A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

# Dehydroglyasperin C suppresses TPA-induced cell transformation in JB6 P+ mouse epidermal cells through direct inhibition of MKK4 and PI3K

*JB6 P+ mouse epidermal cell에서 디하이드로글리아스페린 C의 MKK4, PI3K 저해에 의한 세포 암화 억제 효능*

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# Abstract

Bioactive compounds from plant-derived sources have received substantial attention due to their potential therapeutic and preventive potential against various human diseases. Licorice (*Glycyrrhiza*), one of the most frequently used ingredients in traditional oriental medicine, has been prescribed for treating numerous ailments including inflammatory problems, chronic fatigue syndrome, and even cancer. Glycyrrhizin (a triterpenoid saponin) and chalcones are major constituents of licorice and have been reported to exhibit antioxidant, antiobesity, and antimicrobial activities. Dehydroglyasperin C (DGC) is a major isoflavone found within the root of licorice. However, despite its high content in licorice root, little is known about its physiological properties including potential chemopreventive effects or the underlying molecular mechanisms responsible. In the present study, I investigated the inhibitory effects of DGC on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced cell transformation in JB6P+ mouse epidermal cells. DGC treatment inhibited TPA-induced COX-2 expression and attenuated activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) transcriptional activation. DGC was also observed to inhibit MKK4 and PI3K kinase activity. Taken together, these results suggest that DGC exhibits cancer chemopreventive potential as well as inhibitory effect on neoplastic cell transformation via modulation of the MKK4 and PI3K pathways.

**Key Words: dehydroglyasperin C, cyclooxygenase-2, mitogen-activated protein kinase-kinase 4, phosphatidylinositide 3-kinase, cell transformation**

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# Contents

# Ⅰ. Introduction

Carcinogenesis is a multistage process that arises from the dysregulation of multiple signal transduction pathways [1]. The mitogen-activated protein (MAP) kinases are often implicated in carcinogenesis, and are activated by various tumor promoters that normally convey signals from the cell surface to the nucleus. Activated MAPKs phosphorylate downstream substrates and transcription factors critical for tumor development, including activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) [2]. AP-1 is a transcription factor that plays a critical role in cell transformation, tumor promotion, progression and metastasis [3, 4], and also controls genes involved in inflammation [5]. Similarly, Akt is a major downstream regulator of multiple growth factors that influence cell proliferation, neoplastic transformation and survival [6]. Akt has been reported to be a crucial factor in cell transformation and controls AP-1and NF-κB to induce carcinogenesis [7].

Upstream regulators of MAPKs and the Akt signaling pathway have been recognized as promising targets for chemoprevention. Phosphatidylinositol 3-kinase (PI3K) is a heterodimeric lipid kinase that acts as an upstream regulator of Akt and controls various cellular responses, thereby regulating cell growth, proliferation, and motility [6]. Since PI3K/Akt signaling pathway is frequently overexpressed or hyperactivated in during carcinogenesis, it has also been recognized as a promising target

for cancer prevention and therapy [8]. Likewise, MKK4 and its downstream substrate JNK and p38 have been reported to regulate neoplastic cell transformation [9, 10]. MKK4 plays a major role in transduction of carcinogenic signaling, leading to activation of AP-1 and NF-κB causing inflammation, cell transformation and carcinogenesis [11].

Licorice extracts are commonly used in food, pharmaceutical and tobacco products. Various bioactivity and medicinal applications for licorice extract have been reported, including antioxidant, anti-inflammatory, anti-carcinogenic, hepatoprotective and antimicrobial effects [12-17]. Previous studies have demonstrated the chemopreventive effects of licorice in a DMBA/TPA-induced skin model, as well as a DMBA-induced mammary tumorigenesis model, and benzo[a]pyrene- and N-nitrosodiethylamine-induced lung tumorigenesis models [18-22]. The majority of previous research has been conducted using licorice extract or several constituents including glycyrrhizin, glycyrrhetinic acid, lichochalcone, and isoliquiritigenin [23, 24]. However, licorice contains various other compounds including alkaloids, flavonoids, polysaccharides, polyamines, and triterpenes, the chemopreventive potential for which have not yet been assessed [24, 25]. In addition, though many reports have shown that licorice exhibits chemopreventive effects, its direct molecular target and mechanisms remains to be elucidated. Dehydroglyasperin C (DGC) is present in licorice ethanolic extract, with the highest concentration among

the flavonoids [26]. Recent studies on DGC have highlighted its neuroprotective and anti-proliferative effects on arterial smooth muscle cells [27, 28]. However, there have been no reports on the chemopreventive effects of DGC or its direct molecular targets. In the current study, I have thus sought to examine the chemopreventive effects of DGC and its molecular mechanisms.

# Ⅱ. Materials and Methods

## 2.1. Chemicals

DGC (Figure 1A) was kindly provided by Prof. Soon Sung Lim (Hallym University, Gangwon, Korea). *Glycyrrhiza uralensis* (90 g, 0.9% yield) was dip-extracted from dried and ground roots (1 kg) using an n-hexane/ethanol mix at a ratio of 9:1 (v/v) [29]. TPA was purchased from Sigma Chemical (St. Louis, MO). Eagle's minimum essential medium (MEM), basal medium Eagle (BME), gentamicin and L-glutamine were purchased from GIBCO BRL (Carlsbad, CA) and fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA). The antibodies against phosphorylated MEK (Ser217/221), phosphorylated ERK (Thr202/Tyr204), total ERK, phosphorylated p90RSK (Thr359/Ser363) and total p90RSK were obtained from Cell Signal Biotechnology (Beverly, MA). The antibodies against COX-2 and total MEK1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against β-actin was obtained from Sigma. CNBr-Sepharose 4B, glutathione-Sepharose 4B and [γ-32P]ATP, were purchased from Amersham Biosciences. Polyvinylidene fluoride was purchased from PALL (Port Washington, NY). G418 and the luciferase assay substrate were purchased from Promega (Madison, WI).

