Thesis for the Degree of Master of Science

Anti-cancer Effects of Desmethylanhydroicaritin on U87MG Human Glioblastoma Cells

By

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The Graduate School

Pukyong National University

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(Desmthylanhydroicaritin의 인간 뇌교종 암세포 에서의 항암 효능 확인)

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by

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ABSTARCT

We investigated anti-cancer effects of desmethylanhydroicaritin (DMAI), a major compound of the Chinese herbal medicine *Epimedium*, on Human cancer cell lines, including glioblastoma cancer U87MG cells, human lung cancer A-549 cells, human colorectal cancer HT-29 cells and human gastric cancer AGS cells. Among the all these cancer cell lines, U87MG cells were showed hight sensitive induced cell death by DMAI. DMAI showed cytotoxicity effects on U87MG cells at the concentration of 30 to 40 μM, however did not had any effects on HEK-293 cells. DMAI induced anti-proliferation effects via Ras/Raf/MEK/ERK MAPkinase, PI3K/Akt/mTOR signal pathway and G2/M phase cell cycle arrest by down-regulating p-Cdc 25 C, p-Cdc 2 and cyclin B 1. DMAI led to morphological change and inhibition of filapodia formation through regulation of Rac 1 and Cdc 42. In addition, migration and invasion of U87MG cells were inhibited by DMAI via down-regulation of matrix metalloproteinase (MMP)-2 and -9 expressions and activity. Our results suggest that DMAI has a potential as therapeutic agent against glioblastoma cells.

INTRODUCTION

Human glioblastoma is one of the most frequent and malignant (WHO grade IV) type of brain tumor in adults [1,2]. Glioblastoma is characterized as rapid invasion and long-distance migration [3]. These tumors are highly diffuse, with extensive dissemination of tumor cells within the brain, which hinders complete surgical resection. These aggressive characteristics are associated with a remarkable resistance to therapies available today. Current therapeutic strategies of glioblastoma include surgery, radiation therapy and chemotherapy. However, a median survival of glioblastoma is extremely poor [4,5].

The mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) signaling pathways play key roles on the proliferative, apoptotic and differentiation pathways. Signaling through the Ras/Raf/MEK/ERK MAPK and PI3K/PTEN/Akt/mTOR pathways are carefully orchestrated events generally starting from the cell surface and leading to controlled gene expression within the nucleus [6]. Regulation of these pathways is mediated by series of kinases, phosphatases and various exchange proteins [7]. In addition, dysregulated signaling through these pathways is often the result of genetic alternations in critical components in these pathways (e.g., Rsa, Raf, PI3K, PTEN, Akt) as well

as mutations in upstream growth factor receptors [8]. Also, these pathways interact with various other proteins to the nucleus to control gene expression[8]. Thus, the inhibition proofs of Raf, MEK, PI3K, Akt and mTOR may be useful in cancer treatment

Metastasis is a mechanism to form new tumors in distant tissues from a primary tumor. Migration and invasion are required for cancer metastasis. Invasion is induced by matrix metalloproteinases (MMPs) inducing degradation of the extracellular matrix (ECM) and basement membrane [9,10]. Thus, the inhibition of MMPs is to be a therapeutic strategy for tumor metastasis.

Desmethylanhydroicaritin (DMAI, Figure 1) is a major flavonoid isolated from *Epimedium koreanum*, which has been long used as a traditional Chinese herbal medicine. Previous studies for DMAI have been confirmed for potent inhibitory ability for NF-kB mediated inflammatory gene expression [11,12]. However, anti-cancer effect of DMAI hsve not been studied deeply.

Therefore, this study aimed to investigate the anti-cancer effects of DMAI and its underlying mechanism on anti-proliferation and metastasis in glioblastoma cells, U87MG cells.

MATERIALS AND METHODS

2.1 Isolation of Desmethylanhydroicaritin (DMAI)

The roots (5 kg) of *Sophora flavescens* were refluxed with MeOH for three hours (3x10 L). The total filtrate was concentrated to dyness in *vacuo* at 40°C in order to render the MeOH extract (1.1kg) and this extract was suspended in distilled water and sequentially partitioned with CH_2Cl_2 (114g), EtOAc (124g), *n*-BuOH (305 g), and H2O (524 g) in sequence. The CH_2Cl_2 fraction (70 g) was initially chromatographed over a Si gel column using CH_2Cl_2-MeOH under gradient conditions (CH_2Cl_2→ CH_2Cl_2:MeOH=80:1→1:1, MeOH, gradient) in order to yield DMAI (20mg).

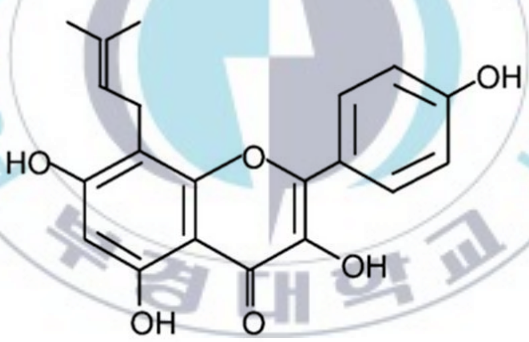


Figure 1. Chemical structure of Desmethylanhydroicaritin(DMAI) which is a major flavonoid isolated from the *Sophora flavescens*

2.2 Cell culture

Human glioblastoma cancer U87MG cells, human gastric cancer AGS cells, human colorectal cancer HT-29 cells, human lung cancer A-549 and human

embryonic kidney cell lines HEK-293, were obtained from American Tissue Culture Collection (Manassas, VA, USA). U87MG cells were incubated with Minimum Essential Medium (MEM), A-549 cells and AGS cells were incubated with RPMI 1640 medium, HT-29 cells and HEK-293 cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM). All media used in this sudy were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PAA Laboratories GmbH, PA Austria). The cells were cultured in 5% CO_2 incubator (Binder) at 37 ℃ in a humidified atmosphere. The culture was sub-cultured every three to four days and routinely checked under an inverted microscope for any contamination.

2.3 Cell viability assay

The effect of DMAI on cell viability was evaluated by WST-1 solution, based on the reduction of the member of metabolically active cells, and the results were expressed as a percentage of the control. Cells were seeded in 96-well microplates at a density of 1×10^4 per well and were cultured for 24 h. After 24 hours, the cells were treated with extracts at various concentrations and incubated for 12 or 24 hours. And then, media were exchanged and WST-1 solution was added 10μl in each well. Cells were incubated with WST-q for 3h at 37℃ for 3 h. Finally the optical density (OD) was measured with an ELISA reader (Molecular Devices, siliconvalley, CA, USA) at 460 nm. The cytotoxic activity of DMAI was expressed as IC50, which is the concentration of compound that causes 50% cell death. DMAI with IC50 of 40 μM or less is

considered active. DMSO was used to dilute the extracts and the final concentration of DMSO in each well was not in excess of 0.5% (v/v). No adverse effect due to presence of DMSO was observed.

