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Curcumin Inhibits the Sonic Hedgehog Signaling Pathway and Triggers Apoptosis in Medulloblastoma Cells

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Medulloblastoma is an aggressive primary brain tumor that arises in the cerebellum of children and young adults. The Sonic Hedgehog (Shh) signaling pathway that plays important roles in the pathology of this aggressive disease is a promising therapeutic target. In the present report we have shown that curcumin has cytotoxic effects on medulloblastoma cells. Curcumin suppressed also cell proliferation and triggered cell-cycle arrest at G_2/M phase. Moreover, curcumin inhibited the Shh–Gli1 signaling pathway by downregulating the Shh protein and its most important downstream targets GLI1 and PTCH1. Furthermore, curcumin reduced the levels of β-catenin, the activate/phosphorylated form of Akt and NF-κB, which led to downregulating the three common key effectors, namely C-*myc*, N-*myc*, and Cyclin D1. Consequently, apoptosis was triggered by curcumin through the mitochondrial pathway via downregulation of Bcl-2, a downstream anti-apoptotic effector of the Shh signaling. Importantly, the resistant cells that exhibited no decrease in the levels of Shh and Bcl-2, were sensitized to curcumin by the addition of the Shh antogonist, cyclopamine. Furthermore, we have shown that curcumin enhances the killing efficiency of nontoxic doses of cisplatin and γ-rays. In addition, we present clear evidence that piperine, an enhancer of curcumin bioavailability in humans, potentiates the apoptotic effect of curcumin against medulloblastoma cells. This effect was mediated through strong downregulation of Bcl-2. These results indicate that curcumin, a natural nontoxic compound, represents great promise as Shh-targeted therapy for medulloblastomas.

Key words: chemosensitization; Bcl-2; piperine; radiosensitization

INTRODUCTION

Medulloblastoma, aggressive tumor of the cerebellum, is the most common malignant brain tumor in children. It represents ~20% of pediatric intracranial neoplasms [1]. Medulloblastoma, like all the other types of cancer, results from alterations in the equilibrium between cell growth and cell death, which drives the proliferation of cerebellar granule neuronal precursors (CGNP) [2]. This equilibrium is under the control of different metabolic pathways. The most important medulloblastoma-related carcinogenesis pathways are Sonic Hedgehog (Shh), WNT/β-catenin, and Akt/nuclear factor-κB (NF-κB) [3]. The Shh signaling pathway plays important roles in the proliferation of the neuronal precursor of the cerebellum and in the genesis of medulloblastoma [4,5]. Shh induces the transcription of two important transcription factors Glioma-associated oncogene homolog 1 (*GLI1*) and patched homolog 1 (*PTCH1*) in many cell types [6]. Furthermore, Shh induces the expression of different important oncoproteins, including the cell-cycle proteins N-*myc*, C-*myc*, and Cyclin D1 [1,7]. Like Shh, N-*myc* activity is necessary for both normal and neoplastic cerebellar growth [8–10]. Amplification of N-*myc* and/or C-*myc* occurs in 5–10% of medulloblastoma cases [11]. The *myc* family of proteins acts as transcription regulators that play key roles in cell-cycle progression, transformation, and angiogenesis [12]. Amplifications as well as high transcriptional levels of MYCC are associated with an unfavorable survival outcome of

Abbreviations: Shh, Sonic Hedgehog; NF-κB, nuclear factor-κB; GLI1, Glioma-associated oncogene homolog 1; PTCH1, patched homolog 1; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PI, propidium iodide; IR, ionizing radiation.

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medulloblastoma patients [13,14]. The other important mediator of the Shh pathway is the anti-apoptosis Bcl-2 protein, which is frequently overexpressed in medulloblastomas [15,16]. The upregulation of Bcl-2 and the consequent inhibition of apoptosis in Shh-dependent medulloblastoma is Akt/NF-κB-related [16]. In fact several lines of evidence suggest that the Atk/NF-κB pathway synergizes with Shh to promote aggressive medulloblastoma [16,17].

Currently, medulloblastoma patients are best treated with surgery, craniospinal radiotherapy and chemotherapy [18–21]. However, these aggressive regimens are associated with serious long-term side effects [1,20]. Furthermore, surgery cannot be achieved in all cases. Therefore, several new therapeutic solutions are currently under investigation. These include the replacement of combined radio- and chemotherapy by potent and more specific chemotherapy [3,22]. Thereby, there is continuous demand for the identification and the development of nontoxic and efficient anti-medulloblastoma agents. In line with this, several dietary constituents have been studied and have in fact shown great preventive and anti-cancer properties, without the adverse side effects of the currently used chemotherapeutic agents [23,24]. Curcumin (diferuloylmethane) is one of the most widely characterized phytochemicals. It is a yellow natural product extracted from the rhizoma of *curcuma longa* (turmeric) that has been used as food spice and colorant. Curcumin is a polyphenolic, nontoxic, pharmacologically active substance that has anti-oxidant, anti-inflammatory, and antiseptic activities. Thereby, it has been used for centuries as therapeutic agent against various diseases [25]. Curcumin has various anti-cancer characteristics that include the inhibition of cell proliferation and angiogenesis as well as the induction of cell death in tumor cells [26]. However, curcumin has only marginal effect on various normal cell types [27]. Furthermore, it has been recently shown that curcumin induces immunorestoration in tumor-bearing animals [28], indicating that the inclusion of this natural product in therapeutic regimens against cancer should be beneficial for great proportion of cancer patients. In fact, curcumin has already been the subject of several clinical trials for potential use as chemotherapeutic agent [25].

In this study we investigated the effect of curcumin on medulloblastoma cells, and we have shown that this natural nontoxic agent inhibits the Shh–Gli1 signaling and has great anti-medulloblastoma effects.

MATERIALS AND METHODS

Cell Lines, Chemicals, and Cell Culture

DAOY cell line was obtained form ATCC, while the other medulloblastoma cells are primary cells cultured in the laboratory. Cisplatin (*cis* diammine-dicloroplatinum II), Curcumin, and piperine (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) and used at the indicated concentrations.

For the development of primary cell cultures, a piece of the medulloblastoma sections, obtained from consented patients, was first placed in a drop of complete media (DMEM:F12, 50:50 medium supplemented with 15% NBCS and 1% antibiotics) before being minced with scalpel blades to very small pieces (<1 mm in size), and then further pipetted to obtain maximal cell dispersion. Subsequently, the pieces were transferred to sterile 60 mm tissue culture plates and then trapped under a sterile glass cover slip. Complete medium was added to cover the minced tissue. Medium was changed weekly and when confluent grown cells were split using 0.25% trypsin. Cell line identity was confirmed prior to use by karyotyping and immunostaining for neuronal cell markers NF-M, HuD, and nestin (data not shown). Cells were used at low passages (4–8).

Cytotoxicity Assay

Cells were seeded into 96-well plates at $0.5–1.10^4$/well and incubated overnight. The medium was replaced with fresh one containing the desired concentrations of the drugs. After 20 h, 10 μl of the WST-1 reagent was added to each well and the plates were incubated for 4 h at 37°C. The amount of formazan was quantified using enzyme-linked immunosorbent assay (ELISA) reader at 450 nm of absorbance.

