Shikonin combined with curcumin synergistically induces apoptosis of glioma cells by inhibiting the PI3K/Akt/NF-κB signaling pathway

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Abstract. Glioblastoma (GBM) is a highly aggressive, recurrent and drug-resistant malignancy of the central nervous system, with a poor prognosis and a median survival rate of <18 months, highlighting the need for the identification of novel therapeutic approaches. The natural products shikonin and curcumin have been reported to exhibit anti-glioma properties and regulate the PI3K/Akt/NF-κB signaling pathway. However, their combined effects and the underlying molecular mechanisms remain largely unclear. Using human glioma U251 cells and U87 cells as *in vitro* models, cell viability was assessed by Cell Counting Kit-8 assay, colony formation was evaluated by colony formation assay and apoptosis was detected by flow cytometry and Hoechst 33258 staining. The present study found that combined treatment with shikonin and curcumin synergistically reduced cell proliferation and colony formation ability (combination index <1), enhanced apoptosis induction and exhibited relatively lower cytotoxicity toward healthy cells. Mechanistically, western blot analysis showed that the combination treatment increased the Bax/Bcl-2 ratio and elevated the protein expression levels of cleaved caspase-9, cleaved caspase-3 and cleaved-poly (ADP-ribose) polymerase, while attenuating Akt (Ser473) and IκBα (Ser32) phosphorylation, and immunofluorescence confocal microscopy revealed reduced p65 nuclear translocation. These findings suggest that shikonin and curcumin may exert synergistic anti-glioma effects *in vitro* through inhibition of the PI3K/Akt/NF-κB signaling axis and activation of the mitochondrial apoptotic pathway. Collectively, the present study provides preliminary cellular evidence indicating that the combination of shikonin and curcumin may represent a potential sensitizing approach for further investigation in GBM treatment.

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Introduction

Glioblastoma (GBM) is the most common and deadliest primary brain tumor, accounting for 14.2% of all primary brain tumors and 50.1% of all gliomas, with an average annual age-adjusted incidence rate of 3.19 per 100,000 population (1,2). Despite standard treatment, the median overall survival remains at 15-18 months, and the 5-year survival rate is only 6.8-18% (3,4). According to the 2021 World Health Organization (WHO) classification, GBM is designated as isocitrate dehydrogenase (IDH)-wildtype grade 4, with lower-grade gliomas (WHO grades 2-3) stratified by IDH and 1p/19q status for risk-adapted treatment [observation, radiotherapy or procarbazine, lomustine and vincristine/temozolomide (TMZ) chemotherapy] (5). The current standard-of-care for GBM remains the Stupp regimen: Maximal safe resection followed by concurrent radiotherapy (60 Gy) with TMZ (75 mg/m^2 daily) and six cycles of adjuvant TMZ (150-200 mg/m^2 on days 1-5 of each 28-day cycle), with tumor-treating fields as an optional adjunct (6). O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation predicts chemosensitivity, however even with this intensive approach, median survival remains <18 months and the 5-year survival rate is only 4-7% (7).

Treatment failure is largely attributed to tumor heterogeneity, blood-brain barrier (BBB) limitations and apoptosis resistance driven by aberrant activation of the PI3K/Akt and NF-κB pathways (7). In recent years, some new and innovative treatment strategies have been developed for GBM, such as light-activated therapeutic platforms, including near-infrared-II phototheranostic agents for precise tumor targeting and deep-tissue imaging, immune-evasive photodynamic nanoplatforms and microenvironment-responsive strategies for repeatable photodynamic therapy (8-10). These platforms have demonstrated potential in precise, spatiotemporally controlled GBM treatment by enabling targeted

drug delivery, multimodal imaging and enhanced antitumor efficacy. However, these approaches still require combination with molecularly targeted agents to overcome intrinsic apoptosis resistance. Therefore, developing new strategies to overcome apoptosis resistance and enhance the efficacy of existing drugs is needed in glioma research.

Natural products exhibit unique advantages in glioma treatment due to their multitargeted effects, low toxicity and good BBB permeability (11). For instance, resveratrol has been shown to suppress GBM stem-like cells through the Janus kinase 2/STAT3 and PI3K/Akt/mTOR pathways (12,13). In addition, quercetin has been shown to target Ras-related C3 botulinum toxin substrate 1 and epigenetically activate p16-inhibitor of cyclin-dependent kinase (14). Berberine has been shown to inhibit migration and angiogenesis through TGF-β1/collagen type XI α 1 chain and VEGFR2/ERK, while promoting mitochondrial damage and mutant p53 degradation (15,16). Furthermore, celastrol has been shown to suppress angiogenesis and induce mitochondrial dysfunction through PI3K/Akt/mTOR and mitofusin 1 downregulation, while synergizing with TNF-associated apoptosis-inducing ligand through Wnt/β-catenin inhibition (17-19). Curcumin is a hydrophobic polyphenol extracted from the rhizome of *Curcuma longa*, with anti-inflammatory, antioxidant, anti-angiogenic and antitumor activities (20,21). Studies have shown that curcumin can inhibit glioma cell proliferation and induce apoptosis by triggering endoplasmic reticulum stress, mitochondrial dysfunction and inhibiting the STAT3 pathway (22,23). Curcumin exhibits very poor water solubility, rapid metabolism *in vivo* and low bioavailability, which means clinical use requires very high doses (usually >8 g per day), greatly limiting its translation (24).

Shikonin, the main lipophilic active ingredient of the Chinese herb *Lithospermum erythrorhizon*, belongs to the naphthoquinone family. Recent studies have found that shikonin exerts antitumor effects through numerous mechanisms, including inducing reactive oxygen species (ROS) bursts, suppressing glycolysis and triggering autophagic cell death (25,26). Notably, shikonin effectively inhibits NF-κB nuclear translocation and downregulates anti-apoptotic Bcl-2 family proteins, thereby reversing tumor resistance to chemotherapy (27). However, shikonin alone is toxic to healthy tissues at higher doses, which prevents dose escalation. For instance, intraperitoneal injection of shikonin at doses of ≥10 mg/kg caused acute death in mice, whereas 1-3 mg/kg was well tolerated, indicating a narrow therapeutic window (28).

Combination therapy has become an important direction in cancer treatment due to its synergistic effects, reduced single-agent dosage and multi-pathway blockade (29). In recent years, many studies have supported the rationality of combining active ingredients from Traditional Chinese Medicine. For example, both shikonin and curcumin affect the PI3K/Akt/NF-κB axis but act on different targets: Shikonin tends to inhibit Akt phosphorylation, while curcumin more effectively blocks IκBα degradation and p65 nuclear translocation (30,31). Therefore, their combination may achieve stronger pathway inhibition through complementary mechanisms.

The PI3K/Akt/NF-κB signaling pathway is abnormally activated in the majority of gliomas (32). Activation of this pathway promotes cell survival and inhibits apoptosis primarily by: i) Suppressing of proapoptotic proteins such as cleaved-poly (ADP-ribose) polymerase (PARP) and caspase-3; ii) upregulating anti-apoptotic proteins Bcl-2 and Bcl-xL; and iii) activating NF-κB to drive the transcription of survival genes (32). In the canonical NF-κB pathway, phosphorylation and degradation of IκBα releases the p65/p50 dimer to enter the nucleus and initiate the transcription of anti-apoptotic targets including Bcl-2, Survivin and cellular FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) (33). Therefore, simultaneously targeting Akt and NF-κB to restore the Bax/Bcl-2 balance may be an effective strategy to overcome apoptosis resistance in glioma.