## 2.2. Cell lines

The JB6 P+ mouse epidermal cells were cultured in monolayers at 37°C in a 5% CO2 incubator in MEM containing 5% FBS and 1% streptomycin. The JB6 mouse epidermal cell lines were stably transfected with AP-1, NF-κB, or COX-2 luciferase reporter plasmids and maintained in MEM supplemented with 5% FBS containing 200 g/ml G418.

## 2.3. Cell viability assay

Cells were seeded in 96-well plates (1000 to 4000 cells per well depending on the cell type) and incubated overnight before treatment. Cell viability was measured using the CellTiter 96® AQueous MTS Reagent (Promega).

## 2.4. Anchorage-independent transformation assay

The effects of DGC on TPA-induced cell transformation were investigated in JB6 P+ cells. Cells ($8\times10^3$/ml) were exposed to TPA with or without DGC in 1 ml of 0.33% BME agar containing 10% FBS or in 3.5 ml of 0.5% BME agar containing 10% FBS. The cultures were maintained at 37 °C in a 5% CO2 incubator for 12 days, after which time the cell colonies were counted under a microscope with the aid of Image-Pro Plus software (v.4; Media Cybernetics, Silver Spring, MD).

## 2.5. Luciferase assay for COX-2, AP-1, or NF-κB transactivation

Confluent monolayers of JB6 P+ cells stably transfected with COX-2, AP-1, or NF-κB luciferase reporter plasmids were trypsinized, and $8\times 10^3$ viable cells were suspended in 100 μl of 5% FBS MEM and added to each well of a 96-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO2. When cells reached 80–90% confluence, they were starved in 0.1% FBS-MEM for an additional 24 h. The cells were then treated for 1 h with DGC (0–20 μM) and then exposed to 20 ng/ml TPA for 24 h. After treatment, cells were disrupted with 100 μl of lysis buffer (0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM dithiothreitol (DTT), and 2 mM EDTA), and the luciferase activity was measured using a luminometer (Luminoskan Ascent, Thermo Electron, Helsinki, Finland).

## 2.6. Western blotting

After the cells ($1.5\times 10^6$) were cultured in a 10 cm dish for 48 h, they were starved in serum-free medium for an additional 24 h to eliminate the influence of FBS on kinase activation. The cells were then treated with DGC (0–20μM) for 1 h before being exposed to 20 ng/ml TPA for different times. The harvested cells were disrupted, and the supernatant fractions were boiled for 5 min. The protein concentration was determined using a DC protein assay kit (Bio-Rad) as described in the manufacturer's manual. Lysate protein (30 μg) was subjected to 10% SDS-PAGE and

electrophoretically transferred to a polyvinylidene fluoride membrane. After blotting, the membrane was incubated with the specific primary antibody at 4°C overnight. Protein bands were visualized by a chemiluminescence detection kit after hybridization with the horseradish peroxidase-conjugated secondary antibody.

## 2.7. Kinase assays

The in-vitro kinase assay was carried out in accordance with the instructions provided by Upstate Biotechnology. In brief, active Src and EGFR protein, Src and EGFR substrate peptide contained in the assay buffer, and [γ-32P]ATP solution diluted with magnesium–ATP cocktail buffer were incubated at 30°C and then aliquots were transferred onto p81 paper and washed with 0.75% phosphoric acid. The radioactive incorporation was determined using a scintillation counter. The effect of DGC (5 or 10 μM) was evaluated by incubating DGC with the Src or EGFR kinase reaction mixtures at 30°C for 10 min. Each experiment was performed three times.

For the MKK4 and MKK3/6 kinase assays, 10 ng/μl active MKK4 or 40 ng/μl active MKK3/6 recombinant murine protein and DGC (5 or 10 μM) were reacted at 30 °C for 10 min. For each reaction, 5 μl of 2x kinase buffer (10 mM MOPS (pH 7.2), 5 mM β-glycerol phosphate, 2 mM EGTA, 0.8 mM EDTA, 10 mM MgCl2, 0.1mM DTT), 5 μl of 250 μM ATP, and 0.2

μg/μl of the inactive JNK2 were added. The mixtures were incubated at 30°C for 15 min. A 5 μl aliquot was removed from the reaction mixture containing 10 μl of 2 mg/ml of ATF-2 substrate peptide, 5 μl of 2x kinase buffer, and 5 μl of 0.16 μCi/μl [32P]ATP solution, and incubated at 30 °C for 15 min. Then, 20 μl aliquots were transferred onto p81 filter paper and washed three times with 1% phosphoric acid for 5 min per wash and once with acetone for 5 min. Radioactive incorporation was determined using a scintillation counter (LS6500; Beckman Coulter). Each experiment was performed three times.

## 2.8. PI3K assay

Active PI3K (100 ng) was incubated with DGC for 10 min at 30°C. The mixture was then incubated with 20 μl of 0.5 mg/ml phosphatidylinositol (Avanti Polar Lipids) for 5 min at room temperature, followed by incubation with reaction buffer [100 mmol/L HEPES (pH 7.6), 50 mmol/L MgCl2, and 250 μmol/L ATP containing 10 μCi of [γ-32P]ATP] for an additional 10 min at 30°C. The reaction was stopped by the addition of 15 μl of 4 N HCl and 130 μl of chloroform/methanol (1:1). After vortexing, 30 μl of the lower chloroform phase were spotted onto a 1% potassium oxalate–coated silica gel plate that had been previously activated for 1 h at 110°C. The resulting 32P-labeled phosphatidylinositol-3-phosphate was separated by TLC, and the radiolabeled spots were visualized

by autoradiography.

## 2.9. *In vitro* immunoprecipitation assay

For the preparation of DGC–Sepharose 4B beads, Sepharose 4B powder was suspended in 1 mM HCl and DGC was added to the coupling solution (0.1 M $NaHCO_3$ and 0.5 M NaCl) and mixed on a rotary shaker at 4°C overnight. The procedure was performed as reported earlier [30]. For the *in vitro* immunoprecipitation assay, MKK4 was incubated with DGC–Sepharose 4B (or Sepharose 4B alone as a control) beads in reaction buffer and mixed on a rotary shaker at 4°C overnight. After incubation, the beads were washed 5 times with washing buffer. Proteins bound to the beads were analyzed by Western blotting.

