

# Amentoflavone Induces Apoptosis and Inhibits NF- $\kappa$ B-modulated Anti-apoptotic Signaling in Glioblastoma Cells

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**Abstract.** *The goal of the present study was to investigate anticancer effect of amentoflavone on glioblastoma cells in vitro. Our results demonstrated that amentoflavone not only significantly reduced cell viability, nuclear factor-kappa B (NF- $\kappa$ B) activation, and protein expression of cellular Fas-associated protein with death domain-like interleukin 1 beta-converting enzyme inhibitory protein (C-FLIP) and myeloid cell leukemia 1 (MCL1), but significantly triggered cell accumulation at the sub-G<sub>1</sub> phase, loss of mitochondrial membrane potential, and expression of active caspase-3 and -8. In order to verify the effect of NF- $\kappa$ B inhibitor on expression of anti-apoptotic proteins, we performed western blotting. We found that the of NF- $\kappa$ B inhibitor or amentoflavone markedly diminished protein levels of MCL1 and C-FLIP. Taken all together, our findings show that*

*amentoflavone induces intrinsic and extrinsic apoptosis and inhibits NF- $\kappa$ B-modulated anti-apoptotic signaling in U-87 MG cells in vitro.*

Glioblastoma is the most common primary malignancy of the adult central nervous system. The 5-year survival rate for patients with glioblastoma is only approximately 4.5% after diagnosis. Patients with glioblastoma have unsatisfactory response to current standard treatments and median survival less than 15 months (1, 2). Therefore, development of adjuvant anticancer therapy may offer benefits for patients with glioblastoma.

Apoptosis, *i.e.* programmed cell death, can be initiated by anticancer agents through extrinsic and intrinsic pathways (3). Nuclear factor-kappa B (NF- $\kappa$ B), a transcription factor, modulates the hallmarks of cancer by inducing expression of its target genes. Expression of anti-apoptotic proteins such as cellular Fas-associated protein with death domain-like interleukin 1 beta-converting enzyme inhibitory protein (C-FLIP) and myeloid cell leukemia-1 (MCL1) are linked to NF- $\kappa$ B activation and associated with resistance to apoptosis induced by anticancer agents in glioblastoma cells (4-6).

Amentoflavone, a flavonoid isolated from *Selaginella tamariscina*, is able to cross the blood-brain barrier and afford neuroprotection against neonatal hypoxic-ischemic brain injury (7). In our previous studies, we demonstrated amentoflavone-induced apoptosis and reduced NF- $\kappa$ B activation in MCF-7 breast cancer cells (8, 9). However, the anticancer effect of

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amentoflavone on glioblastoma cells is ambiguous. The aim of the present study was to investigate the effect of amentoflavone on tumor cell growth and NF- $\kappa$ B-modulated anti-apoptotic mechanism by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, western blotting, and NF- $\kappa$ B reporter gene assay in glioblastoma cells *in vitro*. We also used NF- $\kappa$ B inhibitor (QNZ) to verify the effect of NF- $\kappa$ B inactivation on expression of anti-apoptotic-related proteins.

## Materials and Methods

**Drugs.** Amentoflavone was purchased from Sigma-Aldrich (St. Louis, MO, USA). Amentoflavone was dissolved using dimethyl sulfoxide (0.1% DMSO) as 10 mM stock solution. NF  $\kappa$ B inhibitor (QNZ) was purchased from Apexbio Technology LLC (Houston, TX, USA) and prepared as 1 mM stock solution in 0.1% DMSO.

**Cell culture.** Human U-87 MG glioblastoma cells were kindly provided by Professor Rwei-Ming Chen, Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University and used for this study. Cells were cultured in Minimum Essential Medium Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (10,000 U/ml) and 1% sodium pyruvate (100 mM) at 37°C in a humidified incubator with 5% CO<sub>2</sub> (10).

**MTT assay.** MTT was obtained from Sigma-Aldrich. U-87 MG cells were cultured in 96-well plates at 2×10<sup>4</sup> cells/well overnight. Cells were treated with different concentration of amentoflavone (0-200 μM) for 48 h, and then cell viability was analyzed with MTT assay (11). The absorbance in each well, including the blanks, was measured at 570 nm by Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA).

