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6-Gingerol Induced Apoptosis and Cell Cycle Arrest in Glioma Cells via MnSOD and ERK Phosphorylation Modulation

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Abstract

6-gingerol, a bioactive compound from ginger, has demonstrated promising anticancer properties across various cancer models by inducing apoptosis and inhibiting cell proliferation and invasion. In this study, we explore its mechanisms against glioblastoma multiforme (GBM), a notably aggressive and treatment-resistant brain tumor. We found that 6-gingerol crosses the blood-brain barrier more effectively than curcumin, enhancing its potential as a therapeutic agent for brain tumors. Our experiments show that 6-gingerol reduces cell proliferation and triggers apoptosis in GBM cell lines by disrupting cellular energy homeostasis. This process involves an increase in mitochondrial reactive oxygen species (mtROS) and a decrease in mitochondrial membrane potential, primarily due to the downregulation of manganese superoxide dismutase (MnSOD). Additionally, 6-gingerol reduces ERK phosphorylation by inhibiting EGFR and RAF, leading to G1 phase cell cycle arrest. These findings indicate that 6-gingerol promotes cell death in GBM cells by modulating MnSOD and ROS levels and arresting the cell cycle through the ERFR-RAF-1/MEK/ERK signaling pathway, highlighting its potential as a therapeutic agent for GBM and setting the stage for future clinical research.

Key Words: 6-gingerol, GBM, BBB, MnSOD, ERK, EGFR

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive form of adult brain tumor, classified as a grade IV astrocytoma by the World Health Organization. It constitutes approximately 40% of all primary brain tumors and 78% of malignant tumors in the central nervous system (Miller and Perry, 2007; Louis *et al*., 2016). The standard treatment protocol, which includes surgery followed by concurrent chemoradiation therapy (CCRT), offers an average post-treatment survival of less than 15 months (Stupp *et al*., 2005; Park *et al*., 2010). This grim prognosis is primarily due to the highly invasive nature of glioma cells and their notable resistance to conventional therapies, including radiation and chemotherapy (Li *et al*., 2022; Pandey *et al*., 2022).

The efficacy of radiotherapy is significantly undermined by the presence of cancer stem cells (CSCs), while the effectiveness of chemotherapy is often compromised by the overexpression of O6-methylguanine-DNA methyltransferase (MGMT), which enhances the repair of damaged DNA in GBM cells (Delello Di Filippo *et al*., 2021). These challenges underscore the urgent need for novel therapeutic agents that can

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efficiently cross the blood-brain barrier (BBB) and target tumor cells more effectively.

Ginger (*Zingiber officinale*), a common dietary ingredient, is rich in bioactive phenolic compounds such as gingerols, paradols, shogaols, and gingerdiols, which have shown promising anticancer properties (Simon *et al*., 2020; Promdam and Panichayupakaranant, 2022). Among these, 6-gingerol is particularly notable for its anti-inflammatory, antitumor, and antioxidant activities (Promdam and Panichayupakaranant, 2022; Ahmed *et al*., 2023). This compound has demonstrated potential against various cancers, including breast, cervical, colorectal, and prostate cancers. For instance, 6-shogaol has been shown to inhibit lung cancer cell proliferation by targeting AKT kinase activity and inducing cell cycle arrest (Hung *et al*., 2009; Qi *et al*., 2015; Promdam and Panichayupakaranant, 2022). Similarly, 6-paradol suppresses pancreatic cancer metastasis through modulation of the epidermal growth factor receptor (EGFR) and inactivation of the PI3K/AKT signaling pathway (Jiang *et al*., 2021).

Recent studies have highlighted that 6-gingerol enhances the sensitivity of gastric cancer cells to cisplatin, leading to cell cycle arrest, suppression of migration and invasion via the PI3K/AKT pathway, and increased radiosensitivity through G2/M arrest and apoptosis induction (Promdam and Panichayupakaranant, 2022). Additionally, it exhibits anti-proliferative effects on cervical cancer cells and induces TRAIL-mediated apoptosis in glioblastoma tumor cell lines (Lee *et al*., 2014). In colorectal cancer models, 6-gingerol demonstrates anti-inflammatory, anti-proliferative, and apoptotic effects (Aloliqi, 2022; Promdam and Panichayupakaranant, 2022).

Despite these advances, the specific anticancer properties and mechanisms of action of 6-gingerol in GBM have not been fully elucidated. This study aims to investigate these aspects by examining the ability of 6-gingerol to cross the BBB and elucidate its mechanisms in inducing cell death in GBM cells.

MATERIALS AND METHODS

Materials

Tetramethylrhodamine methyl ester (TMRM) reagent was sourced from Invitrogen (Life Technologies, CA, USA). Bovine serum albumin (BSA), RNase A, propidium iodide (PI), dihydroethidium (DHE), and the ATP Assay Kit were procured from Sigma-Aldrich (St. Louis, MO, USA). The mitogen-activated protein kinase kinase (MEK) Inhibitor U0126 was purchased from Promega (Madison, WI, USA). Mito-TEMPO and the Cell Counting Kit-8 (CCK-8) were obtained from Targetmol (Shanghai, China). The PAMPA-BBB assay kit was acquired from BioAssay Systems (Hayward, CA, USA). The Annexin V-FITC/PI apoptosis detection kit was supplied by Fremont (CA, USA). Antibodies specific to GAPDH were sourced from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for E2F1, E2F3, p21, Cyclin A, Cyclin B, Cyclin E, Cyclin D, phospho-ERK1/2 (T202/Y204), ERK1/2, puma, Bax, Bcl-xL, Caspase-3, Caspase-9, and PARP were procured from Cell Signaling Technology (Beverly, MA, USA). Antibodies for manganese superoxide dismutase (MnSOD), RAS, RAF, phospho-RAF, EGFR, and phospho-EGFR were obtained from ABclonal (Woburn, MA, USA). Secondary horseradish peroxidase (HRP)-conjugated donkey anti-rabbit and donkey anti-mouse antibodies were purchased from Invitrogen (Life Technologies).

Cell culture, cell viability, and colony formation assay

Human GBM cell lines M059K and U251, sourced from the American Type Culture Collection (ATCC), Manassas, VA, USA, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The cultures were maintained at 37°C and 5% CO_2 in a fully humidified environment. All experiments were conducted during the logarithmic growth phase of the cells.

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8). M059K and U251 cells (4×10^3 cells/well) were seeded in 96-well plates overnight and then treated with various concentrations of 6-gingerol at 37°C for 24, 48, and 72 h. Post-incubation, CCK-8 solution was added, and the plates were incubated at 37°C until color development. Absorbance at 450 nm was measured using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA).

The colony formation assay followed established protocols (Tsai *et al*., 2021). Briefly, colonies were counted and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) after staining, air-drying, and solubilization in dimethyl sulfoxide (DMSO). Results are presented as the mean ± standard error (SE) based on three independent experiments.

PAMPA-BBB assay and and analysis of its ability to penetrate the BBB

The PAMPA-BBB assay is a high-throughput method for predicting a drug's ability to penetrate the BBB. This assay evaluates the permeability of drug molecules through an artificial membrane containing porcine brain polar lipids (PBL), which mimic the composition of brain endothelial cells. The test compound diffuses from the donor well through the artificial membrane to the receptor well, and the effective permeability (Pe) is calculated based on the concentration of the compound in the receptor well. Test compounds and commercial drugs were dissolved in DMSO at a concentration of 5 mg/mL and then diluted to 25 μg/mL in PBS buffer (pH 7.4). Porcine polar brain lipid (PBL) was solubilized in dodecane at 20 mg/mL. To set up the assay, 4 μL of the PBL solution was applied to the filter surface of the donor plate. After ensuring the filter was fully saturated, 150 μL of the test compound solution was added to the donor plate, and the acceptor well was filled with 300 μL of PBS buffer. The donor plate was then carefully placed onto the acceptor plate, ensuring proper membrane and liquid contact. The assembly was covered and incubated in darkness at 25°C for 6 h. Post-incubation, the concentrations in the acceptor, donor, and reference wells were determined using a microplate reader, correlating UV absorbance with a standard curve. Effective permeability (Pe) values were then calculated, allowing compounds to be classified based on their potential to cross the BBB and indicating their possible central nervous system activity. Each sample set was tested in triplicate to ensure consistency. Compounds were classified based on their Pe values as follows: CNS+ ($Pe>4.0\times10^{-6}$ cm/s), CNS- ($Pe<2.0\times10^{-6}$ cm/s), and CNS± (Pe between 2.0×10^{-6} cm/s and 4.0×10^{-6} cm/s).

