protein designated isoform III (~60 kD). Isoform III retains some growth suppressive properties, albeit to a limited extent; hence, the presence of this isoform in the absence of isoform I (70 kD) is associated with the development of a mild form of NF2 (33–35). SK-N-AS neuroblastoma cells contain mRNA for wild-type merlin (isoforms I, II, and III; ref. 19) and predominantly express isoform I (~70 kD; Fig. 2B and D).

To study the mechanism of curcumin-induced merlin dephosphorylation (activation), we examined the phosphorylation status of the merlin phosphatase, MYPT1-pp1 δ , which is known to directly dephosphorylate (activate) merlin (7, 36, 37). MYPT1-pp1 δ is similar to merlin in that it is activated by dephosphorylation of specific residues. Treatment of HEI-193 cells with curcumin resulted in a 65% decrease in phosphorylation (thr696) of MYPT1-pp1 δ by 30 minutes (Fig. 3A). One, 2, and 4 hours posttreatment time points were also examined; however, no specific decrease in MYPT1 phosphorylation (when compared with empty liposome) was seen at these time points (data not shown). The effect was also dose dependent (Fig. 3B), with a decrease in phosphorylated MYPT1 seen with doses of curcumin as low as 10 μ mol/L. This is well within the range of curcumin responsible for growth inhibition and apoptosis (Figs. 1A and 3B). Total

MYPT1 levels remained unchanged. Thus, curcumin may contribute to the activation of merlin in schwannoma cells by activating MYPT1-pp1 δ .

We investigated possible upstream regulators of MYPT1 to see whether we could determine which phosphatase was responsible for activating (dephosphorylating) MYPT1. MAPK phosphatase 5 (MKP5), which is known to be upregulated by curcumin in normal prostatic epithelium (38), was not induced by curcumin in HEI-193 or SK-N-AS cells (data not shown); therefore, another phosphatase is likely to be responsible for the dephosphorylation of MYPT1 seen in our cell lines.

Curcumin upregulates hsp70, which binds to merlin in HEI-193 and SK-N-AS cells

Curcumin upregulates stress proteins, including hsp70, *in vivo* and *in vitro* (14–16). Hsp70 is upregulated by curcumin in both HEI-193 and SK-N-AS cells [Fig. 4A and B (i)]. Immunoprecipitation with anti-hsp70 followed by Western blotting with anti-C-terminal merlin reveals the characteristic merlin bands [see Figs. 2 and 4B (i)] and shows that the 2 proteins are coimmunoprecipitated in both cell lines (merlin expression is lower in the NF2 cell line as expected). Curcumin seems to increase the amount of merlin that is coimmunoprecipitated with the anti-hsp70 antibody [Fig. 4B (i), bottom, and (ii)]. Reversing the antibodies used for immunoprecipitation and Western blotting and using an anti-N-terminal merlin antibody yielded the same results [Fig. 4B (ii)]. This is the first time, to our knowledge, that merlin and hsp70 have been shown to interact endogenously. These results confirm findings from a recently published article showing that merlin and hsp70 interact in a purely recombinant system (39).

Induction of hsp70 in HEI-193 schwannoma cells by curcumin

Curcumin increases the levels of reactive oxygen species (ROS) in several tumor cell lines, especially those sensitive to curcumin-induced apoptosis (15, 40, 41). Induction of ROS, in turn, is associated with the upregulation of hsp70 (15). Curcumin-sensitive HEI-193 schwannoma cells treated with curcumin for 48 hours showed a significant increase in ROS production (Fig. 5A).

Curcumin upregulates hsp70 mRNA in chronic myelogenous leukemia cells (42). We investigated whether curcumin upregulates hsp70 mRNA levels in HEI-193 cells. Heat shock and bortezomib, a proteasome inhibitor, were used as positive inducers of hsp70 mRNA. Hsp70 mRNA was significantly upregulated by heat shock ($P < 0.05$) and bortezomib ($P < 0.01$) as expected and also by 10 μ mol/L curcumin ($P < 0.05$), whereas GAPDH mRNA levels remained fairly constant (not significant by the Fisher *t* test; Fig. 5C). NF2 mRNA was increased slightly by curcumin ($P < 0.05$), with a concomitant slight increase in merlin protein expression (Fig. 2D), but not significantly by heat shock and bortezomib. To determine whether ROS were responsible for the induction

