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

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Abbreviations: NF2: Neurofibromatosis 2, hsp70: heat shock protein 70, MYPT1pp1δ: myosin phosphatase target subunit 1, DMSO: dimethyl sulfoxide.
Abstract

Neurofibromatosis (NF) type 2 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 anti-tumorigenic 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 down-regulated the phosphorylation of Akt and ERK 1/2. Curcumin also activated MYPT1pp1δ (a merlin phosphatase), which was associated with dephosphorylation of merlin on serine 518 (ser518), an event that results in the folding of merlin to its active conformation. Additionally curcumin induced apoptosis and generated reactive oxygen species (ROS) in HEI-193 cells. Consequently, heat shock protein (hsp)70 was up-regulated 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 a heat shock protein inhibitor synergistically suppressed schwannoma cell growth. Our results provide a rationale for combining curcumin and KNK437 in the treatment of NF2.
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 menigiomas, spinal schwannomas, and ependymomas. NF2 occurs in 1 of 33,000 live births (1, 2). The mainstay of treatment for NF2 has been surgery, though recently an anti-VEGF (vascular endothelial growth factor) 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 Knudson’s two-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 phosphatidylinositol-3 (PI3) kinase.

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 ser518, abrogating N-terminal/C-terminal domain interaction and subsequent folding of merlin to its “closed” or active conformation. Merlin’s phosphorylation at ser518 can be induced by activation of p21-activated kinase (Pak1) or protein kinase A via two distinct mechanisms (7-10). Protein kinase B (Akt) also directly binds to and phosphorylates merlin on threonine 230 (thr230) and serine 315 (ser315) (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 PIKE-L (PI3-kinase...
enhancer-long) 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 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 up-regulates heat shock protein 70 (hsp70) in several cell lines, including HEI-193 schwannoma cells, which may serve as a means of escape from curcumin-induced apoptosis (14-16). Additionally, the combination of curcumin and the heat shock protein 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 heat shock protein inhibitors.
Materials and Methods

Cell lines  HEI-193 schwannoma cells were obtained from the House Ear Institute (Los Angeles, CA in 2005) and contain a loss of heterozygosity 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 RT-PCR sequencing of this NF2 mutation (2008). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM-F12) supplemented with 15% fetal calf serum (FCS) (GIBCO/BRL, Carlsbad, CA). The SK-N-AS neuroblastoma cell line contains a wild-type NF2 gene (19) and was purchased from ATCC (Manassas, VA), and was 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 μM curcumin. All cells were maintained in a 37°C incubator with 5% CO₂.

Clonogenic assay  Cells were seeded at 1x 10⁵ cells per 60 mm dish (Nunc International, Naperville, IL) and grown overnight. Free or liposomal curcumin was added for five days. Cultures were washed, and fresh medium was added. Cultures were grown for three weeks, stained with crystal red in 0.5% methanol (Sigma-Aldrich, St. Louis, MO), and colony numbers were counted. Statistical significance was assessed by Fisher’s t-test.

Preparation of liposomal curcumin  Preparation of liposomes was carried out as described (20). Curcumin (Sigma) was dissolved in 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 FITC-conjugated Annexin V. Cells were seeded at 1x10⁶/2.0 mL/well of a six-well plate, grown to 70% confluence, treated with various
concentrations of curcumin for 24 hours, then harvested and stained with FITC-Annexin V and propidium iodide according to the manufacturer’s instructions (BD Pharmingen, San Diego, CA). Cells were analyzed by fluorescence-activated cell sorter (FACS) analysis (Becton Dickinson, Bedford, MA).

