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Anti-tumor Effects of Curcumin and ABT-737 in Combination Therapy for Glioblastoma in Vivo

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ABSTRACT

The resistance of tumor cells to ABT-737 can be attributed to alterations in the equilibrium of Bcl-2 family proteins. In this study, the effect of curcumin on the Mcl-1expression and the sensitivity of glioblastoma cells to ABT-737 were examined. Trypan blue assay and colony formation assay were performed to explore the effects of treatments on cell proliferation. MTT assay was performed to measure cytotoxicity. Cell migration was determined using a wound healing assay. Cell apoptosis was measured by Hoechst 33342 staining, ELISA cell death, and caspase-3 activity assay. The expression levels of Mcl-1 mRNA were also tested by qRT-PCR. Our results revealed that combination therapy significantly lowered the IC_{50} value and synergistically decreased the colony formation and migration, cell survival and growth of glioblastoma cells compared with curcumin or ABT-737 alone. Treatment with curcumin clearly inhibited the expression of Mcl-1 mRNA. Moreover, suppression of Mcl-1 mRNA by curcumin was associated with enhancement of apoptosis induced by ABT-737. In conclusion, curcumin has the ability to inhibit the cell proliferation and migration, and activate the intrinsic pathway of apoptosis. Moreover, it can enhance the sensitivity of glioblastoma cells to ABT-737 by suppressing the expression of Mcl-1.

Keywords: ABT-737, Apoptosis, Bcl-2, Curcumin, Glioblastoma, Mcl-1

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Introduction

Glioblastoma multiform is one of the most common and devastating primary brain tumors in humans (1). Despite the use of various therapeutic methods such as surgery, radiotherapy and chemotherapy, the five-year survival rate of patients is less than 10% (2). Therefore, understanding the molecular mechanisms of glioblastoma and development of new therapeutic strategies are essential.

The intrinsic pathway of apoptosis is regulated by Bcl-2 family protein members including anti- and pro-apoptotic proteins (3). The anti-apoptotic members such as Bcl-2 and Bcl- xL share a structural homology domains, namely Bcl-2 homology (BH) 1, 2, 3, 4 (3). The elevated level of Bcl-2 anti-apoptotic proteins is commonly observed in various types of tumor tissues, which is associated with cell survival, migration, increased risk of metastasis and chemoresistance (4). Moreover, studies have shown that the expression of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL are elevated in glioblastoma multiforme that are associated with pathological conditions and drug-resistance (5). Therefore, targeting Bcl-2 proteins has been identified as a potential therapeutic strategy for cancer treatment (4, 5).

ABT-737 is a synthetic pan-Bcl-2 inhibitor that strongly binds to the hydrophobic groove of Bcl-2, Bcl-xL and Bcl-w proteins and inhibits the sequestration of pro-apoptotic BH3-only members such as Bad, Bim, and tBid. However, ABT-737 binds weakly to some other anti-apoptotic Bcl-2 family members, including Mcl-1 and Bfl-1/A1 (5-7). Previous studies have shown that ABT-737 is effective as a single-agent or in combination with other anti-cancer agents against different types of malignancies such as breast cancer, small cell lung cancer, thyroid cancer and ovarian cancer (6, 8-10). Moreover, reports have shown that the increase in the cellular level of Mcl-1 protein is one of the important mechanisms of ABT-737 resistance in cancer cells (6, 7, 11). The resistance can be overcome by combining ABT-737 with Mcl-1 inhibiting or suppressing agents such as sorafenib, YM155 or N-(4-hydroxyphenyl) retinamide (7, 11-13).

Curcumin (diferuloylmethane) is the main curcuminoid produced by plants of *Curcuma longa L.* Curcomin has shown strong anti-cancer property in many types of cancers, including glioblastoma (14, 15). Moreover, previous preclinical studies have demonstrated that curcumin can effectively inhibit the proliferation of glioblastoma cells. Moreover, curcumin can augment the efficacy of chemotherapy, radiotherapy and molecular-targeted therapy in tumor cells (15-17). However, the combination effect of ABT-737 and curcumin on cancer cells has not yet been determined. In this study, we hypothesized that curcumin can sensitize glioblastoma cells to ABT-737 by inhibition of Mcl-1 expression. Therefore, we investigated the effect of curcumin on Mcl-1 expression and sensitivity of the glioblastoma cells to ABT-737 *in vitro.*

Materials and methods

Cell culture

The U373-MG and T98G glioblastoma cells (Pasteur Institute, Tehran, Iran) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, Life technologies, Germany) containing 15% fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin-100 µg/ml streptomycin (Gibco), 2 mM of glutamine, and 1% sodium pyruvate at 37 ˚C in a 95% humidified incubator containing 5% CO2.

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MTT assay

The cytotoxic effects of curcumin and ABT-737 in glioblastoma cells were evaluated using MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay. The assay was divided into five groups: ABT-737, curcumin, ABT-737 and curcumin, solvent control and blank control. Briefly, cells were cultured at a density of 4×10^3 per well in 96-well culture plates and then incubated overnight. Next, the cells were exposed to different concentrations of ABT-737 (0-64 µM) and curcumin (0-64 µM) for 48 h. The cytotoxicity of the treatments was then determined using the MTT Cell Proliferation Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol. The absorbance (A) of each well at 570 nm was read using a microplate reader (Awareness Technology, Palm City, FL, USA) at a reference wavelength of 650 nm. The survival rate (SR) was obtained by the equation as follows: SR (%) = ($A_{Treatment}$ /$A_{Control}$) × 100%. The IC_{50} values were calculated by using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

Drug combination study

The interaction between ABT-737 and curcumin was assessed according to the combination index (CI) principle described by the Chou-Talalay method (18). To assess the interaction, data obtained from the MTT assay was converted to Fraction affected values (Fa; range 0-1; where Fa = 1 represents 0% cell viability and Fa = 0 represents 100% cell viability). The obtained Fa values were then analyzed with the CompuSyn software from Combosyn (Paramus, NJ, USA). CI values: <1, =1 or >1 represent synergistic, additive or antagonistic effect, respectively.