The permeability of 6-gingerol and curcumin across the BBB was evaluated using the online BBB predictor tool available

at https://www.cbligand.org/BBB/index.php (Liu *et al*., 2014). The prediction was performed using the SVM-MACCSFP BBB scoring method provided by the CBLigand program. This method utilizes a support vector machine (SVM) algorithm in combination with MACCS fingerprints (MACCSFP) to calculate the BBB score. Four distinct fingerprints were employed

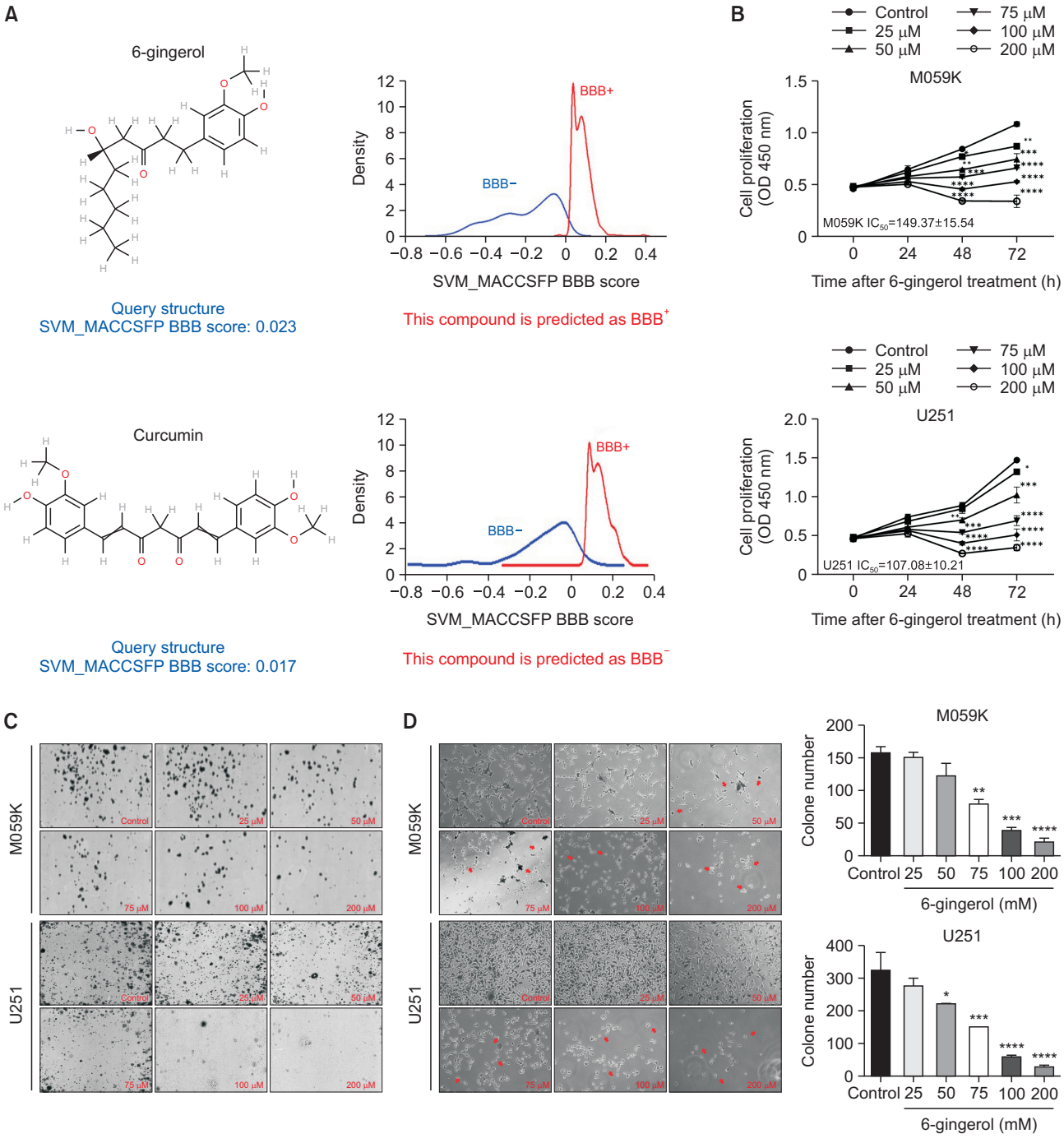

Fig. 1. 6-gingerol exhibits BBB permeability and cytotoxic potential in M059K and U251 cells. (A) Simulations were conducted using Support Vector Machine (SVM) algorithms to predict the potential BBB permeability of 6-gingerol and curcumin. (B) Cell viability was assessed following incubation with varying concentrations of 6-gingerol for 24 to 72 h using the CCK-8 assay. (C) M059K and U251 GBM cells were incubated with different concentrations of 6-gingerol for 16 days, and their ability to form colonies was assessed. The lower panel presents quantitative analyses of colony formation. (D) Concentration-dependent morphological changes, such as cell detachment and cell death, were observed in GBM cells treated with 6-gingerol for 48 h. Light microscopy images (magnification ×100). The scale bar represents 100 mm. The results represent the mean ± SD from three independent experiments; ****p<0.001, ***p<0.005, **p<0.01, *p<0.05 compared to the respective control group.

for the prediction: MACCSFP, OpenbabelFP2, Molprint2DFP, and PubChemFP. The threshold value for the SVM-MACCSFP BBB score was set at 0.020. Compounds with scores exceeding this threshold were considered likely to permeate the BBB.

Cell cycle analysis

For cell cycle analysis, M059K and U251 cells treated with varying concentrations of 6-gingerol for 48 h were harvested, washed with phosphate-buffered saline (PBS, pH 7.2), and fixed in 85% methanol overnight at 4°C. After centrifugation at 1500 rpm for 10 min, the cell pellet was washed twice with PBS and then resuspended in PBS containing 100 μg/mL PI and 20 Units/mL RNase A for 30 min at room temperature. A minimum of 10,000 cells per sample were analyzed using an Attune NxT flow cytometer (Thermo Fisher Scientific), with data processed using Attune NxT Flow Cytometer Software.

FITC-Annexin V apoptosis assay

Apoptosis in M059K and U251 cells treated with various concentrations of 6-gingerol, alone or in combination with U0126 (10 μM), over 48 h, was assessed using the CF®488A Annexin V and PI Apoptosis Kit. Following the manufacturer's instructions, apoptotic cells were labeled with Annexin V-FITC, and late apoptotic or necrotic cells were stained with PI. Data from 10,000 cells were acquired using a BD FACSLyric flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo v10 software (BD Biosciences). The analysis focused on specifically defined regions to precisely evaluate the distribution of cells across different cell cycle phases.

Determination of mitochondrial ROS (mtROS) and mitochondrial membrane potential (ΔΨm)

mtROS production and changes in ΔΨm were assessed using DHE and TMRM, respectively. After treating M059K and U251 cells with various concentrations of 6-gingerol, alone or in combination with U0126 (10 μM) or Mito-TEMPO (5 μM), for 48 h, cells were incubated with 5 μM CellROX® Green Reagent, 250 nM DHE Red Reagent, and 25 nM TMRM for 30 min at 37°C. Cells were then washed with PBS, trypsinized, and analyzed for fluorescence intensity using flow cytometry at excitation/emission wavelengths of 485/530 nm for CellROX, 510/580 nm for DHE, and 488/570 nm for TMRM. Data analysis was performed using Attune NxT Flow Cytometer Software.