**Antibodies and Reagents** Antibodies used for Western blot included anti-N-terminal merlin (A19), anti-C-terminal merlin (aa 336-595), anti-p-Akt (ser473), anti-p-MYPT1 phosphatase (thr696), anti-MYPT1, anti-hsp70 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-p-merlin (ser518) (Rockland Immunochemicals for Research, Gilbertsville, PA), anti-ERK 1/2 and anti-p-ERK 1/2 (Promega, Madison, WI), and anti-Akt (Cell Signaling Technology, Danvers, MA). Anti-β-actin and anti-GAPDH serve as loading controls (Santa Cruz). Free curcumin (Sigma) and KNK437 (EMD Biosciences, San Diego CA) 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, then stimulated with 20% FCS for 30 minutes. Cells were washed with PBS and lysed in solubilization buffer. A modified Lowry protein assay was performed (Pierce Biotechnology, Rockford, IL), and equal amounts of protein were loaded onto 8% SDS-PAGE gels, then transferred to nitrocellulose membranes by electroblotting. Blots were blocked in 3.0% BSA, incubated with primary antibody (22°C for 2 hours), washed, and then incubated with an appropriate secondary antibody conjugated to horseradish peroxidase (BioRad Laboratories, Hercules, CA) and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). For immunoprecipitation experiments, 5x10^6 cells per lane were lysed with 1X NP-40 buffer.
Lysates were pre-cleared with an isotypic control (Santa Cruz Biotechnology) (4°C for two hours), then incubated with specific antibody (2-4 hours at 4°C), centrifuged, washed 4X with cell lysis buffer, resuspended in 2X sample buffer (BioRad), and boiled before loading onto SDS-PAGE gels.

**Real-time PCR** HEI-193 cells were stimulated with 10 μM curcumin for 6 hours. Heat shock (43°C for 4 hours) and bortezomib (1 μM) were used as positive inducers of hsp70 mRNA. Total RNA was extracted using the RNeasy mini-kit from Qiagen (Valencia, CA). Messenger RNA (mRNA) levels for hsp70 and merlin were measured using the TaqMan® RNA–to-CT™ 1-step kit from Applied Biosystems according to the manufacturer’s instructions (Foster City, CA). Primer and probe pairs for the reactions were as follows: hsp70- hs00271229_s1, GAPDH-GapDHhs99999905_m1, NF2- Hs00738978_m1. Reactions were run on an ABI 7500 real-time PCR machine using 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 (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay, according to the manufacturer’s instructions (Sigma). 5 X 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 performed as described (20, 21). Conversion of MTT was measured at 570 nm. Synergy calculations were performed using a fixed 1:2 ratio of curcumin to KNK437 utilizing the method of Chou and Talalay (22, 23). A combination index was calculated using the CalcuSyn software (BioSoft, Cambridge, UK) which utilizes a Monte Carlo method for
determining 95% confidence intervals of the fractional affect vs. combination index plot (dashed lines).

**Measurement of intracellular ROS generation: DCF Fluorescence**

HEI-193 cells were seeded in a 96-well plate (1 X 10^4/0.1 mL/ well) and allowed to adhere for 48 hours. Cells were washed in Hank’s buffered saline solution (HBSS) and incubated with 10 μM carboxy-H_2DCF-DA (2,7,-dichlorofluorescein) (Molecular Probes, Eugene, OR) in the loading medium (1% FCS in F12/DMEM) for 45 minutes. Cells were washed to remove carboxy-H_2DCF-DA and replaced with HBSS containing 20 μM curcumin, 0.3%, H_2O_2, or HBSS alone. DCF fluorescence was measured using 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 \textit{in vitro} and induces apoptosis. To determine if 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 five 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 demonstrated a dose-dependent decrease in colony formation (Fig. 1a). Treatment with 20 $\mu$M liposomal curcumin or 20 $\mu$M pegylated liposomal curcumin resulted in the most significant decrease in colony formation compared to control cells (*p<0.001 by Fisher’s t-test) (Fig. 1a). Curcumin also inhibited the proliferation of HEI-193 cells by MTT assay (data not shown). Because all three forms of curcumin inhibited HEI-193 cells in a comparable manner at concentrations greater than 10 $\mu$M, 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 if HEI-193 cells undergo apoptosis following curcumin treatment, cell-surface phosphatidylserine was measured using FITC-conjugated Annexin V as an indicator of apoptosis. Sixty-seven and 97.9% of HEI-193 cells underwent apoptosis with free curcumin concentrations of 12.5 and 25 $\mu$M, 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 ERK 1/2 in HEI-193 cells.

Curcumin down-regulates several protein kinases and transcription factors necessary for survival pathways, including Akt and NF-κB (27, 30, 31). Akt is a pro-survival serine/threonine kinase that acts in a PI3 kinase-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 μM of liposomal curcumin by 24 hours. Levels of total Akt protein remained unchanged (data not shown and Fig. 2a).

The effect of curcumin on ERK 1/2 kinase, an important component of the MAPK pathways, was also examined. Total levels of ERK1/2 kinase remained fairly stable, whereas 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 μM (data not shown).