**Detection of the sub-G<sub>1</sub> population.** This is a method used to detect cells in the late stage of apoptosis. Propidium iodide (PI) and RNase were purchased from Biovision (Mountain view, CA, USA) and Thermo Fisher Scientific, respectively. U-87 MG cells (5×10<sup>5</sup>) were seeded in 6-well plates and treated with 50 or 100 μM amentoflavone for 48 h before harvesting. Cells were then fixed with 70% ethanol overnight and stained with PI/RNase solution (40 μg/ml PI, 100 μg/ml RNase and 1% Triton X-100 in phosphate-buffered saline (PBS) for 30 min. Cell samples in PI/RNase solution were analyzed by flow cytometry and at least 10,000 single cells for each group were saved (12). The percentage of subG<sub>1</sub> phase cells was measured by FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR, USA).

**Detection of active caspase-3 and -8.** U-87 MG cells were cultured in 6-well plates at 5×10<sup>5</sup> cells/well overnight. Cells were treated with 0, 50 and 100 μM of amentoflavone for 48 h. Afterwards, cells were harvested by centrifugation and washed twice with PBS, and then stained with fluorescein isothiocyanate-Asp(OCH<sub>3</sub>)-Glu(OCH<sub>3</sub>)-Val-Asp(OCH<sub>3</sub>)-fluoromethyl ketone (FITC-DEVD-FMK) working solution (1 μl FITC-DEVD-FMK in 300 μl PBS) or with sulforhodamine-Ile-Glu-Thr-Asp-fluoromethyl ketone (Red-IETD-FMK) working solution (1 μl Red-IETD-FMK in 300 μl PBS) according to the manufacturer's instructions (CaspGLOW™ Fluorescein Active Caspase-3 and Caspase-8 assay kits, respectively; BioVision Inc., Milpitas, CA, USA). Expression of active caspase-3

was evaluated with flow cytometry (FACSCalibur™; Becton-Dickinson, Franklin Lakes, NJ, USA) as described by Chiang *et al.* (3).

**Detection of mitochondrial membrane potential ( $\Psi_m$ ).** 3,3'-Diethylthiopyrocarboxyanine iodide [DiOC<sub>6</sub>(3)] was bought from Enzo Life Sciences (Farmingdale, NY, USA). U-87 MG cells were cultured in 6-well plates at 5×10<sup>5</sup> cells/well overnight. Cells were treated with 0, 50 and 100 μM of amentoflavone for 48 h. Afterwards, cells were harvested by centrifugation and washed twice with PBS, and then stained with DiOC<sub>6</sub>(3) working solution (4 μM DiOC<sub>6</sub>(3) in 500 μl PBS) for 30 min in the dark. Detection of  $\Delta\Psi_m$  was performed by using flow cytometry (FACSCalibur™) as described by Wang *et al.* (13).

**Plasmid transfection.** NF- $\kappa$ B-luciferase2 vector (pNF- $\kappa$ B/luc2) was purchased from Promega (Madison, WI, USA). One million U-87 MG cells were seeded into 10 cm dishes and incubated overnight. Cells were then transfected with pNF- $\kappa$ B/luc2 by using jetPEI™ kit (Polyplus transfection, New York, NY, USA) as previously described (12).

**NF- $\kappa$ B luciferase reporter gene assay.** U-87 MG cells transfected with pNF- $\kappa$ B/luc2 were cultured in 96-well plates at 2×10<sup>4</sup> cells/well overnight and then treated with amentoflavone 0-200 μM or 3 μM QNZ followed by incubation for 48 h. Afterwards, 100 μl of 15 mg/ml D-luciferin was added to each well and plates were incubated for a further 5 min. The signal intensity of each well was acquired by IVIS200 Imaging System (Xenogen, Alameda, CA, USA) for 1 min and quantified into photons per second using Living Image software (Xenogen). D-luciferin was obtained from Gold Biotechnology (St. Louis, MO, USA). Relative NF- $\kappa$ B activity was corrected for cell viability which was analyzed using MTT assay as described previously (13).