Western blot analysis

Cells cultured in 10 cm dishes were treated with various concentrations of 6-gingerol for 48 h, with or without U0126 (10 μM) or Mito-TEMPO (5 μM). Proteins were extracted using RIPA buffer containing protease and phosphatase inhibitors, and total protein content was determined using a Bio-Rad Protein Assay Kit. Proteins (15-50 μg) were separated by SDS-PAGE and transferred onto PVDF membranes, which were then blocked with 5% milk in PBST, incubated with primary antibodies overnight, washed, and incubated with HRP-conjugated secondary antibodies (1:5000 dilution). Band intensities were visualized using a chemiluminescent solution (Pierce, Rockford, IL, USA) and imaged using a MultiGel-21 gel imager (Topbio, Taipei, Taiwan).

Table 1. Predicted drug-like properties, PAMPA-BBB results (Pe: 10^{-6} cm/s), and predicted CNS penetration for 6-gingerol and curcumin

Compounds	P_e ($\times 10^{-6}$ cm/s)[a]	Prediction[b]	BBB permeability[b]
6-gingerol	4.99 ± 0.53	CNS^+	+
Curcumin	1.91 ± 0.03	CNS^-	–
TMZ[c]	4.91 ± 0.41	CNS^+	+

[a]Data are the mean ± SEM of eight independent experiments.
[b][CNS^+, high BBB permeation predicted; Pe (10^6 cm s^{-1})>4.0]; [$CNS^\pm$, BBB permeation uncertain; Pe (10^6 cm s^{-1}) from 2.0 to 4.0]; [CNS^-, low BBB permeation predicted); Pe (10^6 cm s^{-1})<2.0].
[c]TMZ- is an oral alkylating agent used to treat GBM.

Statistical analysis

Data were analyzed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as mean ± standard deviation (SD). Comparisons between groups were made using unpaired Student's t-test, with a *p*-value less than 0.05 considered statistically significant.

RESULTS

Cytotoxic effects of 6-gingerol and validation of a reliable BBB model

Zingiber officinale (ginger) and Curcuma longa (turmeric), both plants of the Zingiberaceae family, contain molecular constituents such as 6-shogaol and 6-gingerol, which exhibit structural similarities to curcumin (Fig. 1A). These similarities suggest potential anticancer properties. To investigate BBB permeability, we utilized the ALzPlatform (Liu *et al*., 2014) and found that curcumin did not meet the criteria for BBB penetration. In contrast, 6-gingerol demonstrated the capability to cross the BBB, with an SVM-MACCSFP BBB score of 0.023, surpassing the threshold of 0.020 required for BBB passage. This prediction was further validated using the PAMPA-BBB assay, confirming the potential of 6-gingerol as a therapeutic agent for GBM (Table 1).

To investigate the influence of 6-gingerol on GBM cell growth, two human GBM cell lines, M059K and U251, were utilized. These cells were treated with 6-gingerol at concentrations of 25, 50, 75, 100, and 200 μM for 24, 48, and 72 h. This selection of concentrations and treatment durations was based on previously observed dose-dependent and time-dependent cytotoxic effects of 6-gingerol (Weng *et al*., 2012; Lee *et al*., 2014; Czarnik-Kwaśniak *et al*., 2019; de Lima *et al*., 2020; Tsai *et al*., 2020; Zhang *et al*., 2021). The results showed that 6-gingerol exhibited dose-dependent and time-dependent cytotoxic effects on both M059K and U251 cell lines. The IC50 values were 107.08 ± 10.21 μM for U251 cells and 149.37 ± 15.54 μM for M059K cells (Fig. 1B). Additionally, a colony formation assay showed dose-dependent inhibition of proliferation in both cell lines (Fig. 1C). Phase-contrast micrographs revealed cell shrinkage and the formation of apoptotic vacuoles with clusters of dying cells (CDC) following 48 h of treatment with 6-gingerol (Fig. 1D).

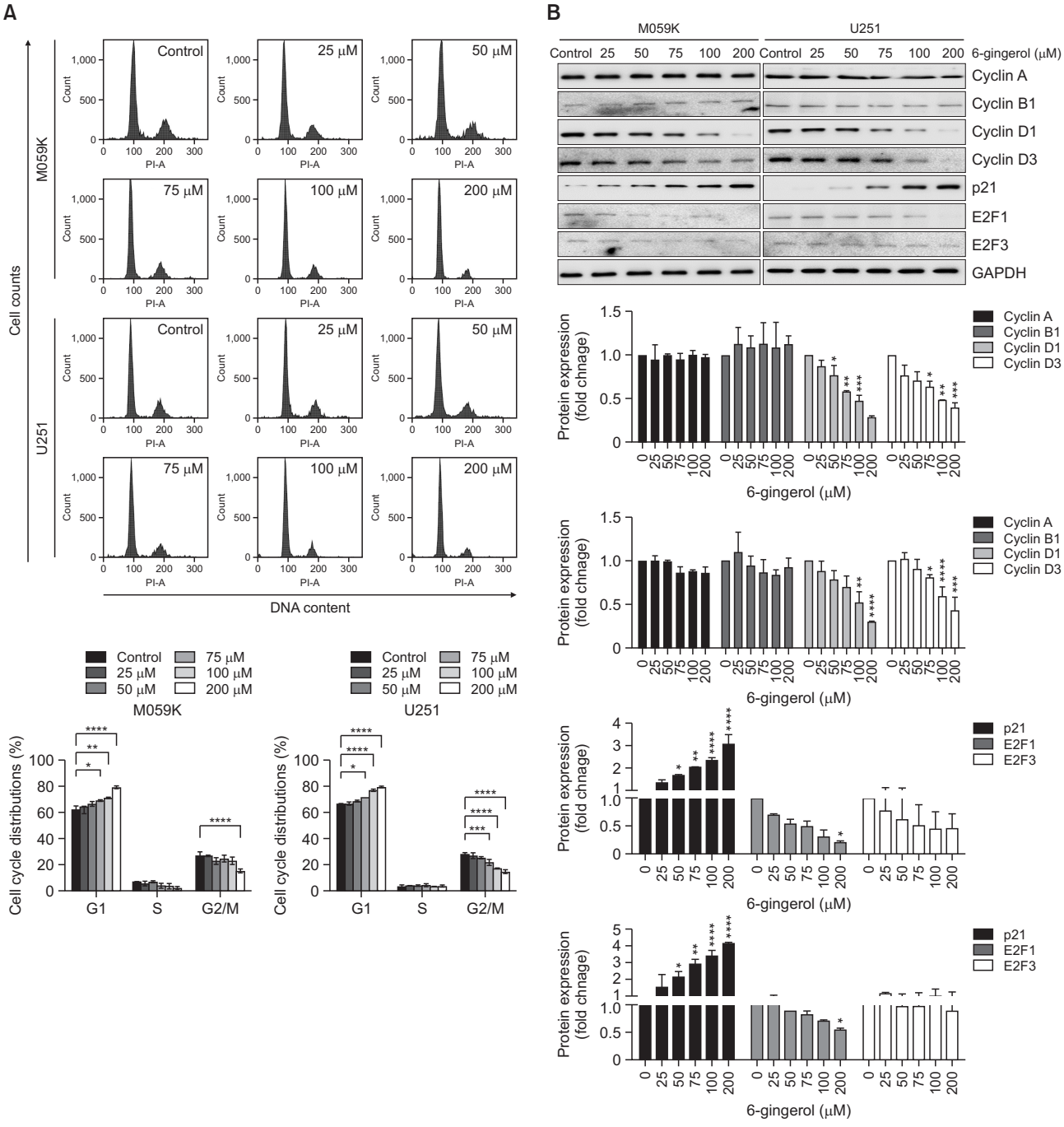

Fig. 2. Cell cycle modulation and protein expression changes in response to 6-gingerol treatment. (A) Treatment of M059K and U251 cells with varying concentrations of 6-gingerol for 48 h, followed by flow cytometry analysis to assess the cell cycle distribution. The graph illustrates the proportion of cells in each stage of the cell cycle. (B) Analysis of protein expression in M059K and U251 cells after 48 h of treatment with 6-gingerol. western blot analysis was conducted for Cyclin A, Cyclin B1, Cyclin D1, Cyclin D3, p21, E2F1 and E2F3, using cell lysates from M059K and U251 cells. The lower panel presents a quantitative analysis of western blot data, illustrating the observed changes in protein expression, with GAPDH used as the internal control. The results represent the mean ± SD from three independent experiments; ****p<0.001,***p<0.005, **p<0.01, *p<0.05 compared to the respective control group.