Cell growth assay

The effect of ABT-737 and curcumin on tumor cell growth was measured by the trypan blue staining. The treatment of 5×10^4 tumor cells with ABT-737 and curcumin, either alone or in combination, was carried out for 5 days in 6-well culture plates. At different time intervals, the cells were collected using trypsinization and subsequently, cell suspensions were treated with 0.4% trypan blue (Merck KGaA, Darmstadt, Germany) for duration of 2 minutes. To measure the number of viable cells (unstained cells), an inverted microscope (Nikon Instrument Inc., Melville, NY, USA) and a hemocytometer were utilized. The calculation of cell viability percentage was established using the following equation: ($N_{Treatment}$ /$N_{Control}$) ×100. At the indicated time intervals, the control group cells were regarded viability rate of 100%.

Quantitative real-time PCR (qRT-PCR)

The RNA extraction kit (Parstous, Tehran, Iran) was used to isolate total RNA from the glioblastoma cell lines after a 48h treatment period. Next, 1µg of purified RNA was reverse transcribed into complementary DNA (cDNA) by use of Easy cDNA Synthesis Kit (Parstous) and oligo-dT primer according to the manufacturer's protocol. To examine the expression of specific genes, qRT-PCR was performed using the SYBR Green qPCR MasterMix (Parstous) on the LightCycler 96 System (Roche Diagnostics GmbH, Mannhein, Germany). The components of the q-PCR reaction system included: 10 µl of SYBR Green qPCR MasterMix, 1 µl of cDNA template, 0.2 µM of each of the primers, and 7 µl of nuclease-free distilled water. The sequence of primers were as follows: forward, 5'-GACATCCGCAAAGACCTGTA-3', and reverse, 5'-GGAGCAATGATCTTGATCTTCA-3', for β-actin, forward, 5'- TAGTTAAACAAAGAGGCTGGGA-3', reverse, 5'-CCTTCTAGGTCCTCTACATGG -3', for Mcl-1, and forward, 5'-ATACCATCGAGACCATG

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CG-3', and reverse, 5'-CCAATGATCCTGTATGTGATCTG-3', for MMP-2. The samples were subjected to an initial incubation step at 95 ºC for 10 min. Subsequently, a total of 35 cycles were carried out, each comprising three distinct temperatures. The initial incubation step at 95 ºC for 10 sec was followed by a second step at 58 ºC for 20 sec and finally a third step at 72 ºC for 20 sec. The relative gene expression level was calculated with the comparative $2^{-(\Delta\Delta Ct)}$ method (19), using β-actin as an internal control.

Scratch wound healing assay

Glioblastoma cells were allowed to grow into 90% confluence in 6-well culture plates. The following day, the cell monolayer was scratched using a sterile micropipette tip and then washed once with PBS to remove cellular debris. IC_{50} concentrations of ABT-737 and curcumin, alone and in combination, were added to the respective wells for 48 h. Three random fields along the scratched line in each well were captured using an inverted microscope.

Colony formation assay

The U373-MG and T98G glioblastoma cells (5×10^3 cells/well) treated with IC_{50} concentrations of ABT-737 and curcumin, alone and in combination in 6-well flat-bottomed plates. The growth culture medium was replenished with fresh medium every 4 days. After 10 days, the cells were fixed with 3.7% paraformaldehyde and then stained with 0.4% crystal violet. Then the colonies were washed, photographed and assessed for colony estimation.

Hoechst 33342 staining

U373-MG and T98G cell lines were seeded at a final concentration of 5×10^4 cells/well into a 6-well plate and then exposed to IC_{50} doses of ABT-737 and curcumin for 48 h. Next, the cells were washed twice with PBS, then fixed in 3.7% formaldehyde and stained with Hoechst 33342 (Beyotime, Jiangsu, China) for 10 min. Cells with condensed or fragmented nuclei were observed under a fluorescence microscope.

Apoptosis ELISA assay

The quantification of cell death was performed using a cell death kit (Roche Diagnostics GmbH) based on ELISA method. This kit enables the quantification of mono- and oligo nucleosomes, which produced by cells undergoing apoptosis. In brief, glioblastoma cells were seeded at a density of 1×10^5 cells/well in 24-well culture plates and treated with ABT-737 and curcumin for 48 h. After lysing the tumor cells, the cell suspensions were centrifuged. Next, 20 µl of the supernatants and 80 µl of the mixture containing anti-DNA-peroxidase and anti-histone-biotin were transferred to individual well of a streptavidin-coated plate. After a 2 h incubation at 25 °C, the wells were underwent washing with incubation buffer. Next, 100 µl of ABTS solution was added to the wells. The reactions were then stopped with ABTS stop solution, and absorbance was quantified immediately by using an microplate reader (Awareness Technology, Palm City, FL, USA) at 405 nm. The results demonstrated fold change in absorbance for the treatment groups when compared to the blank control group.

Caspase-3 activity assay

The activity of caspase-3 was performed by using a Caspase-3 Activity Assay Kit (Abnova Corporation, Taipei, Taiwan). After treatment, the cells were lysed. Resulting cell suspensions were then centrifuged at 12000 rpm for 1 min. Next, 50 µl of reaction buffer was added to 50 µl of cell supernatant. After incubation for 2 h at 37 ºC, the absorbance was measured spectrophotometrically at a wavelength of 405 nm.

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Statistical analysis

Results were presented as the way of the mean ± standard deviation (SD) of at least triplicate experiments. Statistical evaluation between groups was performed using analysis of variance (ANOVA) and Student's *t*-test. GraphPad Prism software (GraphPad, San Diego,CA,USA)was employed to analyze all data. Values of *P* equal to or less than 0.05 were considered statistically significant.