Despite studies have separately reported the inhibitory effects of shikonin or curcumin on glioma cells, to the best of our knowledge, whether their combination produces a synergistic effect and enhances apoptosis through the PI3K/Akt/NF-κB pathway to regulate Bax/Bcl-2, has not been systematically reported. In the present study, human glioma U251 cells were used as a model to systematically evaluate the effects of shikonin combined with curcumin on cell proliferation, colony formation and apoptosis, as well as explore their corresponding underlying molecular mechanisms. The aim was to provide experimental evidence and theoretical support for combination therapy of glioma.

Materials and methods

Materials. Human glioma U251 (cat. no. JY143), U87-MG (cat. no. JY110; GBM of unknown origin) and SVG p12 (cat. no. JY-J1095) cells were purchased from Shanghai Jinyuan Biotechnology Co., Ltd., originally derived from the American Type Culture Collection and authenticated by short tandem repeat profiling. Shikonin and curcumin were obtained from Dalian Meilun Biotechnology Co., Ltd. and dissolved in DMSO as stock solutions. DMEM medium was purchased from Dalian Meilun Biotechnology Co., Ltd. Penicillin-streptomycin solution (100X) and Hoechst 33258 staining solution were purchased from Beyotime Biotechnology. The Cell Counting Kit-8 (CCK-8) assay kit was obtained from Dojindo Laboratories, Inc. The Annexin V/PI Apoptosis Detection Kit was obtained from Wuhan Elabscience Biotechnology Co., Ltd. Antibodies against Bax (cat. no. 2772), Bcl-2 (cat. no. 15071), caspase-9 (cat. no. 9502), PARP (cat. no. 9542), cleaved caspase-3 (cat. no. 9662), β-actin (cat. no. 4967), phosphorylated (p)-Akt (cat. no. 9271), Akt (cat. no. 9272), p-IκBα (cat. no. 2859), IκBα (cat. no. 4814) and p65 (cat. no. 8242) were purchased from Cell Signaling Technology, Inc.

Cell culture. U251, U87-MG and SVG p12 cells were cultured in DMEM medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1X penicillin-streptomycin (100 U/ml penicillin + 100 μg/ml streptomycin; cat. no. C0222; Beyotime Biotechnology). Cells were maintained in a humidified incubator at 37˚C with 5% CO_2.

CCK-8 assay. To evaluate the cytotoxicity of shikonin and curcumin alone or in combination on glioma cells, cell viability was measured using the CCK-8 method. U251 or U87-MG cells in the logarithmic growth phase were seeded

into 96-well plates at a density of $3x10^3$ cells per 100 μl. After cell attachment for 24 h, the medium was replaced. Cells were divided into a control group, shikonin groups (0.5, 0.75, 1 and 1.25 μM), curcumin groups (10, 15, 20 and 25 μM) and combination groups (0.5 μM shikonin + 10 μM curcumin; 0.75 μM shikonin + 15 μM curcumin; 1 μM shikonin + 20 μM curcumin; 1.25 μM shikonin + 25 μM curcumin). After 48 h of treatment in a humidified incubator at 37˚C with 5% CO_2, cell viability was assessed using the CCK-8 kit. Cells were incubated in medium containing 10% CCK-8 solution for 1.5 h, and absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.). Cell viability (%) was calculated as: (Mean absorbance of experimental group/mean absorbance of control group) x100. For combination groups, the combination index (CI) was calculated using CalcuSyn software (http://www.biosoft.com/w/calcusyn.htm). CI>1 indicated antagonism, CI=1 indicated an additive effect and CI<1 indicated synergism. Furthermore, SVG p12 cells were seeded into 96-well plates at a density of $4x10^3$ cells per 100 μl. After cell attachment for 24 h, the medium was replaced. Cells were divided into a control group, shikonin group (0.75 μM), curcumin group (15 μM), LY294002 group (a specific PI3K inhibitor; cat. no. S1737; Beyotime Biotechnology; 20 μM) and combination groups (0.75 μM shikonin + 15 μM curcumin with or without LY294002). After 48 h of treatment, cell viability was assessed as aforementioned.

Plate colony formation assay. U251 cells were digested with trypsin, resuspended, counted and seeded into 6-well plates at 1,500 cells per well. After attachment, the medium was removed, and cells were divided into control, shikonin (0.75 μM), curcumin (15 μM) and combination (0.75 μM shikonin + 15 μM curcumin) groups. Cells were incubated with the indicated treatments at 37˚C for 48 h. Subsequently, the medium was replaced with drug-free DMEM and changed every 3 days for 2 weeks. Colonies were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with 0.1% crystal violet at room temperature for 30 min. Colonies were defined as clusters of ≥50 cells and counted manually under a light microscope (Olympus Corporation).

Hoechst 33258 staining. U251 cells were incubated at 37˚C with 5% CO_2, and treated with the indicated agents [control, shikonin (0.75 μM), curcumin (15 μM) and combination (0.75 μM shikonin + 15 μM curcumin)] for 48 h. U251 cells were then washed three times with PBS and incubated with 1,000 μl Hoechst 33258 staining solution (Beyotime Biotechnology; 5 μg/ml) at 37˚C with 5% CO_2 for 30 min. After washing three times with PBS, cells were observed and imaged under a fluorescence microscope.

Flow cytometry analysis of apoptosis. U251 cells were incubated at 37˚C with 5% CO_2 and treated with the indicated agents [control, shikonin (0.75 μM), curcumin (15 μM) and combination (0.75 μM shikonin + 15 μM curcumin)] for 48 h. U251 cells were digested with 0.25% trypsin- free EDTA (cat. no. C0207; Beyotime Biotechnology) and collected to prepare a suspension of $1x10^6$ cells/ml. A 100 μl aliquot was incubated with Annexin V-FITC and PI staining solutions (cat. no. C1062S; Beyotime Biotechnology) at room temperature in the dark for 15 min, according to the manufacturer's instructions. Apoptosis was analyzed using a BD FACS Accuri C6 flow cytometer (BD Biosciences). Data were processed using FlowJo software (version 10.0, Tree Star, Inc.). Cells positive for Annexin V-FITC and negative for PI were considered early apoptotic, while cells positive for both were considered late apoptotic.

Western blotting analysis. After 48 h of treatment at 37˚C with 5% CO_2, U251 and U87 cells were collected and lysed in RIPA lysis buffer (cat. no. P0013C; Beyotime Biotechnology) containing 1% phenylmethylsulfonyl fluoride. Total protein was extracted and quantified using the BCA method. Equal amounts of protein (32 μg) were separated by 10 or 12% SDS-PAGE and transferred to PVDF membranes at 80 V. Membranes were blocked with 5% nonfat milk at room temperature for 2 h and then incubated with primary antibodies (β-actin at 1:5,000; others at 1:1,000) overnight at 4˚C. The next day, membranes were washed three times with TBST (0.1% Tween-20; 5 min each) and incubated with an HRP-conjugated secondary antibody (1:5,000; cat. no. 7074 for anti-rabbit and cat. no. 7076 for anti-mouse secondary antibody; Cell Signaling Technology, Inc.) at room temperature for 2 h. Protein bands were visualized using ECL reagent (cat. no. P0018M; Beyotime Biotechnology). Densitometric analysis was performed using the gel imaging system (Clinx 6300).

