



# Modulation of Autophagy is a Potential Strategy for Enhancing the Anti-Tumor Effect of Mebendazole in Glioblastoma Cells

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## Abstract

Mebendazole (MBZ), a microtubule depolymerizing drug commonly used for the treatment of helminthic infections, has been suggested as a repositioning candidate for the treatment of brain tumors. However, the efficacy of MBZ needs further study to improve the beneficial effect on the survival of those patients. In this study, we explored a novel strategy to improve MBZ efficacy using a drug combination. When glioblastoma cells were treated with MBZ, cell proliferation was dose-dependently inhibited with an IC<sub>50</sub> of less than 1 μM. MBZ treatment also inhibited glioblastoma cell migration with an IC<sub>50</sub> of less than 3 μM in the Boyden chamber migration assay. MBZ induced G2-M cell cycle arrest in U87 and U373 cells within 24 h. Then, at 72 h of treatment, it mainly caused cell death in U87 cells with an increased sub-G1 fraction, whereas polyploidy was seen in U373 cells. However, MBZ treatment did not affect ERK1/2 activation stimulated by growth factors. The marked induction of autophagy by MBZ was observed, without any increased expression of autophagy-related genes ATG5/7 and Beclin 1. Co-treatment with MBZ and the autophagy inhibitor chloroquine (CQ) markedly enhanced the anti-proliferative effects of MBZ in the cells. Triple combination treatment with temozolomide (TMZ) (another autophagy inducer) further enhanced the anti-proliferative effect of MBZ and CQ. The combination of MBZ and CQ also showed an enhanced effect in TMZ-resistant glioblastoma cells. Therefore, we suggest that the modulation of protective autophagy could be an efficient strategy for enhancing the anti-tumor efficacy of MBZ in glioblastoma cells.

**Key Words:** Mebendazole, Autophagy, Autophagy inhibitor, Chloroquine, Glioblastoma

## INTRODUCTION

Glioblastoma is the most aggressive incurable adult brain tumor, accounting for about 60% of adult brain tumors (Wu *et al.*, 2021). The average 5-year patient survival rate is 7.2%. Despite the development of novel therapies and techniques, little improvement in overall survival (OS) has been achieved, partly due to limited anti-cancer drug penetration through the blood-brain barrier (BBB). Surgery is the most effective treatment for glioblastoma patients, and radiotherapy (RT) is the most accepted adjuvant therapy (Veliz *et al.*, 2015). Temozolomide (TMZ) has been used as the first-choice chemotherapeutic agent for glioblastoma treatment, since it replaced the PCV regimen [a combination of procarbazine, 1-(2-chloroethyl)-3-cy-

clohexyl-1-nitrosourea (CCNU), and vincristine] in 2005 (Stupp *et al.*, 2005). When combined with RT, TMZ showed weaker side effects and greater efficacy compared to PCV. However, the current standard of care using TMZ and RT for glioblastoma still leads to poor prognosis with a median OS of 14.6 months. Thus, many efforts have been made to identify TMZ adjuvants or substitutes, but no promising drugs have been found for curing glioblastoma patients.

Mebendazole (MBZ), a benzimidazole derivative, is widely used as an anthelmintic drug along with albendazole and acts as a microtubule-disrupting agent by binding to the colchicine-binding domain of tubulin (Friedman and Platzer, 1980, Lacleite *et al.*, 1980). The cytotoxic effect of MBZ has been reported in several cancer cells such as melanoma, gastric, and

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lung cancers (Mukhopadhyay *et al.*, 2002; Doudican *et al.*, 2008; Pinto *et al.*, 2015). The survival benefit of MBZ in glioblastoma animal models supported the possibility of MBZ as a potent repositioning drug candidate for glioblastoma treatment (Bai *et al.*, 2011). A correlation between microtubule polymerization inhibition and cytotoxicity was also reported in glioblastoma cells treated with MBZ (De Witt *et al.*, 2017). MBZ has good BBB penetration, whereas vincristine (anti-microtubule agent) has poor BBB penetrance (Boyle *et al.*, 2004; Pantziarka *et al.*, 2014; Bai *et al.*, 2015b). MBZ showed greater efficacy with lesser side effects compared to vincristine in a preclinical model (De Witt *et al.*, 2017), strongly supporting the clinical use of MBZ for treating brain tumors.

Autophagy is the process of constant degradation of damaged or superfluous proteins and organelles under several stressful conditions. During this process, double-membrane vesicles termed autophagosomes, form, engulf damaged protein aggregates and organelles, and then fuse with lysosomes to recycle the components (Choi *et al.*, 2013a). The formation of autophagosomes is initiated by class III PI3K and Beclin 1. Autophagy-related gene 5 (ATG5) and autophagy-related gene 7 (ATG7) are involved as core proteins in the process. Autophagy plays a dual role in tumor suppression and promotion and regulates the stemness of cancer stem cells, tumor recurrence, and resistance to anticancer agents (Yun and Lee 2018; Shin, 2020). Autophagy is activated in tumor cells by chemotherapeutic agents and radiation and serves to protect the tumor cells (Paglin *et al.*, 2001; Kanzawa *et al.*, 2004; Sui *et al.*, 2013). Thus, targeting autophagy has emerged as a promising strategy to enhance anti-cancer efficacy and overcome tumor resistance to various cancer treatments (Xiao *et al.*, 2021).