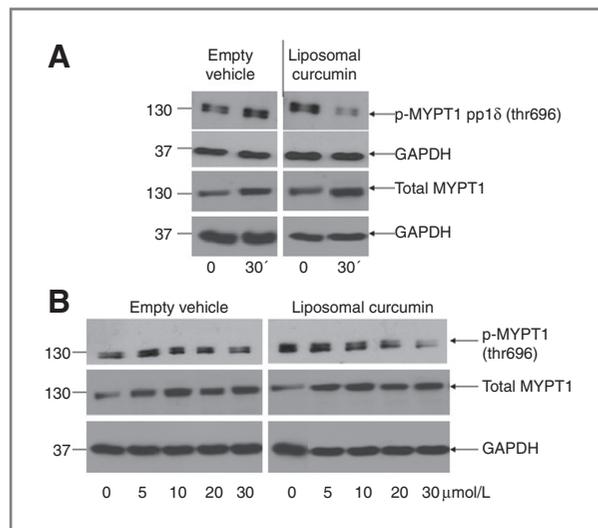


Figure 3. A, dephosphorylation (activation) of MYPT1-pp1 δ phosphatase in HEI-193 cells treated with liposomal curcumin. HEI-193 cells were starved overnight in 0.5% FCS, treated with empty vehicle or liposomal curcumin (30 μ mol/L) for 30 minutes, and then stimulated for 30 minutes with 20% FCS. Changes in the phosphorylation state of MYPT1-pp1 δ (thr696) were examined by Western blot analysis. B, dose response showing dephosphorylation of MYPT1-pp1 δ phosphatase in HEI-193 cells treated with empty liposome or liposomal curcumin for 72 hours. The dephosphorylation of MYPT1-pp1 δ is dose dependent and associated with concomitant dephosphorylation (activation) of merlin (Fig. 2B). For total MYPT1 and GAPDH levels (right), zero time points are from the same gel but were cut and placed next to the liposomal curcumin treatment lanes for ease of comparison.