Immunofluorescence confocal microscopy. U251 cells were seeded onto coverslips in 6-well plates at a density of $5x10^5$ cells per well and treated for 48 h at 37˚C with 5% CO_2. Cells were fixed with 4% paraformaldehyde at room temperature, permeabilized with 0.2% Triton X-100 and blocked with PBS containing 5% BSA (cat. no. P0007; Beyotime Biotechnology) at room temperature for 1 h. After washing, cells were incubated with 3% FBS (Gibco; Thermo Fisher Scientific, Inc.) in PBS for 30 min at room temperature, then incubated with anti-p65 antibody (1:500 dilution) overnight at 4˚C. The next day, cells were incubated with fluorescent secondary antibody (Anti-Rabbit Dylight 594; cat. no. KTD109; Abbkine Scientific Co., Ltd.) in the dark for 60 min at room temperature. Nuclei were counterstained with DAPI (Beyotime Biotechnology; 2.5 μg/ml) at room temperature for 10 min. Images were captured under a laser confocal microscope (Leica Microsystems GmbH) and analyzed using ImageJ software (National Institutes of Health).

Statistical analysis. All experimental data are expressed as the mean ± SD. Data were analyzed using SPSS (version 19.0; SPSS, Inc.). For single-factor comparisons among multiple groups, one-way ANOVA followed by Tukey's post-hoc test was used. For analyses involving two independent variables, two-way ANOVA followed by Tukey's post-hoc test was used. $P<0.05$ was considered to indicate a statistically significant difference

Results

Shikonin and curcumin synergistically inhibit GBM cell proliferation. A CCK-8 assay was used to detect the viability

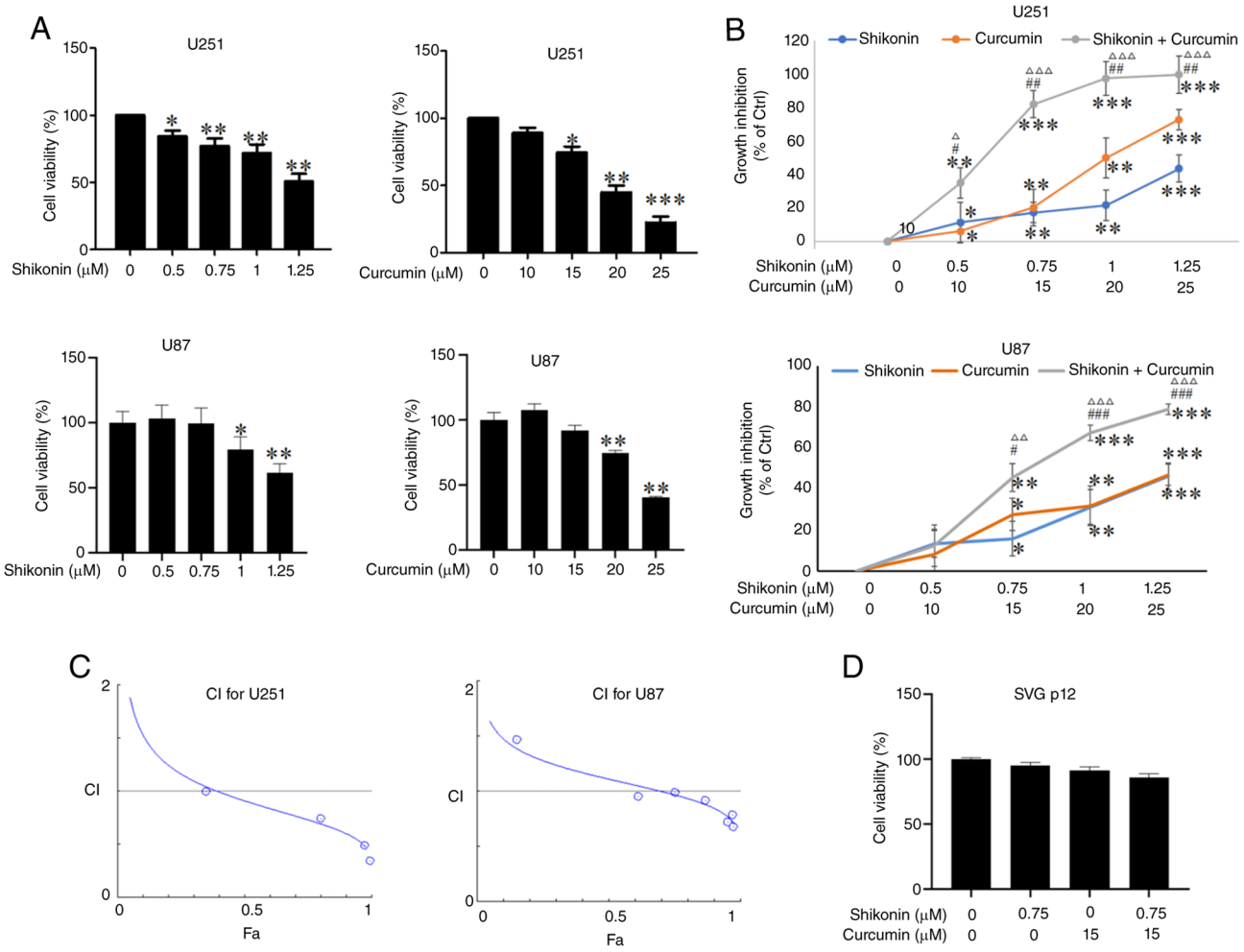


Figure 1. Effects of shikonin and curcumin alone or in combination on the proliferation of glioblastoma cells. (A) A CCK-8 assay was used to detect the cell viability of U251 and (B) U87 cells (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared with the control group (one-way ANOVA). (C) CI for U251 and U87 cells was calculated by CalcuSyn software. (D) CCK-8 assay was used to detect the cell viability of SVG p12 cells. *P<0.05, **P<0.01 and ***P<0.001 compared with the control group (two-way ANOVA). $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 (shikonin vs. shikonin + curcumin). $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 (curcumin vs. shikonin + curcumin). CCK-8, Cell-Counting Kit-8; CI, combination index; Fa, fraction affected.

of GBM (U251 and U87) cells treated with different concentrations of shikonin (0, 0.5, 0.75, 1 and 1.25 μM) and curcumin (0, 10, 15, 20 and 25 μM) alone or in combination for 48 h. Results indicated that both shikonin and curcumin significantly inhibited U251 and U87 cell proliferation in a concentration-dependent manner (Fig. 1A and B). To further evaluate the combined effect, four combination groups were set: 0.5 μM shikonin + 10 μM curcumin; 0.75 μM shikonin + 15 μM curcumin; 1 μM shikonin + 20 μM curcumin; and 1.25 μM shikonin + 25 μM curcumin. CI values calculated by CalcuSyn software were all <1 (Fig. 1C), indicating that shikonin and curcumin synergistically inhibited the proliferation of U251 and U87 cells. Based on these results, 0.75 μM shikonin + 15 μM curcumin was selected for subsequent mechanistic studies. Furthermore, the combination (0.75 μM shikonin + 15 μM curcumin) exhibited minimal toxicity to normal SVG p12 cells compared with U251 and U87 cells under the tested conditions (Fig. 1D), indicating relative selectivity for glioma cells.