Previously, we reported that MBZ induced autophagy in endothelial cells, and inhibited endothelial proliferation, migration, and tube formation (Sung *et al.*, 2019). Autophagy inhibition by siRNAs against Beclin 1 or ATG 5/7 and the autophagy inhibitor chloroquine (CQ) enhanced the anti-proliferative and pro-apoptotic effects of MBZ in endothelial cells. CQ (weakly basic) is widely known as an antimalarial drug and increases vacuolar pH by accumulating in acidic lysosomes, preventing the fusion of autophagosomes with lysosomes, and inhibiting the late stage of autophagy (Poole and Ohkuma, 1981). In glioblastoma patients, the addition of CQ to glioblastoma standard therapy was shown to exert a beneficial adjuvant effect (Briceño *et al.*, 2007). Therefore, in this study, we evaluated the efficacy of the combination of MBZ and CQ in glioblastoma cells as a novel treatment option.

## MATERIALS AND METHODS

### Reagents

Mebendazole (MBZ), chloroquine (CQ), and temozolomide (TMZ) were purchased from Sigma (St. Louis, MO, USA). MBZ and TMZ were dissolved in dimethyl sulfoxide (DMSO). CQ diphosphate was dissolved in phosphate-buffered saline (PBS).

### Cell culture

Human glioblastoma cell lines, U87-MG and LN18 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). U373 and T98G cells were pur-

chased from the Korean Cell Line Bank (KCLB, Seoul, Korea). To obtain TMZ-resistant cells, U87-MG and U373 cell lines were initially cultured in the presence of 25  $\mu$ M TMZ. The concentration was then increased by 2-fold at every two passages until it reached 800  $\mu$ M. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (FBS, Corning, NY, USA) and 1% antibiotics under standard culture conditions of 5% CO<sub>2</sub> and 37°C.

### Cell viability assay

The cells were seeded at  $1 \times 10^3$  cells per well in 96-well plates. After 24 h, fresh medium with or without inhibitor was added and cultured for 48-72 h. For combination treatment,  $1 \times 10^3$  cells were seeded in a 96-well plate. After 24 h, the cells were treated with 50  $\mu$ M TMZ and 0.2  $\mu$ M MBZ, incubated for 24 h, and then treated with 20  $\mu$ M CQ and incubated for 48 h. Cell proliferation was assessed using the MTS Cell Viability Assay Kit (Promega Corp., Madison, WI, USA). MTS reagent (20  $\mu$ L) was added to each well. After 2 h, the plates were read at 490 nm using an ELISA plate reader.

### Cell migration assay

The cell migration assay was performed in a modified Boyden chamber as described previously (Kim *et al.*, 2008). Serum-starved U87 and U373 cells were incubated with MBZ for 30 min and then seeded into the upper chamber ( $2 \times 10^4$  cells). DMEM containing 10% FBS was added to the lower chamber. Cell migration was allowed for 24 h, and then the migrated cells were fixed and stained with Diff-Quik solution (Sysmex Co., Kobe, Japan). The migrated cells were photographed and counted in five randomly selected fields.

### Cell cycle analysis

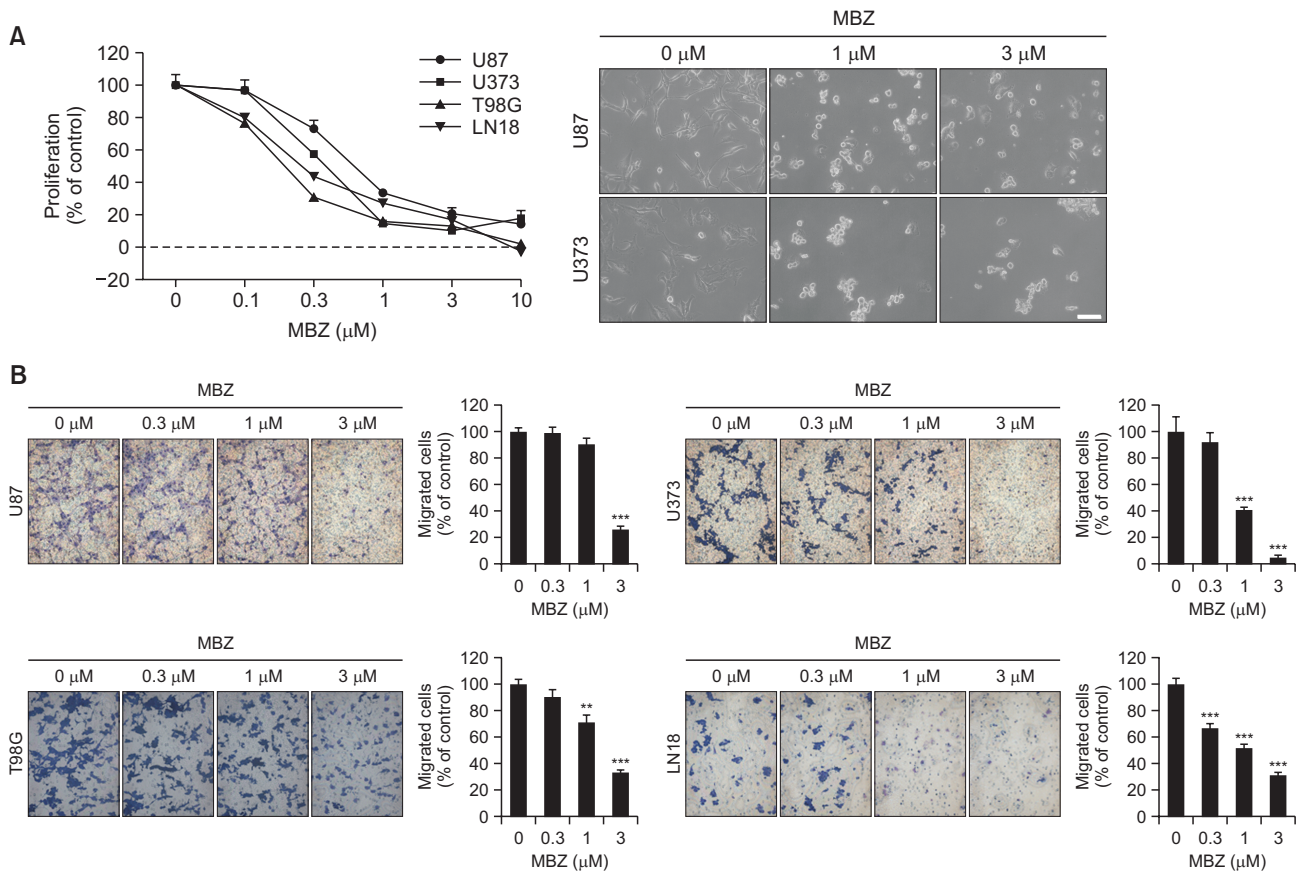
U87 and U373 cells were cultured for 24 h, serum-starved for 4 h, and then treated with MBZ (0, 1, and 3  $\mu$ M) for 24, 48, and 72 h. The cells were collected after trypsin-EDTA treatment, washed with PBS, and fixed with 70% ethanol overnight at  $-20^\circ\text{C}$ . After centrifugation, the cell pellets were resuspended in 1 mL of a hypotonic solution containing 50  $\mu$ g/mL of propidium iodide (PI), 0.1% sodium citrate, 0.1% Triton X-100, and 10  $\mu$ g/mL of RNase and incubated in the dark at room temperature for 30 min. PI fluorescence was measured on a linear scale using a FACScan flow cytometer (FACS Canto II, BD Biosciences, NJ, USA), and cell cycle distribution was analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