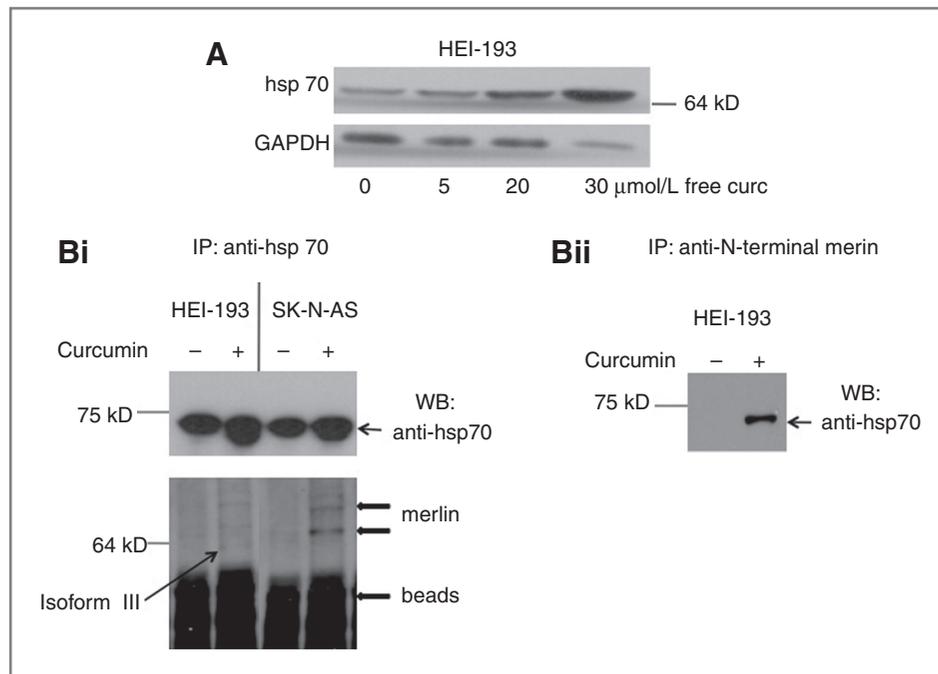


Figure 4. A, hsp70 expression is induced by curcumin in HEI-193 schwannoma cells and is dose dependent. HEI-193 cells were treated with increasing concentrations of curcumin for 72 hours, and hsp70 levels were determined by Western blotting. B, hsp70 and merlin interact endogenously in HEI-193 schwannoma and SK-N-AS neuroblastoma cells. i, Immunoprecipitation (IP) with anti-hsp70 antibody followed by Western blotting (WB) with either anti-hsp70 or anti-C-terminal merlin. Cells were starved overnight in 0.5% FCS before treatment with empty vehicle (–) or 30 $\mu\text{mol/L}$ liposomal curcumin (+) for 72 hours. Cells were then exposed to 20% FCS for 30 minutes and harvested for immunoprecipitation followed by Western blotting. hsp70 protein expression increased in both cell lines after curcumin treatment (top). Merlin is detected in both cell lines after immunoprecipitation with an anti-hsp70 antibody followed by WB with an anti-C-terminal merlin antibody (bottom). Arrow indicates lower band (isoform III) in HEI-193 cells. This band is not present in SK-N-AS. Both cell lines were run on the same gel, but the lanes were cut and placed so that HEI-193 is consistently on the left and SK-N-AS is on the right. ii, IP/WB in HEI-193 cells by an anti-N-terminal merlin antibody for IP followed by WB with anti-hsp70 antibody.

of hsp70, cells were pretreated with the antioxidant NAC for 30 minutes and then treated with curcumin (10 or 20 $\mu\text{mol/L}$) for 24 hours. Antioxidant pretreatment could not reverse the induction of hsp70 by curcumin in HEI-193 cells (data not shown). However, in SK-N-AS cells treated with 30 $\mu\text{mol/L}$ curcumin, the induction of hsp70 protein expression was inhibited when cells were pretreated with NAC (Fig. 5D).

The combination of curcumin and KNK437 synergistically inhibits HEI-193 schwannoma cell growth

Because hsp70 protects tumor cells from apoptosis (14, 15), the induction of hsp70 by curcumin in schwannoma cells might serve as an escape pathway used to avoid the antiproliferative effects of curcumin. Therefore, we examined the ability of an hsp inhibitor to synergize with curcumin in inhibiting schwannoma cell growth.

HEI-193 cells were treated with KNK437 alone (a commercially available hsp inhibitor that suppresses hsp70 mRNA expression; refs. 43, 44), curcumin alone, or the combination for 36 hours and assessed by MTT assay (Fig. 6A). Treatment with curcumin alone resulted in a dose-dependent decrease in proliferation (3.125–50 $\mu\text{mol/L}$), as expected on the basis of our earlier observa-

tions (Fig. 1). Treatment with KNK437 alone did not inhibit HEI-193 cell growth (Fig. 6A, top), whereas the combination of KNK437 and curcumin was strongly synergistic in all combinations tested [Fig. 6A (top) and B (top), CI at $\text{IC}_{50} = 0.15$].

Because hsp70 is upregulated by curcumin in HEI-193 cells, we developed a curcumin-resistant variant (HEI-193 Resistant) that expresses a higher baseline level of hsp70 [Fig. 6C (left), arrows, and lanes 1 and 7]. Baseline hsp70 protein levels increased by 16% in the resistant cell line compared with HEI-193 parental cells, as determined by densitometry. Curcumin upregulated hsp70 in both the parental and resistant cell lines (Fig. 6C, left), but unlike the parental cells, HEI-193 Resistant cells were slightly sensitive to treatment with KNK437 alone (Fig. 6A). The higher level of hsp70 expressed by HEI-193 Resistant protects them from the constant exposure to curcumin, and KNK437 directly targets this means of protection (Fig. 6A). Conversely, it takes a higher concentration (>12.5 $\mu\text{mol/L}$) of curcumin alone to inhibit the proliferation of HEI-193 Resistant compared with HEI-193 parental cells (Fig. 6A). In the resistant cell line, the combination of curcumin and KNK437 was only moderately synergistic [Fig. 6A (bottom) and 6B (bottom), CI at $\text{IC}_{50} = 0.45$]; hence, the higher level of hsp70 expressed in