**Western blotting assay.** U-87 MG cells (3×10<sup>6</sup>) were seeded into 10 cm diameter dishes and grown overnight. Cells were treated with 0, 50 and 100 μM of amentoflavone or 0.3 μM QNZ for 48 h. Total protein cell lysis buffer (120 mM NaCl, 0.5% NP-40, 50 mM Tris-HCl pH 8.0, and 1 mM phenylmethanesulfonyl fluoride) was used to extract total protein from cells. Protein expression of MCL1 and C-FLIP was determined with western blotting assay as previously described (12). C FLIP (catalog no. O15519; anti rabbit) was bought from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody to MCL1 (anti rabbit) was obtained from BioVision Inc. (Milpitas, CA, USA) al that to  $\beta$ -actin (anti rabbit) was purchased from Thermo Fisher Scientific. Protein bands were quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** All statistical analyses were performed using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA). Parametric data are shown as the mean±standard deviation. To compare values between two groups, Student's *t*-test was performed. Differences with a *p*-value less than 0.05 was considered statistically significant.

## Results

**Amentoflavone induced cytotoxicity towards U-87 MG glioblastoma cells.** We used MTT assay to evaluate effect of amentoflavone on cell viability in U-87 MG cells. Figure 1

indicates that amentoflavone treatment (50-200  $\mu\text{M}$ ) significantly inhibited the viability of U-87 MG cells by 23-71% at 48 h as compared to controls. The half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) of amentoflavone was 100  $\mu\text{M}$  under the 48 h treatment schedule.

*Amentoflavone-induced apoptosis is dependent on intrinsic and extrinsic apoptotic signaling pathways in U-87 MG glioblastoma cells.* Amentoflavone-induced apoptotic signaling was analyzed using flow cytometric apoptosis assays. Figure 2A and B show that amentoflavone significantly induced the accumulation of cells in the sub- $G_1$  population and increased the level of active caspase-3 by 14-52% and 24-42%, respectively, at 48 h as compared to controls. We also found amentoflavone significantly triggered the loss of  $\Psi_m$  and the expression of active caspase-8 by 23-53% and 25-50%, respectively, at 48 h as compared to controls (Figure 2C and D).

*Blockage of NF- $\kappa\text{B}$  activation reduces expression of anti-apoptotic proteins MCL1 and C-FLIP in U-87 MG glioblastoma cells.* We used QNZ, a specific NF- $\kappa\text{B}$  inhibitor, to verify the effect of NF- $\kappa\text{B}$  inactivation on expression of anti-apoptotic proteins MCL1 and C-FLIP in U-87 MG cells. NF- $\kappa\text{B}$  reporter gene assay was performed to verify the effect of QNZ on NF- $\kappa\text{B}$  activation. Relative NF- $\kappa\text{B}$  activity was corrected with cell viability. Figure 3A indicates that amentoflavone significantly suppressed NF- $\kappa\text{B}$  activation in a dose-dependent manner by 23-71% at 48 h as compared to the control. We also found 0.3  $\mu\text{M}$  QNZ significantly reduced protein levels of MCL1 and C-FLIP by 87% and 79%, respectively, at 48 h as compared to controls (Figure 3B).

*Amentoflavone diminishes protein expression of MCL1 and C-FLIP through suppression of NF- $\kappa\text{B}$  activation in U-87 MG glioblastoma cells.* Figure 4A shows amentoflavone significantly reduced NF- $\kappa\text{B}$  activation in a dose-dependent manner by 25-87% at 48 h as compared to the control. Amentoflavone significantly reduced protein expression of MCL1 and C-FLIP by 50-80% and 38-57%, respectively, at 48 h as compared to controls (Figure 4B).

## Discussion

Amentoflavone has been reported to trigger apoptosis and reduce NF- $\kappa\text{B}$  activation in breast cancer and melanoma cells (8, 9, 14). The effects of amentoflavone on glioblastoma cells has not been elucidated. Therefore, we evaluated the effect of amentoflavone on tumor cell growth and NF- $\kappa\text{B}$ -modulated anti-apoptotic signaling in U-87 MG cells *in vitro*.