Shikonin and curcumin synergistically suppress colony formation in U251 cells. Plate colony formation assays showed that shikonin (0.75 μM) and curcumin (15 μM) alone significantly inhibited colony formation of U251 cells compared with the control group (P<0.05 and P<0.01, respectively). The combination group exhibited further significantly enhanced inhibition compared with single-agent groups (P<0.01; Fig. 2). These results demonstrated that shikonin combined with curcumin synergistically suppressed colony formation.

Shikonin and curcumin synergistically induce apoptosis in U251 cells. Hoechst 33258 staining showed that nuclei in the control group displayed uniform, faint blue fluorescence. In shikonin and curcumin single-agent groups, partial chromatin condensation, brighter blue fluorescence and fewer apoptotic bodies were observed. In the combination group, apoptotic morphological changes were much more apparent and the number of apoptotic cells was notably higher compared with the single-drug groups (Fig. 3A).

Flow cytometry with Annexin V-FITC/PI double staining was used to quantify apoptosis. The apoptosis rate of the control group was 1.21±0.58%. Shikonin and curcumin alone significantly increased apoptosis to 5.54±2.47 and 6.36±3.24%, respectively (both P<0.05 vs. control). The combination group

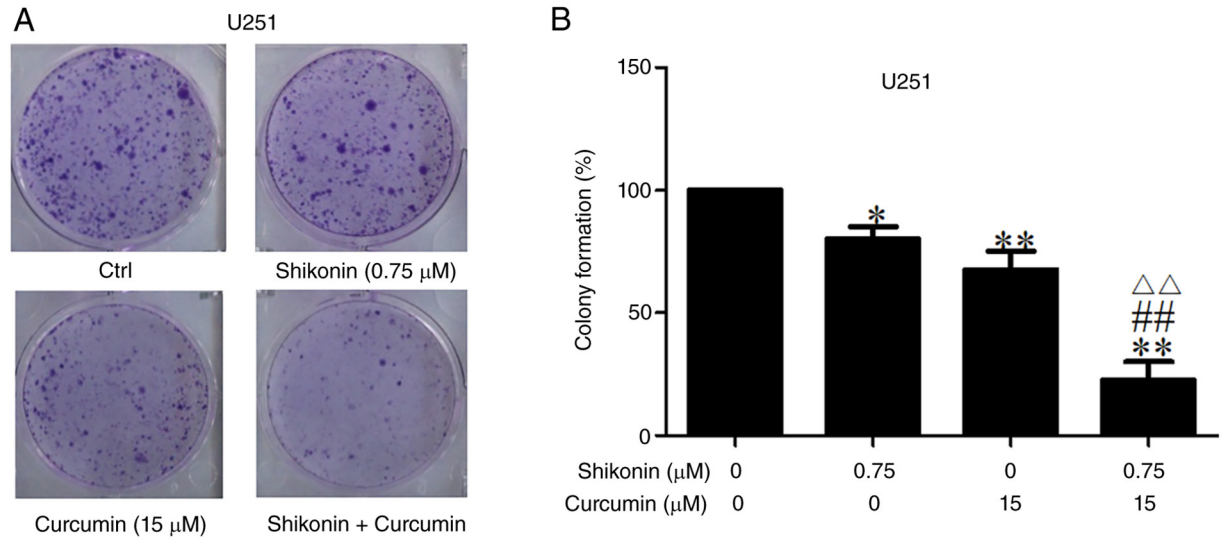


Figure 2. Effects of shikonin and curcumin alone or in combination on colony formation of U251 cells. (A) Representative images of colony formation (n=3). (B) Quantitative analysis of colony formation rate. *P<0.05 and **P<0.01 compared with the control group (one-way ANOVA with Tukey's post hoc test). $^{\Delta\Delta}$P<0.01 (shikonin vs. shikonin + curcumin). $^{\#\#}$P<0.01 (curcumin vs. shikonin + curcumin).