### DAPI staining to visualize apoptosis

To investigate apoptosis after MBZ treatment, the cells were stained with fluorescent 4',6-diamidino-2-phenylindole (DAPI) to detect nuclear condensation and chromatin fragmentation. U87 and U373 cells grown on coverslips were treated with MBZ for 72 h and fixed with 4% paraformaldehyde (PFA) for 10 min. The fixed cells were stained with 1  $\mu$ g/mL of DAPI solution (Sigma) for 10 min and examined under fluorescence microscopy (Observer Z1 inverted, ZEISS, Oberkochen, Germany). The percentage of apoptotic cells was calculated as the number of cells with apoptotic nuclei divided by the total number of cells in three random fields.

### Western blot analysis

Western blotting was performed as described previously



**Fig. 1.** Mebendazole inhibits the proliferation and migration of glioblastoma cells. (A) Glioblastoma cells (U87, U373, T98G, and LN18) were treated with the indicated concentrations of MBZ. After 48 h, the MTS assay was performed (left panel). The percentage of proliferation was calculated based on the cell proliferation of the untreated control group. U87 and U373 cells were treated with MBZ (1 and 3 μM) for 24 h and then observed by light microscopy (right panel). Scale bars: 100 μm. (B) Glioblastoma cells (U87, U373, T98G, and LN18) pre-treated with the indicated concentrations of MBZ for 30 min were allowed to migrate into the bottom chamber with medium containing 10% FBS for 24 h. Then, the migrated cells were counted per view field. \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

(Choi *et al.*, 2013b). Briefly, total cellular extracts were prepared by lysing cells in lysis buffer, followed by separating proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferring them to nitrocellulose membranes. The membranes were incubated with the designated primary antibodies against phospho-ERK1/2 (pThr<sup>202</sup>/Tyr<sup>204</sup>) (Cell Signaling Technology, Beverly, MA, USA), ERK1/2 (Cell Signaling Technology), LC3B (Cell Signaling Technology), Beclin 1 (Cell Signaling Technology), ATG5 (Cell Signaling Technology), ATG7 (Cell Signaling Technology), and β-actin (GeneTex, Irvine, CA, USA). Then, the membranes were incubated with the respective secondary antibodies (GeneTex). The immunoreactive bands were visualized using a chemiluminescent substrate (ECL kit) (GE Healthcare, Piscataway, NJ, USA) and the band intensity was measured using ImageJ software (National Institutes of Health, MD, USA).