Many cytotoxic agents inhibit growth of glioblastoma cells by inducing apoptosis (15). Apoptotic signaling pathways are divided into intrinsic (mitochondria) and extrinsic (death

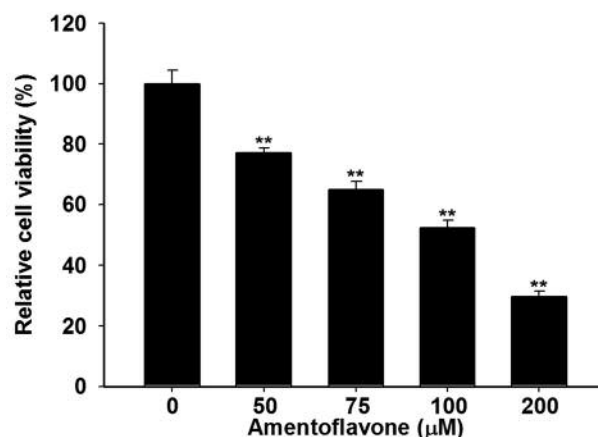


Figure 1. U-87 MG cells ( $2 \times 10^4$ ) were seeded in 96-well plates and treated with 0, 50, 100, 150, 200  $\mu\text{M}$  amentoflavone in 0.1% dimethylsulfoxide for 48 h. The viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. \*\*Significantly different at  $p < 0.01$  vs. 0  $\mu\text{M}$ .

receptors) pathways (16). Temozolomide (TMZ), an oral O6-methylating agent used for treatment of glioblastoma, has been shown to trigger intrinsic and extrinsic apoptosis by inducing DNA lesion in O6-methylguanine. Active caspase-8 is key mediator of the extrinsic apoptotic signaling pathway (17). DNA fragmentation initiated by active caspase-3 can be triggered *via* intrinsic and extrinsic apoptotic signaling pathways. High expression of cleaved caspase-8 and active caspase-3 are positive prognostic indicators for patients with glioma (1, 18). Figure 2B and D indicate amentoflavone significantly induced expression of active caspase-3 and -8, while Figure 2C shows amentoflavone significantly induced loss of  $\Psi_m$ , all associated with apoptosis.

Constitutive activation of NF- $\kappa\text{B}$  was observed in glioma and correlated with glioma grade (19). High expression of NF- $\kappa\text{B}$  contributes tumor progression and modulates acquired resistance to treatment in glioblastoma (20). TMZ not only induces apoptosis but also triggers NF- $\kappa\text{B}$  activity in glioblastoma cells. Therapeutic efficacy of TMZ is limited because of overexpression of NF- $\kappa\text{B}$ -modulated anti-apoptotic proteins; inhibition of NF- $\kappa\text{B}$  activation sensitizes glioblastoma cells to TMZ (21, 22). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a potential anticancer agent because it induces apoptosis leading to tumor growth inhibition in cancer (23). MCL1 and C-FLIP are disruptors of intrinsic and extrinsic apoptotic pathways, respectively. Protein expression of MCL1 and C-FLIP are modulated by NF- $\kappa\text{B}$  activation in cancer (12). Han *et al.* showed that silencing NF- $\kappa\text{B}$  reduced C-FLIP expression and enhanced TRAIL-induced apoptosis in glioblastoma cells (24). Murphy *et al.* also demonstrated that silencing MCL1 increased sensitivity to