## 2.10. Statistical analysis

Where necessary, data are expressed as means ± S.E.M. and analysis of variance (ANOVA) was used to perform statistical analysis for single comparisons. A probability value of $p < 0.05$ was used as the criterion for statistical significance. All analyses were performed using Statistical Analysis Software (SAS, Inc., Cary, NC).

# Ⅲ. Results

## 3.1. DGC inhibits TPA-induced neoplastic transformation of JB6 P+ cells

I first examined the inhibitory effects of DGC on TPA-induced neoplastic transformation of JB6 P+ cells. Treatment with DGC significantly suppressed TPA-induced neoplastic transformation of JB6 P+ cells (Fig. 1B, left panel), with DGC at 10 μmol inhibiting TPA-induced cell transformation by 53% (Fig. 1B, right panel). The inhibition of cell transformation by DGC was not caused by cytotoxicity, with the MTS assay indicating that an effective concentration range for inhibiting cell transformation did not affect the viability of JB6 P+ cells (Fig. 1C).

## 3.2. DGC suppresses TPA-induced COX-2 promoter activity and protein expression in JB6 P+ cells

COX-2 is a major enzyme triggering the inflammatory response and induces tumor progression and neoplastic transformation [31]. I therefore sought to evaluate whether DGC affects TPA-induced up-regulation of COX-2. Luciferase assays revealed that DGC inhibits TPA-induced COX-2 promoter activity in JB6 cells stably transfected with a COX-2 luciferase reporter plasmid (Fig. 2B). DGC also inhibited the TPA-induced COX-2 protein expression in a dose-dependent manner (Fig. 2A). Together these results indicate that DGC regulates TPA-induced COX-2 up-regulation at

the transcriptional level and that DGC may contribute to the antitumor-promoting activity of licorice.

### 3.3. DGC suppresses TPA-induced transactivation of AP-1 and NF-κB in JB6 P+ cells

I next measured the effect of DGC on transactivation of AP-1 and NF-κB using JB6 P+ cells stably transfected with an AP-1 or NF-κB luciferase reporter plasmid. Consistent with the results for COX-2 expression, DGC inhibited TPA-induced transactivation of AP-1 (Fig. 2C) or NF-κB (Fig. 2D) in a dose-dependent manner, an event that may contribute to the antitumor and anti-inflammatory activities of DGC.

### 3.4. DGC inhibits TPA-induced phosphorylation of p38, JNK, and Akt but has no effect on phosphorylation of Src, EGFR, Raf, MEK, ERK, or p90RSK in JB6 P+ cells

AP-1 and NF-κB activation are mediated by MAPK-signaling intermediates including ERK, JNK and p38 [32]. To elucidate the manner by which DGC modulates AP-1 and NF-κB activities, I examined the effects of DGC on TPA-induced phosphorylation of MAPKs. I found that DGC suppresses TPA-induced phosphorylation of p38, and JNK, but not MKK4, MKK3/6, MEK1/2, Raf, ERK, Src, or EGFR (Figure 3). DGC also strongly suppressed the phosphorylation of Akt, a major downstream

substrate of PI3K (Fig. 3A). These results suggest that the inhibition of JNKs, p38, and Akt by DGC leads to the suppression of AP-1 and NF-κB transactivation, resulting in decreased COX-2 expression.

### 3.5. DGC inhibits MKK4 and PI3K activity *in vitro*

As DGC treatment was observed to strongly suppress JNK and Akt signaling pathways, I investigated the effects of DGC treatment on the kinase activity of MKK4 and PI3K. Kinase assay data revealed that DGC strongly suppresses MKK4 and PI3K activity *in vitro* (Fig. 4 and Fig. 5A). However, DGC had no effect on MKK6 activity (Fig. 5C).

### 3.6. DGC directly binds to MKK4

I next sought to determine whether DGC interacts directly with MKK4. Direct physical binding of DGC to MKK4 was demonstrated by *in vitro* immunoprecipitation assay (Fig. 5B).