Cell Proliferation Assay

Cells were seeded into 96-well plates at $0.5–1 \times 10^4$/well and incubated overnight. The medium was replaced with fresh one containing 40 μM of curcumin and incubated for different time intervals (0, 24, 48, and 72 h). WST-1 reagent was added to each well. Except for the 0 h, the plates were then incubated for 4 h at 37°C. The amount of formazan was quantified using ELISA reader at 450 nm of absorbance.

Irradiation

Cells were grown to 60% confluency, and then were exposed to a Cobalt source used at a dose rate of 0.60 Gy/min.

Cellular Lysate Preparation

Cells were washed with phosphate-buffered saline (PBS) and then scraped in RIPA buffer (150 mM of NaCl, 1 mM of EDTA, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 50 mM of Tris–HCl (pH 7.5)), supplemented with protease inhibitors. Lysates were homogenized and then centrifuged at 14 000 rpm at 4°C for 15 min in an Eppendorf microcentrifuge. The supernatant was removed, aliquoted, and stored at −80°C.

Immunoblotting

SDS–PAGE was performed using 12% separating minigels and equal amounts of protein extract (30 μg) were loaded. After protein migration and transfer onto polyvinylidene difluroide membrane (PVDF), the membrane was incubated overnight with the appropriate antibodies:

NF-κB (F-6), Shh (H-160), β-catenin (9F2), N-*myc* (3C 165), Cyclin D1 (HD11), Survivin (C-19), Atk (b-1), p-Akt (104A282), Bcl-2 (C-2), Bax (B-9), pro-caspase3 (H-277), pro-caspase9 (F-7), PARP (H-250), NF-M (1A2), HuD (E1), Nestin (10C2), GAPDH (FL-335) and β-actin (C-11) and C-*myc* (C-19) from Santa Cruz, CA.

RNA Extraction, cDNA Synthesis, and RT-PCR Assay

Total RNA was extracted using the Tri® Reagent (Sigma) and the yield was quantitated spetrophotometrically. Following the manufacturer's instructions, single stranded cDNA was synthesized using 200 ng of total RNA, the MMLV reverse transcriptase and the oligo dT_{18} (Roche, San Francisco, CA). The cDNA was amplified for 40 cycles under the following conditions: melting temperature (95°C) for 50 s, annealing temperature (54°C) for 50 s, and extension temperature (72°C) for 1 min. The PCR products were separated by electrophoresis on a 2% agarose gel at 80 V for an hour. The sequences of the primers were as follow:

GLI1, Fw: ACC CGG GGT CTC AAA CTG; Rv: GGC TGA CAG TAT AGG CAG AGC
PTCH1, Fw: GAC GCC GCC TTC GCT CTG; Rv: GCC CAC AAC CAA GAA CTT GCC
β-actin, Fw: CCCAGCACAATGAAGATCAAGATCAT; Rv: ATCTGCTGGAAGGTGGACAGCGA

Quantification of Protein and RNA Expression Levels

The expression levels of RNAs and proteins were measured using the densitometer (BIO-RAD, Hercules, CA; GS-800 Calibrated Densitometer). Films were scanned and protein signal intensity of each band was determined. Next, dividing the obtained value of each band by the values of the corresponding internal control allowed the correction of the loading differences. The fold of induction was determined by dividing the corrected values that corresponded to the treated samples by that of the nontreated one (time 0).

Annexin V/PI and Flow Cytometry

Confluent cells were either treated with DMSO and used as control or challenged with different agents, whereupon cells were incubated in DMEM/F12 medium with supplements. Detached and adherent cells were harvested 72 h later, centrifuged and resuspended in 1 ml of PBS. Cells were then stained by propidium iodide (PI) and Alexa Fluor 488 Annexin V, using Vibrant Apoptosis Assay kit #2 (Molecular probe, Eugene, OR). Stained cells were analyzed by flow cytometry. The percentage of cells was determined by the FACScadibur apparatus and the Cell Quest Pro software from Becton Dickinson (San Jose, CA). For each cell culture three independent experiments were performed using 10^4 cells in each experiment.

Cell-Cycle and Cell Death Analysis by Flow Cytometry

Cells were treated with DMSO or curcumin, and then harvested and resuspended in 1 ml of PBS before being fixed by drop wise addition of 3 ml of 100% methanol. Fixed cells were centrifuged, resuspended in 50 μl of RNase (1 mg/ml) and incubated for 30 min at room temperature, followed by addition of 1 ml of 0.1 mg/ml of PI. Cells were analyzed for DNA content by flow cytometry (Becton Dickinson). The percentage of cells in various cell-cycle phases was determined by using Cell Quest software (Becton Dickinson).

RESULTS

Curcumin Has Cytotoxic and Anti-Proliferative Effects on Medulloblastoma Cells

We investigated the cytotoxic effect of curcumin on different medulloblastoma primary cells and the DAOY cell line using the WST-1 assay. Cells were seeded in triplicates into microtiter plates and treated with increasing concentrations of curcumin for 24 h, and then the cytotoxic effect was measured. For each cell culture, at least three independent experiments were carried out. Figure 1A shows dose-dependent effect of curcumin on four different medulloblastoma cells (MED-1, MED-4, MED-5, and DAOY). While MED-1 cells exhibited high resistance to curcumin, the three other cells showed different sensitivity to the agent. The LC_{50} (the concentration that leads to 50% survival) were 20, 25, and 28 μM for the DAOY, MED-5, and MED-4, respectively (Figure 1A). This shows that curcumin is cytotoxic against most medulloblastoma cell cultures and that the DAOY cell line is the most sensitive.

Subsequently, the MED-5 cells were treated with 40 μM curcumin for different periods of time (0, 24, 48, and 72 h) and curcumin-dependent cell killing was assessed using flow cytometry. Figure 1B shows that cell death increased in a time-dependent manner, reaching the maximum proportion (58%) after 72 h of treatment. This confirms the cytotoxic effect of curcumin against medulloblastoma cells and shows that its effect is time dependent.

Next, we investigated the effect of curcumin on medulloblastoma cell proliferation using the WST-1 cell proliferation assay. Cells were seeded into microtiter plates and were either mock treated or challenged with 40 μM of curcumin for different periods of time. Figure 1C shows that while the number of nontreated cells increased in