2.4 Protein extraction and Western blot analysis

U87MG cells were treated with DMAI. The treated cells were washed with iced-cold 1x phosphate-buffered saline (PBS) and collected in lysis buffer [(50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS and cocktail of proteinase inhibitors (PMSF, EDTA, aptotinin, leupeptin, prostatin A; Intron biotechnology, Gyeonggi, Korea)] on ice. After incubation on ice for 30 min, the insoluble materials were removed by centrifugation at 14,000 rpm for 20 min. The protein contents of the cell lysates were determined by a Protein Quantification Kit (Commasie Blue Brilliant solution®) (Dojindo Molecular Technologies, Rockvile, MD, USA) with bovine serum albumin (BSA) as standard. The absorbance was measured at 595 nm. An aliquot form each sample was boiled for 5 min and then resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). And then proteins were electrotransferred to a nitrocellulose membrane (PALL Life Sciences, Pensacola, MI, USA) and blocked in PBST buffer (135 μM NaCl, 2.7 mM KCl, 4.3 mM $NaPO_4$, 1.4mM KH_2PO_4 and 0.5% Tween-20) containing 5% nonfatmilk (w/v). After blocking, membranes were then incubated with primary antibodies against GAPDH, Cleaved caspaser-3, Cleaved PARP, Epidermal Growth Factor Receptor (EGFR), Phospho-EGFR^{Tyr1068}, Ras,

Phospho-C-Raf^{Ser338}, Phospho-MEK1/2$^{Ser217/221}$, Phospho-ERK1/2$^{Thr202/Tyr204}$, P38MAPK, SAPK/JNK, Erk2, Phospho-P38MAPK$^{Tyr180/Tyr182}$, Phospho-SAPK/JNK$^{Thr180/Tyr183}$, β−actin, PI3K p110α, PI3K p110β, PI3K p110γ, PTEN, Akt, mTOR, p70 S6 kinase, Phospho-Akt^{Ser473}, Phospho-mTOR^{Ser2481}, Phospho-p 70 S6 kinase^{Ser371}, Phospho-Cdc25C^{Ser216}, Phospho-Cdc2^{Tyr15}, CyclinB1, Rac1, Cdc42, MMP-2, MMP-9 for overnight at 4 °C. Membranes were next incubated with HRP-conjugated secondary antibodies (Cell signaling technology) for 60 min. All membranes were visualized using West Save Gold ECL (Ab Frontier) and exposed to Hyperfilm (GE Healthcare). GAPDH was used as a loading control.

2.5 Wound healing assay

U87MG (5.0 x 10^5cells) were seeded in the chamber of an IBIDI culture insert (IbidiGmbH, Amkleferspitz19, 82152Martinsried, Germany) consisting of two reservoirs separated by a 500μm wall and incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h. After incubation, the inserts were gently removed and cells were cultured with medium to facilitate cell migration. Cell migration was recoded by phase contrast microscopy over a 12 h time course treating DMAI. On-line based wimasis image analysis was used to carry out a quantitative analysis of cell migration.

2.6 Transwell invasion assay

The invasion of tumor cells was assessed in matrigel coated trans-well chambers

with 6.5 mm polyvinyl/pyrolidone-free polycarbonate filter of 8 μm pore size (Corning life science, MA, USA), as described previously [Hung and Chang, 2008]. 5 x 10^4 cells (U87MG) and test compounds with different concentration were suspended in 200 μl of serum free media, placed in the upper trans-well chamber, and incubated for 24 h at 37 °C. Then, the cells on the upper surface of the filter were completely wiped away with a cotton swab, and the lower surface cells were fixed with 4 % formaldehyde, stained with crystal violet. After staining, lower surface cells were lysed with 2 % SDS for 1 h and the lysate was measured using a microplate reader at 570 nm.

2.7 Immunofluorescence staining

Cultured U87MG cells on cover-glass bottom dish were incubated for 30 min with 20 μM DMAI. After incubation, the cells were pre-treated with 1 μg/ml DAPI for 20min at 37 °C, then fixed with 4% formaldehyde (JUNSEI Chemical Co., JAPAN) for 15 min at room temperature and blocked for 1 h in a including 5 % mouse and rabbit normal serum (Santa Cruz Biotechnology Inc.) with 0.3 % Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Fixed and blocked cells were incubated with the primary antibodies (β-actin, Phospho ERK $1/2^{Thr202/Tyr204}$) for 3h and washed three times with PBS buffer. After washing, the cells were treated with 0.1μg/ml of anti-mouse IgG (H+L), F(ab´)2 fragment (Alexa Fluor®555Conjugate) and anti-rabbit IgG (H+L), F(ab´)2 fragment (Alexa Fluor® 488 Conjugate) for 1 h at room temperature. Stained cells were mounted on the slide with Prolong Gold Antifade Reagent (Invitrogen, GrandIsland, NY,

USA) and measured under a CarlZeiss LSM710 confocal laser scanning Microscope (CarlZeiss, Jena, Germany).

2.8 FACS analysis

Cells were harvested by trypsinization and fixed in 70 % ethanol overnight at 4°C. The cells were then resuspended in PBS buffer containing 0.2mg/ml RNase A and incubated for 1 h at 37 °C. The cells were stained with 40μg/ml propidium iodide at room temperature for 30 min in the dark. The distribution of G2/M DNA was analyzed using a FAS verse (Becton-Dickinson, Mountain View, CA, USA).

2.9 Gelatin zymography

U87MG cells were seeded onto 24-well cell culture plate and incubated with MEM containing 10 % FBS. When the cells had grown to approximately 90 % confluency, the cells were incubated with various concentrations of DMAI for 24 h. After 24 h, the culture medium was collected to process for analysis of influence of DMAI on MMP activities. Protein samples were mixed with 2X sample buffer (0.5M Tris-HCl, pH 6.8, Glycerol, 10 % SDS and 0.1 % Bromophenol Blue) and applied to a 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 0.1% gelatin. After electrophoresis, the gels were incubated in 2.5 % Triton X-100 for 30 min at room temperature twice, equilibrated for 30 min in Zymogram developing buffer (50mM Tris, pH 7.5, 200mM $NaCl_2$ and 0.2% Brij35) and

replaced with fresh Zymogram developing buffer and inctbated for 18 h at 37 °C. The gels were stained for 1 h in 0.5 % Coomassie Blue R-250 (30 % ethanol, 10 % acetic acid) and destained with a Coomassie R-250 destaining solution (50 % methanol, 10 % acetic acid). Proteolytic areas appear as clear bands against a dark background.

2.10 Statistical analysis

Data are presented as the mean ± standard deviation for the indicated number of separate experiments. The mean of the control was compared with the mean of the each individual treatment group by one-way ANOVA followed by Tukey's test using the statistical software Sigma Plot v.12.3 (Systat Software Inc., San Jose, CA, USA), and a statistically significant difference was set at $p < 0.05$)