**Data and statistical analysis**

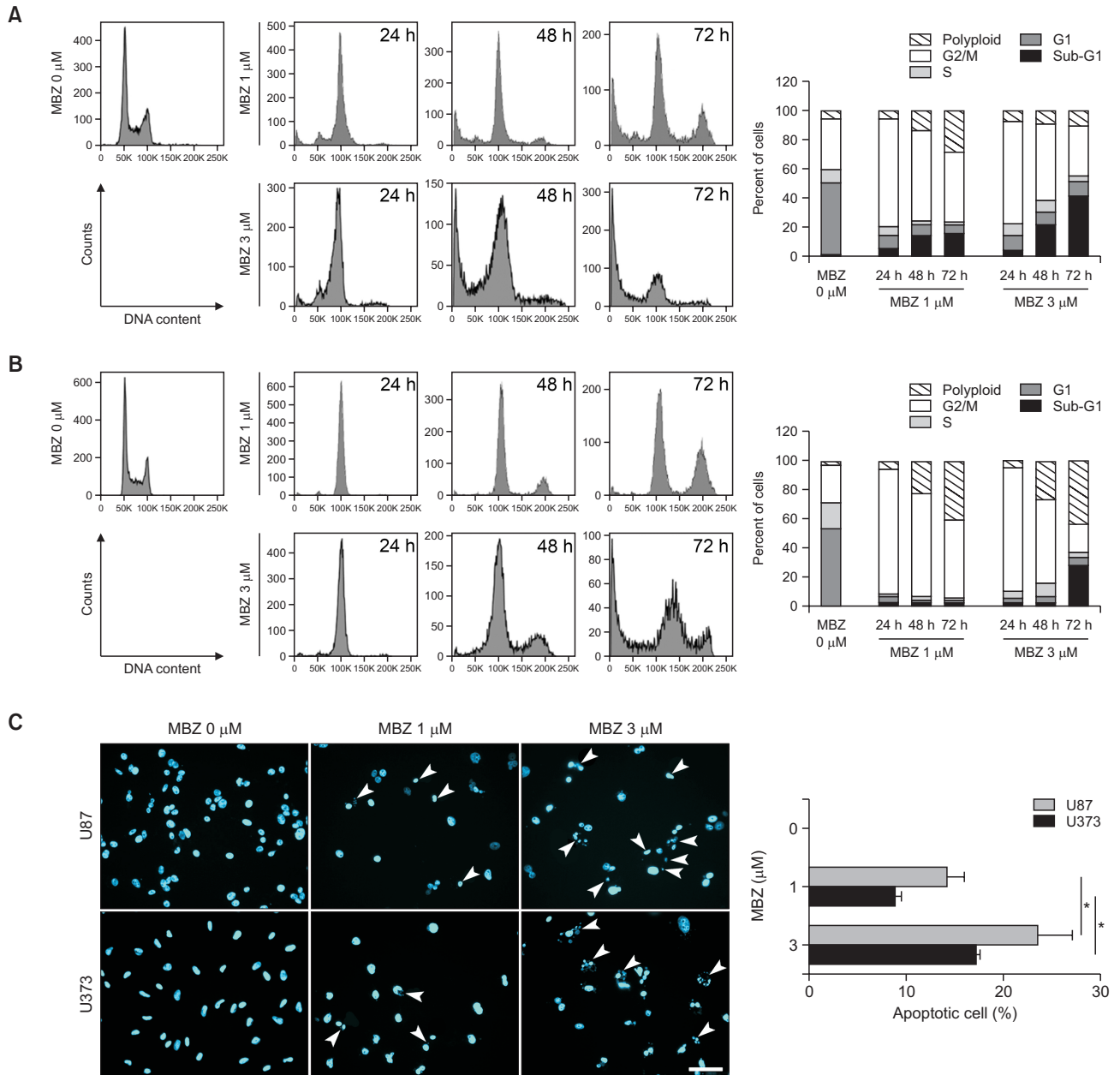
All experiments were repeated three times, and statistical values were presented as means ± standard error of the mean (SEM) of representative experiments. Differences between groups were analyzed by student’s t-test for comparison of two groups and one-way ANOVA with Turkey test for

comparison of three or more groups using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) for most experiments.  $p$ -values less than 0.05 were considered as statistically significant.

**RESULTS**

**Mebendazole inhibits the proliferation and migration of glioblastoma cells**

We investigated whether MBZ inhibited the proliferation of human glioblastoma cells under growth conditions. We tested U87, U373, T98G, and LN18 glioblastoma cell lines with different molecular backgrounds. As shown in Fig. 1A, all of the cells tested showed a dose-dependent inhibition of cell proliferation with IC<sub>50</sub> values of 0.22-0.65 μM. Treatment with 1 μM MBZ led to cell shrinkage at 24 h in both U87 and U373 cells, due to its anti-microtubule action. Upon MBZ treatment, the cells shrank, aggregated, or floated in the culture media. Next, we performed a modified Boyden chamber assay to examine whether MBZ inhibited the migration of glioblastoma cells. As shown in Fig. 1B, MBZ significantly inhibited 10% FBS-induced glioblastoma cell migration in a dose-dependent man-



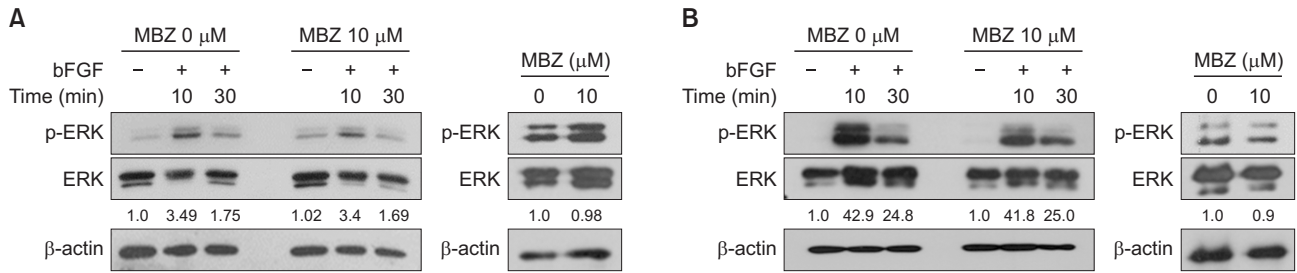
**Fig. 2.** Mebendazole induces G2-M arrest, polyploidy, and apoptosis in U87 and U373 cells. (A, B) U87 (A) and U373 (B) cells were treated with MBZ (1 and 3 μM) for 24, 48, and 72 h. The cell cycle distribution was analyzed by flow cytometry (left panel). Comparative quantification of cell percentages in different cell cycle phases is shown by a bar diagram (right panel). (C) U87 cells and U373 treated with MBZ (1 and 3 μM) for 72 h were fixed with 4% PFA and stained with DAPI to observe chromatin condensation and nuclear fragmentation (arrowheads). The percentage of apoptotic cells was calculated as the number of cells with apoptotic nuclei divided by the total number of cells in three random fields. Scale bar: 50 μm. \**p*<0.05.