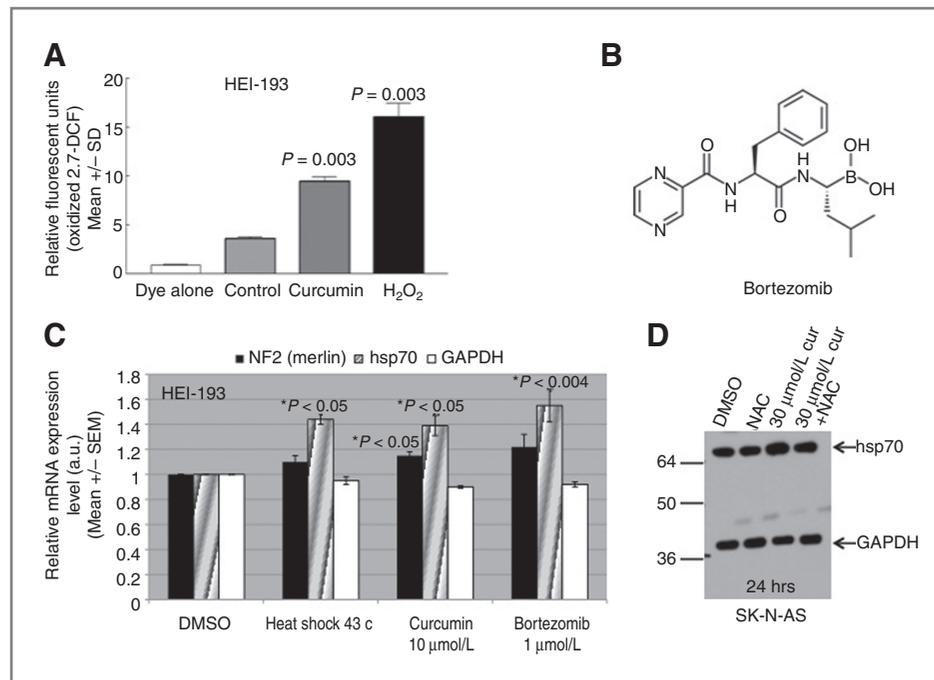


Figure 5. A, curcumin induces release of ROS in HEI-193 cells. Cells were treated with HBSS containing 20 μmol/L curcumin, 0.3%, H₂O₂, or HBSS with dye alone for 48 hours. Measurement of oxidized, fluorescent DCF, or ROS is represented by relative fluorescent units on the y-axis. Control contained curcumin only and no cell extract. The experiment was carried out in duplicate. *P* values were determined by the Fisher *t* test. B, the chemical structure of bortezomib. C, Hsp70 mRNA is induced by curcumin in schwannoma cells as shown by real-time PCR. HEI-193 cells were treated with 10 μmol/L curcumin for 6 hours. Heat shock and bortezomib (1 μmol/L) were used as positive inducers of hsp70 mRNA. mRNA from DMSO-treated cells were used as baseline and given the arbitrary value of 1.0. arbitrary unit (a.u.). D, SK-N-AS cells were pretreated with the antioxidant NAC (5 mmol/L) for 30 minutes, stimulated with 30 μmol/L curcumin for 24 hours, and cell lysates analyzed by Western blotting. NAC inhibited hsp70 upregulation by 30 μmol/L curcumin. Cur, curcumin.

HEI-193 Resistant cells seems to provide some protection from the combination of curcumin plus KNK437.

Hsp70 protein levels decreased following treatment with KNK437 alone (Fig. 6C, lanes 3 and 4 and 9 and 10) and increased after treatment with curcumin alone, as expected in both cell lines (Fig. 6C, lanes 2 and 8); however, they did not change significantly with the combination of curcumin and KNK437, compared with treatment with KNK437 alone (Fig. 6C, lanes 3 and 4 vs. lanes 5 and 6). In HEI-193 Resistant cells, the combination of curcumin and KNK437 did not inhibit hsp70 expression as much as KNK437 alone (Fig. 6C, compare lanes 9 and 10 with lanes 11 and 12). Higher concentrations of KNK437 may be required to see more of a decrease in hsp70 levels and a more synergistic effect when used in combination with curcumin in HEI-193 Resistant cells (Fig. 6C, compare lanes 5 and 6 with 11 and 12).