# Figure 1

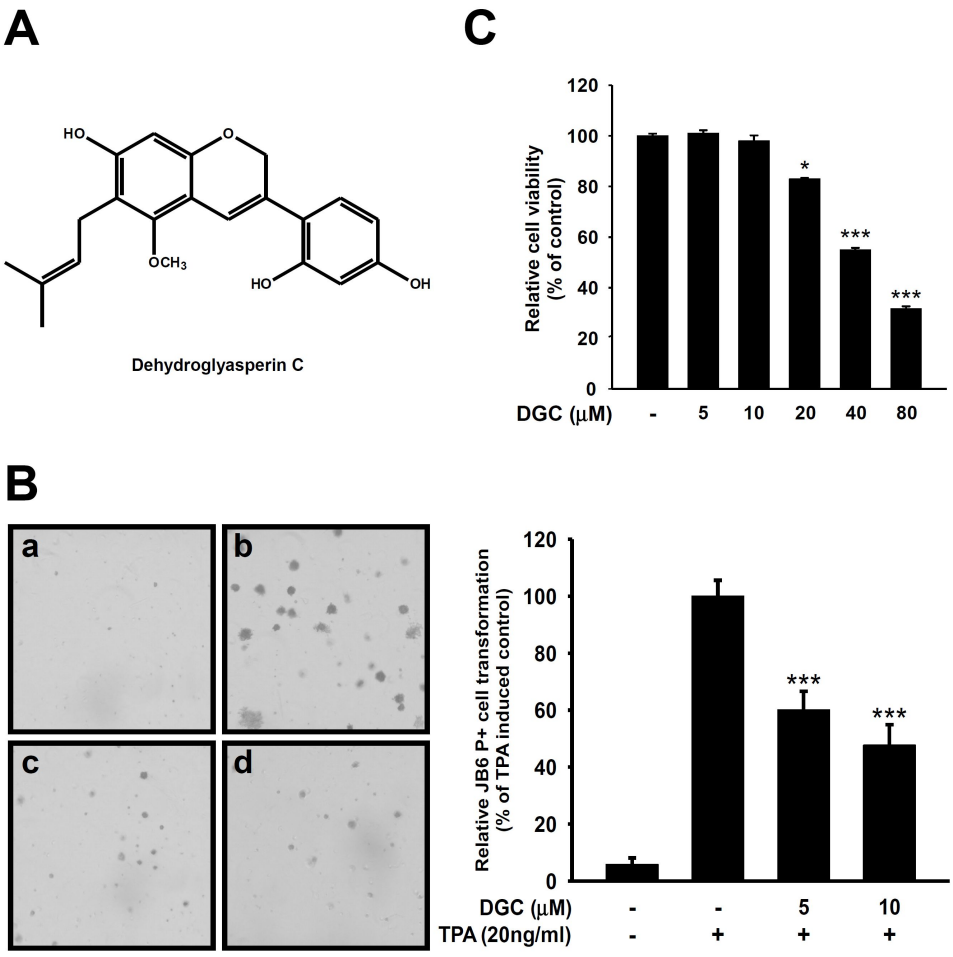

A
HO
O
OCH3
HO
OH
Dehydroglyasperin C
C
Relative cell viability (% of control)
120
100
80
60
40
20
0
*
***
***
DGC (μM) - 5 10 20 40 80
B
a
b
c
d
Relative JB6 P+ cell transformation (% of TPA induced control)
120
100
80
60
40
20
0
***
***
DGC (μM) - - 5 10
TPA (20ng/ml) - + + +

**Figure 1. Effects of DGC on TPA-induced neoplastic transformation in JB6 P+ cells.**

(A) Chemical structure of Dehydroglyasperin C. (B) DGC inhibits TPA-induced cell transformation. JB6 P+ cells were treated as described in Materials and Methods. *a*: untreated control, *b*: TPA alone, *c*: TPA with 5 μg/ml DGC, *d*: TPA with 10 μg/ml DGC. The effects of DGC on JB6 P+ cell transformation, presented as percent inhibition relative to TPA-induced cells in soft agar. (C) Effect of DGC on JB6 P+ cell viability. JB6 P+ cells were treated with DGC at the indicated concentrations for 24 h, and viability assessed using an MTS assay. Absorbance was measured at 492 and 690 nm.

# Figure 2

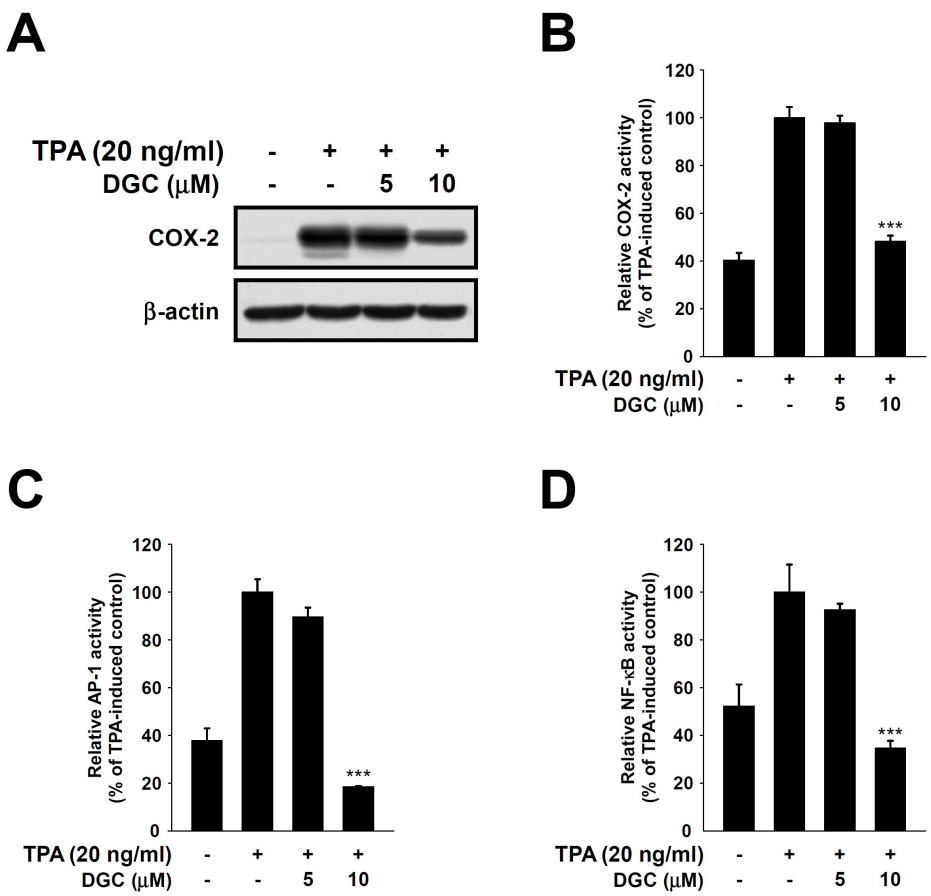

A
TPA (20 ng/ml) - + + +
DGC (μM) - - 5 10
COX-2
β-actin
B
Relative COX-2 activity (% of TPA-induced control)
120
100
80
60
40
20
0
***
TPA (20 ng/ml) - + + +
DGC (μM) - - 5 10
C
Relative AP-1 activity (% of TPA-induced control)
120
100
80
60
40
20
0
***
TPA (20 ng/ml) - + + +
DGC (μM) - - 5 10
D
Relative NF-κB activity (% of TPA-induced control)
120
100
80
60
40
20
0
***
TPA (20 ng/ml) - + + +
DGC (μM) - - 5 10

**Figure 2. Effect of DGC on TPA-induced COX-2 expression and COX-2, AP-1 and NF-κB luciferase activity in JB6 P+ cells.**

(A) DGC inhibits TPA-induced COX-2 expression in JB6 P+ cells. Data are representative of 3 independent experiments that gave similar results. (B) Apigenin suppresses TPA-induced COX-2 promoter activity. JB6 P+ cells were stably transfected with a luciferase reporter plasmid bearing COX-2, AP-1, and NF-κB. Cells were cultured as described in the Materials and Methods (***, $P < 0.001$, for differences between groups treated with both DGC and TPA versus TPA treatment alone).