3. RESULTS

3.1 DMAI induces anti-proliferation in glioblastoma

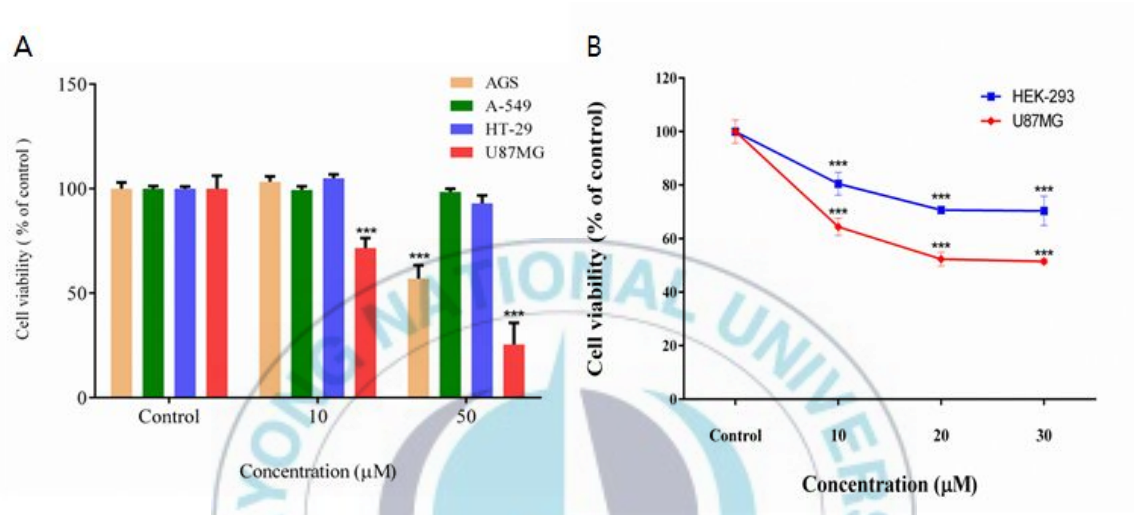


Figure 2. The effects of DMAI on cell proliferation. (A) Cancer cell lines, including AGS cells, AGS-549 cells, HT-29 cells and U87MG cells were treated with indicated concentration of DMAI. (B) DMAI had anti-proliferation effects on U87MG cancer cells, but did not affect cell viability of non-cancerous HEK-293 cells. Each bar represents the mean ± SD of three independent experiment. Asterisks are significant difference from control by ANOVA, *P<0.01 **P<0.05 ***P<0.001

To investigate anti-proliferative effects of DMAI, we performed WST-1 assay on human glioblastoma cancer U87MG cells, human gastric cancer AGS cells, human colorectal cancer HT-29 cells, human lung cancer A-549 cells and non-cancerous HEK293 cells. As shown in Figure 2 A, DMAI inhibited cell growth in cancer cell lines in a dose-dependent manner. Among these cell lines,

DMAI particularly decreased cell viability of U87MG cells than in other cancer cell lines. The IC50 of DMAI was observed between 40 and 50 μM and morphological changes were observed in dose-dependent manner (Figure 3 A). However, DMAI did not show any significant effect of non-cancerous HEK293 cells (Figure 2 B).

3.2 DMAI induces apoptosis in glioblastoma

As shown in figure 2 A, DMAI has anti-proliferation effects in cancer cell lines which exhibited most effective inhibition on cell proliferation of U87MG (Figure 2 B). To determine whether DMAI affects nuclear condensation and formation of apoptosome in U87MG, DAPI (4′,6-diamidino-2-phenylindole dihydrochloride hydrate) staining and western blot analysis were performed. The results of DAPI staining indicated that U87MG cells treated with 20 μM of DMAI showed changes at nuclear membrane. A classical hall marker of apoptotic cells, nuclear condensation (apoptosome formation), was observed in U87MG cells treated with 30 μM DMAI for 24 h (Figure 3 B). In addition, the expression of cleaved caspase-3 and cleaved PARP proteins, key effector proteins which induce nuclear condensation and apoptosome, were down-regulated in a dose-dependent manner (Figure 3 C).

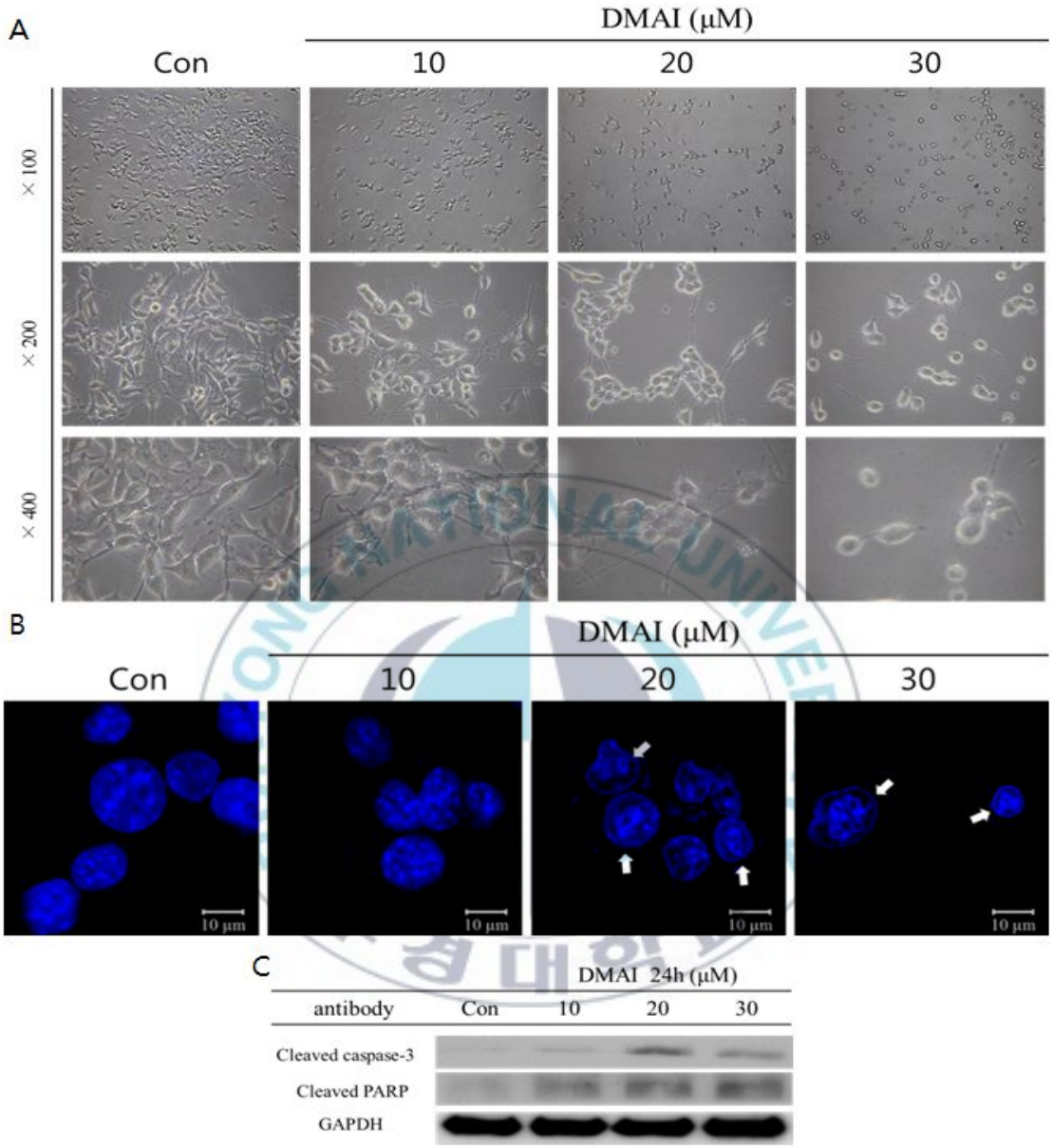


Figure 3. The effects of DMAI on morphological changes and apoptosis induction. (A) U87MG cells were treated with DMAI in a dose-dependent manner for 24h, and imaged the cell morphology under a phase contrast inverted microscope at ×100, ×200 and ×400 magnification. (B) The cells containing condensed chromatin or exhibiting fragmented nuclei are observed following DAPI staining. (C) Apoptosis related proteins by western blot analysis after DMAI treatment.