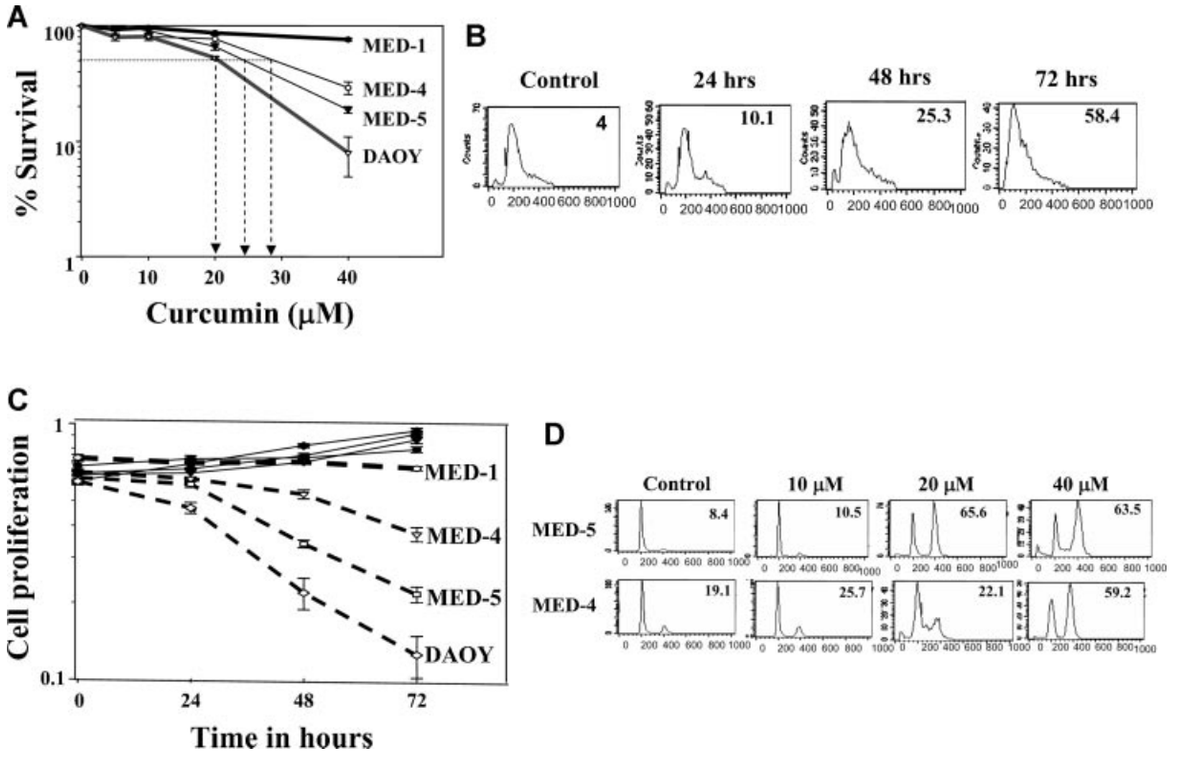

Figure 1. Cytotoxic and anti-proliferative effects of curcumin on medulloblastoma cells. (A) Exponentially growing cells were cultured in 96-well plates and treated with the indicated curcumin concentrations for 72 h. Cell death was analyzed using the WST-1 assay. The arrows indicate the LC_{50} for each cell line and the error bars represent standard deviations. (B) MED-5 cells were treated with 40 μM of curcumin and reincubated for the indicated periods of time. Cell death was assessed by flow cytometry. The numbers in the boxes represent the proportion of cell death. (C) Cells were cultured in 96 wells plates and challenged with curcumin (40 μM) for the indicated periods of time, and then cell proliferation was assessed by the WST-1 assay. Dashed lines: treated cells, continues lines, nontreated cells. (D) Cells were either mock treated or challenged with the indicated concentrations of curcumin for 72 h. The cell cycle status was analyzed by flow cytometry. The numbers represent the proportions of G_2/M cells.

a time-dependent manner, the number of curcumin-treated cells decreased gradually reflecting curcumin-dependent inhibition of cell proliferation. Like for apoptosis the effect was more pronounced on DAOY cells, while was not significant against the MED-1 cell culture and intermediate for MED-4 and MED-5 (Figure 1C).

After showing the effect of curcumin on cell proliferation we sought to investigate its effect on the cell cycle of the MED-4 and MED-5 cell cultures that were treated with different concentrations for 3 days. Figure 1D shows a dose-dependent accumulation of cells at the G_2/M phase of the cell cycle reaching after 3 days of treatment 59% and 63% in MED-4 and MED-5, respectively. Similar proportion of G_2/M cells was reached at only 20 μM of curcumin in MED-5 cells (Figure 1D). This shows that curcumin triggers G_2/M cell-cycle arrest in medulloblastoma cells, which led to growth inhibition.

Curcumin Inhibits the Sonic Hedgehog Signaling Pathway in Medulloblastoma Cells

The Shh signaling pathway is a major regulator of the equilibrium between cell proliferation and cell death, and therefore is implicated in the development of the cerebellar tumor medulloblastoma [1,29]. Thereby, we sought to study the effect of curcumin on this important medulloblastoma-related pathway. To this end, MED-5 cells were treated with 40 μM of curcumin for different periods of time (0–24 h), and then cellular lysates were prepared and used for Western blot analysis using specific antibodies and GAPDH as internal control. Figure 2A shows that the expression level of the Shh protein was downregulated 12.5-fold after 8 h of treatment, reaching a level as low as 8% of the basal level. To further appreciate the curcumin effect on the oncogenic Shh signaling pathway, we studied its effect on the direct downstream targets of the Shh protein, namely *PTCH1* and *GLI1*. Therefore, MED-5 cells were either sham treated or challenged with 40 μM of curcumin for 8 h. Total RNA was purified and the levels of the *PTCH1* and the *GLI1* mRNAs were assessed by RT-PCR. Figure 2B shows that the level of both genes decreased significantly in response to curcumin treatment. Indeed, the level of *GLI1* decreased more than five times and the *PTCH1* level decreased more than 2-fold

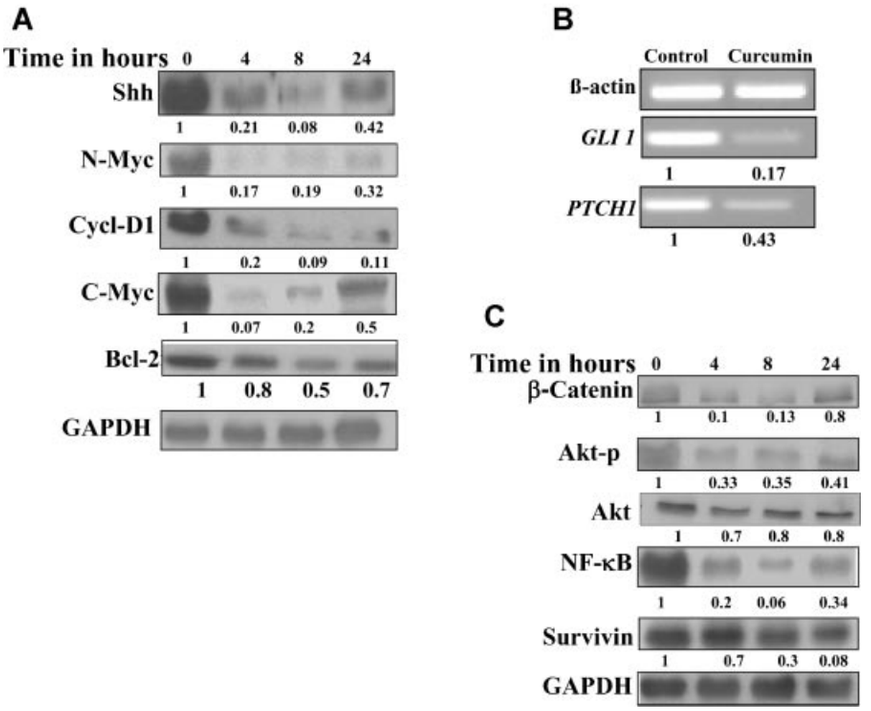

Figure 2. Curcumin inhibits the Shh–Gli1 signaling pathway. MED-5 cells were not treated or challenged with curcumin (40 μM) and then reincubated for the indicated periods of time. (A and C) Whole cell extracts were prepared and used for immunoblot analysis using the indicated antibodies. (B) Total RNA was extracted from these cells, and the cDNA was synthesized and used to evaluate the mRNA expression for the indicated genes using the RT-PCR technique. β-Actin was used as internal control. The numbers under the bands represent the corresponding expression levels as compared to the basal level (time 0, control).

as compared to the control nontreated cells (Figure 2B).