Results

Curcumin and ABT-737 exhibited potent cytotoxicity against glioblastoma cells

To determine the cytotoxic effect of curcumin and ABT-737 on glioblastoma cells, a combination treatment of curcumin and ABT-737 on U373-MG and T98G cells was studied. The results of MTT assay revealed that treatment with either curcumin or ABT-737 alone significantly inhibited the cell survival dose-dependently (Figure 1, relative to the blank control). As shown in Table 1, the IC_{50} values for curcumin and ABT-737 for 48 h of treatment were 52. 1 and 18 µM in U373-MG cells, and 16.6 and 34 µM in T98G cells, respectively. Moreover, combination treatment with curcumin and ABT-737 led to further decrease in the cell survival rate and IC_{50} values ($p<0.05$, relative to single therapy).

The interaction between curcumin and ABT-737 exhibited a synergistic effect in glioblastoma cells

To determine whether the combination effect of curcumin and ABT-737 on cell survival is synergistic effect, the CI analysis was performed using CompuSyn software. The results demonstrated that the combination effect of curcumin (0–64 nM) with ABT-737 (0-64 µM) on glioblastoma cells was synergistic (CI<1) across all concentrations. Our data also revealed that the best synergistic effect of 48 h was observed at 64 µM curcumin in combination with 64 µM ABT-737 (CI=0.68), with Fa level of 0.98 in U373-MG cells (Figure 1). Also, in T98G cells the best synergistic effect was obtained at 16 µM curcumin in combination with 16 µM ABT-737 (CI=0.69), with Fa level of 0.65 (Figure 1).

Curcumin augmented the growth inhibitory effect of ABT-737 in glioblastoma cells

We next investigated the combination effect of curcumin and ABT-737 on glioblastoma cells. The cells were treated with curcumin and ABT-737 for 24-120 h, and next, the cell viability was assessed using trypan blue dye exclusion assay. Results demonstrated that compared with the blank control group, the viability of the cells in curcumin, ABT-737 and combinatorial group, significantly decreased time-dependently. Twenty-four hours after treatment, the cell viability of U373-MG cells in curcumin, ABT-737 and combinatorial groups decreased to 77.5 %, 76.3 % and 69.4 % after 24 h, and to a further 41.3 %, 38.8 % and 37.2 %, respectively, at the end of the assay ($p<0.05$; Figure 1). Similar results were observed in the T98G cell line. Results show that the growth inhibitory effect was enhanced when the two compounds were combined, as evidenced by the lower IC_{50} value used in combined group.

Curcumin reversed the effect of ABT-737 on Mcl-1 expression

The effect of curcumin and ABT-737 on the Mcl-1 and MMP-2 mRNA expression was determined by qRT-PCR method. The results showed that 48 h treatment of the cells with curcumin decreased MMP-2 and Mcl-1 mRNA expression (compared to the blank control group, $p<0.05$). In addition, exposure of cells with ABT-737 did not result in a notable change in mRNA levels of MMP-2, while it upregulated the expression of Mcl-1. The combination treatment resulted in a significant reduction in the expression of MMP- 2 when

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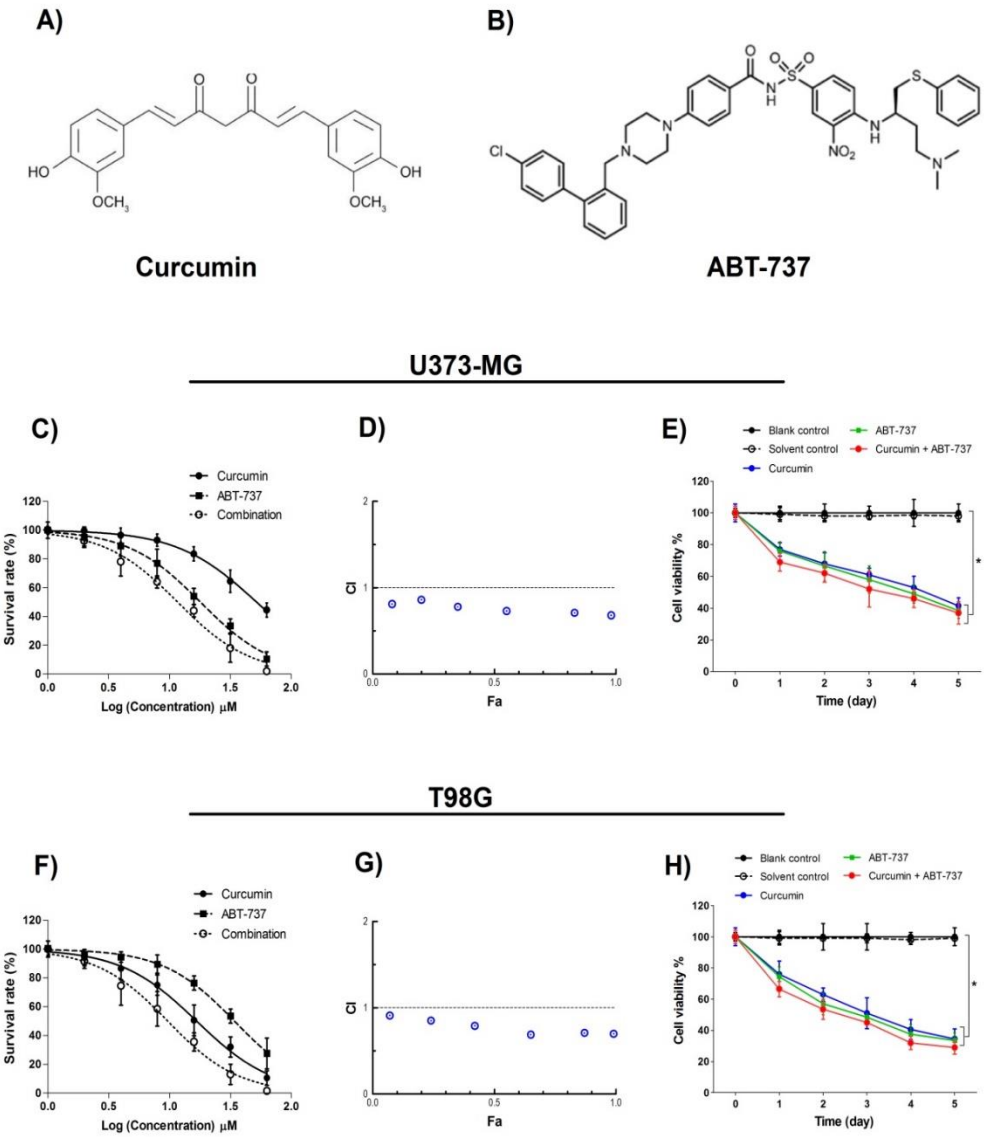