3.3 DMAI inhibits Raf/MEK/ERK MAPK pathway in U87MG cells

Mitogen-activated protein kinase (MAPK) signaling cascades have been proved to have essential roles in the regulation of a wide variety of cellular processes, including cell growth, migration, proliferation, differentiation, apoptosis and cell cycle arrest. In glioblastoma, Ras/Raf/MEK/ERK pathway is required for cell proliferation. Recently researches have shown that some agents inhibiting this signal pathway, such as Sorafenib, an inhibitor of Raf, which is able to inhibit proliferation and thereby induce apoptosis in glioblastoma[13]. In this study, effects of DMAI on the expression of ERK, JNK and p38 MAP kinases were investigated. Western blot analysis showed that the protein expression of ERK, JNK and P38MAP kinases was not significantly changed in time-dependent manner (Figure 4 A). The phosphorylation of JNK and P38MAPkinase were not significantly reduced in DMAI treatment, while a phosphorylation of ERK was down-regulated in time-dependent manner (Figure 4 A). These results suggest that DMAI selectively regulate ERK activation through modulating its phosphorylation in glioblastoma cells. To confirm whether DMAI affects Raf/MEK/ERK pathway, key proteins of Raf/MEK/ERK pathway, Ras, Epidermal Growth Factor Receptor (EGFR), Raf and MEK were investigated by western blot analysis. As shown in Figure 4 B, the expression of EGFR was not significantly changed in time-dependent manner. However, the phosphorylation of EGFR and expression of Ras were significantly decreased in time-dependent manner. Phosphorylation of C-Raf and MEK was also reduced

in a time-dependent manner (Figure 4 C). Furthermore, expression level of phosphorylated ERK and its translocation to nucleus were decreased in immunofluorescence staining of phospho ERK 1/2 (Figure 4 D).

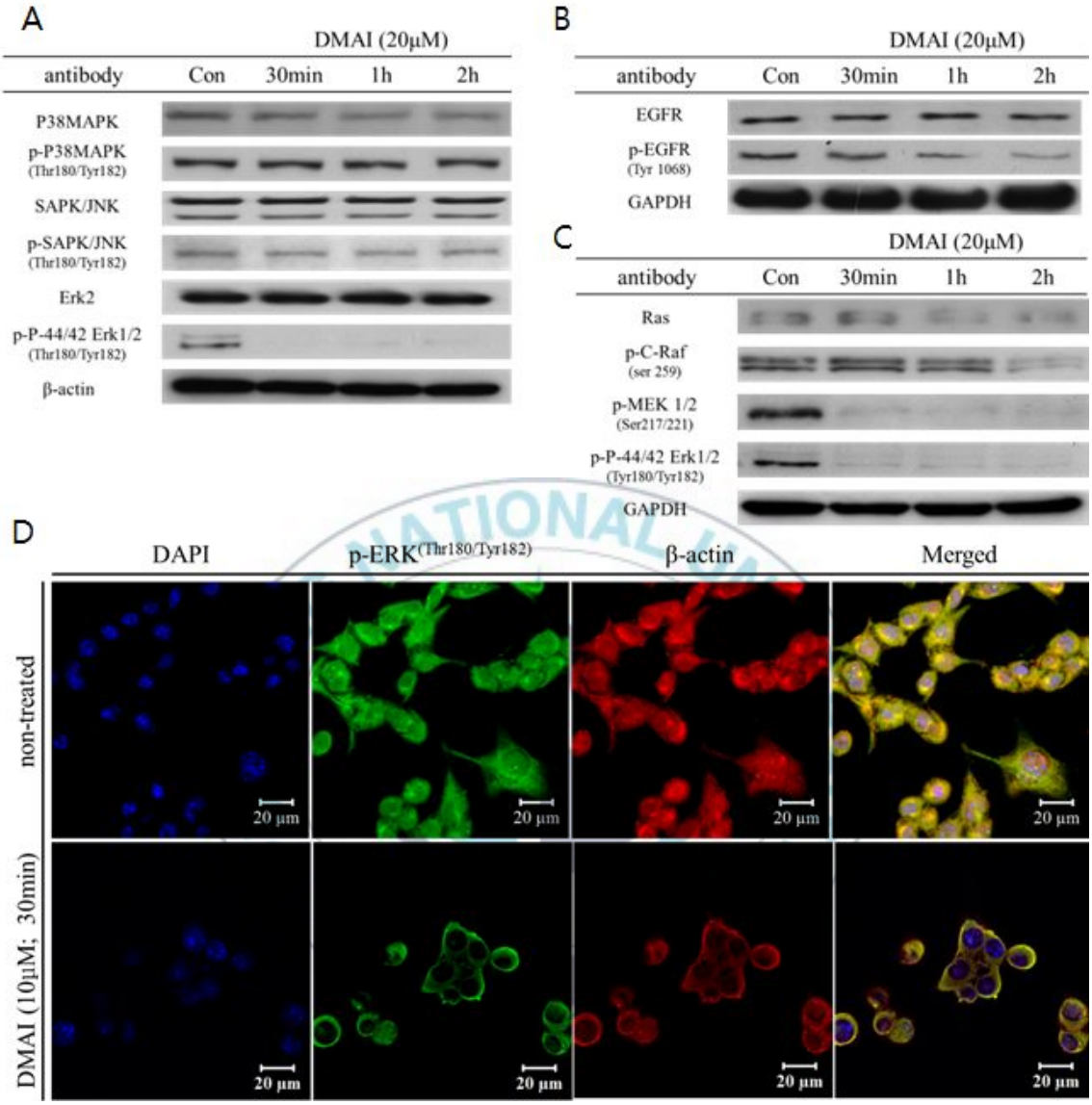


Figure 4. DMAI inhibits EGFR/Ras/Raf/MEK/ERK pathway. (A) U87MG cells were treated with time-dependent manner for 20μM DMAI, followed by western blot analysis for total and phosphorylated MAPK members (P38, JNK, ERK). (B) and (C) Western blot analysis for EGFR/Ras/Raf/MEK/ERK pathway related proteins after DMAI treatment. (D) U87MG cells were treated with DMAI, and examined by immunofluorescence staining to detect the level of phosphor-ERK (green) with DAPI (nucleic; blue) and β-actin (cytoskeletal; red) using confocal laser scanning microscope (×400)

3.4 DAMI inhibits the PI3K/AKT/mTOR/p70S6K pathway in U87MG cells

To investigate ether possible mechanisms of DMAI, the PI3K/Akt/mTOR/p70 S6K signaling pathways was determined in U87MG cell lines. As shown in Figure 5 A, an expression of PI3K p110α was significantly decreased, whereas P3K p110β and p110γ were notchanged in a time-dependent DMAI treatment. There wasno great chnge on an expression of Akt, mTOR and p 70 S6 kinase. In contrast, phosphorylation of those three proteins was diminished in response to DMAI treatement (Figure 5 B). In addition, it was observed that distributed phosphorylated Akt was reduced by DMAI, using immunofluorescent staining (Figure 5 C). These results indicated that DMAI can inhibit PI3K/Akt/mTOR/p70S6K pathway by down-regulating phosphorylation of proteins involved in this pathway.