To further elucidate the effect of curcumin on the Shh signaling pathway, we studied the effect of curcumin on the main Shh and Gli1 downstream targets, N-*myc*, C-*myc*, and Cyclin D1. Cyclin D1 and N-*myc* are important mediators of Shh-induced proliferation and carcinogenesis [30]. Importantly, curcumin led to a sharp decrease in the expression of these three proteins during the first 4 h of treatment (Figure 2A). The levels of C-*myc*, Cyclin D1, and N-*myc* were reduced 14-, 11-, and 6-fold, respectively (Figure 2A). Since the anti-apoptosis Bcl2 protein is also an important mediator of Shh in medulloblastoma [15] and its transcriptionally upregulated by Shh through the Gli1 transcription factor [31], we sought to investigate the effect of curcumin on this protein. Figure 2A shows 50% decrease in the level of Bcl-2 after 8 h of treatment. These results present the first evidence that curcumin inhibits the Shh–Gli1 signaling in medulloblastoma cells.

Next, we investigated whether the inhibition of the Shh signaling pathway affected the levels of β-catenin and NF-κB, two major transcription factors that are implicated in medulloblastoma carcinogenesis and interact with Shh [16,17,32]. Interestingly, 8 h of curcumin treatment led to a sharp decrease in the level of the NF-κB protein and the phosphorylated/active form of the Akt kinase, reaching levels that represents only 6% and 35% of the basal level, respectively (Figure 2C). Likewise, the level of the transcription factor β-catenin was strongly downregulated in response to curcumin reaching a level 10-fold lower than the corresponding basal level, after only 4 h of treatment (Figure 2C).

Among the cancer markers that are under the control of the NF-κB pathway there is the anti-apoptosis protein survivin. Figure 2C shows that curcumin treatment led to the downregulation of survivin as well. The reduction in the level of this protein was time dependent in medulloblastoma cells reaching a level 12-fold lower after 24 h of treatment (Figure 3C).

It is noteworthy that the effect of curcumin on these proteins and pathways was also observed in the DAOY cell line (data not shown), indicating that this effect could be considered as general to most medulloblastoma cells.

Together, these results indicate that curcumin has great inhibitory effect on the Shh signaling pathway and its downstream medulloblastoma-driven effectors.

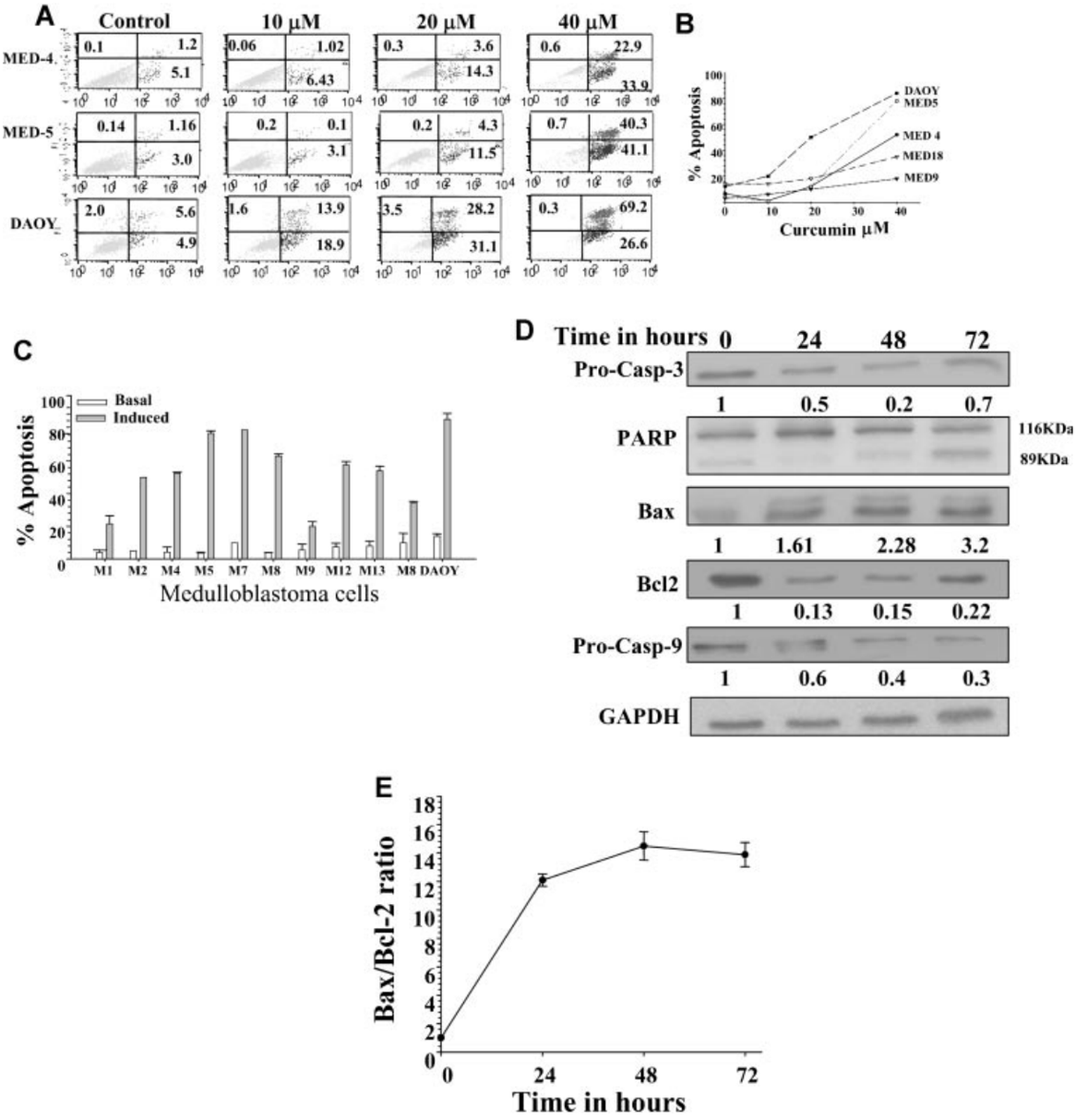

Figure 3. Curcumin triggers apoptosis through the internal pathway in medulloblastoma cells. Sub-confluent cells were either mock treated or challenged with the indicated concentrations of curcumin for 72 h and then cell death was analyzed using the Annexin V/PI flow cytometry assay. (A) Charts, with the numbers indicating the proportion of apoptotic and necrotic cells. (B) Graph representing the dose-dependent apoptosis in the indicated cells. (C) Histogram, cells were treated with 40 μM curcumin for 72 h. M for MED. (D) The MED-5 cells were treated with 40 μM curcumin for the indicated periods of time, and then cell extracts were prepared and used for immunoblotting analysis using the indicated antibodies. the numbers under the bands represent the corresponding expression levels as compared to the basal level (time 0). (E) Graph showing the Bax/bcl-2 ratio. Error bars represent standard deviations of three different experiments.