Fig.1. Effect of curcumin and ABT-737 on cell growth and survival. The glioblastoma cells were exposed to the curcumin (A) and ABT-737 (B) at concentrations outlined in the study. To determine the survival rate of the cells, the MTT assay was performed after a 24-hour incubation period. GraphPad software was employed to generate cell survival curves by utilizing the obtained data (C and F). The mean±SD of three independent experiments is depicted in the data provided within this study. By utilizing CalcuSyn software, the combination index (CI) values were calculated based on the examination of Fa values derived from the MTT assay (D and G). E and H show the growth curves of the breast cancer cells.

Table 1. IC_{50} values of the curcumin and ABT-737, alone and in combination, in glioblastoma cell lines.

	IC_{50} (μM)	
	U373-MG	**T98G**
Curcumin	52.1	16.6
ABT-737	18	34
Combination	11.6*	9.6*

Data are expressed as the mean±SD (n=3). *p<0.05 versus single treatment

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compared to both the control group and cells treated solely with ABT-737 (Figure 2). The effect of combination therapy on MMP-2 mRNA levels was lower than the curcumin treated cells. The expression levels of Mcl-1 mRNA in combination group demonstrated a noticeable variation compared with single treatment group and control group (Figure 2). The findings demonstrated that EGCG has the ability to counteract the elevated levels of Mcl-1 that are caused by ABT-737. The mRNA expression levels between the solvent control and blank control groups did not show any significant variation. The findings were consistent across both U373-MG and T98G cell lines, indicating that the treatment had comparable effects on both cell lines (Figure 2).

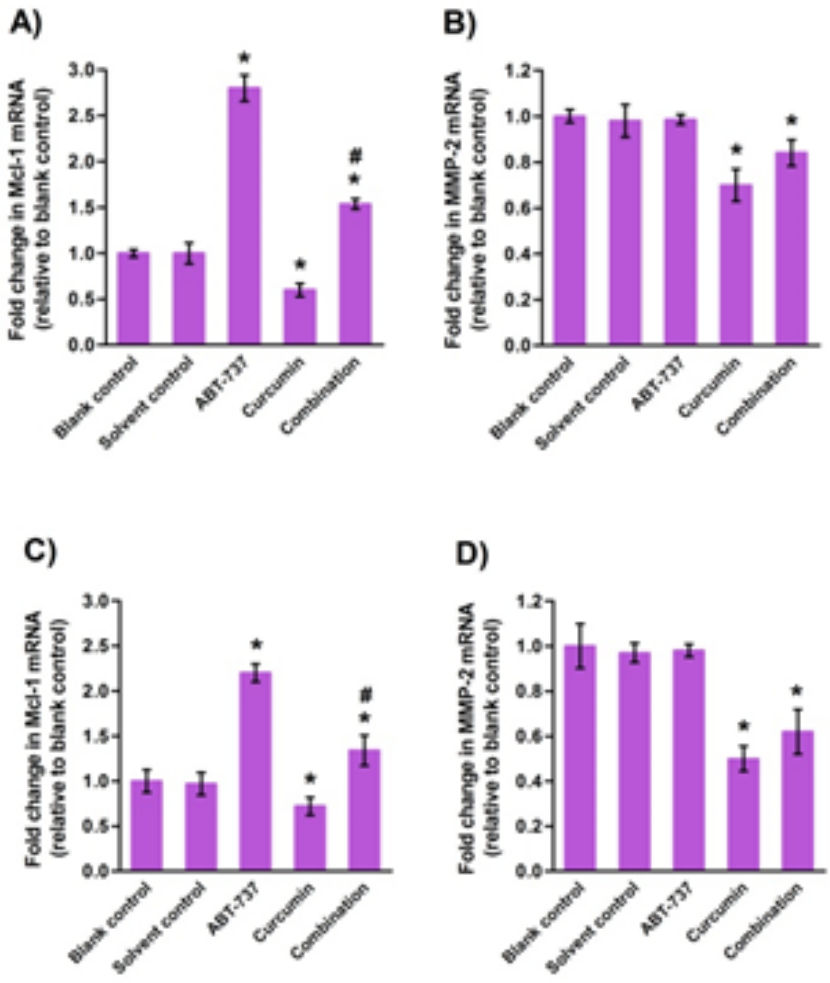

Fig.2. The analysis of glioblastoma cells using RT-qPCR. The U373-MG and T98G glioblastoma cells were exposed to the curcumin and ABT-737 (IC50 doses) for 24 h. The evaluation of Mcl-1 and MMP-2 mRNA expression levels in U373-MG and (A and B) and T98G (C and D) cells was carried out using the RT-qPCR technique and the $2^{-(\Delta\Delta Ct)}$ method. The mean value along with the standard deviation (SD) is presented as results (n=3). $^{\#}p< 0.05$ relative to single treatment; $^{*}p<0.05$ relative to blank control.