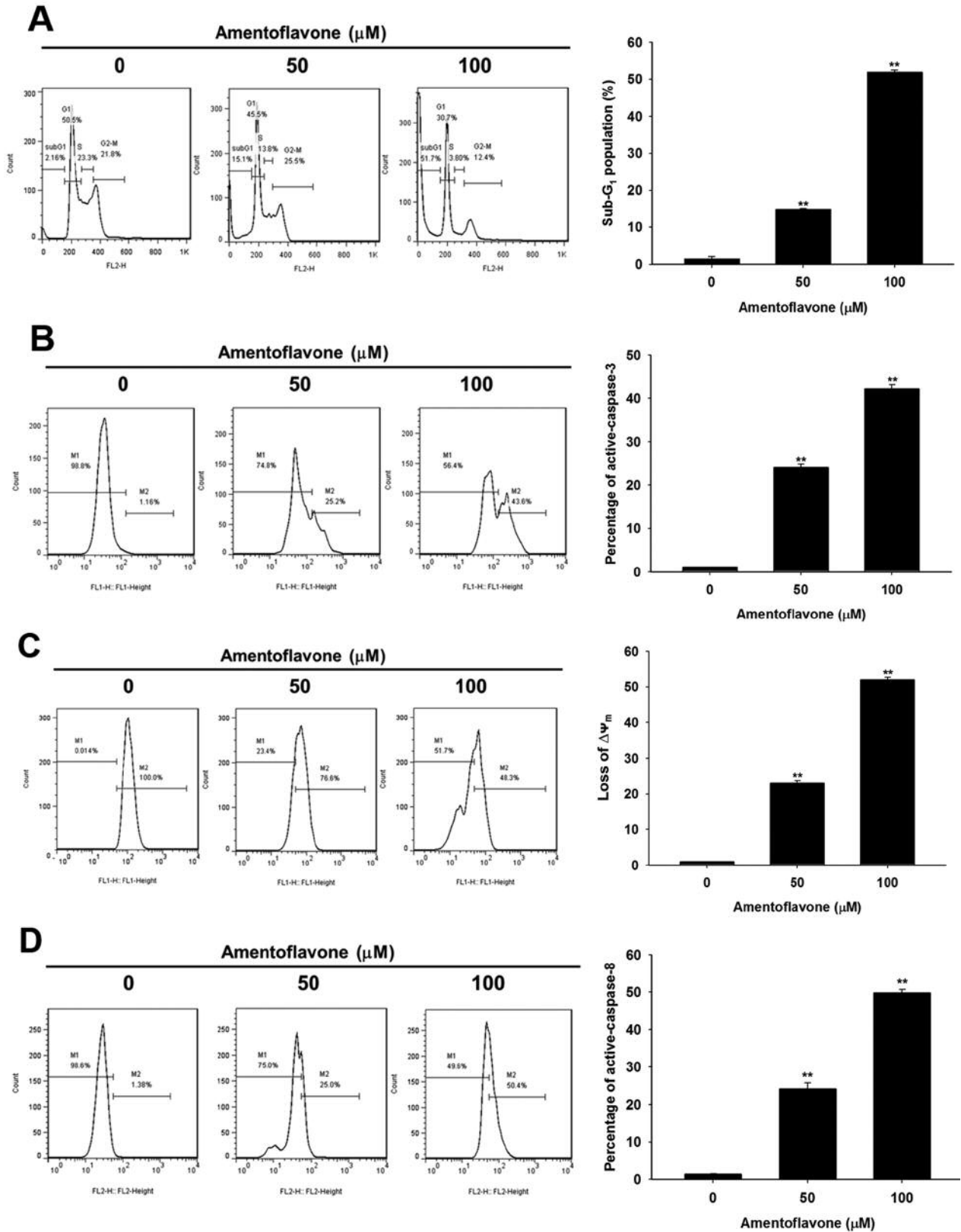


Figure 2. U-87 MG cells were treated with 0, 50, 100  $\mu\text{M}$  amentoflavone for 48 h and prepared for cell cycle and caspase-3 flow cytometry (A, B), or analyzed for mitochondrial membrane potential and caspase-8 (C, D). \*\*Significantly different at  $p < 0.01$  vs. 0  $\mu\text{M}$ .