Discussion

NF2 is a debilitating disease that develops as the result of loss of the *NF2* tumor suppressor gene. Because *NF2* is a single-gene disorder, it follows that an appropriate pathway might be targeted to restore functional growth suppression to cells that lack wild-type *NF2*. Inhibition of

the growth of these tumors may greatly decrease the high morbidity associated with NF2. Various explanations could account for the function of merlin as a tumor suppressor, including contact inhibition of proliferation through activation of the Hippo tumor suppressor signaling pathway and promotion of the endocytosis of various signaling receptors (7, 45), but its exact role has not yet been delineated.

Curcumin induces apoptosis and G₂-M arrest in cancer cells by generating superoxides, increasing caspase-3, caspase-7, and PARP cleavage, downregulating Akt phosphorylation, and upregulating p53 phosphorylation (27, 28). Our data show that Akt phosphorylation is downregulated in schwannoma cells following curcumin treatment. Furthermore, Akt-mediated phosphorylation of merlin on ser315 and thr230 leads to ubiquitination and degradation of merlin, which may be inhibited by curcumin treatment (11). p-ERK 1/2 is also downregulated by curcumin in schwannoma cells, indicating a role for curcumin-induced modification of the MAPK pathway to modulate the proliferation of these cells. Our observations suggest that curcumin treatment may restore tumor suppressor activity to isoform III by causing dephosphorylation and folding of merlin to its active conformation (Fig. 2B). Our data also suggest that curcumin may activate merlin via activation of the merlin phosphatase

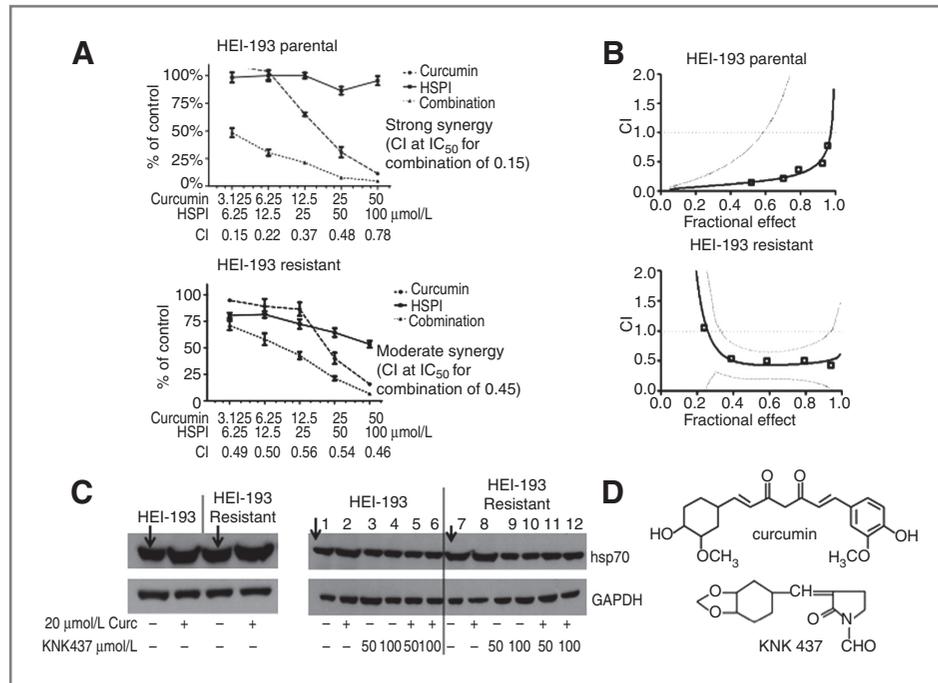


Figure 6. A, inhibition of HEI-193 cell growth by curcumin, KNK437, or the combination by MTT assay. Cells were treated with increasing doses of curcumin and KNK437 individually or in combination for 36 hours. Conversion of MTT is expressed as change in cell growth compared with untreated (control) cultures, which were assigned 100% growth. Results shown are the mean and SEM of 3 experiments. B, formal synergy analysis of HEI-193 parental schwannoma cell inhibition and HEI-193 Resistant cells with the combination of curcumin (range: 3.13–50 μmol/L) and KNK437 (range: 6.25–150 μmol/L). Synergy calculations were conducted as described in Materials and Methods. CIs of 1 or less represent increasing synergy, whereas values of 1 or more represent increasing antagonism. C, HEI-193 Resistant cells express higher levels of hsp70 protein than parental HEI-193 (arrows). HEI-193 parental or Resistant cells were treated with 20 μmol/L curcumin alone or curcumin plus 50 or 100 μmol/L KNK437, lysed, and subjected to Western blot analysis. D, the chemical structures of curcumin and KNK437.