Curcumin enhanced the ABT-737-mediated suppression of glioblastoma cell migration

The wound-healing assay was employed to examine the impact of curcumin and ABT-737, both individually and in combination, on the migration of glioblastoma cells. The results demonstrated that both

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curcumin and ABT-737 alone were successful in inhibiting cell migration. However, when these two compounds were combined, the same inhibitory effect was achieved at a lower dose (Figure 3). This suggests that curcumin can enhance the inhibitory effects of ABT-737 on the migration of glioblastoma cells. The quantitative analysis of wound healing was visually depicted in Figure 3.

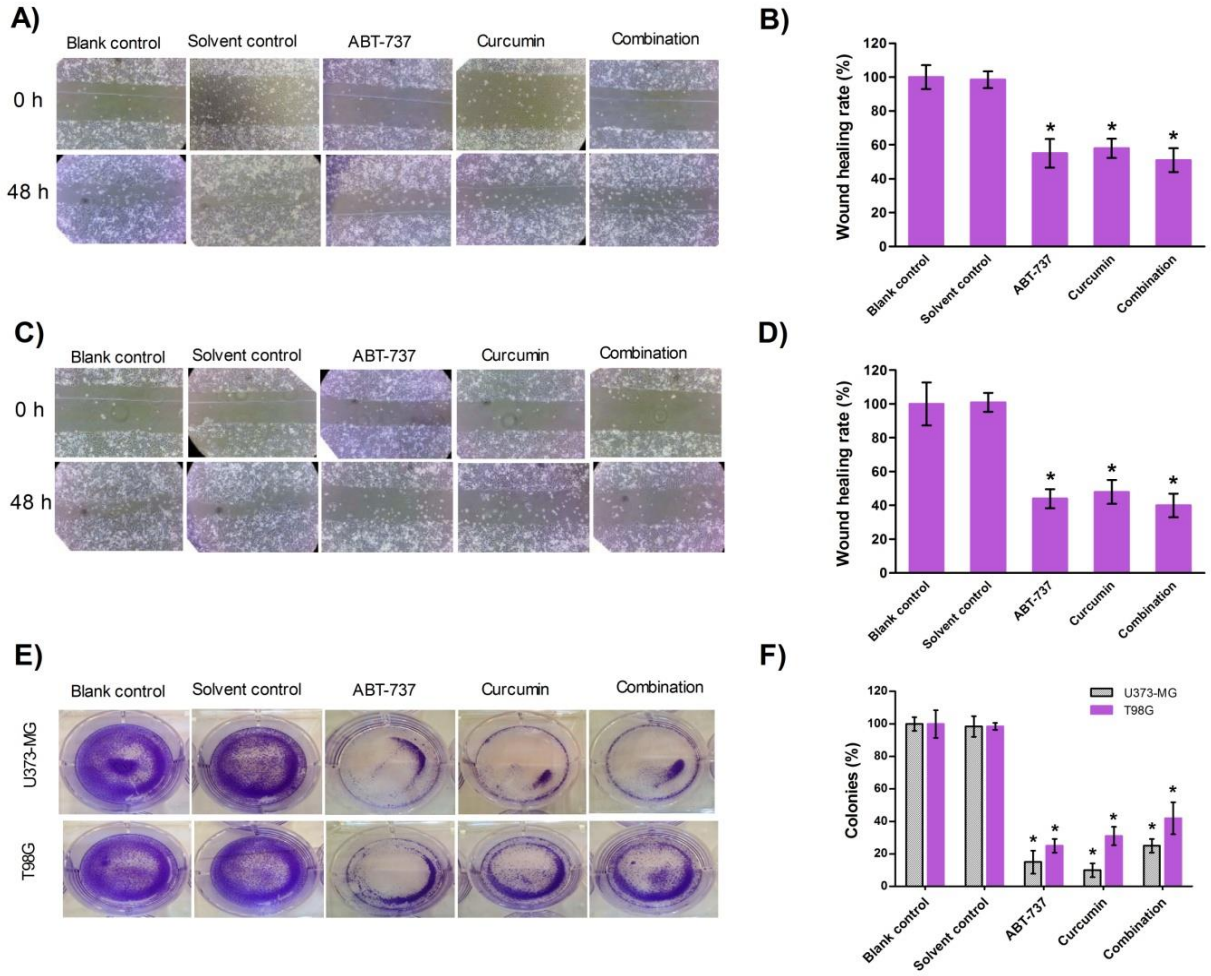

Fig.3. Effect of curcumin and ABT-737 on colony formation and migration of glioblastoma cells. The cells were treated with IC_{50} doses of curcumin and ABT-737 for 48 h. The migration of U373-MG (A and B) and T98G cells (C and D) was assessed by measuring wound closure areas 48 h after treatments. E and F show the effect of curcumin and ABT-737 on colony formation in glioblastoma cells. The cell colonies were stained with crystal violet and the number of colonies was observed after 48 h. Results were representative of three independent experiments.

Curcumin inhibited the colony formation rate of glioblastoma cells

The colony formation assay was employed to assess the antitumor effects of curcumin and ABT-737 on U737-MG and T98G cells. The results indicated a significant decrease in the colony formation ability of both cell lines following exposure to curcumin and ABT-737 (Figure 3). Furthermore, when the two compounds were used in combination, a marked reduction in colony formation was observed in comparison to the blank control group ($p<0.05$), suggesting a synergistic effect in inhibiting colony formation in both U737-MG and

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T98G cells. The IC_{50} dose of combination treatment is found to be lower than IC_{50} dose of either compound treated individually. As a result, it can be inferred that the combination treatment processes a more potent impact on colony formation in comparison to the single treatment.