Curcumin Triggers Apoptosis Through the Mitochondrial Pathway in Medulloblastoma Cells

Since the inhibition of the Shh-dependent signaling pathway triggers apoptosis in medulloblastoma [33,34], we investigated whether curcumin triggers apoptosis in these cells. To this end, the Annexin V/PI staining technique followed by flow cytometry was used. Sixty percent confluent cells were treated with different concentrations of curcumin for 3 days, and then were stained and sorted. Figure 3A shows four groups of cells, viable cells that excluded both Annexin V and PI (Annexin V−/PI−), bottom left; early apoptotic cells that were only stained with Annexin V (Annexin V+/PI−), bottom right; late apoptotic cells that were stained with both Annexin

V and PI (Annexin V+/PI+), top right and necrotic cells that were only stained with PI (Annexin V−/PI+), top left. The proportion of apoptosis was considered as the sum of both early and late apoptosis after deduction of the proportion of spontaneous apoptosis. Curcumin triggered essentially apoptosis in all medulloblastoma cells. This effect increased in a dose-dependent manner, and cells showed different responses, with the highest proportion of apoptosis induced in the DAOY cell line (Figure 3A and B). This parallels the cytotoxic result presented in Figure 1A, indicating that the curcumin-dependent cytotoxicity is mediated mainly through the apoptotic cell death pathway, with only marginal necrosis. It is noteworthy that there is a great correlation between the proportion of cell death detected by WST-1 (Figure 1A) and by Annexin V (Figure 3A and B).

To shed more light on the effect of curcumin on medulloblastoma cells we treated 11 medulloblastoma cells (10 primary cultures and the DAOY cell line) with 40 μM of curcumin for 3 days and then the apoptotic response was assessed. Figure 3C shows that curcumin has differential effect on medulloblastoma cells. While eight cells exhibited high sensitivity with more than 40% cell death through apoptosis, three cell cultures showed high resistance to the drug. MED-5, MED-7, and DAOY were the most sensitive with 80% cell death, whilst MED-1, and MED-9 were the most resistant with about 30% apoptotic cells (Figure 3C).

To confirm the induction of apoptosis by curcumin in medulloblastoma cells and determine the apoptotic route that curcumin-dependent Shh inhibition activates, the MED-5 cells were treated with 40 μM of curcumin and harvested after different time periods (0, 24, 48, and 72 h). Whole cell extracts were prepared and 50 μg of extracted proteins were used to evaluate the levels of different pro- and anti-apoptotic proteins using the immunoblot technique and specific antibodies. β-Actin and GAPDH were used as internal controls. First, we assessed the effect of curcumin on the caspase-3 and PARP proteins (two principal markers of apoptosis). Figure 3D shows that the level of pro-caspase 3 decreased fivefolds after 48 h of curcumin treatment. Concomitantly, the level of the cleaved form of the PARP protein increased significantly after 72 h of treatment. This clearly shows the induction of apoptosis by curcumin in medulloblastoma cells. Next, we assessed the levels of Bax and Bcl-2 proteins and have found that while the level of Bax increased in a time-dependent manner, the level of Bcl-2 decreased sharply after only 24 h of treatment and then increased slightly (Figure 3D). This resulted after 48 h of treatment in 15-fold increase in the Bax/Bcl-2 ratio (Figure 3E), showing that curcumin triggers apoptosis through the mitochondrial pathway. To confirm this, we assessed the level of the pro-caspase 9 in these cells, and showed that the level of this protein decreased in a time-dependent manner reaching a level more than threefold lower after 72 h of treatment (Figure 3D). Together, these results demonstrated that curcumin triggers apoptosis in medulloblastoma cells through the internal mitochondrial pathway via Bcl-2 decrease.

The Shh Antagonist Cyclopamine Sensitizes Curcumin-Resistant Medulloblastoma Cells

To further elucidate the role of the Shh pathway in curcumin-dependent induction of apoptosis in medulloblastoma cells, we investigated the effect of curcumin on the Shh pathway in the curcumin-resistant MED-1 cells. Figure 4A shows that curcumin did not significantly affect the expression of the Shh and its important downstream protein Cyclin D1 in MED-1 cells. This indicates that the resistance of these cells to curcumin could be due to the inability of curcumin to inhibit the Shh signaling pathway. To investigate this possibility we explored whether cyclopamine, a natural antagonist of the Shh signaling pathway [35], could sensitize MED-1 cells to curcumin.

Cells were treated with curcumin (20 μM), cyclopamine (10 and 20 μM), and the combinations of both agents, for 72 h and then the proportion of apoptosis was determined. Figure 4B shows that while the effect of the single agents is only marginal the combination of curcumin and cyclopamine triggered high proportion of apoptotic cells. Indeed, more than 80% of cells died through apoptosis when cyclopamine (20 μM) was added to curcumin (20 μM) (Figure 4C). Similar results were obtained with the MED-9 cells (data not shown). This clearly shows that MED-1 and MED-9 cells are resistant to curcumin-dependent inhibition of the Shh signaling and that curcumin and cyclopamine have synergistic effect in inducing apoptosis in medulloblastoma cells.

Interestingly, in the curcumin-resistant MED-1 cells, wherein curcumin increased slightly the level of the Shh protein, the levels of Cyclin D1 and Bcl-2 were not downregulated as in the curcumin-sensitive MED-5 cells (Figure 2) but rather increased following treatment with curcumin (40 μM) (Figure 4D). This shows that the pro-apoptotic effect of cucumin in medulloblastoma cells could be mediated through the downregulation of Shh and the anti-apoptosis protein Bcl-2. Similarly, the anti-apoptosis protein survivin was also highly upregulated following treatment of MED-1 cells with curcumin (Figure 4D), which provides further explanation to the high resistance of these cells to curcumin.

Curcumin Potentiates the Apoptotic Effects of Cisplatin and γ-Rays

Ionizing radiation (IR) and cisplatin are the major therapeutic agents for medulloblastoma [1]. Therefore, we sought to test the ability of curcumin, as