ner. Overall, the effect of MBZ to inhibit migration ( $IC_{50}$ =0.88-2.25 μM) was less than its effect to inhibit proliferation. Thus, these results suggest that MBZ inhibits the proliferation and migration similarly in all the types of glioblastoma cells.

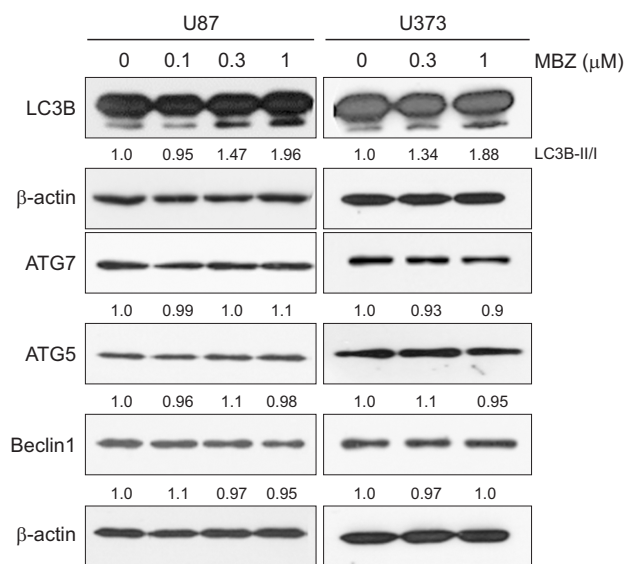
### Mebendazole induces G2-M arrest and apoptosis in glioblastoma cells

Since anti-microtubule agents induce G2-M cell cycle arrest and apoptosis (Kaur *et al.*, 2014), we examined whether MBZ

affected cell cycle progression in glioblastoma cells. U87 and U373 cells were treated with MBZ (1 and 3 μM) for 24, 48, and 72 h prior to DAPI staining and FACS analysis. An increase in G2-M arrest was observed in MBZ-treated U87 and U373 cells at 24 h (Fig. 2A, 2B). Then, the number of polypliod cells and cells in the sub-G1 fraction was increased over time from 24 h to 72 h. Treatment with 1 μM MBZ caused marked apoptosis in U87 cells (16.6% at 72 h), whereas few apoptotic U373 cells were seen at 1 μM MBZ treatment (3.1% at 72 h). Apoptosis



**Fig. 3.** Mebendazole does not inhibit the ERK1/2 activation pathway in glioblastoma cells. (A, B) U87 (A) and U373 (B) cells were incubated with MBZ (10 μM) for 1 h and then stimulated with bFGF (10 ng/mL) for the indicated time. U87 and U373 cells were incubated with MBZ (10 μM) in DMEM medium containing 10% FBS for 24 h (long-term). Then, the cells were harvested and analyzed by Western blotting to determine the phosphorylation of ERK1/2 and ERK1/2. β-actin was examined as the loading control. The numbers indicate the phospho-ERK1/2 (Thr202/Tyr204)/ERK1/2 ratio.



**Fig. 4.** Mebendazole induces autophagy in U87 and U373 cells. Cells were treated with the indicated concentrations of MBZ for 24 h. Then, the cells were harvested and analyzed by Western blotting to determine the protein levels of ATG5, ATG7, Beclin 1, and LC3B I/II. β-actin was examined as the loading control. The level of each protein was normalized to β-actin. The numbers indicate expression relative to that in the untreated control group.

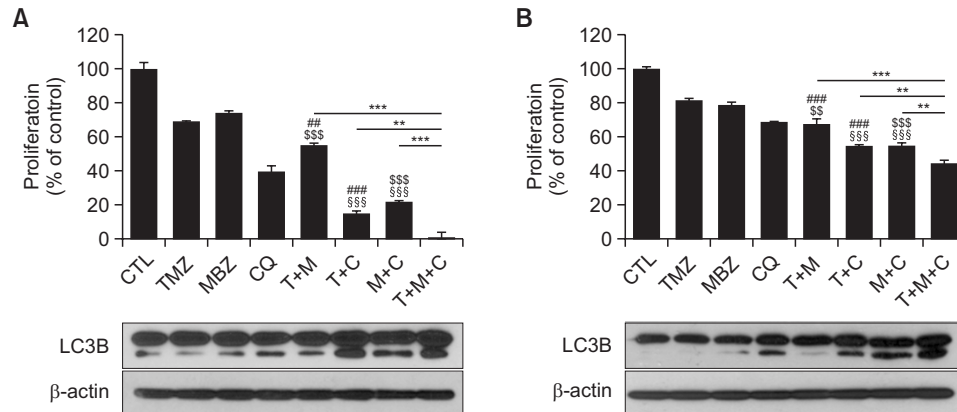
was greater in U87 cells treated with MBZ for 48 h and 72 h (15.2% and 16.6%, respectively, in the sub-G1 fraction), while a marked increase in the polyploid fraction was observed in U373 cells (22.3% at 48 h, 40.1% at 72 h). Longer MBZ treatment (72 h) of U373 cells at a higher concentration (3 μM) resulted in a marked increase in the sub-G1 fraction (28.8%). Consistent with the increases in the sub-G1 fraction by MBZ treatment, dose-dependent increases in chromatin condensation and nuclear fragmentation of the attached cells were observed in U87 and U373 cells treated with 1 and 3 μM MBZ (Fig. 2C). Upon treatment with MBZ, up to 24% of the U87 cells and 17% of the U373 cells showed chromatin condensation and nuclear fragmentation of the attached cells. The results indicate that MBZ induces G2-M arrest and apoptosis in glioblastoma cells.