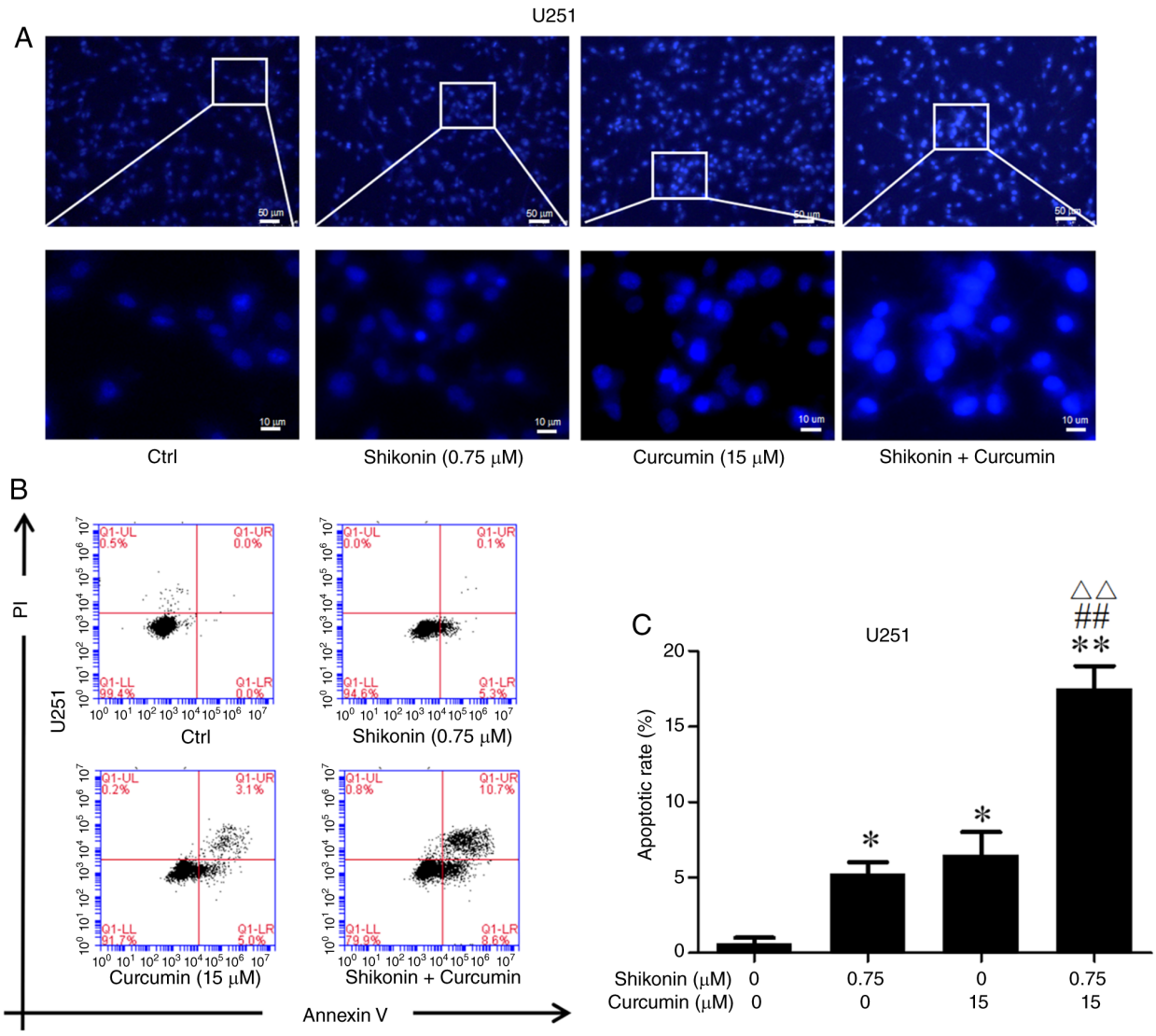


Figure 3. Effects of shikonin and curcumin alone or in combination on apoptosis of U251 cells. (A) Hoechst 33258 staining (n=3). Scale bar: 50 mm (upper panels) and 10 mm (lower panels). (B) Flow cytometry analysis (n=3). (C) Quantitative analysis of apoptosis rate. *P<0.05 and **P<0.01 compared with the control group (one-way ANOVA with Tukey's post hoc test). $^{\Delta\Delta}$P<0.01 (shikonin vs. shikonin + curcumin). $^{\#\#}$P<0.01 (curcumin vs. shikonin + curcumin).

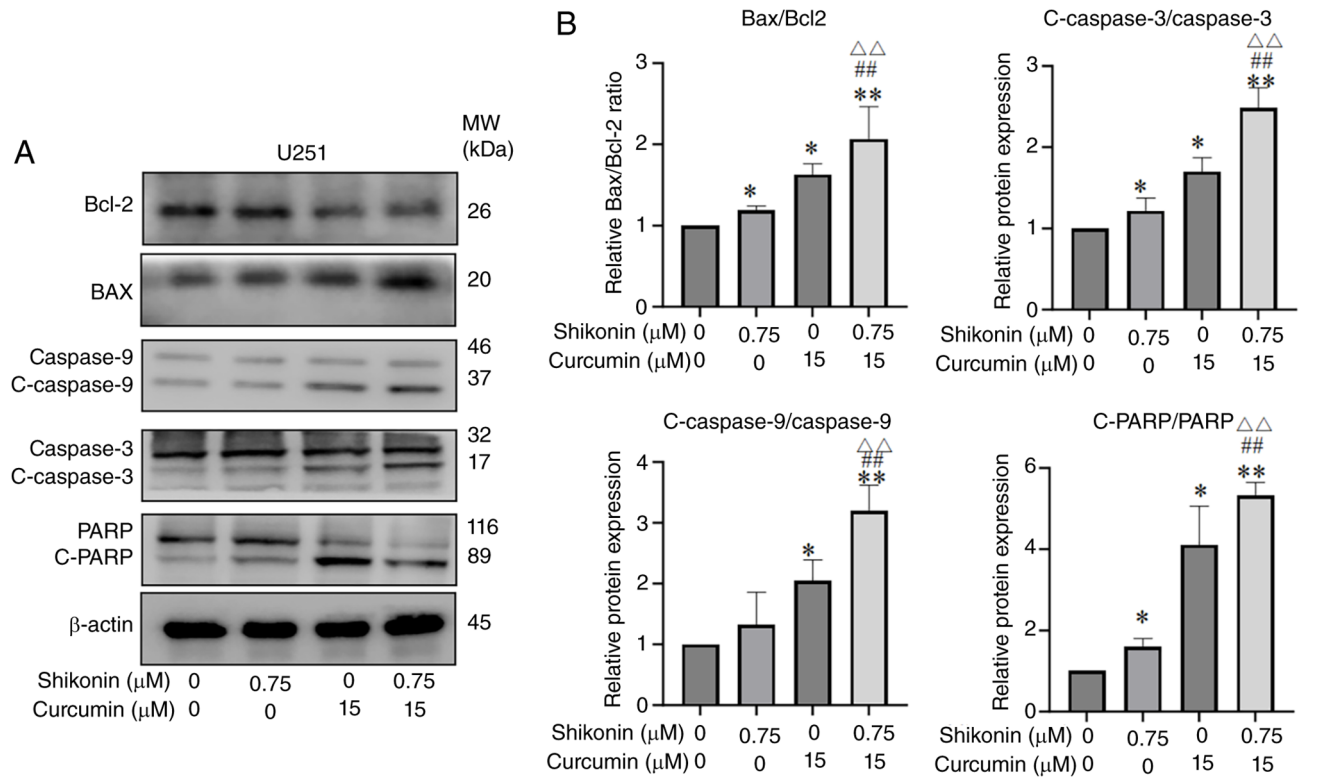


Figure 4. Effects of shikonin and curcumin alone or in combination on apoptosis-associated proteins in U251 cells. (A) Western blotting images of apoptosis-associated proteins in U251 cells (n=3). (B) Semi-quantitative analysis of Bax/Bcl-2 ratio. Relative protein expression levels of C-caspase 3/9 and C-PARP. *P<0.05 and **P<0.01 compared with control group (one-way ANOVA with Tukey's post hoc test). $^{\Delta\Delta}$P<0.01 (shikonin vs. shikonin + curcumin). $^{\#\#}$P<0.01 (curcumin vs. shikonin + curcumin). PARP, poly (ADP-ribose) polymerase; C-, cleaved; MW, molecular weight.

reached 17.87±3.15%, which was significantly higher compared with either single treatment (P<0.01; Fig. 3B and C). These results demonstrated that shikonin + curcumin synergistically induced apoptosis in U251 cells.

Shikonin and curcumin synergistically activate the mitochondrial apoptotic pathway. Western blotting was used to detect key proteins in the apoptotic pathway. Compared with the control group, shikonin or curcumin alone significantly increased the Bax/Bcl-2 ratio and the expression of cleaved caspase-3 and cleaved-PARP (P<0.05). Although shikonin alone did not significantly increase cleaved caspase-9 levels, curcumin alone did significantly increase cleaved caspase-9 expression (P<0.05). The combination group further significantly elevated the protein levels compared with single-agent groups (P<0.01) (Fig. 4). These results indicated that the combined treatment synergistically induced apoptosis through the mitochondrial apoptotic pathway.

Shikonin and curcumin synergistically suppress the PI3K/Akt/NF-κB signaling pathway. Western blotting was used to detect the expression and phosphorylation of key proteins in the PI3K/Akt/NF-κB pathway. Compared with the control group, shikonin or curcumin alone significantly reduced the protein levels of p-Akt (Ser473) and p-IκBα (Ser32; P<0.05), while total Akt and total IκBα exhibited no marked changes. The combination group significantly decreased phosphorylation levels of p-Akt and p-IκBα compared with the control (P<0.01), notably more than shikonin or curcumin alone (Fig. 5A and B). To further investigate whether the PI3K/Akt/NF-κB pathway contributes to the observed effects, additional experiments were performed using LY294002, a specific PI3K inhibitor that blocks PI3K-mediated phosphorylation of Akt and thereby suppresses downstream NF-κB activation. The results demonstrated that treatment with LY294002 (20 μM) alone significantly decreased cell viability in U251 and U87 cells (P<0.01), mimicking the effects of the shikonin and curcumin combination. Notably, when the combination treatment was co-administered with LY294002, no significant further enhancement of reduced cell viability was observed compared to the combination alone (P>0.05; Fig. 5C), suggesting that the combination and inhibitor act on the same pathway. These findings implied that the pro-apoptotic effect of the combination was associated with suppression of the PI3K/Akt/NF-κB signaling axis.