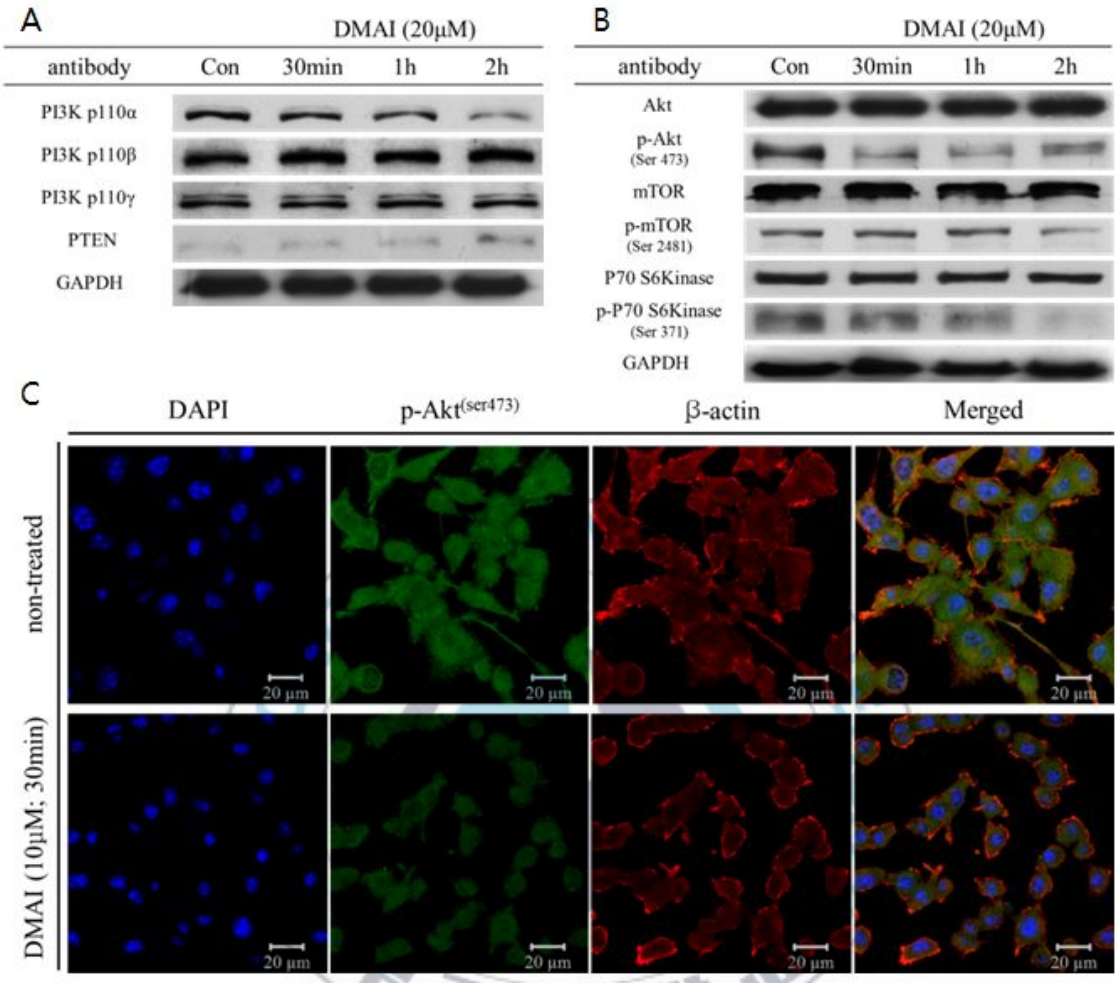
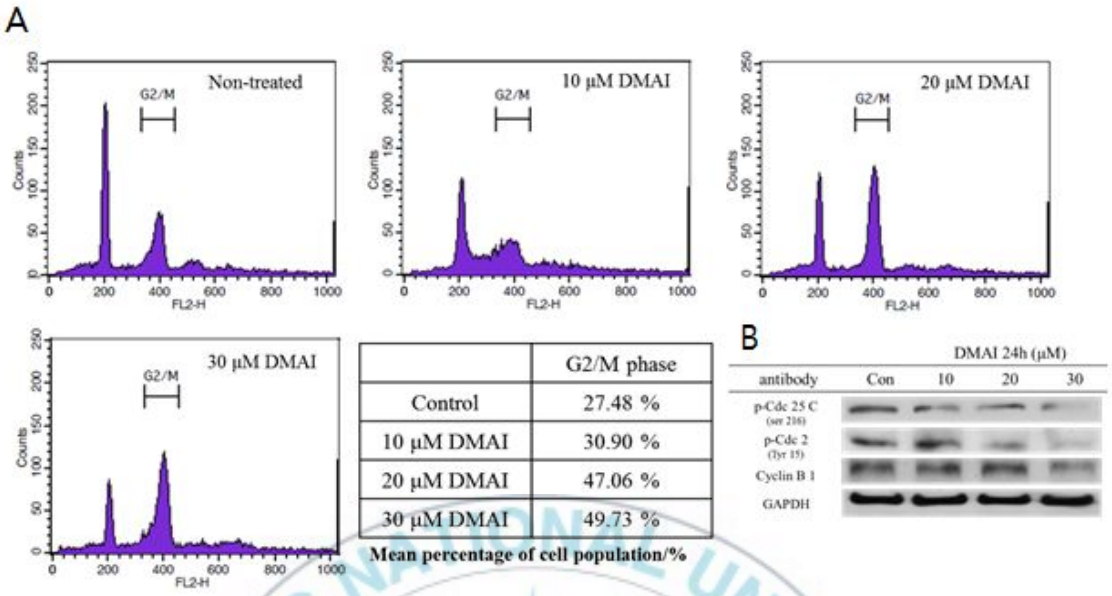


Figure 5. DMAI inhibits to PI3K/Akt/mTOR/p70S6kinase signal pathway. (A) U87MG cells were treated with time-dependent manner (o h, 30 min, 1h and 2 h) for 20 μM DMAI, followed by western blot analysis for PI3K p110α, PI3K p110β, PI3K p110γ and PTEN. (B) Western blot analysis for Akt/mTOR/p70S6kinase related proteins after DMAI treatment. (C) U87MG cells were treated with DMAI, and examined by immunofluorescence staining to detect the level of phospho-Akt (green) with DAPI(nucleic; blue) and β-actin (cytoskeletal; red) using confocal laser scanning microscope (×400)

3.5 DMAI induces G2/M cell cycle arrest on U87MG cells

Previously, it was confirmed that essential components for cell growth and cell proliferation signal, Ras/Raf/MEK/ERK MAPK and PI3K/Akt/mTOR/p70S6kinase signal pathways, were suppressed by DMAI treatment. To determine whether DMAI affects to cell-cycle distribution and related checkpoint factors, western blot analysis and FACS analysis were performed. The results showed that DMAI treated cells were accumulated progressively in the G2/M phase (Figure 6 A). Compared with the control, treatment with DMAI has resulted an increased proportion of G2/M phase cells in a dose-dependent manner 30.90%, 47.06%, and 49.73%. Besides, DMAI treatment resulted in a down-regulated in phosphorylation of Cdc 25 C, Cdc 2 and expression of Cyclin B 1 (Figure 6 B). All the above observations suggest that G2/M phase arrest also accounts for the anti-proliferative effect of DMAI in U87MG cells.



	G2/M phase
Control	27.48 %
10 μM DMAI	30.90 %
20 μM DMAI	47.06 %
30 μM DMAI	49.73 %

Mean percentage of cell population/%

Figure 6. DMAI induces cell-cycle arrest in U87MG cells. (A) Cells were treated with 10, 20 and 30 μM DMAI for 24 h and then the cell cycle distribution was measured using flow cytometery (B) Western blot analysis for G2/M transition related proteins after DMAI treatment.