6-gingerol induces G1 arrest in GBM cells

Given the observed dose-dependent and time-dependent cytotoxic effects, we further investigated the impact of 6-gingerol on the cell cycle of GBM cells to understand the underlying mechanisms of its anticancer activity. Treatment with 6-gingerol resulted in an increased number of M059K and U251 cells in the G1 phase in a dose-dependent manner (Fig. 2A). This G1 arrest was associated with the accumulation of p21 and decreased levels of E2F1 (Fig. 2B). Additionally, there was a dose-dependent suppression of Cyclin D1 and Cyclin

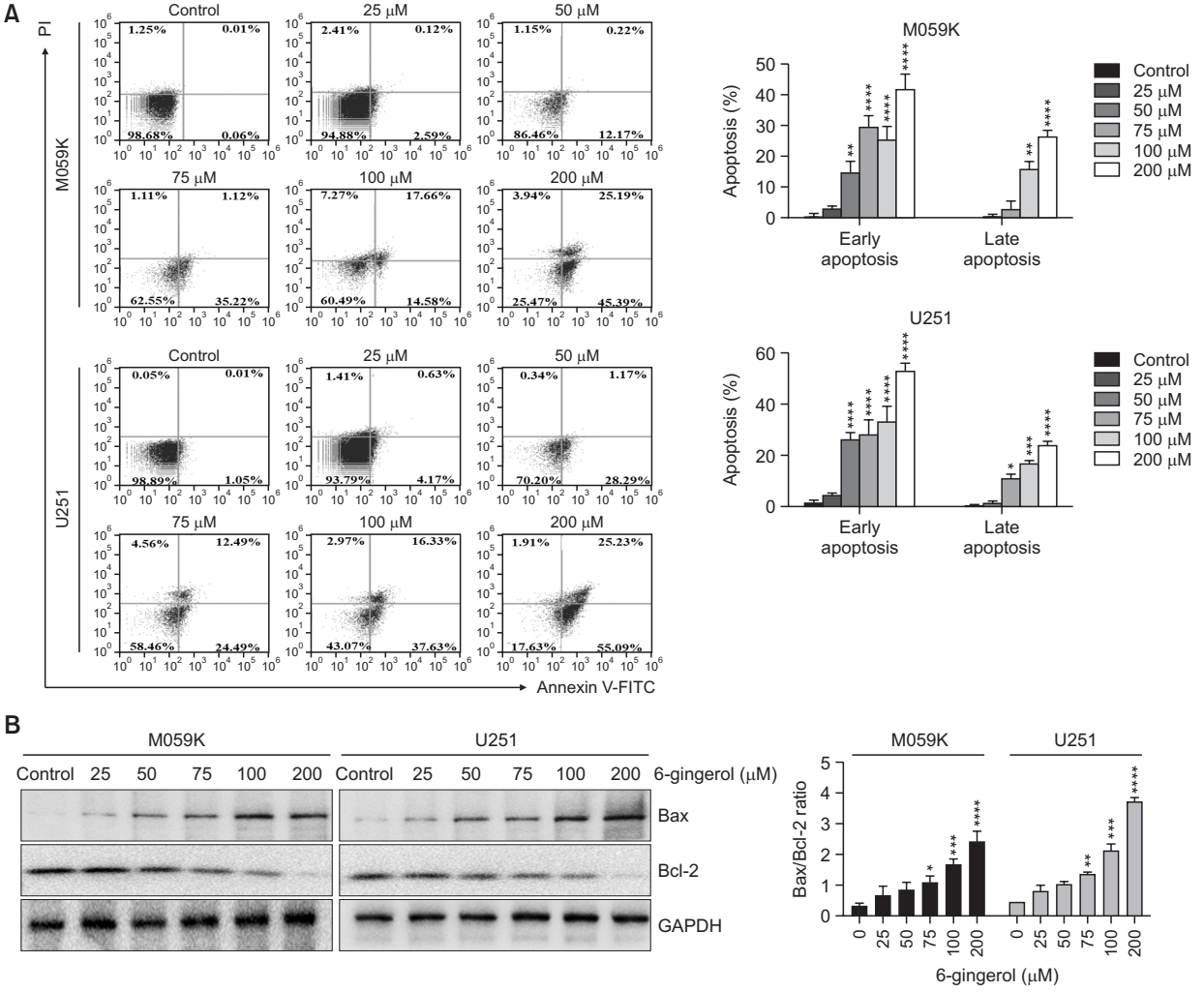

Fig. 3. Apoptosis induction and protein expression changes in response to 6-gingerol exposure. (A) After a 48 h exposure to 6-gingerol, apoptosis in M059K and U251 cell lines was assessed using cytofluorimetry. The proportion of apoptotic cells was determined through Annexin V-FITC and propidium iodide staining. The right panel illustrates the percentages of cells in early apoptosis (Annexin V+/PI-) and late apoptosis (Annexin V+/PI+), calculated as Annexin V-positive cells for statistical analysis. (B, C) The expression of pro- and anti-apoptotic proteins in M059K and U251 cells was analyzed following a 48 h exposure to 6-gingerol. This analysis included anti-Bax and Bcl-2, Cleaved Caspase-3, Cleaved Caspase-9, and PARP/cleaved PARP. Western blot analysis of lysates from M059K and U251 cells was conducted. GAPDH served as the internal control. The right panel provides a quantitative assessment of western blot data, indicating observed changes in protein expression. All results are presented as mean ± SD from three independent experiments; ****p<0.001, ***p<0.005, **p<0.01, *p<0.05 compared to the respective control.

D3, with no significant changes observed in Cyclin A and Cyclin B1 levels. These findings suggest that 6-gingerol effectively induces cell cycle arrest at the G1 phase, contributing to its cytotoxic effects on GBM cells.

6-Gingerol induces apoptosis in GBM cells

To determine if 6-gingerol-induced cell growth inhibition is mediated by apoptosis, we used flow cytometry with annexin V and PI staining. Annexin V detects early and late apoptosis, while PI detects late apoptosis and necrosis. Early apoptotic cells are annexin V positive and PI negative (lower right quadrant, Annexin V^+/PI^-), whereas late apoptotic or necrotic cells are positive for both annexin V and PI (upper right quadrant, Annexin V^+/PI^+). This analysis confirmed that 6-gingerol induces apoptosis in GBM cells. A significant increase in the percentage of apoptotic cells was observed in M059K and U251 cells compared to controls (Fig. 3A). This was consistent with increased activities of caspases -3 and -9 and elevated levels of cleaved PARP (Fig. 3B). There was also a decrease in Bcl-2 expression and an increase in Bax expression following exposure to 6-gingerol (Fig. 3C). Collectively, these results indicate that 6-gingerol induces apoptosis in GBM cell lines.

mtROS scavengers prevent 6-gingerol-induced mtROS elevation, ΔΨm disruption, and apoptosis

6-gingerol may induce ROS accumulation and ferroptosis (Liu *et al*., 2022). To further elucidate the effects of 6-gingerol on oxidative damage in GBM cells, we focused on mitochondrial function. The impact of 6-gingerol on ΔΨm and mtROS was assessed. Compared to controls, treatment with 6-gin-