Shikonin and curcumin synergistically inhibit p65 nuclear translocation. Immunofluorescence confocal microscopy was used to detect the localization of NF-κB p65. In the control group, activated p65 (p-p65) was found to be primarily located in the nucleus, exhibiting strong red fluorescence. The nuclear fluorescence intensity of p-p65 was reduced in shikonin and curcumin single-agent groups. In the combination group, nuclear localization of p-p65 was significantly reduced (P<0.001; Fig. 6). These results indicated that shikonin + curcumin synergistically inhibited p65 nuclear translocation and blocked NF-κB transcriptional activity (Fig. 7).

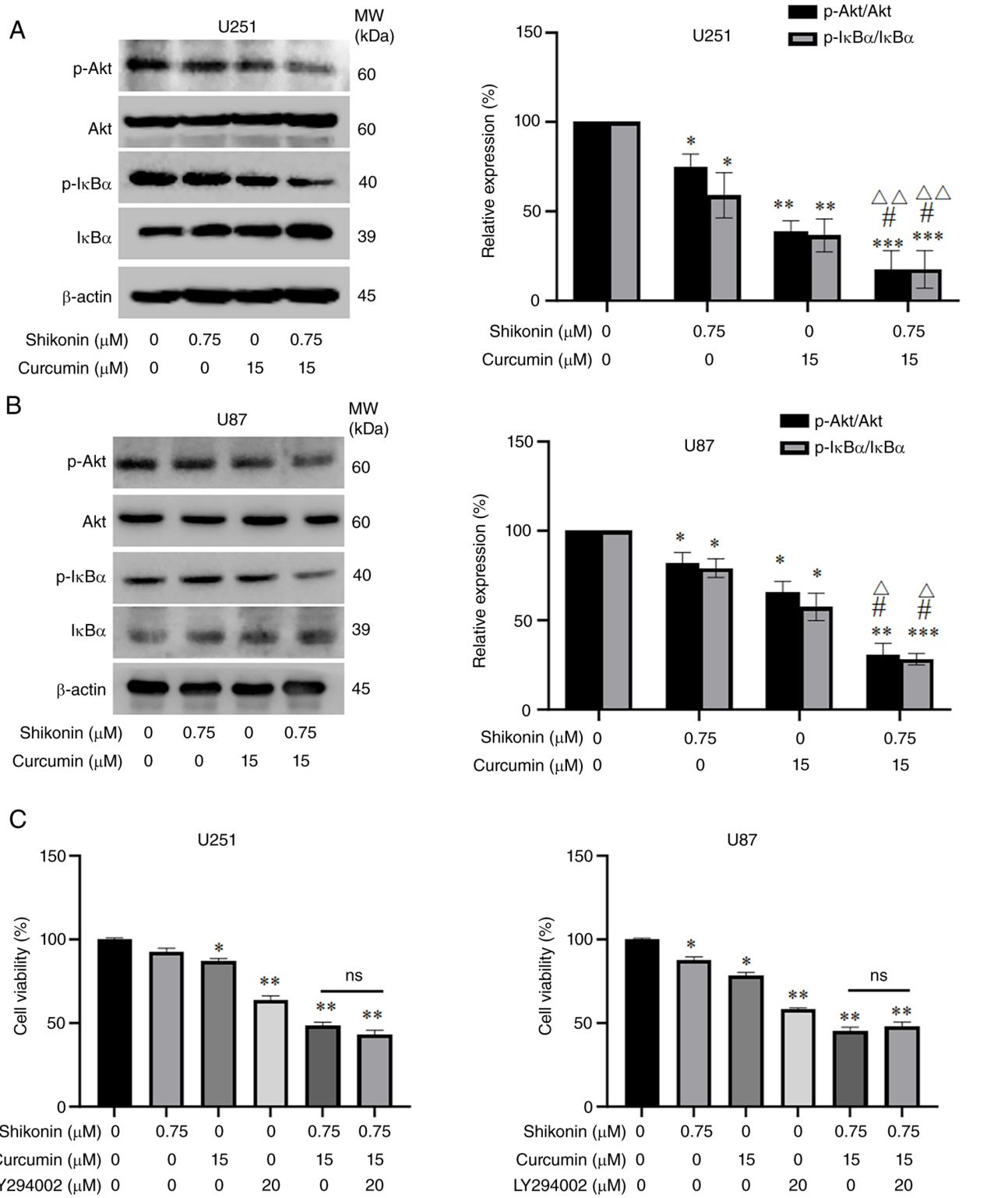


Figure 5. Effects of shikonin and curcumin alone or in combination on the PI3K/Akt/NF-κB signaling pathway in glioblastoma cells. (A) Western blotting images and semi-quantitative analysis of relative protein expression for U251 cells (n=3). (B) Western blotting images and semi-quantitative analysis of relative protein expression for U87 cells (n=3). (C) Cell Counting Kit-8 assay was used to detect the cell viability of U251 and U87 cells with LY294002 (a specific PI3K inhibitor) treatment (n=5). *P<0.05, **P<0.01 and ***P<0.001 compared with control group (one-way ANOVA with Tukey's post hoc test). $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.01 (shikonin vs. shikonin + curcumin); $^{\#}$P<0.05 (curcumin vs. shikonin + curcumin). ns, not significant; MW, molecular weight; p-, phosphorylated.

Discussion

The present study systematically demonstrated that shikonin combined with curcumin synergistically inhibited proliferation and notably induced apoptosis in human glioma U251 cells *in vitro*. The molecular mechanism may involve synergistic inhibition of the PI3K/Akt/NF-κB signaling pathway, reduced levels of p-Akt and p-IκBα, blocked p65 nuclear translocation, increased Bax/Bcl-2 ratio, activation of the mitochondrial apoptotic pathway and sequential cleavage of caspase-9,