Curcumin dephosphorylates (activates) merlin via activation of MYPT1-pp1δ, 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 panel), indicating that it precedes the dephosphorylation of ERK and Akt. These experiments were repeated at least three times with doses of curcumin ranging from 5-30 μM. Time points included 8, 24, 48, and 72 hours. Densitometric analysis of three
representative Western blots shows a significant decrease in p–merlin starting at 10 μM 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 is a truncated protein, identical to the normally occurring merlin protein designated isoform III (approximately 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) (19), and predominantly express isoform I (approximately 70 kD) (Figure 2b. and d.).

To study the mechanism of curcumin-induced merlin dephosphorylation (activation), we examined the phosphorylation status of the merlin phosphatase, MYPT1-pp1δ, which is known to directly dephosphorylate (activate) merlin (7, 36, 37). MYPT1-pp1δ 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-pp1δ by 30 minutes (Fig. 3a.). One, two, and four hours post-treatment 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 p-MYPT1 seen with doses of curcumin as low as 10 μM. 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-pp1δ.
We investigated possible upstream regulators of MYPT1 to see if we could determine which phosphatase was responsible for activating (dephosphorylating) MYPT1. Mitogen-activated protein (MAP) kinase phosphatase 5 (MKP5), which is known to be up-regulated 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 up-regulates hsp70, which binds to merlin in HEI-193 and SK-N-AS cells. Curcumin up-regulates stress proteins, including hsp70, in vivo and in vitro (14-16). Hsp70 is up-regulated by curcumin in both HEI-193 and SK-N-AS cells (Fig. 4a. and b., panel A). Immunoprecipitation with anti-hsp70 followed by Western blot with anti-C-terminal merlin reveals the characteristic merlin bands (see Fig. 2. and Fig. 4b., panel A), and demonstrates that the two proteins are co-immunoprecipitated in both cell lines (merlin expression is lower in the NF2 cell line as expected). Curcumin appears to increase the amount of merlin that is co-immunoprecipitated with the anti-hsp70 antibody (Fig. 4b., panel A, bottom and panel B). Reversing the antibodies used for IP and Western blot and using an anti-N-terminal merlin antibody yielded the same result (Fig. 4b., panel B). 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 paper demonstrating 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 up-regulation 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 up-regulates hsp70 mRNA in chronic myelogenous leukemia (CML) cells (42). We investigated whether curcumin up-regulates 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 up-regulated by heat shock (p<0.05) and bortezomib (p<0.01) as expected, and also by 10 μM curcumin (p<0.05), while GAPDH mRNA levels remained fairly constant (not significant using Fisher’s 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 if ROS were responsible for the induction of hsp70, cells were pretreated with the antioxidant, NAC for 30 minutes then treated with curcumin (10 or 20 μM) 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 μM 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. Since 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 curcumin’s anti-proliferative effects. Therefore, we examined the ability of a heat shock protein (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) (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 to 50 μM) as expected based on our earlier observations (Fig. 1.). Treatment with KNK437 alone did not inhibit HEI-193 cell growth (Fig. 6a., top panel), whereas the combination of KNK437 and curcumin was strongly synergistic in all combinations tested (Fig. 6a., top and 6b., panel A, CI at IC50 = 0.15).

Since hsp70 is up-regulated 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 panel, arrows, and lanes 1&7). Baseline hsp70 protein levels increased by 16% in the resistant cell line compared to HEI-193 parental cells as determined by densitometry. Curcumin up-regulated hsp70 in both the parental and resistant cell lines (Fig. 6c., left panel), 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 μM) of curcumin alone to inhibit the proliferation of HEI-193 Resistant compared to 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., panel B, CI at IC50 = 0.45), hence, the higher level of hsp70 expressed in HEI-193 Resistant cells appears to provide some protection from the combination of curcumin plus KNK437.

Hsp70 protein levels decreased following treatment with KNK437 alone (Fig. 6c., lanes 3&4 and 9&10) and increased after treatment with curcumin alone as expected in both cell lines (Fig. 6c., lanes 2&8), however, they did not change significantly with the
combination of curcumin and KNK437, compared to treatment with KNK437 alone (Fig. 6c., lanes 3&4 versus lanes 5&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&10 with lanes 11&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&6 with 11&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 merlin’s function 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, down-regulating Akt phosphorylation, and up-regulating p53 phosphorylation (27, 28). Our data show that Akt phosphorylation is down-regulated 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 down-regulated by curcumin in schwannoma cells, indicating a role for curcumin-induced modification of the MAP kinase 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, MYPT1-pp1δ, or prevent merlin’s degradation via down-regulation 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 since 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. Additionally, changes in the phosphorylation of ser315 and thr230 after curcumin treatment should be examined since 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 the results section. These include down-regulation of p-Akt and p-ERK1/2 by curcumin, and synergy with hsp70 inhibitors.