3.6 DMAI inhibits the formation of filapodia in glioblastoma cells through down-regulation of Cdc42 and Rac expression

As shown in Figure 3 A, it was confirmed that DMAI induces cell morphological change in dose-dependent manner. Changes in cell shape and plasticity in skeletal dynamics are critically involved in cell adhesion, migration, invasion and the whole process of metastasis [14,15]. Several studies have shown that an actin polymerization which leads to filapodia assembly and an actin depolymerization play essential roles in cell motility [16,17]. To explore the molecular events associated with actin cytoskeletal assembly in response to DMAI treatment, the formation filapodia in U87MG cells was assessed by staining with Rhodamine-phalloidin, which specifically bindes F-actin [18]. The results indicated that filapodia assembly in U87MG was abolished by DMAI (Figure 7 A) and expression of cell division cycle 42 (Cdc 42) and Rac 1, which are essential for cell motility and filapodia formation [19] was down-regulated in dose-dependent manner in western blot analysis (Figure 7 C, d). Besides, expression level of Rac 1 was decreased by immunostaining of Rac 1 (Figure 7 B).

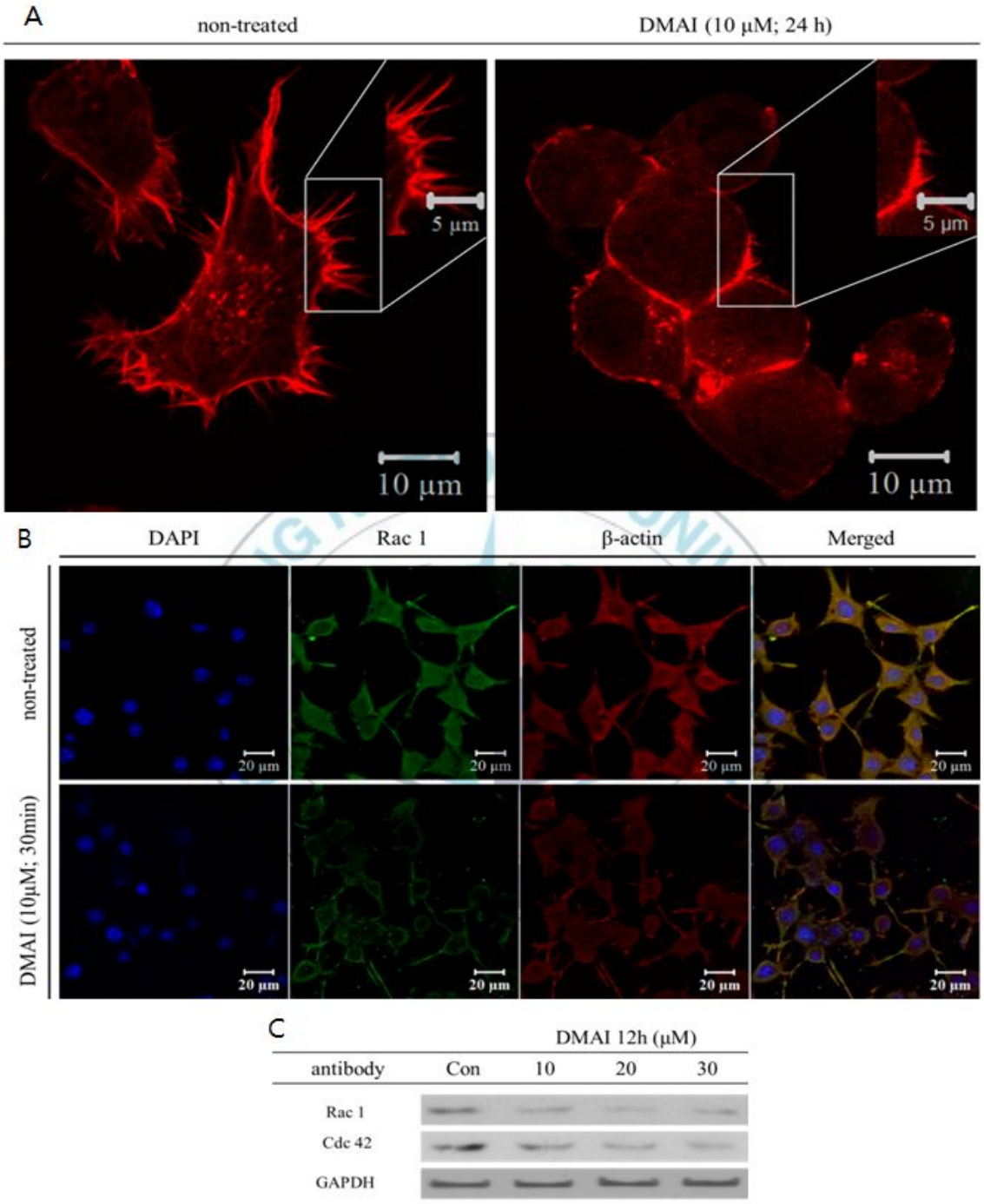


Figure 7. DMAI disrupts actin assembly and filapodia formation. (A) U87MG cells were assessed by fluorescence staining with Rhodamine phallodin, a substance which

specifically detects F-actin. Incubation of U87MG cells with DMAI (10μM) for 24h (right) (B) U87MG cells were treated with 10 μM DMAI, and examined by immunofluorescence staining to detect the level of Rac 1(green) and β–actin (cytoskeleton; red) using confocal scanning microscope (×400) (B) U87MG cells were treated in a dose-dependent manner (0, 10, 20, 30 μM) DMAI for 12h, followed by western blot analysis for Rac1 and Cdc 42.

3.7. DMAI inhibits invasion and migration of U87MG cells

Glioblastoma patients die more often from invasion and migration [20], therefore the inhibition of invasion and migration abilities is a useful treatment of tumor progression and malignant. In previous results, it is confirmed that DMAI inhibits filapodia through down-regulation of actin assembly protein, Rac 1 and Cdc 42. To investigate the anti-invasion and anti-migration effects of DMAI, U87MG cells were examined using wound healing assay and trans-well invasion assay. Wound healing assay showed that entire wound areas in DMAI treated group were not markedly decreased in comparison with the control group (Figure 8 C). In trans-well invasion assay, invasive cells were decreased in a dose-dependent manner (Figure 8 D). In addition, MMP-2 and MMP-9 are secreted to degrade the extracellular matrix for cell invasion and migration in metastatic cancer cells [21]. In zymography assay the activity of MMP-2 and MMP-9 was reduced when cells were treated with DMAI for 12 h consitent with this result, the expression of both two proteins was reduced in western blot analysis. (Figure 8 A, B).