# Figure 3

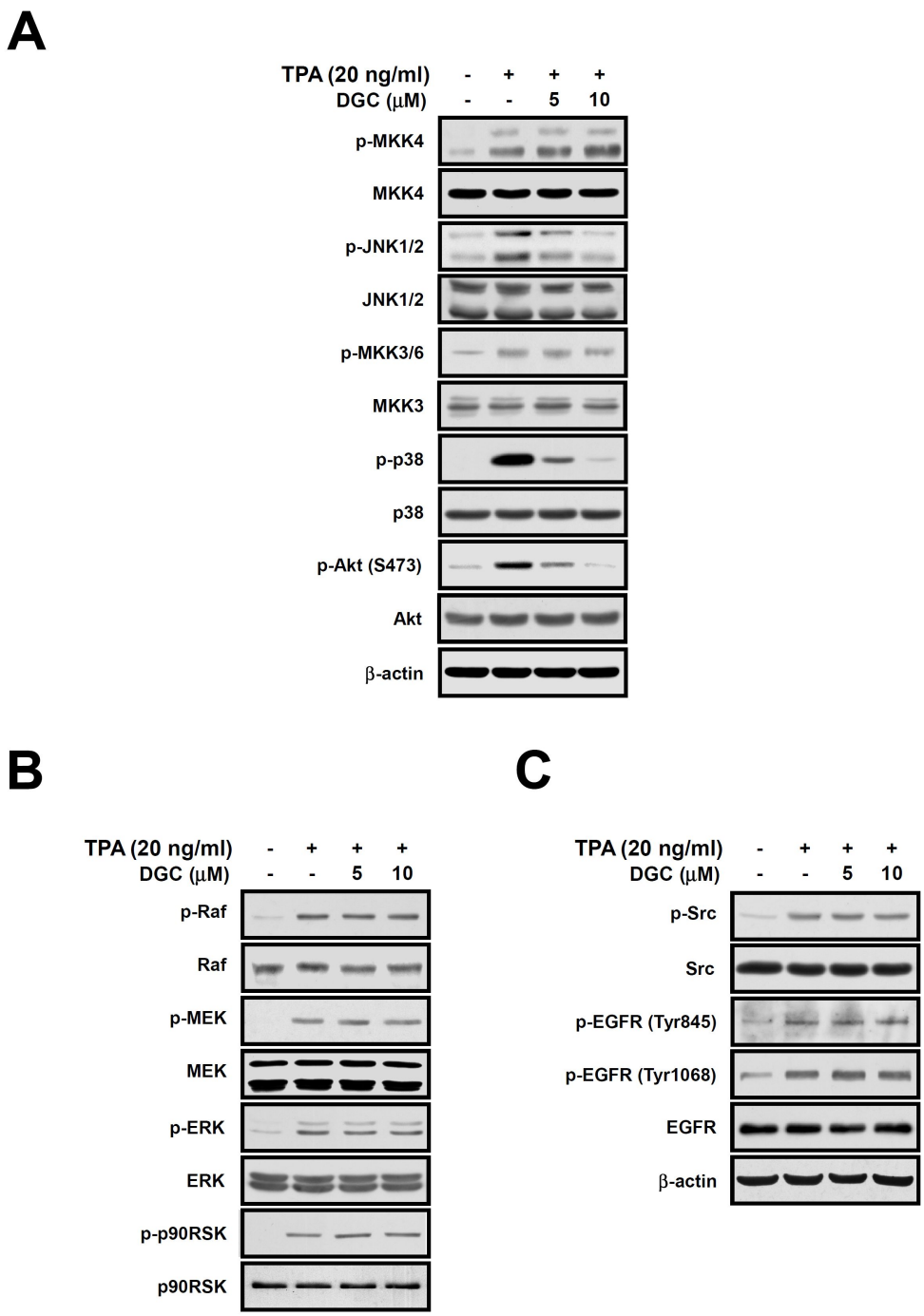

A
TPA (20 ng/ml) - + + +
DGC (μM) - - 5 10
p-MKK4
MKK4
p-JNK1/2
JNK1/2
p-MKK3/6
MKK3
p-p38
p38
p-Akt (S473)
Akt
β-actin
B
TPA (20 ng/ml) - + + +
DGC (μM) - - 5 10
p-Raf
Raf
p-MEK
MEK
p-ERK
ERK
p-p90RSK
p90RSK
C
TPA (20 ng/ml) - + + +
DGC (μM) - - 5 10
p-Src
Src
p-EGFR (Tyr845)
p-EGFR (Tyr1068)
EGFR
β-actin

**Figure 3. Effect of DGC on TPA-induced cell signaling in JB6 P+ cells.**

(A) DGC inhibits TPA-induced phosphorylation of JNK, p-38 and Akt, but not MKK4 or MKK3/6. (B and C) DGC does not affect TPA-induced phosphorylation of Raf, MEK, ERK, p90RSK, Src or EGFR. JB6 P+ cells were starved in 0.1% FBS-MEM and at the indicated concentrations for 1 h before stimulation with TPA (20 ng/ml). Data are representative of three independent experiments that gave similar results.