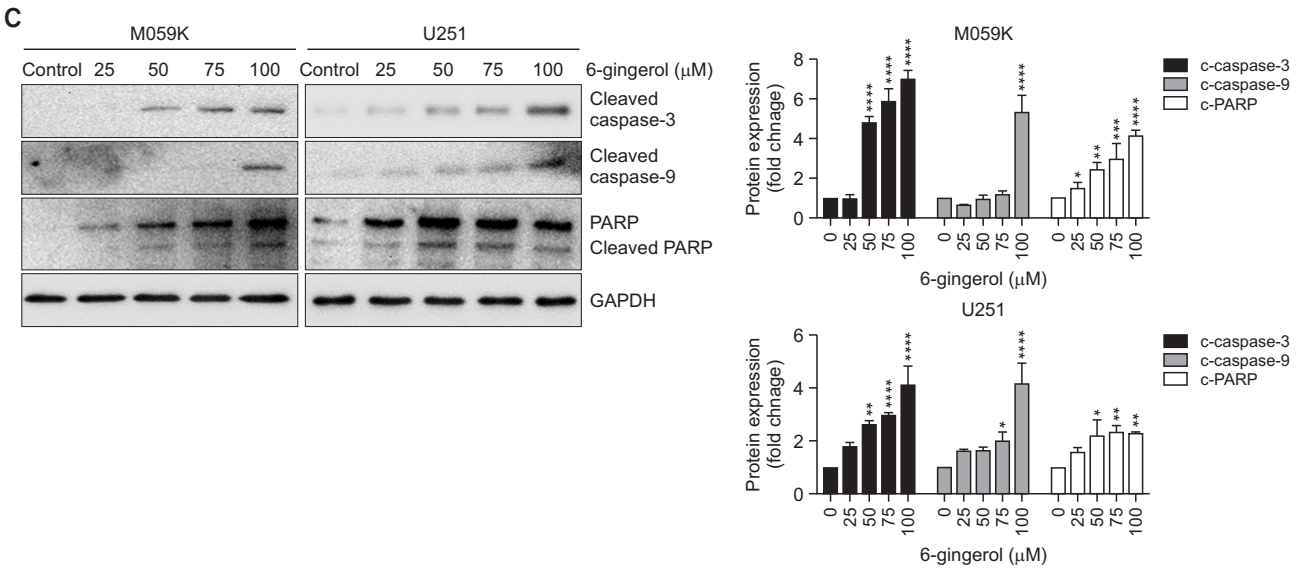

Fig. 3. Continued.

gerol (25-200 μM) resulted in a reduction in TMRM fluorescence, indicating a dose-dependent loss of ΔΨm. This loss of ΔΨm was accompanied by increased levels of mtROS, detected using DHE, suggesting that 6-gingerol induces a dose-dependent elevation in oxidative stress (Fig. 4A, 4B). Decreased levels of the antioxidant enzyme MnSOD correlated with the increased mtROS (Fig. 4C). Co-treatment with Mito-TEMPO, a mitochondria-targeting antioxidant, significantly reduced mtROS production and preserved ΔΨm in cells exposed to 6-gingerol (Fig. 4D, 4E). Additionally, pretreatment with Mito-TEMPO suppressed apoptosis markers, including cleaved caspase-3 and -9 (Fig. 4F). These findings underscore the role of ROS-dependent mitochondrial dysfunction in 6-gingerol-induced apoptosis.

6-Gingerol inhibits the MAPK/ERK signaling

The MAPK pathway is pivotal in the regulation of apoptosis (Yuan *et al*., 2013). Since the MAPK pathway has been reported as a regulator of mitochondrial ROS production (Valko *et al*., 2007), we investigated whether ROS-dependent mitochondrial dysfunction in 6-gingerol-induced apoptosis is mediated through the activation of MAPK/ERK signaling, thereby promoting ROS production in GBM cells. We examined the effect of 6-gingerol on the phosphorylation levels of ERK, JNK, and p38. Western blot results indicated that phosphorylated ERK and p38 levels decreased in 6-gingerol-treated GBM cells, while the phosphorylation level of JNK remained unchanged (Fig. 5A). Notably, the increase in the ERK/p-ERK ratio suggests that 6-gingerol primarily impacts the ERK pathway. Based on these data, we further evaluated the involvement of MAPK/ERK activation in 6-gingerol-induced cell cycle arrest, apoptosis, and mtROS scavenging using the MAPK/ERK inhibitor U0126. As shown in Fig. 5B, treatment with the ERK inhibitor U0126 enhanced the effects of 6-gingerol on Cyclin D1 and p21. Additionally, co-treatment with U0126 and 6-gingerol preserved MnSOD levels but did not inhibit the cleavage of caspases 3 and 9, resulting in apoptosis (Fig. 5C). These findings suggest that 6-gingerol induces apoptosis and cell cycle arrest in GBM cells primarily through the activation of the ERK pathway and the modulation of MnSOD and caspase activities.

Inhibition of EGFR signaling pathway by 6-gingerol in GBM cells

The signaling activity of the EGFR is crucial for regulating apoptosis and proliferation. Activation of EGFR leads to the autophosphorylation of specific tyrosine residues. Once activated, EGFR binds to growth factor receptor-bound protein 2 (GRB2) and recruits SH2 domain-containing transforming protein (SHC). SHC then binds to GRB2, which subsequently binds to son of sevenless homolog 1 (SOS1). SOS1 activates RAS, which in turn activates RAF-1. RAF-1 phosphorylates MEK 1/2, leading to the activation of ERK 1/2, culminating in various biological responses. EGFR plays a pivotal role in coordinating apoptosis and proliferation by mediating the cell-to-cell propagation of ERK activation.

The effect of 6-gingerol on EGFR expression and phosphorylation was investigated. Examination of the upstream regulators of ERK, specifically the EGFR-RAS-RAF axis, showed that 6-gingerol treatment significantly decreased phosphorylated EGFR, RAS levels, and phosphorylated RAF (Fig. 6A). This suggests that 6-gingerol exerts its cytotoxic effects through the RAF-1/MEK/ERK pathway.

The role of the EGFR agonist in this signaling pathway was further investigated by analyzing the expression of these signaling molecules in the presence of EGF and 6-gingerol. Increased p-EGFR and p-ERK1/2 expression was noted upon EGF treatment, observed after 30 min, suggesting the role of an EGFR agonist in the activation of this signaling pathway. However, 6-gingerol reversed the effect of EGF, indicating that the antiproliferative and anti-apoptotic activities of 6-gingerol against GBM cells involve modulation of EGFR signaling (Fig. 6B).