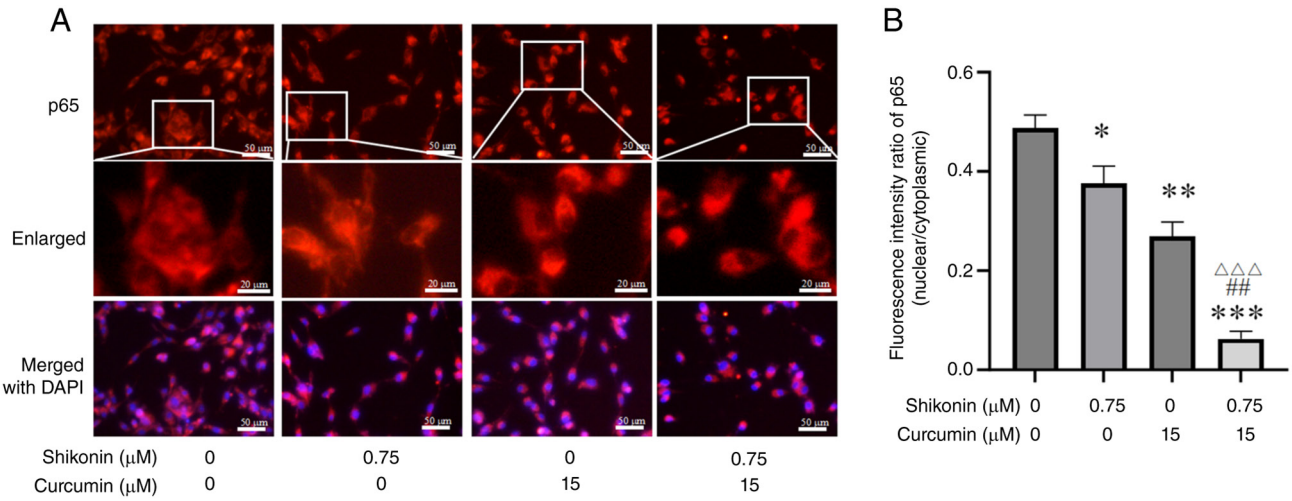


Figure 6. Effect of shikonin and curcumin alone or in combination on the nuclear localization of p65. (A) The nuclear localization of p65 in U251 cells was detected by immunofluorescence confocal microscopy (n=3). Scale bar: 50 mm (upper panels), 20 mm (middle panels) and 50 mm (lower panels). (B) Quantitative analysis of the fluorescence ratio of p65 in the nucleus and cytoplasm. *P<0.05, **P<0.01 and ***P<0.001 compared with the control group (one-way ANOVA with Tukey's post hoc test). $^{\Delta\Delta\Delta}$P<0.001 (shikonin vs. shikonin + curcumin). $^{\#\#}$P<0.01 (curcumin vs. shikonin + curcumin).

caspase-3 and PARP. While the present study focused on establishing the mechanistic synergy between shikonin and curcumin, the lack of direct comparison with standard-of-care agents such as TMZ represents a limitation. Such evaluations will be important in assessing the translational relevance of this combination in future studies. Collectively, the present findings provide a mechanistic rationale for the further development of this combination as a potential sensitizing strategy for glioma therapy.

The PI3K/Akt/NF-κB pathway serves a key role in glioma development and is frequently dysregulated (34,35). Activated Akt promotes IκBα degradation by phosphorylating IKKα/β, allowing NF-κB p65/p50 to enter the nucleus and drive transcription of anti-apoptotic genes such as Bcl-2, Survivin and c-FLIP, enhancing cell survival and chemoresistance (32,33). Therefore, simultaneously targeting Akt and NF-κB is considered a promising strategy to overcome apoptosis resistance.

Natural products have attracted interest due to their multitarget regulatory properties (11). Previous studies have reported that shikonin induces both apoptosis and necroptosis in human glioma cells by inhibiting EGFR phosphorylation and decreasing phosphorylation of EGFR downstream molecules, including Akt, P44/42MAPK and phospholipase C γ1 (36), while curcumin regulates the expression of IKKβ to block NF-κB activation (37). However, to the best of our knowledge, whether their combination can synergistically suppress the PI3K/Akt/NF-κB axis to enhance apoptosis, has not been systematically investigated. The present results showed that combined treatment did further reduce p-Akt and p-IκBα levels and markedly decreased p65 nuclear translocation, suggesting dual blockade at the Akt upstream and IKK levels, leading to synergistic inhibition.

The Bcl-2 family is a core regulator of the mitochondrial apoptotic pathway, and the balance between pro- and anti-apoptotic members determines cellular sensitivity to apoptosis (38). Bax promotes cytochrome *c* release, whereas Bcl-2 inhibits apoptosis by sequestering Bax (39). An increased Bax/Bcl-2 ratio is a key switch for mitochondrial apoptosis. Previous studies have shown that shikonin upregulates Bax and downregulates Bcl-2 partly by inhibiting NF-κB (40) and curcumin acts similarly through NF-κB inhibition (41). However, their combined synergistic regulation of Bax/Bcl-2 has been previously unclear. The present data showed that the combination increased the Bax/Bcl-2 ratio to 2-3 times that of single agents and strongly activated caspase-9, caspase-3 and PARP, demonstrating robust synergistic activation of the mitochondrial apoptotic pathway.

Caspase cascade activation is central to the execution phase of apoptosis. Cleaved caspase-3 and PARP are widely used markers of apoptosis induction in glioma studies. Shikonin has been reported to activate caspase-3 via the NF-κB pathway (40) and curcumin can trigger the caspase-9/caspase-3 cascade (42). However, direct evidence of their combined synergistic activation is lacking. The present results showed that the combination group exhibited markedly higher levels of cleaved caspases and PARP compared with single treatments, consistent with the apoptosis rate detected by flow cytometry.

Combination therapy with natural products has gained increasing attention. The synergistic anti-glioma effect of shikonin and curcumin likely arises from their complementary actions on the PI3K/Akt/NF-κB signaling axis. Shikonin modulates the PI3K/Akt pathway and suppresses NF-κB activation through regulation of toll-like receptor 4/NF-κB/MAPK and PI3K/Akt cascades, inhibiting Akt (Ser473) and IκBα (Ser32) phosphorylation and blocking p65 nuclear translocation (43). Curcumin similarly exerts anti-cancer effects through direct inhibition of the IKK complex and PI3K/Akt/mTOR pathway, suppressing NF-κB activation through PTEN upregulation and reduced PI3K/Akt-mediated phosphorylation (44). Emerging evidence has indicated that curcumin combined with other natural products enhances anti-tumor effects through concurrent suppression of NF-κB activation mediated by PI3K/Akt and associated pathways (45), which provides a comparative theoretical basis for the combined synergy of shikonin and curcumin. In the present study, all combination groups (0.5/10, 0.75/15, 1/20 and 1.25/25 μM shikonin/curcumin) exhibited