MYPT1-pp1δ or prevent the degradation of merlin via downregulation of Akt activity.

However, if the activation of merlin by curcumin is solely dependent on the dephosphorylation of ser518, then merlin may not become activated in the majority of NF2 tumors, as these tumors frequently have truncating mutations resulting in a merlin protein with no intact ser518. The molecular profiling of mutations in the *NF2* gene could be used to match NF2 patients with a potential treatment. This strategy is already frequently used in many cancers. In addition, changes in the phosphorylation of ser315 and thr230 after curcumin treatment should be examined, as these residues lie more toward the N-terminus and may remain intact in a larger proportion of patients. Dephosphorylation of these residues also contributes to merlin activation; however, there are no commercially available antibodies for these residues at the present time. Also, effects other than the dephosphorylation of ser518 may be responsible for the growth inhibitory activity of the combination of curcumin and hsp70 inhibitors, as outlined in Results. These include downregulation of p-Akt and p-ERK 1/2 by curcumin and synergy with hsp70 inhibitors.

Merlin and hsp70 coimmunoprecipitated in both schwannoma and neuroblastoma cells (Fig. 4B). These experiments were repeated at least 4 times in 2 different

cell lines with consistent results—that is, that hsp70 and merlin interact. This is the first time to our knowledge that this interaction has been shown for endogenous hsp70 and merlin. Hsp70 is a chaperone protein that responds to the conformational changes of proteins. It assists in correct folding of proteins and escorts improperly folded proteins to the proteasome for degradation (46). It is plausible that dephosphorylation of merlin by curcumin, along with increased hsp70 levels, permits and promotes the interaction between hsp70 and merlin. Regardless, the high levels of hsp70 in our curcumin-induced cells may enhance resistance (14–16).

In our experiments, curcumin induced significant apoptosis and generated ROS in HEI-193 cells (Fig. 5A). Induction of ROS is associated with upregulation of hsp70, and it has been suggested that cell lines that generate high levels of hsp70, and perhaps other hsps, are more resistant to curcumin-induced apoptosis, whereas cell lines that cannot mount a significant stress response are destined to undergo apoptosis due to the induction of ROS (15). Interestingly, primary and non-transformed cell lines are resistant to curcumin-induced apoptosis and produce very low levels of ROS following curcumin treatment (15). We generated an HEI-193 Resistant cell line and showed that it expresses higher levels of hsp70 than the parental cell line and that the

combination of curcumin and KNK437 is only moderately synergistic in its antiproliferative effects in these cells whereas the combination is strongly synergistic in the parental cell line. Cycloheximide and actinomycin D both inhibit hsp70 induction by curcumin, suggesting that both transcription and translation are required for the induction of hsp70 in these cells (data not shown). We showed by real-time PCR and Western blotting that curcumin upregulates the expression of hsp70 in schwannoma cells.

Curcumin is an effective inhibitor of schwannoma cell growth; however, the induction of hsp70 in these cells could attenuate the response to curcumin and lead to escape from curcumin-induced apoptosis. The combination of curcumin plus KNK437 is synergistic in its ability to inhibit schwannoma cell growth. Although curcumin has limited bioavailability, a clinical study in pancreatic cancer showed biological and antitumor effects (47). Furthermore, liposome-encapsulated and bioavailable

absorbable forms of curcumin are under development (20, 26, 48–50). Taken together, these data provide a rationale for the combination of curcumin and hsp70 inhibitors in the treatment of NF2.

Disclosure of Potential Conflicts of Interest

R. Kurzrock has a patent on liposomal curcumin and holds royalty or milestone payments related to patent. No potential conflicts of interest were disclosed by the other authors.

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