Curcumin augmented the apoptotic impact of ABT-737 in glioblastoma cells

To determine whether the cytotoxicity of agents on glioblastoma cells was related to the induction of apoptosis, the U373-MG and T98G cells were treated with the IC_{50} doses of curcumin, ABT-737 and their combination for 48 h. Then, we conducted Hoechst 33342 staining and ELISA cell death assay. The presence of apoptotic cells exhibiting alteration in nuclear morphology is evident in both the curcumin and ABT-737 groups (Figure 4). Conversely, no such changes were observed in control groups, highlighting the distinct effects of these compounds. Moreover, there was no notable distinction in the number of apoptotic cells between the combination group and the groups that received single compounds.

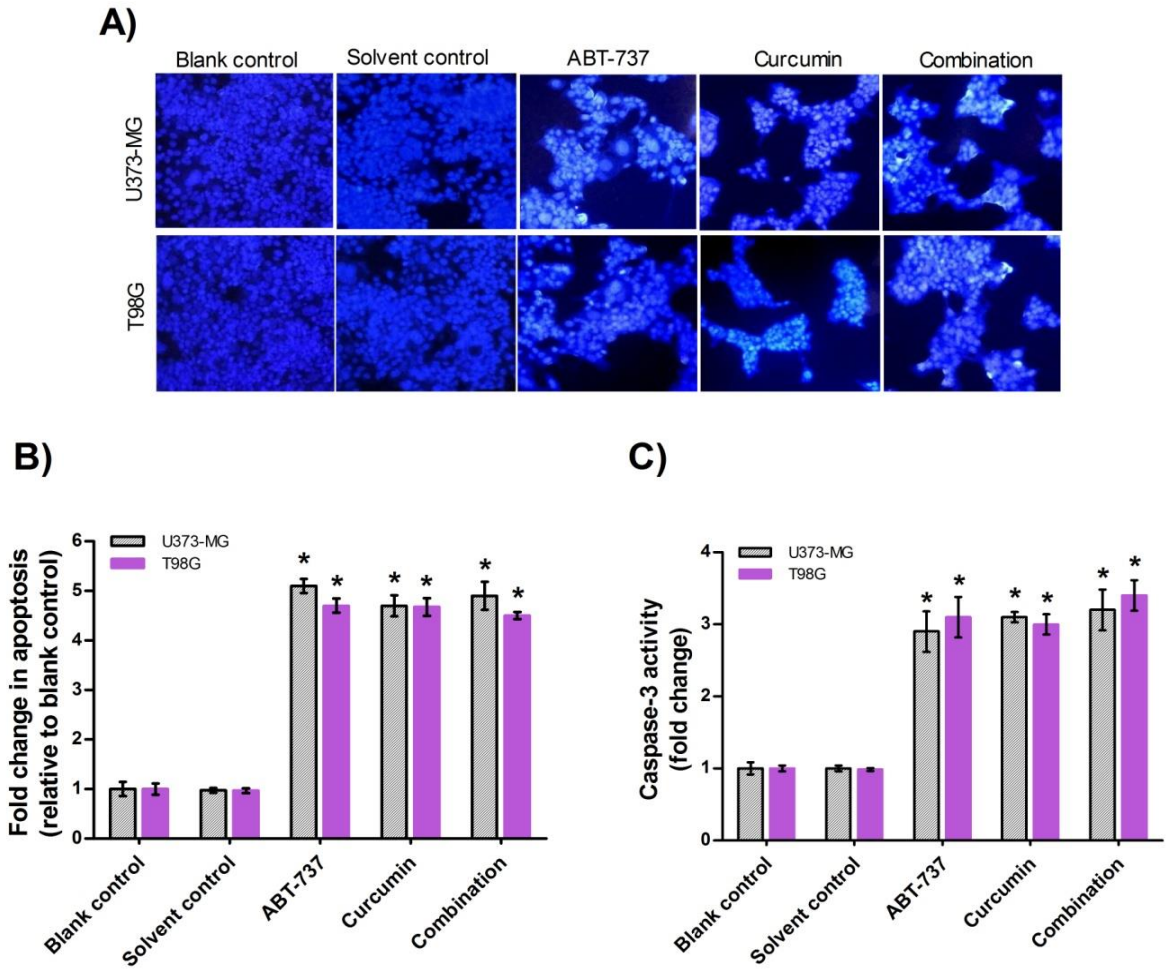

Fig.4. Induction of apoptosis by curcumin and ABT-737 in glioblastoma cells. U373-MG and T98G cells were exposed to IC_{50} doses of curcumin and ABT-737 for 48 h. Nuclear morphological changes were determined using Hoechst33342 staining and a fluorescence microscope (A). Fold change in apoptosis was assessed using ELISA cell death assay (B). C, show caspase-3 activity of glioblastoma cells after 48 h treatment.

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The results of the ELISA cell death assay showed that at 48 h exposure of the U373-MG cells to curcumin and ABT-737, the extent of apoptosis significantly increased by 4.65 and 4.70 fold, respectively, relative to the blank control group ($p<0.05$). Furthermore, when the two compounds were combined, the apoptosis level was elevated to 4.52 fold at the specified time point ($p<0.05$). No significant changes in apoptosis were observed in the solvent control group when compared to the blank control group ($p>0.05$). Similar results were observed in the T98G cell line.

The IC_{50} dose of combination treatment is found to be lower than IC_{50} dose of either compound treated individually. As a result, it can be inferred that the combination treatment processes a more potent impact on initiating apoptosis in comparison to the single treatment.

Caspase-3 activity enhanced after treatment of the cells with curcumin and ABT-737

To elucidate the molecular mechanism underlying apoptosis induced by curcumin and ABT-737, we conducted a caspase-3 activity assay. Results revealed a significant increase in caspase-3 activity in cells treated with curcumin and ABT-737, when compared to the control cells ($p<0.05$). Interestingly, when the cells were exposed to a combination of these two compounds, the level of caspase-3 activity observed was not significantly different from the caspase-3 activity observed in cells exposed to either compound alone ($p>0.05$, Figure 4).