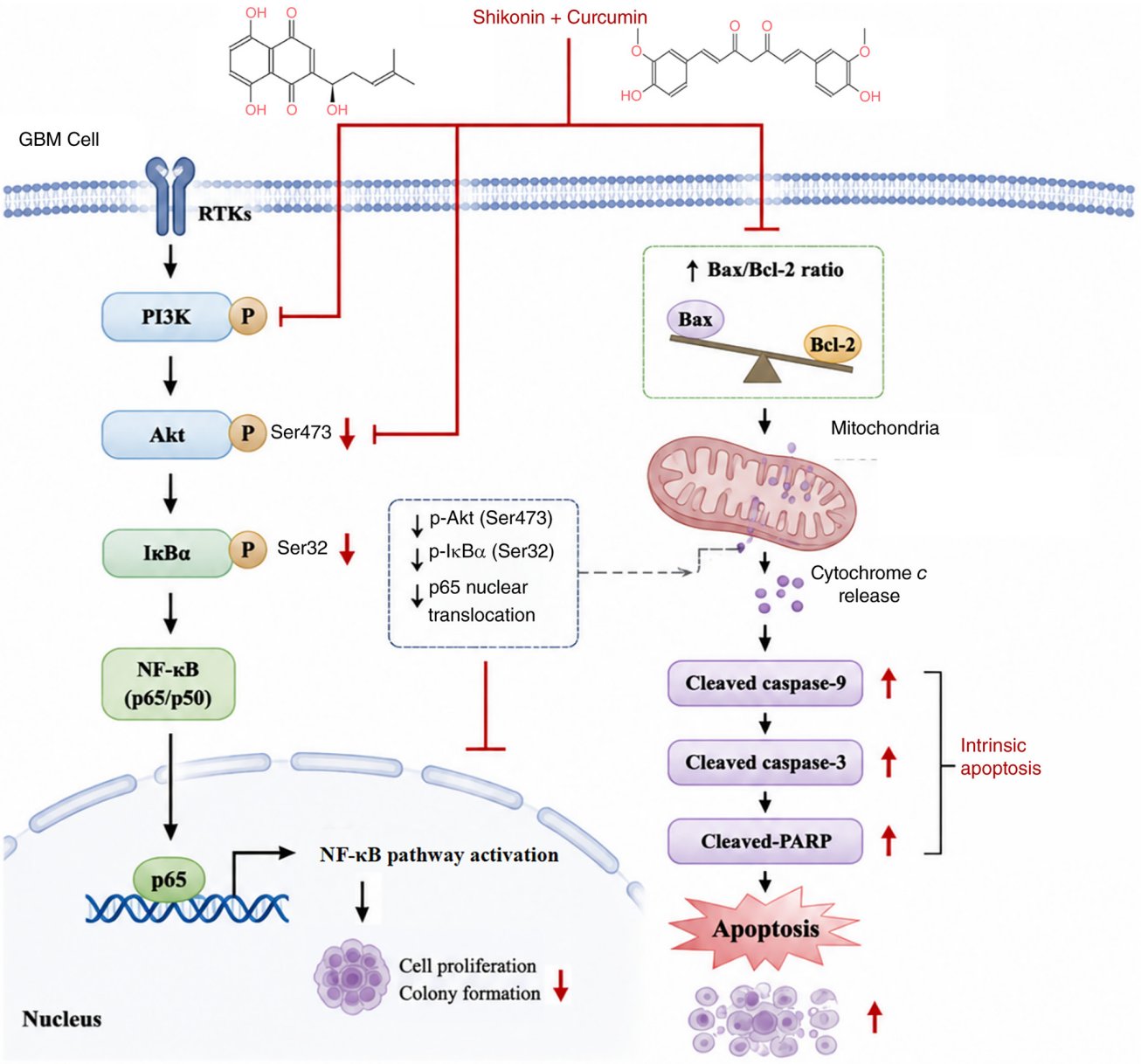


Figure 7. Schematic illustration summarizes the signaling pathways inhibited by the combination of shikonin and curcumin in GBM. GBM, glioblastoma; p-, phosphorylated; PARP, poly (ADP-ribose) polymerase; RTKs, receptor tyrosine kinases.

CI<1, demonstrating strong synergism. The concentration used in mechanistic experiments (0.75 μM shikonin + 15 μM curcumin) is lower than their respective IC_{50} values, suggesting that combination therapy can greatly reduce effective doses and potentially lower toxicity to normal neural cells. The synergy between shikonin and curcumin likely arises from dosage-dependent interactions, complementary molecular targets and convergence on common signaling pathways. Both compounds independently suppress PI3K/Akt phosphorylation and IκBα degradation but target distinct nodes: Shikonin modulates PI3K/Akt cascades (43), while curcumin inhibits the IKK complex and enhances PTEN expression (44). Their combined application produces convergent inhibition of the PI3K/Akt/NF-κB axis at multiple levels, amplifying p65 nuclear translocation blockade, shifting the Bax/Bcl-2 ratio, activating the mitochondrial apoptotic cascade and enhancing apoptosis. This complementary multi-target mechanism, rather than simple dose addition, provides a molecular rationale for the observed synergy (CI<1).

The potential integration of shikonin and curcumin with current GBM standard treatments warrants consideration. Preclinical evidence has indicated that curcumin sensitizes GBM cells to TMZ by modulating MGMT expression, enhancing apoptosis and increasing ROS production (22,46). Similarly, shikonin has been shown to synergize with TMZ in GBM-derived cells, suppressing proliferation and migration through PI3K/Akt inactivation and inhibition of glial-to-mesenchymal transition (47). Regarding radiotherapy, curcumin enhances radiation sensitivity under both low- and high-linear energy transfer radiation conditions by increasing ROS levels and apoptosis (48), while shikonin reduces the nuclear entry of DNA repair-associated enzymes, thereby increasing radiation sensitivity (49,50). These findings suggest that combining shikonin and curcumin with standard therapies

may represent a promising sensitizing strategy, warranting further *in vivo* and clinical investigation.

Despite the present *in vitro* data demonstrating promising anti-glioma activity, the safety profiles of shikonin and curcumin warrant careful consideration. Curcumin has demonstrated favorable tolerability in early clinical studies at oral doses up to 8,000 mg/day, as emerging case reports have indicated that hepatotoxicity may occur, particularly with bioavailability-enhanced formulations containing piperine (51,52). Shikonin exhibits relatively low acute toxicity following oral administration in animal models; however, toxicity increases with parenteral administration, highlighting the importance of dose and route selection in future translational studies (53).

The present study exhibits a number of limitations. Firstly, experiments were only performed at the *in vitro* level. No *in vivo* animal experiments were performed to evaluate efficacy and safety. This absence of rescue experimentation means that the causality of shikonin + curcumin combinations with PI3K/Akt/NF-κB signaling needs to be further established. The inhibitor data (LY294002) are correlative, thus causal validation through pathway activation or overexpression is required in future studies. In addition, both shikonin and curcumin suffer from poor water solubility and low bioavailability (24,26). To address these gaps, future research plans should aim to include the establishment of glioma xenograft models in mice to validate *in vivo* efficacy, systematic pharmacokinetic and toxicity evaluations to assess drug safety and disposition and the development of nanocarrier-based formulations (such as liposomes or exosomes) to improve solubility and targeted delivery *in vivo*. Despite these limitations, the present study has important translational potential. Glioma stem cells highly express NF-κB and Akt, leading to resistance to TMZ (6,54). By dual blockade of this pathway, shikonin + curcumin may reverse TMZ resistance. Further studies using RNA-sequencing or proteomics may therefore identify key downstream molecules and provide new targets for precision glioma therapy.

Overall, the present study demonstrated that shikonin combined with curcumin synergistically inhibited proliferation and colony formation, whilst markedly inducing apoptosis in human glioma U251 cells *in vitro*. The underlying mechanism was associated with suppression of the PI3K/Akt/NF-κB pathway, decreased phosphorylation of Akt and IκBα, reduced p65 nuclear translocation, increased Bax/Bcl-2 ratio, activation of the mitochondrial apoptotic pathway and sequential cleavage of caspase-9, caspase-3 and PARP. These findings systematically clarify the molecular mechanism by which shikonin enhances curcumin-induced apoptosis in glioma cells, provide experimental evidence for their combination as a novel chemosensitizing strategy for glioma and lay a theoretical foundation for future *in vivo* validation and clinical translation.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XZ and JZ conceived and designed the experiments. MZ, YH, ZY and YS performed the experiments. XZ, MZ, YH, ZY and YS conducted the statistical analysis. XZ and JZ wrote the manuscript. XZ and JZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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