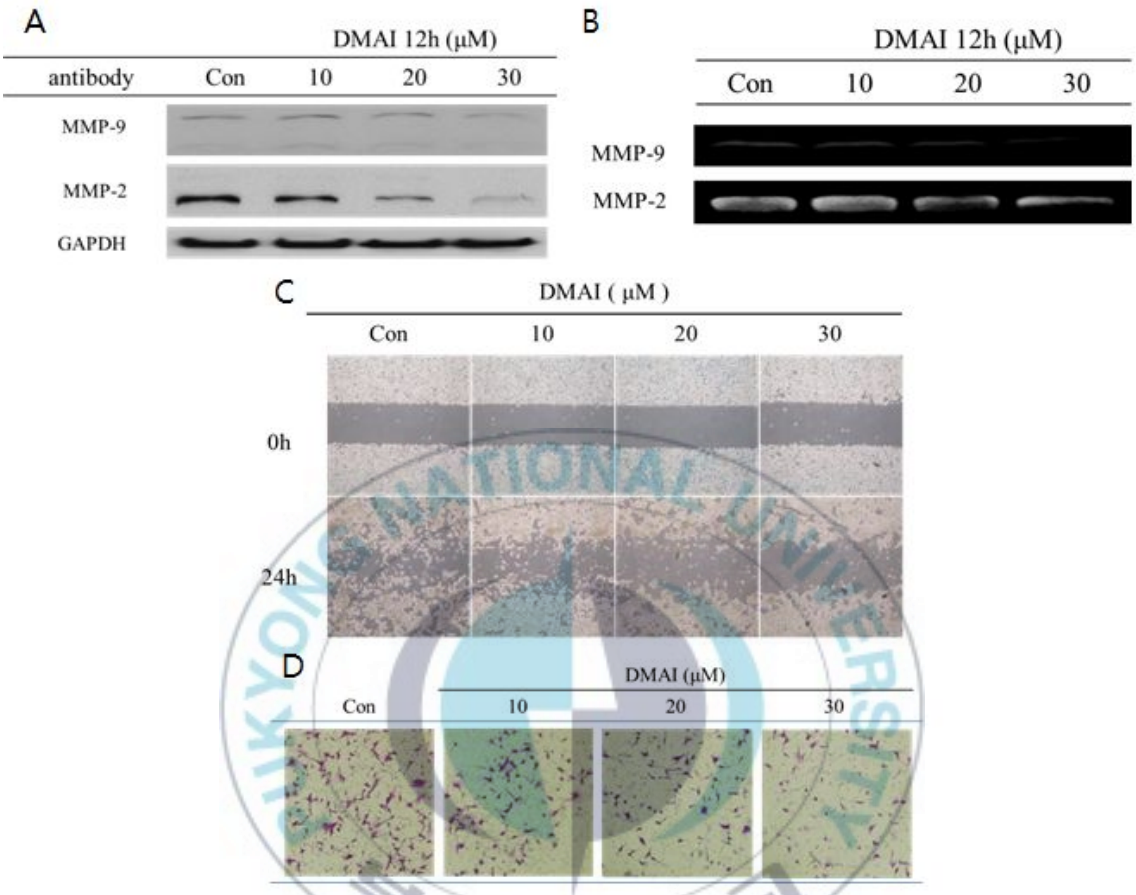


Figure 8. DMAI inhibited migration and invasion of U87MG cells. U87MG cells were treated with 0, 10, 20, 30 μM with DMAI for indicated time. (A) Western blot results showing MMP-2 and MMP-9 expression and (B) gelatin gel zymography results showing MMP-2 and MMP-9 activity. (C) Wound healing assay and (D) trans well invasion assay results showing migration and invasion of U87MG cells for 24 h in a dose-dependent manner (×40)

4. DISCUSSION

This study demonstrates that Desmethylanhydroicaritin isolated from *Epimedium*, induces down-regulation of Ras/Raf/MEK/ERK MAPkinase and PI3K/Akt/mTOR pathways, destruction of filapodia formation and anti-metastasis, leading to anti-cancer effects in human glioblastoma U87MG cells.

DMAI had anti-proliferation effect on human cancer cell lines by WST-1 assay (Figure 2 A). Especially, most inhibitory effect on cell proliferation was indicated in U87MG human glioblastoma cells, withoyt any inhibitory effects on non-cancerous HEK-293 human kidney cells (Figure 2 B). Furthermore, DMAI induced severe cell morphological changes and nucleic condensation and fragmentation in U87MG cells (Figure 3 A and B). Nucleic condensation and fragmentation are related to apoptotic cell death, we measured protein expression associated with apoptosis cell death using western blot analysis. Expression of cleaved caspase-3 and cleaved PARP proteins were increased by DMAI treatment in a dose-dependent manner (Figure 3 c). It had been know that PARP helps cells to maintain their viability by DNA repair [22]. However, when PARP is cleaved by cleaved caspase-3 which by cleaves a variety of cellular substrate to induce apoptosis, it facilitates cellular disassembly and it is proposed as a marker of cells undergoing apoptosis [23, 24]. Therefore, these results suggest that DMAI particularly decreases cell viability of U87MG cells by anti-proliferative effects and induction of apoptotic cell death. Because cleaved caspase-3 and cleaved PARP proteins were increased by DMAI treatment in a dose-dependent manner (Figure 3 C).

The Ras/Raf/MEK/ERK MAPKinase and PI3K/Akt/mTOR pathways have critical roles in several cellular processes, including cell growth, cell cycle, motility, survival and proliferation, and are tightly controlled by various physiological mechanisms [25]. These signal pathways are activated by mutated receptor tyrosine kinase (RTK) [26,27]. In human glioblastoma, the mutation of RTKs occurs frequently including platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), suggesting an autocrine or paracrine loop activation, activating mutation in ERBB2(member of the EGFR family) and activating mutation in hepatocyte growth factor receptor (MET) [27-29]. These RTKs alteration leads to highly invasive and over growth feature of glioblastoma cells, resulting in the failure to clean up the tumor tissues by surgery and radiotherapy [30]. Therefore, suppressing this signal is important to develop new anti-cancer therapeutic compound for human glioblastoma. In MAPK pathway, DMAI selectively inhibited to ERK activation on glioblastoma cells by down-regulation in phosphorylation of protein, not P38 and JNK (Figure 4 A). In addition, DMAI effected key upstream components of ERK pathway by inhibiting phosphorylation of EGFR, MEK, C-Raf and expression of Ras by down–regulation in time-dependent manner (Figure 4 B and C). Furthermore, the expression level of phosphorylated ERK and its translocation to nucleus were decreased in immunofluorescence staining of phosphor ERK 1/2 (Figure 4 D). In PI3K/Akt/mTOR pathway, DMAI inhibited expression of PI3K p 110α, but does not affect expression of PI3K p110β and PI3K p110γ (Figure 5 A). Expression of PTEN, a major negative regulator of the PI3K and Akt

signaling [31,32], was also up-regulated in time-dependent manner (Figure 5 A). Phosphorylation of Akt, mTOR and P70S6Kinase was down-regulated in time-dependent manner (Figure 5 B). Furthermore, expression level of phosphorylated Akt was decreased in immunofluorescence staining of phosphor Akt (Figure 5 C). It was further examined whether this inhibitory effect of DMAI affects to cell-cycle distribution and related checkpoint factors. Cdc 25 C and Cdc 2 proteins have important roles in an entry of eukaryotic cells into mitosis [33]. Cdc 25 C is a phosphatase protein responsible for activating Cdc 2 through dephosphorylation of tyrosine 15 (Tyr 15) and phosphorylation of Serine (Ser) 345 [34]. When Cdc 25 C and Cdc 2 are activated, these proteins binds to cyclin B 1 which is key regulatory substrate to form mitosis-promoting factor (MPF) [34,35]. As shown in Figure 6, phosphorylation of Cdc 25 C, Cdc 2 and expression of Cyclin B 1 were down-regulated in dose-dependent manner and the G2/M cell cycle arrest was detected by facs analysis. In conclusion, DMAI exhibits anti-proliferation effects via down regulation of Ras/Raf/MEK/ERK signaling pathway, PI3K/Akt/mTOR signaling pathway and G2/M phase cell cycle arrest mediated by decreases in Cdc 25 C, Cdc 2 and Cyclin B 1 in U87MG human glioblastoma cells.