### Mebendazole has no inhibitory effect on the ERK1/2 pathway in glioblastoma cells

Previously, MBZ was reported to interfere with vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) by competing with ATP (IC<sub>50</sub>=4.3 μM), thereby inhibiting the autophosphorylation of the Y1175 residue (Bai *et al.*, 2015a). This site provides a binding site for phospholipase C gamma (PLCγ), eventually leading to the phosphorylation of ERK1/2 (Takahashi *et al.*, 2001). Thus, there is a possibility that MBZ could also interfere with growth factor-induced signaling pathways by competing with ATP. Therefore, we examined whether MBZ could inhibit growth factor-induced ERK activation. In the experiment, we used a relatively high dose of MBZ to treat glioblastoma cells prior to growth factor stimulation. As shown in Fig. 3A, no significant inhibition of ERK activation was observed after bFGF stimulation in U87 cells pre-treated with 10 μM MBZ for 1 h. A similar pattern was observed in U373 cells pre-treated with MBZ (Fig. 3B). No change in phospho-ERK1/2 levels was observed when the cells were treated with 10 μM MBZ for a longer time (24 h) under culture conditions containing 10% FBS. Therefore, the results indicate that MBZ has no effect on the growth factor-stimulated ERK1/2 signaling pathway in glioblastoma cells.

### Mebendazole induces autophagy in glioblastoma cells

Benzimidazole drugs, flubendazole and albendazole, have been reported to induce autophagy in tumor cells (Zhang *et al.*, 2015, Jung *et al.*, 2022). Previously, we also demonstrated that MBZ increased LC3-positive puncta and LC3B-II/LC3B-I ratios in human umbilical vein endothelial cells time- or dose-dependently (Sung *et al.*, 2019). Here, we examined whether MBZ induced autophagy in glioblastoma cells. Western blot analysis of MBZ-treated U87 and U373 cells showed a dose-dependent increase in the LC3B-II/LC3B-I ratio (Fig. 4). When the expression level of Beclin 1, the main regulator of autophagy initiation, was examined by Western blot analysis, we found that MBZ treatment had no effect on Beclin 1 expression. The expression of ATG5 and ATG7, core proteins involved in autophagosome formation was also not affected by MBZ treatment. Thus, the results indicate that MBZ induces autophagy in glioblastoma cells without upregulating autophagosome formation-related proteins.



**Fig. 5.** Triple combination enhances the anti-proliferative effect of MBZ in glioblastoma cells. (A, B) U87 (A) and U373 (B) cells were treated with TMZ (50  $\mu$ M) and MBZ (0.2  $\mu$ M) for 24 h and then treated with CQ (20  $\mu$ M) for 48 h, and MTS analysis was performed. The percentage of proliferation was calculated based on the cell proliferation in the untreated control group (CTL). In parallel, the cells were harvested and analyzed by Western blotting to determine the level of LC3B I and II (bottom). \*\* $p$ <0.01 and \*\*\* $p$ <0.001; ### $p$ <0.001 and ### $p$ <0.001 vs. TMZ treatment; \$\$\$ $p$ <0.001 vs. MBZ treatment; \$\$\$ $p$ <0.001 vs. CQ treatment.

### Effect of the triple combination of TMZ, MBZ, and CQ on U87 and U373 cells

Previously, we demonstrated the synergistic efficacy of the combination of TMZ (autophagy inducer) and CQ (late-stage autophagy inhibitor) in glioblastoma cells (Lee *et al.*, 2015). Thus, we were interested in whether the inhibitory efficacy of autophagy-inducing MBZ could be enhanced by CQ in glioblastoma cells. Indeed, the inhibition of proliferation was enhanced by the combination of MBZ and CQ in U87 cells (data not shown). A previous preclinical model study showed that the combination of MBZ and TMZ extended survival further than TMZ treatment alone (Bai *et al.*, 2011). The main inhibitory mechanism of TMZ (DNA-alkylating agent) is different from that of MBZ, and both drugs induce autophagy. Thus, we can expect improved efficacy when TMZ and MBZ are combined with late-stage autophagy inhibitors. Therefore, we examined the efficacy of the triple combination of TMZ, MBZ, and CQ as a novel therapy to improve the efficacy of standard therapy in glioblastoma patients. As shown in Fig. 5A, in U87 cells, the combination of MBZ and CQ or TMZ and CQ significantly decreased proliferation compared to single treatments alone. The highest inhibition was achieved by the triple combination. In the Western blot assessment of autophagy, all cells co-treated with CQ showed a marked increase in the LC3B-II/LC3B-I ratio, whereas single treatment with TMZ or MBZ showed lower conversion of LC3B-II to LC3B-I. In U373 cells, the addition of CQ to MBZ or TMZ treatment also showed an enhanced inhibitory effect compared to MBZ or TMZ treatment alone (Fig. 5B). The triple combination treatment also showed better outcomes than double combination treatments in U373 cells. Accordingly, high conversion of LC3B-II to LC3B-I was observed in the triple combination treatment. The results indicate that the triple combination of TMZ, MBZ, and CQ is highly effective in inhibiting TMZ-sensitive glioblastoma cell growth.