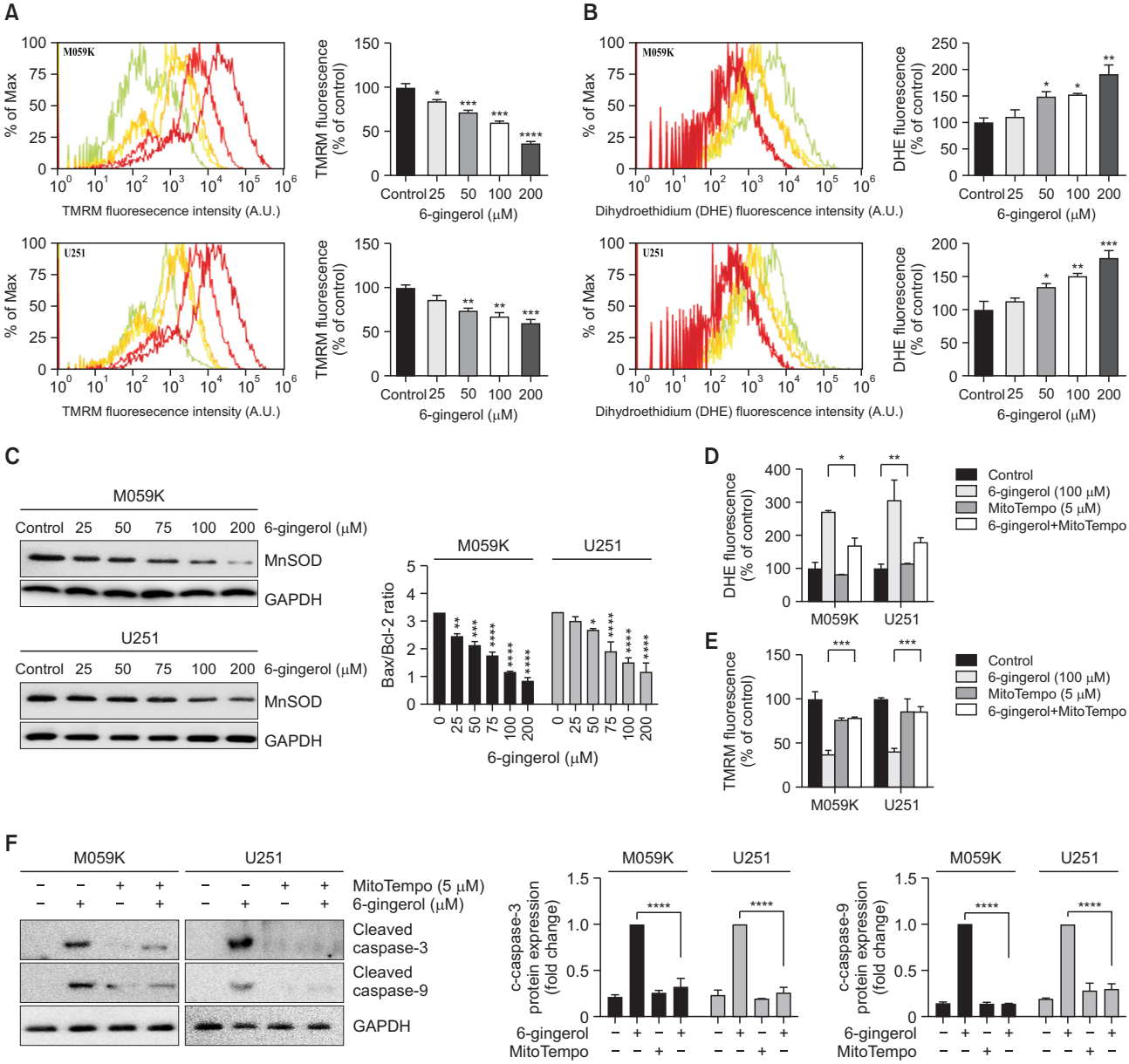

Fig. 4. Mito-TEMPO pretreatment partially reverses 6-gingerol-induced mtROS accumulation, ΔΨm reduction, and apoptosis. (A) The impact of 6-gingerol treatment on the reduction of ΔΨm in M059K and U251 cells after 48 h was evaluated using cell-permeable cationic TMRM and flow cytometry. The right panel presents the percentages of M059K and U251 cells with disrupted ΔΨm, which increased with escalating concentrations of 6-gingerol. (B) mtROS levels were assessed by DHE staining and analyzed via flow cytometry in M059K and U251 cells following a 48 h treatment with various concentrations of 6-gingerol. The right panel illustrates the quantification of mtROS accumulation induced by 6-gingerol. (C) Cell lysates were subjected to western blot analysis using an anti-SOD2 antibody, with GAPDH as the internal control. The right panel displays quantified SOD2 protein levels normalized to GAPDH. (D, E) Flow cytometry histograms depict alterations in ΔΨm in M059K and U251 cells. Following a 2 h incubation with 5 μM Mito-TEMPO, cells were treated with or without 100 μM 6-gingerol for 48 h. Results show a reduction in intact ΔΨm alongside an increase in mtROS levels compared to untreated cells. (F) Cleaved Caspase-3 and -9 protein expression were analyzed in M059K and U251 cells. The right panel provides a quantitative assessment of western blot data, indicating observed changes in protein expression. All data are presented as mean ± SD from three independent experiments; ****p<0.001, ***p<0.005, **p<0.01, *p<0.05 compared to the respective control.

DISCUSSION

Brain cancer, particularly GBM, is the most prevalent malignancy within the central nervous system and ranks among the top causes of cancer-related deaths globally (Miller and Perry, 2007; Louis *et al*., 2016). The current standard treatment, primarily utilizing drugs like temozolomide (TMZ), faces significant challenges due to toxicity and the emergence of resistance (Delello Di Filippo *et al*., 2021). This has prompted a search for innovative treatments, with recent research fo-

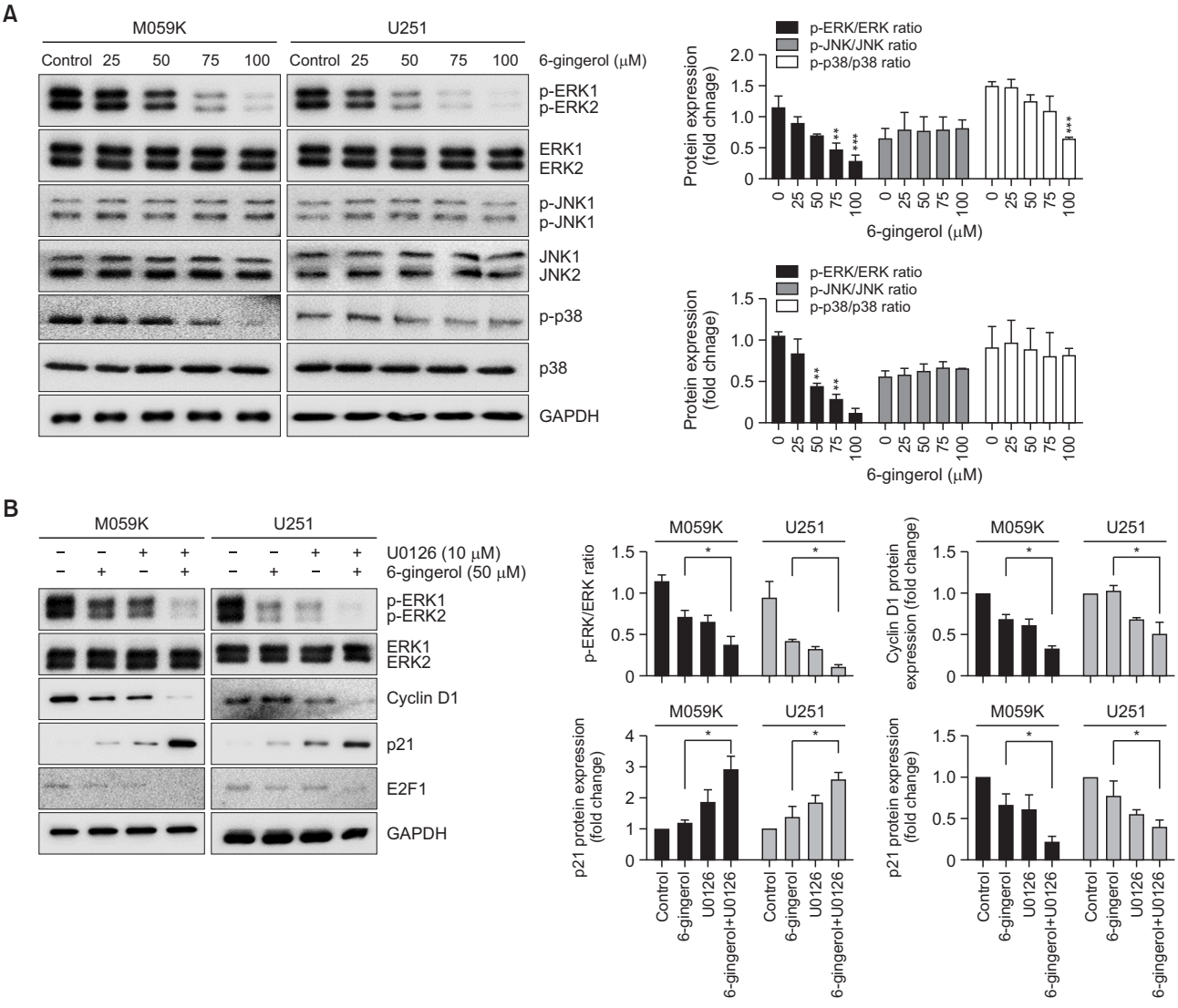

Fig. 5. 6-gingerol triggers apoptosis and suppresses cellular proliferation through ERK signaling pathway activation. (A) M059K and U251 cells were exposed to varying concentrations of 6-gingerol for 48 h. Subsequently, cells were harvested, and the levels of ERK, p-ERK, JNK, p-JNK, p38, and p-p38 proteins were analyzed through western blotting. (B) Another set of cells was treated with 100 mM 6-gingerol or 10 mM U0126 for 48 h, alongside their respective control conditions. Protein expression levels of ERK, p-ERK, p21, Cyclin D1, and E2F1, which are related to cell cycle regulation, were examined. (C) In both M059K and U251 cells, protein levels of SOD2 and apoptosis-related markers, including Cleaved Caspase-3 and -9, were assessed. GAPDH was used as the internal control. The right panel provides quantitative measurements derived from western blot data, illustrating observed changes in protein expression. All presented data represent the mean ± standard deviation obtained from three independent experiments; ****p<0.001, ***p<0.005, **p<0.01, *p<0.05 compared to their respective control conditions.

cusing on mechanisms like autophagy and ROS generation as potential therapeutic targets (Buccarelli *et al*., 2021; Li *et al*., 2022; Pandey *et al*., 2022; Sanati *et al*., 2022; Tong *et al*., 2023).