It was observed that DMAI induced morphological changes on U87MG cells in dose-dependent manner (Figure 2 A), hence it was thought that DMAI is involved in cytoskeletal changing. Cytoskeltal changing is regulated by Rac 1 and Cdc 42 and it was essential for development of cell motility. Rac 1 and Cdc 42 lead to the formation of membrane ruffles via filapodia assembly and

depolymerization, have been shown to regulate a vast spectrum of biological function, including cell cytoskeleton rearrangement and cell polarity during migration and invasion [19]. In Rhodamine-phalloidin staining, the formation of filapodia was inhibited by DMAI treatment (Figure 7 A). It has been revealed that Cdc 42 has key role for filapodia formation and its extension and Rac 1 is one of Rho GTPase families which is recruited to plasma membrane at the leading edge to increase membrane ruffles. In this study, expression level of both Cdc 42 and Rac 1 were down-regulated in a dose-dependent manner. (Figure 7 C). Also, Expression level of Rac 1 was decreased in immunofluorescence staining (Figure 7 B). These results demonstrate that DMAI inhibites cell motility of U87MG cells via inhibition of filapodia formation. To prove that filapodia destruction by DMAI results in decreases in cell migration, wound healing assay was performed. Wound healing assay showed that entire wound areas in DMAI treated cells group were not markedly decreased in comparison with the control group (Figure 8 C). Consequently, DMAI diminished cell migration through down regulation of Cdc 42 and Rac 1 which lead to inhibite the formation of filapodia at leading edges of cell membrane.

One of the most representative characteristics of glioblastoma is invasive ability, diffusing into intact brain regions, which hinders elimination of the cancer by surgery [20,37]. Due to this invasive ability of metastatic cancer cells, it is necessary to degrade extracellular matrix by matrix metalloproteinases(MMPs), zinc-dependent endopeptidases. MMPs degrade the extracellular matrix and promote invasion and metastasis of cancer cells [38,39],

therefore, inhibition of invasion and migration could be an effective strategy for treatment of glioblastoma. In trans-well invasion assay, invasive cells were decreased in dose-dependent manner (Figure 8 D). Protein expression and activities of MMP-2 and MMP-9 were decreased by DMAI in dose-dependent manner (Figure 8A and 8B). It is thought that decreased portein expression cause reduction of MMP-2 and MMP-9 enzymatic activities. Therefore, our results suggest that DMAI inhibited invasion and migration of U87GM cells via negative regulation of MMP-2 and MMP-9 expression.

Taken together, these results demonstrated that DMAI has particularly anti-proliferation effects on U87MH human glioblastoma cells via regulating Ras/Rad/MEK/ERK MAPK, PI3K/Akt/mTOR signal pathways, and G2/M phase cell cycle arrest effects by down-regulation of p-Cdc 25 C, p-Cdc 2 and cyclin B 1. It is also founded that DMAI inhibitory effects on filapodia formation and reduction of MMPs expression and activities contribute to decreases in cell migration and cell invasion, respectively. As shown in Figure 9. proposed signaling pathway related to anti-cancer effects of DMAI is displayed as schematic representation. Our results may give new insights into the molecular mechanisms of DMAI regulation anticancer effects and provide a potential way to treat glioblastoma.

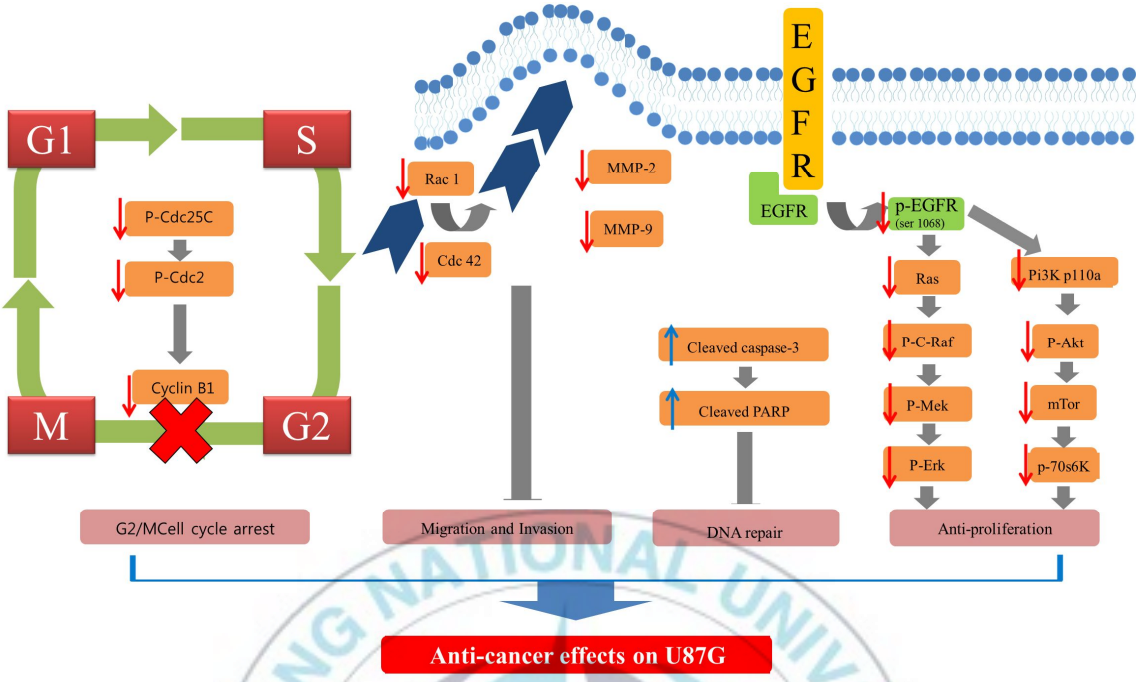


Figure 9. Our results demonstrated that proposed signaling pathway of DMAI induced anti-cancer effects on U87MG cells.

5. 국문초록

Glioblastoma (인간 뇌교종 암)는 사람의 뇌에 발생하는 암으로 침윤성과 전이성이 매우 높은 암으로 다발성적인 특징이 있으며 기기를 이용한 완벽한 제거가 어렵다. 다른 치료 방법으로는 항암제나 방사선 치료가 있으나 부작용이 크며 glioblastoma의 약물에 의한 저항성으로 인해 효과가 미비한 것으로 알려져 있다.

Desmethylanhydroicaritin은 *Sophora flavescens* (고삼) 에서 분리된 물질로, 현재까지 NF-kB pathway 관여와 anti-inflammation 효능이 있다는 것이 밝혀진 상태이나 항암 효능에 대해서는 아직 까지 구체적으로 알려진 바가 없다. 따라서, 본 연구는 desmethylanhydroicaritin의 항암 효능을 알아보고 glioblastoma 의 새로운 항암제로서의 개발 가능성을 알아보았다. 우선, desmethylanhydroicaritin를 이용하여 여러 암세포(위암, 뇌암, 폐암, 췌장암)에 cell cytotoxicity assay를 실시한 결과 glioblastoma에서 가장 높은 세포 독성이 나타났으며, apoptotic cell death를 유도함을 확인하였다. 그리고 세포 생존에 관여하는 MAPK pathway 중 Raf/MEK/Erk MAPK pathway와 PI3K/Akt/mTOR pathway를 억제시키며, G2에서 M기로의 세포 주기 진행을 억제됨을 확인하였다. 또한 암세포의 이동성과 관련된 filapodia의 형성이 붕괴되고 침윤 과정에 필수적인 matrix metalloproteinase -9과 -2의 발현과 활성이 감소됨을 확인 할 수 있었다. 이를 통해 desmethylanhydroicaritin이 glioblastoma 특이적으로 세포 성장을 억제와 전이성을 억제 효과가 있다고 제안할 수 있다.

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