Discussion

Despite the availability of treatment options like surgery, radiation therapy, and chemotherapy, patients diagnosed with GBM continue to face a discouraging future. The prognosis remains bleak, with an average survival time of around 1-2 years. The main factor contributing to this grim reality is the inherent resistance of GBM cells to different chemotherapy drugs (2). This emphasizes the importance of developing novel drugs or treatment plans to confront these challenges and enhance the prognosis for all individuals. The upregulation of anti-apoptotic proteins has been observed to be connected with accelerated cellular growth, heightened resistance to drugs, decreased occurrence of cell apoptosis, and an unfavorable prognosis in patients diagnosed with GBM (4, 5). Moreover, studies indicate that the rise in Mcl-1 protein levels within cells plays a crucial role in the development of resistance to ABT-737 in cancer cells. This resistance can be effectively countered by utilizing a combination of ABT-737 with agents that inhibit or suppress Mcl-1 (6, 7, 11, 13). In this study, the effect of curcumin on the growth, apoptosis and sensitivity of the glioblastoma cells to ABT-737 has been explored.

The results of our study demonstrated that treatment with either curcumin or ABT-737 alone significantly inhibited the survival and triggered apoptosis. Combination treatment with curcumin and ABT-737 led to a significant decrease in the IC_{50} value and synergistically lowered the cell survival rate relative to curcumin or ABT-737 alone. The IC_{50} dose required for the combination treatment was found to be lower than the IC_{50} dose needed for either compound when used alone. This indicates that our results suggest a more pronounced impact on cell growth and apoptosis when the two agents are combined, as opposed to when they are administered individually. To date, multiple research studies have explored the correlation between the Mcl-1 gene expression levels in cancer cells and their resistance to ABT-737. For example, Woo et al. (11) investigated the effect of YM155, a survivin inhibitor, and ABT-737 on cellular apoptosis in

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glioma, renal cell carcinoma and lung cancer cells. They found that YM155 effectively improved the sensitivity of the tumor cells to ABT-737 through down-regulation of Mcl-1 expression. Maji et al. (20) found that oral squamous cell carcinoma cells that have resistance to ABT-737 over-expressed the Mcl-1 and, knocking down Mcl-1 could overcome this resistance. Kiprianova et al. (21) explored the STAT3/Mcl-1 signaling pathway as a target to overcome the resistance of glioblastoma cells to the ABT-737. They found that STAT3 is a key upstream regulator of Mcl-1. Moreover, they showed that sorafenib, a multikinase inhibitor, targets Mcl-1 in a STAT3-dependent manner, and thereby sensitizes glioma cells to treatment with ABT-737. Tahir et al. (22) conducted a research study focusing on the impact of Bcl-2 family members on the cellular reaction of various small-cell lung cancers (SCLC) cell lines to ABT-737. They found that ABT-737-sensitive SCLC cell lines were identified by their increased expression of Bcl-2, Bcl-xL, Bim, and Noxa, and decreased expression of Mcl-1. Wang et al. (23) demonstrated that A-1210477, a specific inhibitor of Mcl-1, was effective in overcoming resistance to ABT-737 in AML cells that exhibited increased levels of Mcl-1. The combined treatment of A-1210477 and ABT-737 led to a synergistic induction of apoptosis in AML cells. Our data are in agreement with the above reports and show that curcumin can increase the sensitivity of the glioblastoma cells to ABT-737 by inhibition of the Mcl-1expression.

We also investigated the impact of curcumin and ABT-737 on gene expression. Our analysis using qPCR revealed that ABT-737 resulted in an increase in the expression of Mcl-1 mRNA. However, no significant effect on the expression of MMP-2 mRNA was observed. On the other hand, curcumin was found to decrease the expression levels of both MMP-2 and Mcl-1 mRNA in cancerous cells. Moreover, when the two compounds, curcumin and ABT-737, were used in combination, an intriguing interaction was observed. Curcumin was able to counteract the upregulation of Mcl-1 mRNA caused by ABT-737. These changes in gene expression were further associated with important cellular process. The alterations in gene expression were found to be linked to the inhibition of cell proliferation, reduced colony formation, and decreased cell migration. Additionally, the sensitivity of glioblastoma cells to ABT-737 was found to increase after treatment with curcumin. In line with our research, various studies have been conducted to investigate the effect of curcumin on gene expression and cellular processes of tumor cells. A previous study demonstrated that curcumin has the ability to inhibit the growth of tumors and trigger programmed cell death by blocking pathways that promote cell survival. These pathways include STAT3, AKT, and NF-kB, which are crucial for the survival of CLL B cells. Additionally, curcumin was found to suppress the expression of anti-apoptotic proteins Mcl-1 and XIAP, while simultaneously increasing the levels of the pro-apoptotic protein Bim (24). In another study, the impact of NVP-BEZ235, a dual PI3K/Akt and mTOR inhibitor, and curcumin on human renal carcinoma Caki cells was investigated. The findings from this study demonstrated that curcumin effectively triggered apoptosis in cells treated with NVP-BEZ235. Furthermore, the combined administration of NVP-BEZ235 and curcumin induced apoptosis by down-regulating Bcl-2 mRNA at the transcriptional level in a p53-dependent manner, as well as downregulating Mcl-1 protein at the post-transcriptional level (25). Dhandapani et al. (26) investigated the role of curcumin on survival and drug resistance in human and rat glioma cell lines. The results of the research indicated that curcumin reduces cell survival independently of p53 and caspase, by inhibiting the AP-1 and NF-kB signaling pathways through the prevention of constitutive JNK and Akt activation. Moreover, curcumin was observed to enhance the sensitivity of glioma