### Inhibition of TMZ-resistant glioblastoma cell growth by the combination of MBZ and CQ

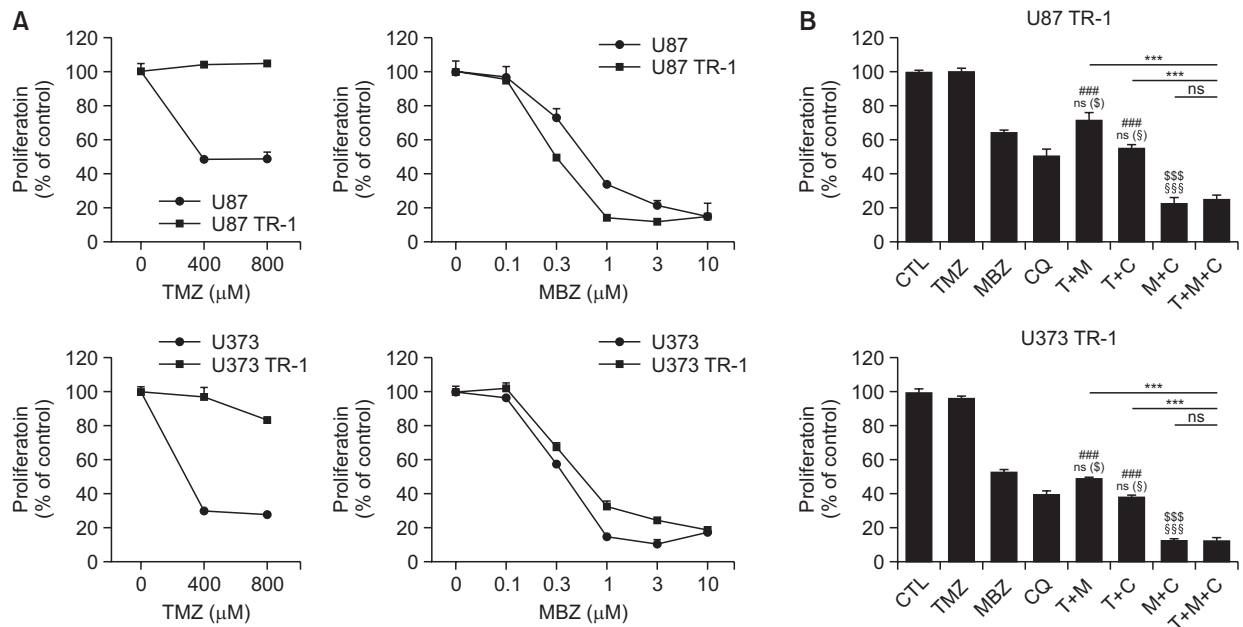
At present, TMZ is the first-choice chemotherapeutic agent to cure glioblastoma. However, glioblastoma develops resistance to TMZ through pathways such as O6-methylguanine

methyltransferase (MGMT), alkylpurine-DNA-N-glycosylase (APNG), base excision repair (BER), and others (Zhang *et al.*, 2012; Singh *et al.*, 2021). Thus, we tested whether the combination of MBZ and CQ could be an effective treatment to overcome TMZ resistance. First, we obtained TMZ-resistant cells ( $IC_{50} \geq 400$   $\mu$ M) by culturing U87 and U373 cells in the presence of gradually increasing concentrations of TMZ for more than one month (Fig. 6A, left panel). When the TMZ-resistant cells were treated with MBZ alone, the treated cells showed a dose-dependent inhibitory effect on cell proliferation at levels ( $IC_{50} = 0.3$ - $0.58$   $\mu$ M) similar to those of TMZ-sensitive U87 and U373 cells (Fig. 6A, right panel). Furthermore, the combination of MBZ and CQ showed enhanced anti-proliferative effects in the TMZ-resistant cells (Fig. 6B). Thus, these results suggest that the combination of MBZ and CQ is effective in inhibiting the proliferation of TMZ-resistant cells.

## DISCUSSION

Benzimidazole anti-helminthics can exert cytotoxicity by binding to nematode tubulin molecules at low concentrations, thereby inhibiting tubulin polymerization and microtubule formation in a fashion similar to that of colchicine (Friedman and Platzer, 1980; Lacey, 1988). MBZ also inhibited tubulin polymerization at 1  $\mu$ M in human glioblastoma cells and elicited *in vivo* anti-tumor effects in preclinical glioblastoma animal models (Bai *et al.*, 2011). MBZ can be an attractive adjuvant candidate for TMZ because MBZ has good BBB penetration. In the present study, we found that MBZ induced autophagy in glioblastoma cells and that combined treatment with MBZ and CQ increased the anti-proliferative effects on glioblastoma cells, like the synergistic effect of combined TMZ and CQ treatment (Lee *et al.*, 2015). We also found that when combined with TMZ, a standard therapeutic agent for glioblastoma, the inhibitory effect of the combination of MBZ and CQ was enhanced. Notably, we demonstrated that the MBZ and CQ combination could provide an effective treatment for TMZ-resistant glioblastoma.