# Figure 4

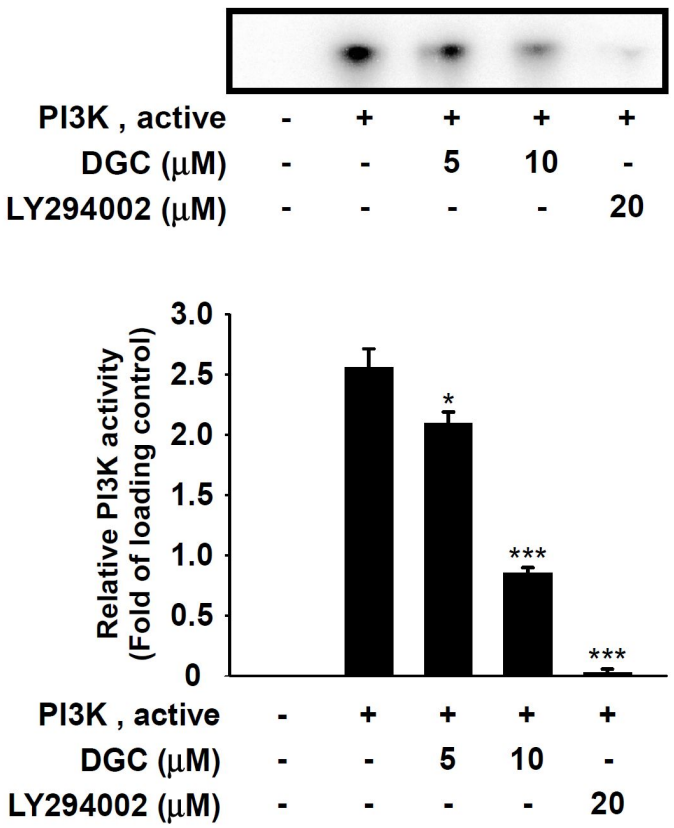

PI3K , active - + + + +
DGC (μM) - - 5 10 -
LY294002 (μM) - - - - 20
Relative PI3K activity (Fold of loading control)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
***
***
PI3K , active - + + + +
DGC (μM) - - 5 10 -
LY294002 (μM) - - - - 20

**Figure 4. Effect of DGC on PI3K activity.**

DGC inhibits PI3K activity in a dose-dependent manner. PI3 kinase assay was performed as described in Material and Methods (*, $P < 0.05$, ***, $P < 0.001$, significant differences between groups treated with both DGC and PI3K active protein and the group treated with PI3K active protein alone). Lane 1: negative control, Lane 5: positive control (LY294002).

# Figure 5

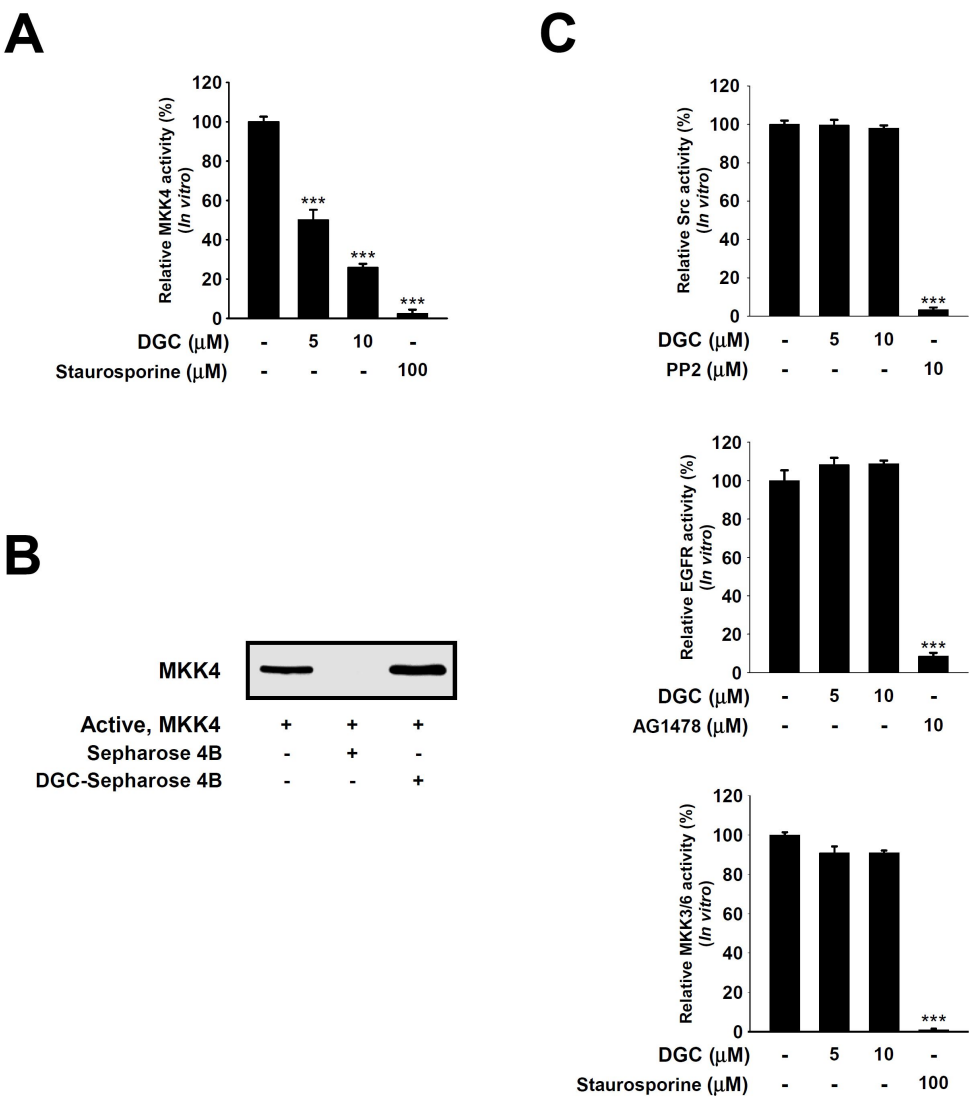
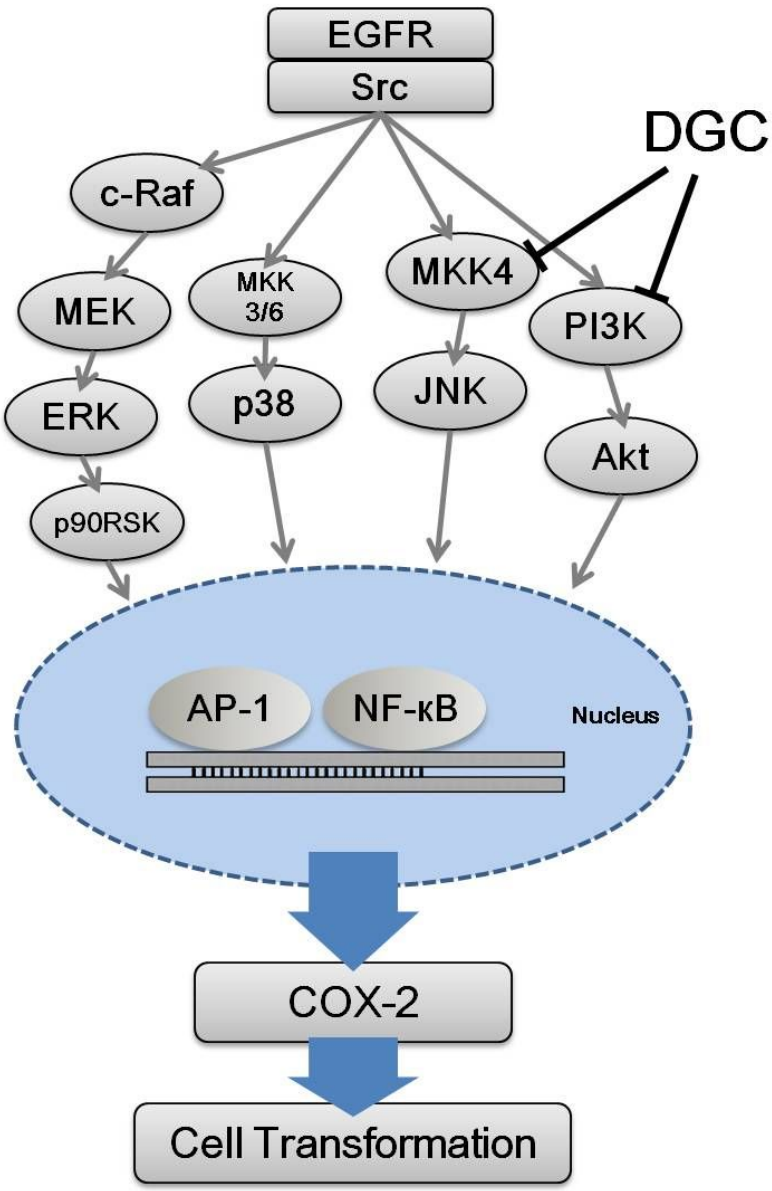