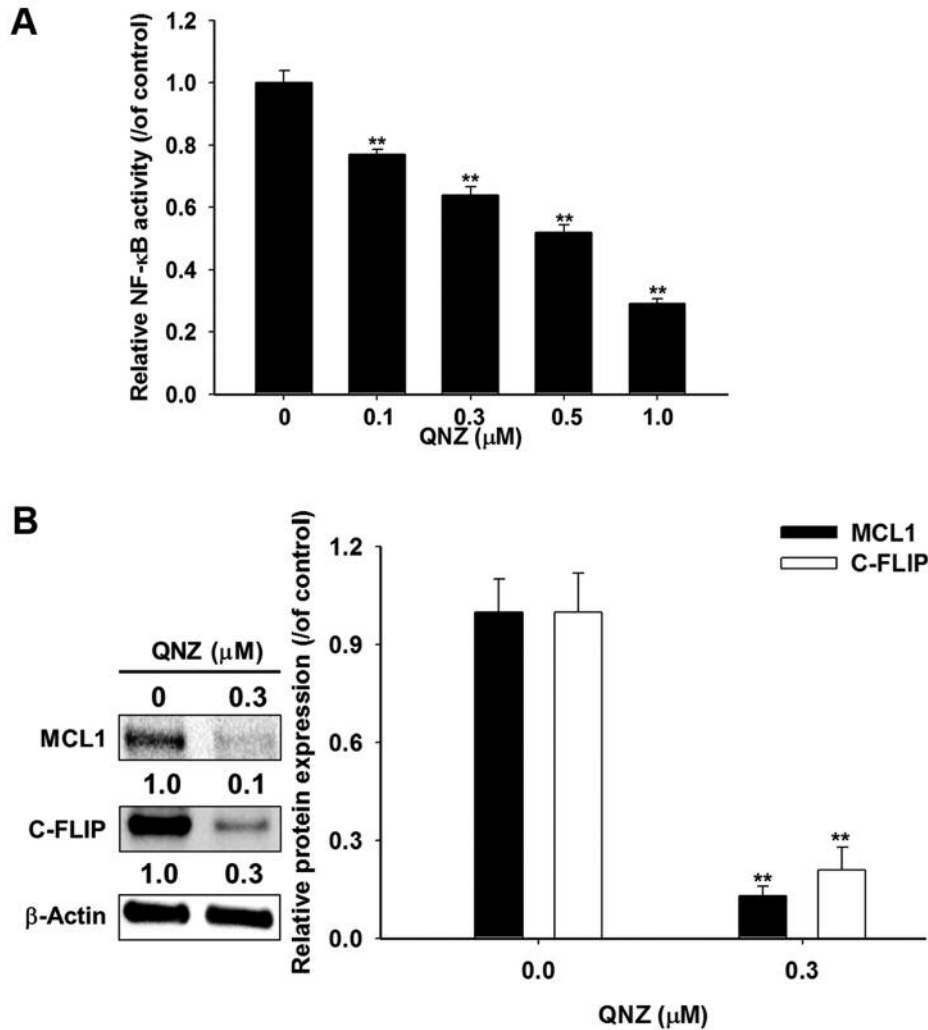


Figure 3. A: U-87 MG cells were treated with 0-1 μM of the nuclear factor-κB (NF-κB) inhibitor QNZ for 48 h and NF-κB activation was evaluated by bioluminescence imaging. B: U-87 MG cells were treated with 0 and 0.3 μM QNZ for 48 h and harvested for western blotting assay using Image J system to quantify the protein level of myeloid cell leukemia 1 (MCL1) and cellular Fas-associated protein with death domain-like interleukin 1 beta-converting enzyme inhibitory protein (C-FLIP). \*\*Significantly different at  $p < 0.01$  vs. 0 μM.

TRAIL in glioblastoma cells (6). We confirmed such effects by finding that NF-κB inhibitor reduced NF-κB activation and protein expression of MCL1 and C-FLIP in U-87 MG cells (Figure 3). We also found amentoflavone, as an inhibitor of NF-κB signaling, reduced expression of NF-κB-modulated anti-apoptotic proteins MCL1 and C-FLIP (Figure 4A and B).

## Conclusion

In conclusion, the present study demonstrated that amentoflavone not only induced intrinsic and extrinsic apoptosis, but also reduced NF-κB-modulated anti-apoptosis signaling in glioblastoma cells *in vitro*. We suggest

amentoflavone as a potential adjuvant which may provide benefit for treatment of patients with glioblastoma.

## Financial & Competing Interests Disclosure

The Authors declare no competing financial interests.