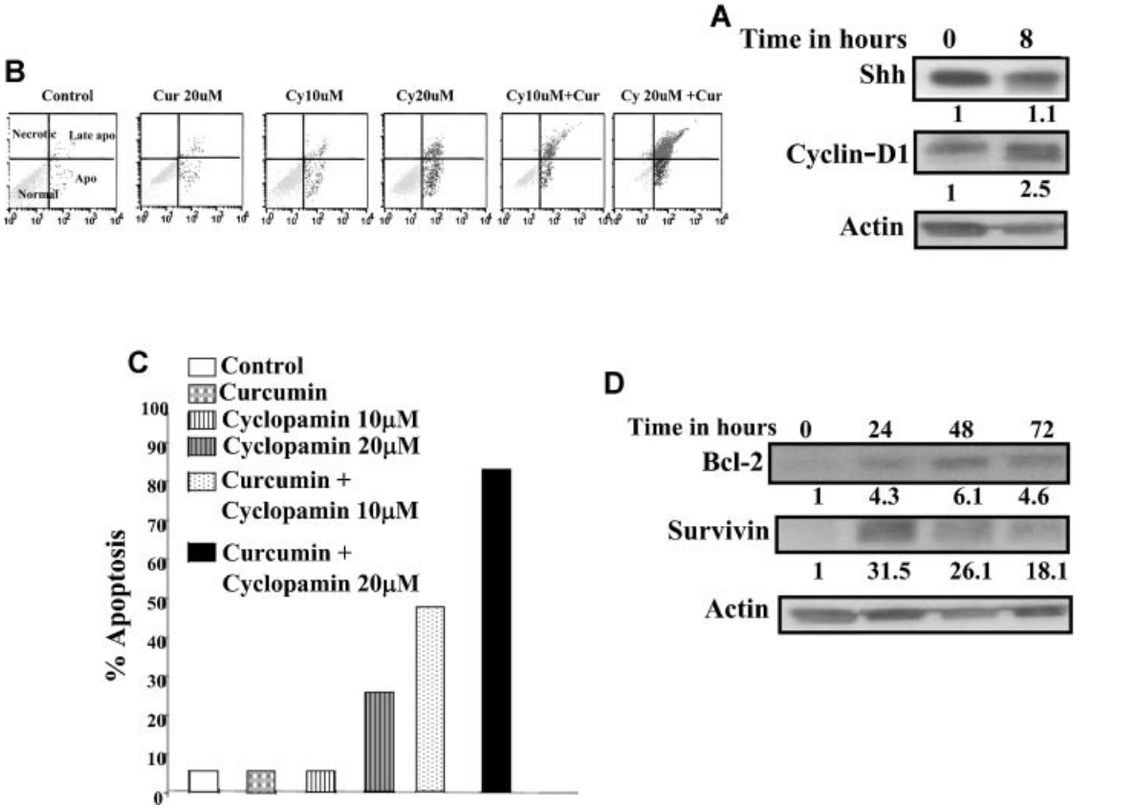

Figure 4. Cyclopamine sensitizes curcumin-resistant medulloblastoma cells. (A and D) Curcumin (40 μM) treated MED-1 cells were incubated for the indicated periods of time and proteins were extracted and used for immunoblot analysis using the indicated antibodies. The numbers under the bands represent the corresponding expression levels as compared to the basal level (time 0). (B) MED-1 cells were either mock treated or challenged with curcumin or cyclopamine or the combination of both. Cells were then reincubated for 72 h and cell death was assessed by Annexin V/PI flow cytometry. The numbers in the charts represent the proportions of necrotic and apoptotic cells. (C) Histogram showing the proportion of apoptotic cells.

inhibitor of the Shh pathway, to potentiate the effect of the DNA damaging agents γ-rays and cisplatin in triggering apoptosis in medulloblastoma cells. Therefore, MED-5 cells were either mock treated and used as control or challenged with curcumin (20 μM), γ-rays (5 Gy), cisplatin (6 μM), and the combination of curcumin with cisplatin or with γ-rays, and then the proportion of induced apoptosis was assessed by the Annexin V/flow cytometry technique (Figure 5A and B). Importantly, while the proportion of apoptotic cells was only marginal in response to each of the single agents, the combination of curcumin with cisplatin or with IR triggered apoptosis in more than 70% of cells, showing a great synergistic effect between curcumin and both cisplatin and IR (Figure 5A and B). This clearly shows that curcumin can potentiate the effect of both γ-rays and cisplatin in inducing apoptosis in medulloblastoma cells. Similar results were obtained with the DAOY cell line (data not shown). To elucidate the molecular basis of this potentiation we investigated the effect of the single agents and the combinations on the levels of Bax and Bcl-2 in MED-5 cells using the immunoblotting technique. Figure 5C shows that while the effect of curcumin and cisplatin on the level of Bcl-2 was only marginal, γ-rays downregulated Bcl-2 level 5-fold, 48 h postirradiation. Interestingly, the combination of curcumin with cisplatin led to a time-dependent downregulation of Bcl-2 reaching a level 10-fold lower after 72 h of treatment. Likewise, curcumin enhanced the γ-ray effect on Bcl-2 leading to a level 20-fold lower only 24 h postirradiation (Figure 5C). On the other hand, the level of Bax did not significantly change following all the treatments (data not shown). These results show that curcumin potentiates the action of cisplatin and γ-rays in triggering cell death through apoptosis by enhancing the downregulation of the anti-apoptosis Bcl-2 protein.

Piperine Potentiates the Effect of Curcumin in Triggering Apoptosis in Medulloblastoma Cells

It has been previously shown that piperine increases the curcumin bioavailability by 2000% [36]. To test whether this natural product can have

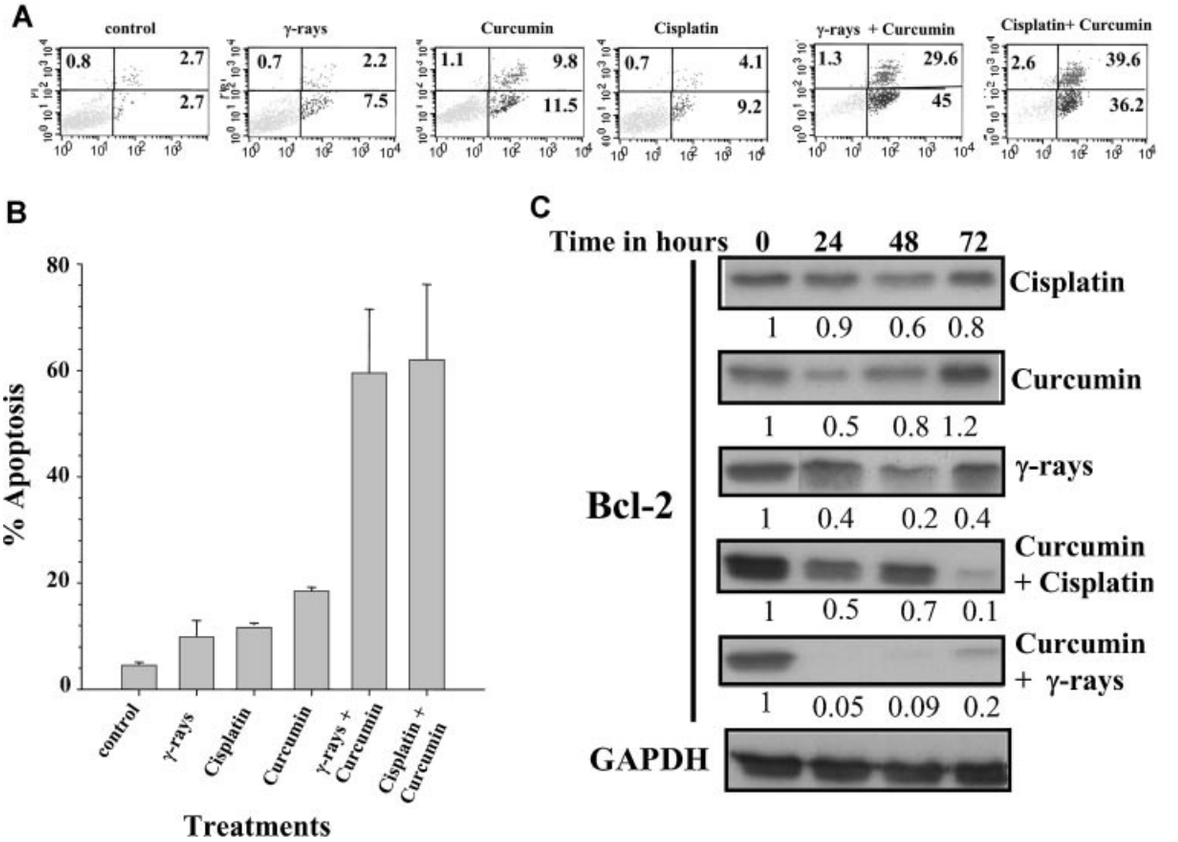

Figure 5. Curcumin enhances the cytotoxic effect of cisplatin and γ-rays through strong Bcl-2 decrease. MED-5 cells were either mock treated or challenged with curcumin (20 μM) or cisplatin (6 μM) or γ-rays (5 Gy) or the combination of curcumin with either cisplatin or γ-rays. Cells were then reincubated for different periods of time as indicated. Cell death was assessed by Annexin V/PI flow cytometry and the level of Bcl-2 was evaluated by immunoblotting. (A) Charts with the numbers representing the proportions of necrotic and apoptotic cells. (B) Histogram showing the proportion of apoptotic cells. The error bars represent standard deviations. (C) Immunoblots, the numbers under the bands represent the corresponding expression levels as compared to basal level (time 0).