Merlin and hsp70 co-immuno-precipitated in both schwannoma and neuroblastoma cells (Fig. 4b.). These experiments were repeated at least 4 times in two 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 up-regulation of hsp70, and it has been suggested that cell lines that generate high levels of hsp70, and perhaps other heat shock proteins, 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 a curcumin-resistant cell line (HEI-193 Resistant) and demonstrated 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 anti-proliferative effects in these cells, while the combination is strongly synergistic in the parental cell line. Cycloheximide and Actinomycin D each 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 demonstrated by real-time PCR and Western blot that curcumin up-regulates 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 demonstrated biologic and anti-tumor 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.
References


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Figure legends

**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 five days and colonies were counted (n=5). (b) HEI-193 cells were treated for 24 hours with media alone (control), 12.5 μM, or 25 μM free curcumin. Apoptosis was assessed by staining with FITC-conjugated Annexin V and propidium iodide.

Abbreviations: Empty = empty vehicle; lip cur = liposomal curcumin; P-L curc = pegylated liposomal curcumin.

**Figure 2.** (a) Concentration-dependent inhibition of p-Akt and p-ERK expression in HEI-193 cells treated with liposomal or free curcumin for 72 hours. Protein expression was examined by Western blot. (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 ~60 kD in HEI-193; isoform I runs at ~70 kD in SK-N-AS. (c) Densitometry of p-merlin bands from three experiments (HEI-193). Only one experiment was available for analysis at 5 μM. S.E.M. = standard error of the mean. Student’s t-test was used for statistical analysis. (d) Levels of total merlin remain fairly constant in both cell lines (48-hour curcumin treatment).

**Figure 3.** (a) Dephosphorylation (activation) of MYPT1-pp1δ 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 μM) for 30 minutes, then stimulated for 30 minutes with 20% FCS. Changes in the phosphorylation state of
MYPT1-pp1δ (thr696) were examined by Western blot analysis. (b) Dose response showing dephosphorylation of MYPT 1-pp1δ phosphatase in HEI-193 cells treated with empty liposome or liposomal curcumin for 72 hours. The dephosphorylation of MYPT1-pp1δ is dose-dependent and associated with concomitant dephosphorylation (activation) of merlin (Fig. 2b.). For total MYPT1 and GAPDH levels (right hand panel), 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 determined by Western blot. (b) Hsp70 and merlin interact endogenously in HEI-193 schwannoma and SK-N-AS neuroblastoma cells.

Panel A: Immuno-precipitation (IP) with anti-hsp70 antibody followed by Western blot (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 μM liposomal curcumin (+) for 72 hours. Cells were then exposed to 20% FCS for 30 minutes and harvested for IP followed by WB. Hsp70 protein expression increased in both cell lines after curcumin treatment (top). Merlin is detected in both cell lines after immuno-precipitation 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.
Panel B: IP/WB in HEI-193 cells using an anti-N-terminal merlin antibody for IP followed by WB with anti-hsp70 antibody.

**Figure 5.** (a) Curcumin induces release of ROS in HEI-193 cells. Cells were treated with HBSS containing 20 μM curcumin, 0.3%, H$_2$O$_2$, or HBSS with dye alone for 48 hours. Measurement of oxidized, fluorescent 2′,7′-dichlorofluorescein (DCF) or ROS is represented by relative fluorescent units on the y axis. “Control” contains curcumin only and no cell extract. The experiment was performed in duplicate. P values were determined using Fisher’s t test. S.D. = standard deviation. (b) The chemical structure of bortezomib. (c) Hsp70 mRNA is induced by curcumin in schwannoma cells as demonstrated by real-time PCR. HEI-193 cells were treated with 10 μM curcumin for 6 hours. Heat shock and bortezomib (1μM) 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. Abbreviation: a.u. = arbitrary units. (d) SK-N-AS cells were pre-treated with the anti-oxidant NAC (5mM) for 30 minutes, stimulated with 30 μM curcumin for 24 hours, and cell lysates analyzed by Western blot. NAC inhibited hsp70 up-regulation by 30 μM curcumin.