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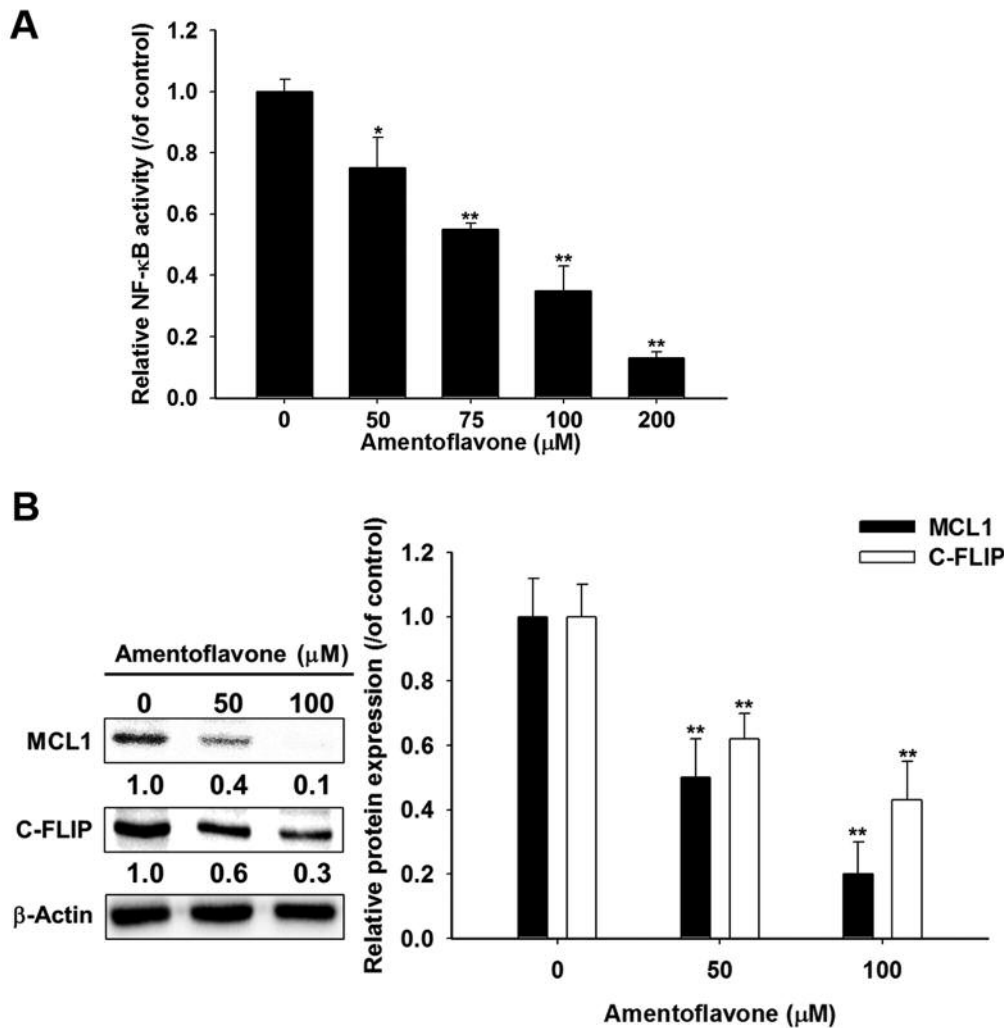


Figure 4. A: U-87 MG cells ( $2 \times 10^4$ ) were seeded in 96-well plates and treated with 0-200  $\mu\text{M}$  amentoflavone in 0.1% dimethylsulfoxide (DMSO) for 48 h. Nuclear factor-kappa B (NF- $\kappa\text{B}$ ) activation was evaluated by reporter gene assay. Relative NF- $\kappa\text{B}$  activity of each group was normalized with viability and calculated through dividing by the value for the control group treated with 0.1% DMSO. B: Cells were treated with 0, 50, 100  $\mu\text{M}$  amentoflavone for 48h and harvested for western blotting assay. The quantification of protein expression of myeloid cell leukemia 1 (MCL1) and cellular Fas-associated protein with death domain-like interleukin 1 beta-converting enzyme inhibitory protein (C-FLIP) was measured by Image J. \*\*Significantly different at  $p < 0.01$  vs. 0  $\mu\text{M}$ .

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