Combretastatin A-4 (CA-4), for instance, induces G2 arrest in U-87 cells by increasing cellular ROS levels (Roshan *et al*., 2023). Herbal medicines such as alpha-lipoic acid and auraptene have shown potential as alternative or combinational therapies. Specifically, the alpha-lipoic acid/auraptene combination has been found to suppress U87 cell viability, induce apoptosis, and cause G2/M cell cycle arrest (Izadi *et al*., 2023). Among herbal compounds, 6-gingerol and curcumin are known for their rich anti-inflammatory and antioxidant properties (Manju and Nalini, 2005; Dugasani *et al*., 2010), as evidenced by consistent findings in animal studies (Ramadan *et al*. 2011). Furthermore, when combined with traditional chemotherapeutics, curcumin has been shown to enhance the effectiveness of cancer treatments while mitigating side effects and resistance (Afshari *et al*., 2023).

Despite extensive research on curcumin, 6-gingerol has shown similar efficacy in inhibiting lung cancer cells, suggesting its potential as an anti-cancer agent (Eren and Betul, 2016). Moreover, 6-gingerol uniquely induces autophagy-dependent ferroptosis by inhibiting the expression of ubiquitin-specific

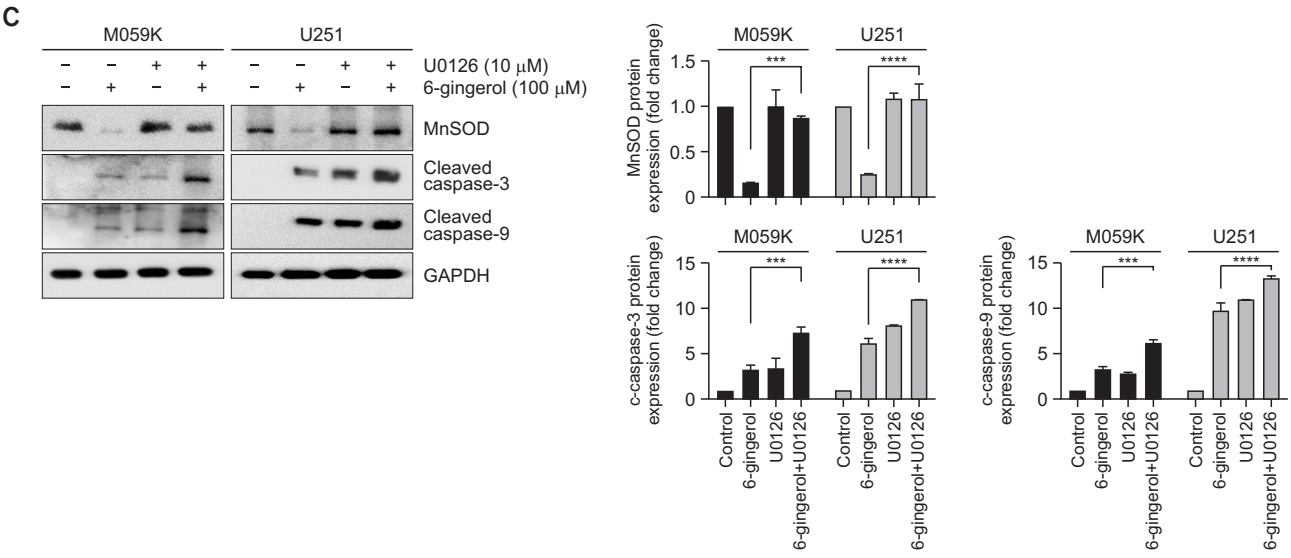

Fig. 5. Continued.

peptidase 14 (USP14) (Tsai *et al*., 2020). However, the inability of curcumin to penetrate the BBB limits its utility in treating glioblastoma. In contrast, 6-gingerol can cross the BBB in rodents and attenuate brain injury induced by subarachnoid hemorrhage (Tang *et al*., 2022), highlighting its potential for GBM research and treatment. Our results from the PAMPA-BBB assay further confirm the penetrating ability of 6-gingerol.

Recent studies have unveiled the potential of 6-gingerol in sensitizing gastric and epithelial ovarian cancer cells to cisplatin, reducing the proliferation of cervical cancer cells, and inducing TRAIL-mediated apoptosis in glioblastoma cell lines (Czarnik-Kwaśniak *et al*., 2019; Salari *et al*., 2023). Additionally, 6-gingerol induced both cellular and mtROS and caused DNA damage in breast cancer cell lines (Sp *et al*., 2021). Similarly, our study confirmed that 6-gingerol induced cell death and inhibited GBM cell proliferation by reducing MnSOD activity. Modulating mitochondrial function and oxidative stress-related genes has emerged as a promising strategy to induce apoptosis in GBM cells. For instance, Elesclomol (a copper-transporting therapeutic agent targeting mitochondria) increases mtROS levels in GBM stem cells and GBM-derived endothelial cells, leading to cell death (Buccarelli *et al*., 2021; Tarin *et al*., 2023; Tong *et al*., 2023).

Traditionally, MnSOD is viewed as a tumor suppressor (Zhong *et al*., 1997; Oberley, 2005). However, recent research suggests that MnSOD may increase the aggressiveness of tumor cells through two mechanisms: enhancing resistance to anoikis, which is crucial for preventing detached cells from surviving and forming metastases (Kamarajugadda *et al*., 2013), and sustaining the Warburg effect, a metabolic phenomenon that enhances tumor aggressiveness (Hart *et al*., 2015). Moreover, the addition of an ERK inhibitor mitigates the mtROS production induced by 6-gingerol and counters its growth-inhibitory effects in GBM cells, suggesting that its actions may be mediated through the inhibition of ERK-MnSOD signaling mediated by NF-κB and TP53 (Li *et al*., 2010; Robb and Stuart, 2010).

The RAS/RAF/MEK/ERK signaling pathway is crucial in cancer development and progression, involving the G-protein RAS and kinases RAF, MEK, and ERK (Song *et al*., 2023). Activation begins with ligand binding to receptor tyrosine kinases, leading to the sequential activation of RAS, RAF, MEK, and ERK (Roskoski, 2018; Wu *et al*., 2019). This pathway is frequently activated in cancers, including brain cancer (Xing, 2013), and serves as a critical downstream signal of EGFR (Koustas *et al*., 2017). In malignant glioblastoma, the pathway is hyperactive due to overexpression and/or increased activity of upstream regulatory factors such as EGFR and EGFRvIII (Saxena *et al*., 2008; Lo, 2010a, 2010b). Our study investigated the inhibitory effects of 6-gingerol on the EGFR/RAS/RAF/MEK/ERK pathway in GBM cells. Results showed that 6-gingerol dose-dependently reduced the levels of p-EGFR, RAS, p-RAF, p-MEK1/2, and p-ERK1/2. This suggests that 6-gingerol acts as a tumor suppressor by enhancing antiproliferative and pro-apoptotic effects through pathway inhibition. To determine if 6-gingerol functions as an EGFR blocker, we examined key signaling molecules in the presence of EGF (an EGFR agonist) and 6-gingerol. EGF treatment increased p-EGFR and p-ERK1/2 levels, confirming pathway activation, but 6-gingerol reversed EGF effects, indicating it effectively inhibits EGFR signaling. Targeting the EGFR/RAS/RAF/MEK/ERK pathway is essential due to its role in cell growth, apoptosis prevention (McCubrey *et al*., 2007), and its association with drug resistance (Garnett and Marais, 2004). Our findings suggest that 6-gingerol inhibits the growth of malignant glioblastoma cells by suppressing this pathway, leading to increased mtROS production and promoting apoptosis. Concurrently, 6-gingerol increases p21 expression, inducing cell cycle arrest in GBM cell lines.