A
Relative MKK4 activity (%)
(In vitro)
120
100
80
60
40
20
0
***
***
***
DGC (μM) - 5 10 -
Staurosporine (μM) - - - 100
B
MKK4
Active, MKK4 + + +
Sepharose 4B - + -
DGC-Sepharose 4B - - +
C
Relative Src activity (%)
(In vitro)
***
DGC (μM) - 5 10 -
PP2 (μM) - - - 10
Relative EGFR activity (%)
(In vitro)
***
DGC (μM) - 5 10 -
AG1478 (μM) - - - 10
Relative MKK3/6 activity (%)
(In vitro)
***
DGC (μM) - 5 10 -
Staurosporine (μM) - - - 100

**Figure 5. Effect of DGC on MKK4, Src, EGFR, and MKK3/6 kinase activity and MKK4 binding activity.**

(A) DGC inhibits MKK4 kinase activity in a dose-dependent manner. (B) DGC binds directly to MKK4. The binding of DGC to MKK4 was confirmed by Western blotting. Lane 1: MKK4 as input control, Lane 2: negative control (unconjugated Sepharose beads), Lane 3: MKK4 immunoprecipitated with DGC-Sepharose 4B affinity beads. (C) DGC has no effect on Src, EGFR or MKK3/6 kinase activity (kinase assay was performed as described in Material and Methods). Effect of DGC on kinase activity expressed as percent inhibition relative to the level of activity in the untreated control.

# Figure 6


EGFR
Src
DGC
c-Raf
MEK
ERK
p90RSK
MKK 3/6
p38
MKK4
JNK
PI3K
Akt
AP-1
NF-κB
Nucleus
COX-2
Cell Transformation

# Ⅳ. Discussion

Recent evidence suggests that a number of naturally-occurring dietary phytochemicals can exert potential chemopreventive effects to delay the onset of carcinogenesis [11, 33]. Cell transformation constitutes a critical step toward carcinogenesis, and COX-2 is a major activator of this event. COX-2 is in turn regulated by eukaryotic transcription factors including NF-κB and AP-1, leading to the idea that inhibition of AP-1 and/or NF-κB may allow for the suppression of cell transformation [33, 34]. This strategy could be addressed by inhibiting upstream kinases, including members of the MAPK family that are responsible for the activation of AP-1 and NF-κB.

Research data indicate that MAP kinase phosphorylation triggers signals from the cell surface, activating various transcription factors, thereby contributing to the regulation of target gene expression. JNKs and p38 are generally recognized as stress-activated MAP kinases. Several kinds of MKKs can phosphorylate JNKs, and p38-MKK3 and MKK6 activate p38, whereas MKK4 can activate both JNKs and p38 MAPK [35]. Increased levels of MKK4 are positively related to the increased proliferation and invasion of several cancer cell lines [36]. In this study, I confirmed that DGC effectively suppresses the phosphorylation of JNK but not MKK4. However, the downstream effects of MKK4 kinase activity were substantially inhibited by DGC treatment. JNK phosphorylation decreases

when MKK4 activity is suppressed in many cell types. However, the effect of MKK4 on p38 varies, and is dependent upon cell type and the nature of the stimuli [35]. Therefore, identification of the molecular target of DGC that regulates p38 would provide a critical insight into its mechanism of action.

As DGC has previously been observed to inhibit PI3K kinase activity, it is plausible that an alternative molecular target of DGC in our in-vitro studies is PI3K. Phosphoinositide 3-kinase (PI3K) and its downstream target Akt/protein kinase B (PKB) are major regulators of NF-κB activation and COX-2 expression [37, 38].