Despite many efforts, glioblastoma is still an incurable dis-



**Fig. 6.** The combination of MBZ and CQ is effective in TMZ-resistant glioblastoma cells. (A) TMZ-resistant cells (U87 TR-1 and U373 TR-1) and parental cells (U87 and U373 cells) were treated with TMZ at high concentrations (400 and 800 μM) for 72 h or with MBZ (0.1, 0.3, 1, 3, and 10 μM) for 48 h. Then, the MTS assay was performed. The percentage of proliferation was calculated based on the cell proliferation in the untreated control group (CTL) (left panel). (B) TMZ-resistant cells were treated with TMZ (50 μM) and MBZ (0.2 μM) for 24 h and then treated with CQ (20 μM) for 48 h, and MTS analysis was performed. The percentage of proliferation was calculated based on the cell proliferation in the untreated control group (CTL). \*\*\* $p < 0.001$ ; ### $p < 0.001$  vs. TMZ treatment; \$\$\$ $p < 0.001$  vs. MBZ treatment; \$\$\$ $p < 0.001$  vs. CQ treatment; ns, no significant difference; ns (\$), no significant difference vs. MBZ treatment; and ns (\$), no significant difference vs. CQ treatment.

ease with an extremely poor patient prognosis and a median overall survival of 14.6 months, even in patients who receive standard therapy (surgery+TMZ+RT) (Stupp *et al.*, 2005). TMZ is the first-choice chemotherapeutic agent with significant survival benefits in glioblastoma patients (Stupp *et al.*, 2009). Autophagy is noted as a resistance mechanism of TMZ in glioblastoma cells since autophagy induced by TMZ treatment functions as a survival and protective mechanism (Sui *et al.*, 2013). Since autophagy induction by chemotherapy may be a stress response that protects cancer cells from apoptosis, the inhibition of autophagy is regarded as a promising target for overcoming drug resistance. Several cohort studies showed that the expression profiles of autophagy-related genes were correlated with the OS of glioblastoma patients, and various autophagy-related gene sets have been reported as prognostic biomarkers for glioblastoma patients (Zhang *et al.*, 2017; Wang *et al.*, 2019, 2020). In our results, MBZ treatment induced autophagy in glioblastoma cells without affecting the expression levels of Beclin 1, ATG5, or ATG7 genes that are involved in autophagosome formation. These results suggest that MBZ treatment may increase the autophagic flux in glioblastoma cells. Notably, these genes were not included in the reported prognostic autophagy biomarkers. In the future, it will be interesting to determine whether the expression of these autophagy biomarkers is correlated with MBZ sensitivity.

To enhance the anti-tumor effect of autophagy-inducing chemotherapeutics, CQ, a late-stage autophagy inhibitor, has been applied in pre-clinical and clinical studies. The inhibition of autophagy by CQ has been reported to enhance the

cytotoxic effects of both RT and chemotherapy (Golden *et al.*, 2014; Hori *et al.*, 2015), and a recent clinical study also showed favorable toxicity and promising OS from treatment with CQ combined with TMZ and RT (Compter *et al.*, 2021). Previously, we reported the synergistic effect of the combination of TMZ and CQ (Lee *et al.*, 2015). In this study, we showed autophagy induction by MBZ and the increased efficacy of combined MBZ and CQ treatment. We also provided evidence that the triple combination of TMZ, MBZ, and CQ produced the greatest inhibition of tumor cell proliferation. Furthermore, we found that the combination of MBZ and CQ was effective in inhibiting TMZ-resistant cell proliferation. Although autophagy induction is a resistance mechanism against chemotherapy, CQ seems to enable cells to overcome drug resistance by inhibiting late-stage autophagy. Targeting autophagy against drug resistance has also been shown in several cases, including the effectiveness of CQ treatment in imatinib-resistant chronic myeloid leukemia (Bellodi *et al.*, 2009; Chang and Zou, 2020). Therefore, we could suggest that the combination of MBZ and CQ would be a good strategy for treating glioblastoma patients with or without TMZ resistance. In the case of patients sensitive to TMZ, a triple combination could achieve the best outcome.

Since glioblastoma cells do not express VEGFR2, we were not able to assess the ability of MBZ to inhibit VEGFR2. However, it is possible that MBZ could also compete with ATP for other receptor kinases. We were also interested in whether MBZ could inhibit growth-factor-mediating signaling through other mechanisms. In this study, we found that MBZ caused G2-M arrest by disrupting microtubule polymerization but did not inhibit growth factor-mediated ERK1/2 signaling pathways.

The IC<sub>50</sub> of MBZ for inhibiting VEGFR2 is hardly achievable at clinical doses (Munst *et al.*, 1980). Thus, the inhibitory mechanism of MBZ may not be related to growth factor-mediated signaling.

Glioblastoma is not curable due to genetic heterogeneity and the restricted passage of drugs through the BBB (Wu *et al.*, 2021). However, MBZ induces autophagy, penetrates the BBB, and demonstrates favorable toxicities. Its inhibitory activity is derived from tubulin binding rather than the specific inhibition of signaling, suggesting that it can evade the development of drug-specific resistance in genetically heterogeneous glioblastoma. Thus, adding MBZ to standard therapy will benefit glioblastoma patients. In addition, MBZ in combination with autophagy inhibitors could provide a solution to overcome TMZ/RT resistance. In the future, more potent and less toxic autophagy inhibitors are anticipated for combination with MBZ to effectively cure glioblastoma.

## CONFLICT OF INTEREST

The authors have no competing interests to declare.

## ACKNOWLEDGMENTS

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