Our study also highlights 6-gingerol ability to cross the BBB and exert potent cytotoxic effects on GBM cells. It induces G1 cell cycle arrest and apoptosis by modulating key cell cycle regulators and increasing pro-apoptotic signals. Specifically, 6-gingerol enhances G1 phase arrest through upregulation of p21 and downregulation of E2F1, along with suppression of Cyclin D1 and Cyclin D3. It also triggers apoptosis by increas-

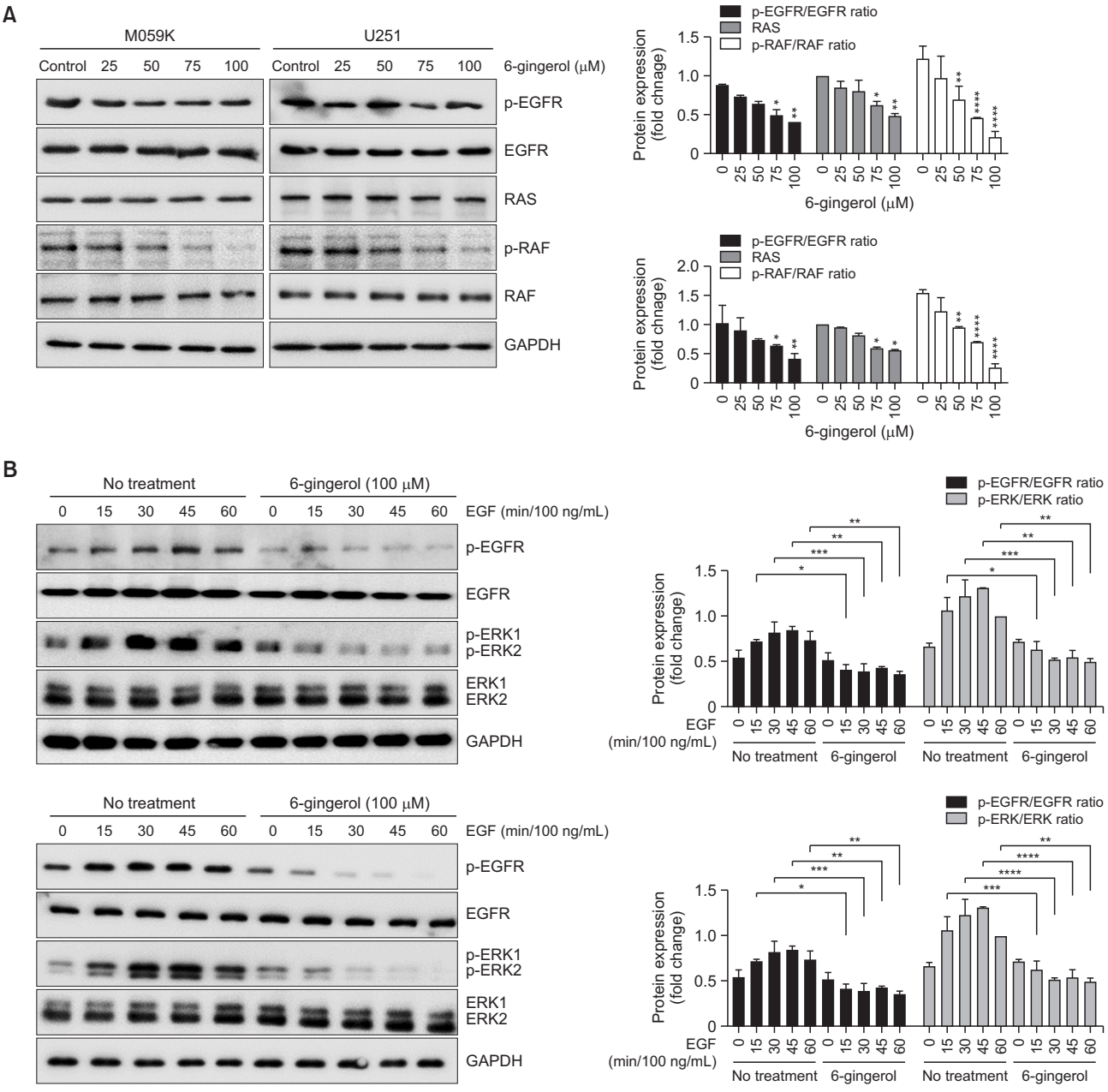

Fig. 6. 6-gingerol disrupts the EGFR/RAS/RAF pathway, leading to the inhibition of MEK/ERK expression. (A) M059K and U251 cells were exposed to varying concentrations of 6-gingerol for 48 h. Subsequently, the cells were harvested, and western blotting was conducted to assess the protein levels of EGFR, p-EGFR, RAS, RAF, and p- RAF. (B) Western blot analysis of EGFR, p-EGFR, ERK, and p-ERK in cell lysates from cells exposed to EGF (100 ng/mL) or 6-gingerol for different durations (0, 15, 30, 45, and 60 min). GAPDH served as the internal control. The right panel provides quantitative measurements derived from western blot data, indicating observed changes in protein expression. All data presented are represented as the mean ± standard deviation from three independent experiments; ****p<0.001, ***p<0.005, **p<0.01, *p<0.05 compared to their respective control conditions.

ing caspase-3 and -9 activities and altering the balance of Bcl-2 and Bax. Furthermore, 6-gingerol impacts mitochondrial function by inducing mtROS and disrupting ΔΨm, promoting oxidative stress-mediated apoptosis (Fig. 7).

Significantly, 6-gingerol inhibition of the EGFR/RAS/RAF/ERK signaling pathway underscores its mechanism of action, presenting a comprehensive approach to suppressing GBM cell proliferation and survival. These results underscore the potential of 6-gingerol as a multifaceted therapeutic candidate for GBM, warranting further investigation into its clinical applications.

CONFLICT OF INTEREST

The authors assert that there are no known financial con-

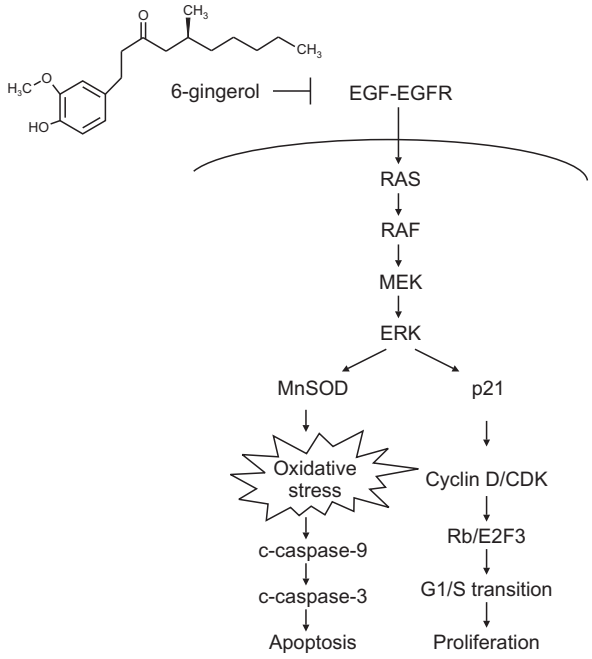

Fig. 7. Inhibition of cell proliferation and induction of apoptosis in GBM cells via the EGFR/RAS/RAF/MEK/ERK pathway by 6-gingerol. 6-gingerol Inhibits GBM cell growth by suppressing the EGFR/RAS/RAF/MEK/ERK Pathway, enhancing mitochondrial ROS production, and facilitating apoptosis. Concurrently, 6-gingerol augments p21 expression, resulting in cell cycle arrest in GBM Cell lines. This dual mechanism acts as an anti-tumor grow.

flicts of interest or personal relationships that could be perceived as affecting the integrity of the work presented in this paper.

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AUTHOR CONTRIBUTIONS

Sher-Wei Lim: Methodology, Conceptualization, Writing – review & editing. Wei-Chung Chen: Methodology, Investigation, Formal analysis, Writing – review & editing. Huey-Jiun Ko: Methodology, Writing – review & editing, Validation, Data curation. Yu-Feng Su: Visualization, Supervision, Methodology. Chieh-Hsin Wu: Project administration, Writing – review & editing. Fu-Long Huang: Data curation, Writing – review & editing. Chien-Feng Li: Project administration, Resources. Cheng Yu Tsai: Project administration, Methodology, Resources, Writing – review & editing.

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