enhancer effect on curcumin-dependent apoptosis induction, both products were used separately or in combination against MED-1, MED-4, MED-5, and DAOY cells, and apoptosis was assessed as described above. We have found that piperine triggers apoptosis in medulloblastoma cells in a dose-dependent manner. In fact 100 and 200 μM of piperine increased the proportion of apoptotic cells from 10% to 31% and 57%, respectively (data not shown). Furthermore, piperine enhanced the killing effect of curcumin on all cells tested (Figure 6A and B). The effect could be considered as additive for MED-4 and DAOY. However, it is rather synergistic for MED-1 and MED-5 cells. Importantly, the combination of both agents triggered apoptosis in more than 70% of the highly resistant MED-1 cell culture (Figure 6B). This suggests that the combination is very effective against resistant medulloblastoma cells.

To elucidate the molecular basis of the potentiation of the curcumin effect by piperine, we investigated the effect of the combination on the level of the Bax and Bcl-2 proteins in the curcumin-resistant MED-1 cells. Figure 6C shows that while the effect of the single agents is insignificant on the expression levels of Bax and Bcl-2, the combination of curcumin and piperine led to 4-folds increase in the level of Bax and 100-fold decrease in the level of Bcl-2, which resulted in 400-fold increase in the ratio Bax/Bcl-2 (Figure 6D). This shows that piperine enhances the apoptotic effect of curcumin by increasing its effect in downregulating the anti-apoptosis protein Bcl-2.

DISCUSSION

In the present report, we present clear evidence that curcumin could constitute a potent anti-medulloblastoma agent for the following reasons:

(1) Curcumin has an outstanding safety profile. Indeed, different phase I clinical trials have shown that curcumin is safe when consumed at doses as high as 12 g per day for 3 months [25,37].
(2) Curcumin crosses the brain blood barrier and reaches the brain. Indeed, it has been shown that

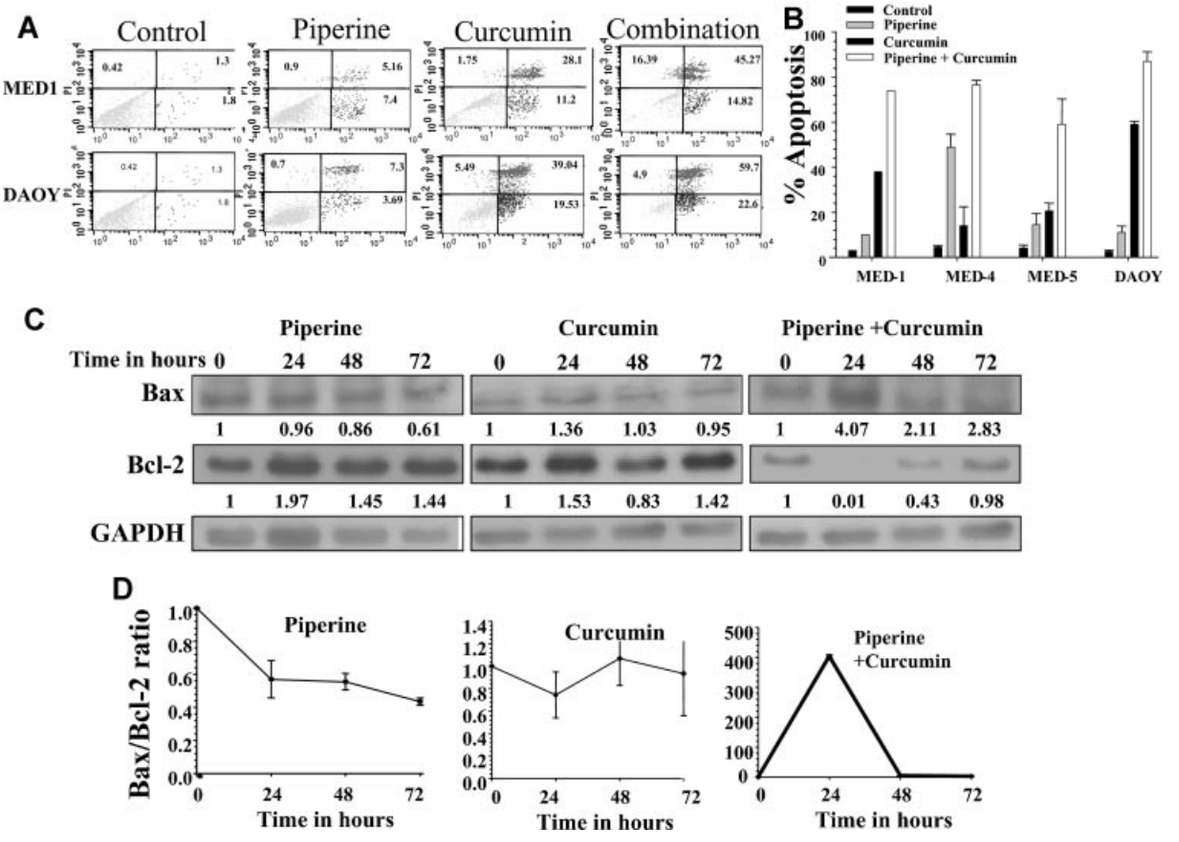

Figure 6. Piperine potentiates the pro-apoptotic effect of curcumin through strong downregulation of Bcl-2 in medulloblastoma cells. Cells were either mock treated or challenged with curcumin (20 μM) or piperine (100 μM) or the combination of curcumin with piperine. (A) Cells were then reincubated for 72 h and cell death was assessed by Annexin V/PI flow cytometry. Charts, the numbers represent the proportions of necrotic and apoptotic cells. (B) Histogram showing the proportion of apoptotic cells. The error bars represent standard deviations. (C) MED-1 cells were reincubated for the indicated periods of time and then cell lysates were prepared and used for immunobloting analysis using the indicated antibodies. The numbers under the bands represent the induction/reduction levels of the Bax and Bcl-2 proteins. (D) Graphs showing the Bax/Bcl2 ratios.

curcumin can reach different regions of the brain including the cerebellum in rats [38] and mice [39–41]. In fact, accumulating data in various experimental models have shown that dietary curcumin has neuroprotective effects and therefore is a strong candidate for use in the prevention or treatment of major disabling age-related neurodegenerative diseases, like Alzheimer's, Parkinson's, and stroke. These promising results have already led to ongoing pilot clinical trials [42].