**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 to untreated (control) cultures, which were assigned 100% growth. Results shown are the mean and S.E. M. of three 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 μM) and KNK437 (range 6.25-150 μM). Synergy calculations were performed as described in Materials and Methods. Combination indices ≤1 represent increasing synergy, while values ≥1 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 μM curcumin alone or curcumin plus 50 or 100 μM KNK437, lysed, and subjected to Western blot analysis. (d) The chemical structures of curcumin and KNK437.
Figure 2.

a. HEI-193

<table>
<thead>
<tr>
<th>p-Akt (ser473)</th>
<th>Akt</th>
<th>(\beta)-actin</th>
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<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>20 (\mu)M lip curc</td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>p-ERK 1/2</th>
<th>ERK 1/2</th>
<th>(\beta)-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>10 (\mu)M free curc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b. HEI-193

<table>
<thead>
<tr>
<th>p-merlin</th>
<th>merlin</th>
<th>GAPDH</th>
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</thead>
<tbody>
<tr>
<td>64 kD</td>
<td>64 kD</td>
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</tr>
</tbody>
</table>

V 10 20 30 \(\mu\)M curc 8 hrs

<table>
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<tr>
<th>(\beta)-actin</th>
<th>GAPDH</th>
</tr>
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<tbody>
<tr>
<td>50 kD</td>
<td>37 kD</td>
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V 10 20 30 \(\mu\)M curc 48 hrs

HEI-193 SK-N-AS

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<thead>
<tr>
<th>p-merlin (ser518)</th>
<th>(\beta)-actin</th>
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<td>64 kD</td>
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<table>
<thead>
<tr>
<th>(\beta)-actin</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 kD</td>
<td>37 kD</td>
</tr>
<tr>
<td>37 kD</td>
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</tbody>
</table>

V 10 20 30 \(\mu\)M curc 48 hrs

c. HEI-193

<table>
<thead>
<tr>
<th>DMSO</th>
<th>5 (\mu)M</th>
<th>10 (\mu)M</th>
<th>20 (\mu)M</th>
<th>30 (\mu)M</th>
<th>curc</th>
</tr>
</thead>
<tbody>
<tr>
<td>120%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Mean (\pm) S.E.M.</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

d. HEI-193 SK-N-AS

<table>
<thead>
<tr>
<th>merlin</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>64 kD</td>
<td></td>
</tr>
</tbody>
</table>

V 10 20 30 \(\mu\)M curc 48 hrs

merlin 64 kD

GAPDH 37 kD

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Figure 3.

(a) Empty vehicle vs. liposomal curcumin.

- **p-MYPT1** pp1δ (thr696)
- GAPDH
- Total MYPT1
- GAPDH

(b) Empty vehicle vs. liposomal curcumin.

- **p-MYPT1** (thr696)
- Total MYPT1
- GAPDH

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Figure 4.

a. HEI-193

hsp 70

GAPDH

0 5 20 30 μM free curc

64 kD

b.

A. IP: anti-hsp 70

<table>
<thead>
<tr>
<th>HEI-193</th>
<th>SK-N-AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>curcumin</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

75 kD

anti-hsp 70

B. IP: anti-N-terminal merlin

<table>
<thead>
<tr>
<th>HEI-193</th>
</tr>
</thead>
<tbody>
<tr>
<td>curcumin</td>
</tr>
</tbody>
</table>

75 kD

anti-hsp 70

64 kD

merlin

Isoform III

beads
Figure 6.

(a) HEI-193 parental

Strong synergy (CI at IC50 for combination of 0.15)

Curcumin HSP70 Combination

% of control

Curcumin HSP70 Combination

Combination index

0.15 0.22 0.37 0.48 0.78

(b) HEI-193 parental

Combination index

Fractional Effect

0.0 0.2 0.4 0.6 0.8 1.0

(c) HEI-193 Resistant

Moderate synergy (CI at IC50 for combination of 0.45)

Curcumin HSP70 Combination

% of control

Curcumin HSP70 Combination

Combination index

0.49 0.50 0.56 0.64 0.46

(d) HEI-193 Resistant

Curcumin hsp70 GAPDH

20µM Curc KN437 µM
Molecular Cancer Therapeutics

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

Laura S Angelo, Ji Y Wu, Feng Meng, et al.

Mol Cancer Ther Published OnlineFirst September 8, 2011.

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