The targeted inhibition of kinase signaling pathways by small molecules is a core strategy in chemoprevention and chemotherapy. In recent years, multi-target kinase inhibitors have become of increasing interest to clinicians, with some recently approved for anticancer therapy [39]. Highly specific inhibitors for single targets is thought to contribute to drug resistance in cancer patients by stimulating the activation of alternative pathways. Therefore, the broad inhibition of multiple targets, rather than a single specific target, could represent a more effective strategy for the treatment of cancer and other disorders. Sorafenib and sunitinib are anticancer drugs that act as small molecular inhibitors of Raf, vascular endothelial growth factor receptor, platelet-derived growth factor receptor, FLT-3 and c-Kit [40, 41]. Both drugs are used to treat renal cell carcinoma

but have also been approved for the treatment of hepatocellular carcinoma and gastrointestinal stromal tumors, respectively. This demonstrates that the broad effects of multi-kinase inhibitors may facilitate their application toward multiple medical conditions.

However, a potential drawback for the use of multi-target kinase inhibitors is the possibility of undesirable side effects. Clinical safety is a pivotal concern for all small-molecule inhibitors, and multi-target kinase inhibitors are no exception. Phytochemicals like DGC are generally regarded as safe due to their long history of human consumption. The rationale follows that if humans have been ingesting such compounds and their analogues for centuries, it lowers the likelihood that unexpected and potent side effects will arise. This is in comparison to synthetic rationally-designed compounds that are entering the human body for the first time in evolutionary history. Further studies into the bioavailability and other pharmacodynamic parameters of DGC are needed, before its widespread use can be considered.

In summary, this study has shown that DGC is a potent inhibitor of tumor promoter-induced neoplastic transformation of JB6 P+ cells. DGC also inhibits UVB-induced COX-2 expression in JB6 P+ cells by blocking the MKK4 and PI3K pathways, and subsequently suppresses AP-1 and NF-κB activities. Taken together, these results suggest that MKK4 is a promising molecular target for the suppression of neoplastic transformation,

which can be effectively targeted by DGC. These findings provide insight into the molecular action of DGC and indicate the potential for DGC to be further developed as a novel chemopreventive agent.

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# VI. 국문초록

천연식물 유래 물질들은 예로부터 여러 질병에 대하여 치료 및 예방 효능이 있다고 알려져 왔다. 감초는 동양 의학에서 전통적으로 가장 많이 쓰여온 약재로써 피부습진, 소화성궤양, 피부감염, 발진 등의 여러 염증성 질환을 치료하는데 효능이 있다고 알려져 있다. 감초에 가장 많이 함유되어 있는 글리시리진 (glycyrrhizin)과 칼콘류 (chalcones)는 항산화능, 항비만, 그리고 항미생물능을 갖는다고 연구가 많이 이루어져 있다. 디하이드로글리아스페린 C (dehydroglyasperin C, DGC)는 감초에 함유되어 있는 이소플라본(isoflavone) 성분 중 하나로 많은 양이 함유되어 있음에도 불구하고 암예방에 대한 생리학적 연구는 매우 부족한 실정이다. 본 연구에서는 DGC 의 화학적 세포암화 예방 효능 및 분자적 표적을 규명하고 분자생물학적 작용 기작을 쥐의 피부상피세포인 JB6 P+ 세포주에서 규명하고자 하였다. DGC 는 JB6 P+ 세포에서 대표적인 외인성 발암물질인 12-O-tetradecanoylphorbol-13-acetate (TPA)에 의한 악성화를 저해하였으며, TPA 로 유도된 cyclooxygenase-2 (COX-2)의 발현을 억제하였다. 또한, 전사인자인 activator protein-1 (AP-

1)과 nuclear factor-κB (NF-κB)의 활성 역시 DGC 처리에 의해 감소하였다. 암화과정을 조절하는 주요 신호전달 체계인 MAPKs 및 PIKs 의 인산화를 western blot 과 kinase assay 로 확인해 본 결과, AP-1 과 NF-κB 의 활성감소가 DGC 가 mitogen-activated protein kinase kinase 4 (MKK4)와 phosphatidylinositide 3-kinases (PI3Ks)를 직접적으로 저해하기 때문임을 확인하였다.

본 연구 결과는 DGC 를 강력한 화학적 암예방 및 염증예방 물질로 제시할 수 있으며 이것은 세포암화과정에 주요하게 작용하는 세포신호전달기작 중 MKK4 와 PI3K 의 활성을 직접적으로 억제함에 기인하는 것임을 제시한다. 이는 DGC 의 세포암화 과정을 예방하는 활성에 대한 직접적인 분자적 표적을 규명한 것으로 향후 천연물 유래 기능성 성분의 세포내 신호전달 조절 기작에 대한 새로운 연구방향을 제시한다.

주요어: 디하이드로글리아스페린 C, cyclooxygenase-2, mitogen-activated protein kinase-kinase 4, phosphatidylinositide 3-kinase, 세포암화

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# 감사의 글

석사과정 2 년이라는 시간은 참 길고도 짧은 것 같습니다. 신입생 때에는 언제 이 시간이 흐르나 하고, 마지막 학기 때는 언제 이 시간이 다 흘렀나 하고 되돌아 봅니다. 먼저, 저의 석사과정 동안 한결 같이 따뜻하고 올곧은 성품으로 학문뿐만 아니라 삶의 자세에서부터 멘토가 되어 주신 이형주 교수님께 깊은 감사를 드립니다. 또한 발상의 전환으로 항상 새롭게 생각하는 훈련을 하도록 도와주신 이기원 교수님 감사합니다. 다사다난했던 두 해를 함께 해준 나의 두 동기 영아랑 재각이, 그리고 차례대로 나의 사수님이 되어주신 지만오빠, 상균오빠, 그리고 정성근 박사님 감사합니다. 석사기간 동안 많은 시간 함께 했었던 세미랑 민정이, 승준오빠, Lee Farrand, 그리고 지금도 함께하고 있는 터프한 은정언니, 마음의 소리 상권오빠, 손초딩 조은오빠, 아이쇼퍼홀릭으로 사이가 돈독해진 영진언니 모두 고맙습니다. 무엇보다도 항상 기도로 저를 응원해 주시는 사랑하는 할머니, 어머니와 아버지, 지아언니, 형부, 그리고 석사 기간 내내 옆에서 함께 울고 웃어준 쏘울메이트 호령이와 나의 오랜 친구들에게도 고맙다는 인사를 전합니다.