(3) We have shown here that curcumin inhibits medulloblastoma cell proliferation by enabling the arrest of the cell-cycle at G_2/M phase.

(4) Curcumin is known to influence several biochemical and molecular pathways that play important roles in the development and progression of various types of cancer [37,43]. In the present study we present the first evidence that curcumin inhibits the Shh signaling. Indeed, curcumin down regulated the Shh protein and also its main targets in the pathway Gli1 and PTCH1 (Figure 2). The Shh pathway plays essential roles in the cerebellar development and neoplastic transformation [4,5]. Furthermore, activation of the Shh signaling promotes medulloblastoma from both neuronal progenitors and stem cells [44,45]. Moreover, blockade of the Shh pathway led to medulloblastoma growth inhibition [33]. Thereby, the curcumin-dependent inhibition of Shh signaling could be of great therapeutic value against medulloblastomas. Besides, we have shown that curcumin downregulates the expression of many important cancer proteins implicated in different signaling pathways that have been found deregulated in various cancers, including medulloblastoma. Indeed, curcumin inhibited two other important medulloblastoma-dependent carcinogenesis pathways Akt/NF-κB and β-catenin (Figure 2C). In fact it has been recently shown that the AKT signaling pathway is activated in medulloblastomas, which exhibited significant expression of phosphorylated Akt [46]. In the

present study we have shown that curcumin downregulates the phosphorylated/active form of Akt, which is able to increase the incidence of Shh-induced medulloblastoma [17]. Similarly, the level of the oncogenic transcription factor β-catenin was also downregulated by curcumin in medulloblastoma cells. Several lines of evidence indicate functional interaction between the two pathways Shh and Wnt/β-catenin [47]. It has been recently shown that the Wnt/β-catenin signaling is required for Hh pathway-driven tumorigenesis [32]. Therefore, it is very important to block Wnt signaling, given its activation in medulloblastomas [21].

Interestingly, the activation of these three pathways Akt/NF-κB, Shh, and Wnt/β-catenin converge in the upregulation of C-*myc*, N-*myc*, and Cyclin D1, three oncoproteins that play key roles in the development of medulloblastomas [1,7,10,29]. High levels of these three oncoproteins were considered to be bad prognosis because they are related to unfavorable therapeutic outcome [19]. N-*myc* proto-oncogene, a member of the *myc* family of transcriptional activators [48], is required for cerebellar granule neuron precursor proliferation [8,9], and therefore it is an important player in the carcinogenesis of medulloblastoma. Our data show that curcumin has strong inhibitory effect on these proteins in medulloblastoma cells, which further supports the inhibition of the three important medulloblastoma-driven pathways and the efficient anti-medulloblastoma effect of curcumin. The fact that these pathways have anti-apoptosis effects, suggests that the pro-apoptotic action of curcumin against medulloblastoma cells is mediated through the inhibition of these pathways and the downstream anti-apoptosis protein Bcl-2.

(5) We have also shown here that in most cases, curcumin triggered cell death in high proportions of medulloblastoma cells mainly through the apoptotic pathway. This seems to take place by downregulating different anti-apoptosis proteins, including Bcl-2 and survivin. Survivin is a potent anti-apoptosis protein that is differentially expressed in cancer and therefore constitutes an important anti-cancer target [49]. Moreover, high expression of survivin plays important role in resistance to chemo- and radiotherapy and has been shown to be related to unfavorable outcome for medulloblastomas [50]. Importantly, our data have shown that 24 h of curcumin treatment can reduce survivin to 30% of the basal level in medulloblastoma cells. Similar effect has been shown on different other cancer cell lines [25]. Interestingly, when the levels of Bcl-2 and survivin were assessed in curcumin-resistant medulloblastoma cells MED-1 and MED-9 an increase rather than decrease was observed. Concomitantly, no decrease in the level of the Shh protein and its downstream target Cyclin D1 was observed, suggesting that curcumin-dependent induction of apoptosis is mediated through Shh/Bcl-2 downregulation. Indeed, the natural Shh antagonist cyclopamine, sensitized the MED-1 cells to curcumin (Figure 4).

These results also showed that these two plant-derived Shh-antagonist products have synergistic effect against medulloblastoma, and this could be of great value for the treatment of these aggressive tumors. Indeed, both agents target pathways that are crucial for medulloblastoma survival. Furthermore, cyclopamine has been considered for the treatment of medulloblastoma patients [51,52].

(6) Curcumin sensitized medulloblastoma cells to γ-rays and cisplatin, two widely used agents for the treatment of these neoplasms [1,20]. Indeed, the combination of curcumin with nontoxic doses of each agent led to 80% cell death in different medulloblastoma cells. This effect has been mediated through a strong downregulation of the anti-apoptosis protein Bcl-2 (Figure 5C). It has been recently shown that curcumin enhanced the anti-tumor effects of gemcitabine and radiation in the PC3 human prostate cancer cell line through downregulating the MDM2 oncogene [53] and pancreatic cancer cells in vitro and in vivo [54]. In another study, curcumin potentiated taxol-induced apoptosis by downregulating NF-κB and Akt [55]. Together, these results show that curcumin can be used to potentiate the activity and reduce the nondesirable side effects of some chemotherapeutic agents and IR.

(7) Curcumin-dependent induction of apoptosis was enhanced by piperine, an enhancer of curcumin bioavailability. The fact that curcumin can induce the expression of different important tumor suppressor proteins and inhibit the expression of various key oncoproteins indicates that this agent could be considered as great anti-medulloblastoma product. However, curcumin is limited in its clinical utility owing to its low bioavailability. Indeed, several reports have shown that curcumin is rapidly degraded. Major reasons contributing to the low plasma and tissue levels of curcumin appear to be due to poor absorption, rapid metabolism, and rapid systemic elimination [56]. Interestingly, it has been shown that piperine, another natural polyphenolic nontoxic agent that inhibits hepatic and intestinal glucuronidation, can significantly enhance the curcumin absorption level and bioavailability in both rats and humans by

154% and 2000%, respectively [36]. Importantly, we have shown here that piperine is cytotoxic and enhances also the killing effect of curcumin against medulloblastoma cells. Strikingly, piperine sensitized the cell cultures that were resistant to the killing effect of curcumin. We have also shown that the synergistic effect of curcumin and piperine was mediated through down regulating the most important anti-apoptosis protein, Bcl-2. Indeed, the combination of both agents decreased by 100-fold the level of this protein (Figure 6). This finding is of great importance, since it has been recently shown that Bcl-2 is an important mediator of the Shh activity in medulloblastoma [15]. The fact that curcumin downregulates both Shh and Bcl-2 suggests that this agent in combination with piperine could constitute a great anti-medulloblastoma agent.

Together, these findings provide clear evidence that curcumin inhibits the Shh–Gli1 signaling and triggers cell growth inhibition and the induction of cell death through the internal apoptotic pathway in medulloblastoma cells. In addition, it enhances the killing effect of cisplatin and γ-rays and targets pathways crucial for tumor survival. Furthermore, piperine enhances curcumin anticancer effect showing that the combination of these two natural and safe products could be of great value if included in the medulloblastoma therapeutic regimens.

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