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cells to various chemotherapeutic agents (doxorubicin, camptothecin, cisplatin, and etoposide) and radiation, which was correlated with a decrease in the expression of Bcl-2 and IAP family members. Another study showed that curcumin potently inhibited glioblastoma cell proliferation as well as migration and invasion in by downregulating the JAK-STAT3 pathway (27). Zanotto-Filho et al. (28) in a study examined the effect of curcumin on glioblastoma cells in vitro and in vivo. Results show that curcumin markedly inhibited proliferation and migration and induced cell death in glioblastoma cells. Moreover, curcumin inhibited the PI3K/Akt and NF-kB survival pathways, suppressed the anti-apoptotic NF-kB-regulated protein Bcl-xL and induced mitochondrial pathway of apoptosis. Furthermore, curcumin synergistically enhanced the apoptotic effects of chemotherapeutics doxorubicin and cisplatin in glioblastoma cells. The findings from the above reports align with our own data, indicating that the use of curcumin may enhance the sensitivity of cancer cells to chemotherapy drugs like ABT-737 by triggering apoptosis.

The mitochondrial or intrinsic pathway of cellular apoptosis is activated by internal stimuli that are detrimental to the cell. These stimuli prompt the release of cytochrome c and the activation of caspases-9 (29). In contrast, the extrinsic pathway is initiated by external ligands binding to death receptors on the cell's surface. This binding triggers the activation of caspase-8. Both the intrinsic and extrinsic pathways converge at caspase-3, a key player in the apoptotic process. Once caspase-3 is activated, it sets off a proteolytic cascade, activating other caspases and initiating a series of events that ultimately lead to apoptosis (29). The regulation of intrinsic pathway is performed by the pro- and anti-apoptotic members of the Bcl-2 family proteins (30). In apoptotic conditions, the pro-apoptotic members such as Bak and Bax are activated. Activated Bak and Bax cause the mitochondrial outer membrane permeability (MOMP), release of cytochrome c into the cytoplasm, and subsequently activation of caspases. The anti-apoptotic proteins such as Bcl-2 and Mcl-1, when not sequestered by pro-apoptotic members, inhibit apoptosis (31). ABT-737, a synthetic compound designed to mimic the BH3 domain, demonstrates a high level of binding affinity towards the Bcl-2, Bcl-xL, and Bcl-w proteins. Conversely, its interaction with the Mcl-1 protein is notably weaker. Studies have shown that an increase in Mcl-1 expression is associated with reduced effectiveness of ABT-737 and the emergence of resistance to this compound. As a result, targeting Mcl-1 has been proposed as a strategy to augment the responsiveness to ABT-737 (5-9).

The effects of curcumin on cellular behavior are mediated through its ability to influence the expression of various proteins. Notably, curcumin has been shown to impact the levels of Bcl-xL, XIAP, Mcl-1, and Bcl-2, which are crucial regulators of cell survival and death. These alterations are achieved through the activation of signaling pathways, including AKT, NF-kB, and STAT3. By modulating these pathways, curcumin exerts its inhibitory effects on cell proliferation while simultaneously triggering apoptosis (32). Research findings have demonstrated that curcumin possesses the ability to trigger apoptosis via the extrinsic and intrinsic pathways. Despite these observations, the precise mechanism by which curcumin induces apoptosis remains ambiguous (33). In this study, we illustrated the ability of curcumin to reduce the expression of Mcl-1. This alteration in gene expression was shown to have a strong association with the onset of cellular apoptosis. Furthermore, the decrease in Mcl-1 levels was linked to an increased sensitivity to ABT-737. The results obtained from our research demonstrate that curcumin possesses the capacity to not only initiate the process of cell death but also enhance the susceptibility of glioblastoma cells towards

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chemotherapeutic medications such as ABT-737. This particular impact is accomplished through the modification of Mcl-1 expression, a protein that plays a crucial role in promoting cell survival.

In this study, we also investigated the impact of the compounds on cell migration. Matrix metalloproteinases (MMPs) are a group of enzymes that have a significant role in the advancement of cancer, particularly in processes such as tumor invasion, neoangiogenesis, and metastasis. The MMPs are responsible for breaking down the extracellular matrix, which enables cancer cells to migrate and invade surrounding tissues. Previous research has shown that elevated levels of MMPs are associated with poor overall survival rates in various types of solid tumors, including breast, lung, colon, gastric, pancreatic, and prostate cancer (34). Our findings from this study demonstrated that the administration of either ABT-737 or curcumin resulted in a reduction in the rate of cell migration. Specifically, the inhibition of cell migration by curcumin was linked to the suppression of the MMP-2 gene expression in glioblastoma cells. These results are consistent with previous studies and provide further evidence that curcumin plays a role in inhibiting cell migration and metastasis by downregulating the expression of MMP-2 (32). This suggests that curcumin may have potential therapeutic implications in the management of cancer progression and metastasis.

To summarize, our findings highlight the promising therapeutic potential of combining curcumin and ABT-737 in the treatment of glioblastoma. This combination treatment not only resulted in a significant reduction in the IC_{50} value, but also demonstrated synergistic effects in inhibiting colony formation, impeding cell growth, and promoting cell death. Moreover, curcumin exhibited its ability to inhibit cell migration and induce apoptosis by downregulating MMP-2 and Mcl-1, respectively. Interestingly, the expression of Mcl-1 was found to increase after treatment with ABT-737. Furthermore, curcumin effectively enhanced the apoptotic effect of ABT-737 by suppressing Mcl-1. These findings provide valuable insights into potential therapeutic strategies for glioblastoma treatment. In the future, the effect of curcumin on the sensitivity of glioblastoma cells to ABT-737 at *in vivo* and clinical trial studies can be conducted. The most important challenges in this regard may be the issue of curcumin's half-life in plasma and its low delivery to the cell, which can be solved by nanoparticles.

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