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석사학위 논문

# The mechanistic study of noble compounds on the inhibition of epithelial cell transformation and tumorigenesis in triple negative breast cancer

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# 상피세포 형질전환 및 삼중음성 유방암 발생을 억제하는 신규 화합물의 기전 연구

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이 논문을 약학 석사학위 신청 논문으로 제출함

2016년 10월

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# 김민수의 석사학위논문을 인준함

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# Contents

# List of Figures

# List of Abbreviations

| | |
|---|---|
| **AP-1** | Activator protein-1 |
| **BrdU** | 5-bromo-2'-deoxyuridine |
| **c-Fos** | AP-1 transcription factor subunit C |
| **DMSO** | Dimethyl sulfoxide |
| **EGF** | Epidermal growth factor |
| **ERK1/2** | Extracellular signal-related kinase1/2 |
| **HER2** | Human epidermal growth factor receptor 2 |
| **MAPK** | Mitogen-activated protein kinasse/extracellular signal-regulated kinase kinase |
| **MEK** | Mitogen-activated protein kinase/extracellular signal-regulated kinase |
| **PARP** | Poly (ADP-ribose) polymerase |
| **PBS** | Phosphate-buffered saline |
| **TNBC** | Triple negative breast cancer |
| **TUNEL** | Terminal deoxynucleotidyl transferase |
| **SDS-PAGE** | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |

# [국문 초록]

## 상피세포 형질전환 및 삼중음성 유방암 발생을 억제하는 신규 화합물의 기전 연구

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삼중 음성 유방암을 가지고 있는 환자의 경우 일반적인 항암 치료제로는 극복하기가 어렵다. 그 원인은 아직 뚜렷한 표적이 발굴 되지 않았기 때문이다. 그러므로 치료를 위한 새로운 표적 단백질을 발굴 하는 것이 매우 중요하다. 최근 연구 들 에서 B-RAF의 돌연변이가 삼중 음성 유방암에서 매우 중요한 역할을 한다고 보고되고 있다.

본 연구에서는 B-RAFV600E 돌연변이를 표적으로 하는 M7과 M8이라는 신규 화합물을 이용하여, 삼중 음성 유방암 세포주인 MDA-MB231 세포에서 endothelial growth factor (EGF) 에 의한 증식과 성장을 억제할 수 있는지 규명 하였다. M7과 M8은 EGF에 의해 유도된 mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK), extracellular signal-related kinase1/2 (ERK) 그리고 c-Fos 와 같은 신호전달 단백질의 인산화를 억제하였다. 또한 M7과 M8은 EGF에 의해 촉진된 c-Fos 와 AP-1 promoter 활성을 농도 의존적으로 억제 시켰다. 특히, JB6 C141 세포에서 M7과 M8은 EGF에 의해 유도하는 상피세포형질전환을 억제하였다. In vivo tumorigenicity assay에서도 M7과 M8을 처리한 4T1 세포를 쥐의 유선에 주입하여 4T1의 종양성장을 비교하였는데, 대조군과 비교했을 때 매우 효과적으로 종양의 생장을 억제하는 것을 관찰 할 수 있었다. 나아가 M7과 M8은 MDA-MB231 세포주에서 p53의 인산화를 유도하여 세포 주

기의 진행을 억제시키며 또한, 세포자멸과 관계있다고 알려진 poly (ADP-ribose) polymerase (PARP) 와 caspase-3의 cleaved를 증가시켜 세포의 사멸을 유도하는 역할을 하는 것으로 관찰 되었다.

결론적으로, 위와 같은 결과를 통하여 M7 과 M8 화합물이 MAPK 신호경로를 차단시키며, 또한 세포주기 중 G1 arrest를 유도하여 삼중 음성 유방암의 증식과 성장을 억제한다. 따라서 B-RAFV600E 돌연변이체가 삼중 음성 유방암의 새로운 표적 단백질로서의 가능성을 제안한다.

# Ⅰ. Introduction

The cause of triple negative breast cancer (TNBC) has not yet been identified. Therefore, many researchers focused about breast cancers features, such as gene mutation or abnormal expressed proteins. Classically, breast cancer is classified into three groups based upon the expression pattern of three receptor proteins [1]. The first type is estrogen receptor (ER) positive breast carcinoma, which is overexpressed ER. Second, progesterone receptor positive (PgR) breast carcinoma refers to a condition in which PgR is overexpressed [2]. It is reported that about 50% patient`s breast tumor samples, which have on both premenopausal and postmenopausal statues, had high levels of ER and PR expression [3]. Indeed, ER positive breast cancer respond to hormone therapy, such as tamoxifen [4]. Tamoxifen acts a competitive inhibitor of ER [4]. When tamoxifen binds to ER, tamoxifen-ER complex is blocked from active transformation and then inhibit abnormal the gene expression of ER downstream [5]. The third group is human epidermal growth factor receptor 2 (HER2) positive breast carcinoma, which is characterized by overexpression of HER2 receptor in breast cancer cells [6]. HER2 belongs to the receptor tyrosine kinase (RTK) of transmembrane receptors, which also include HER1 (EGFR), HER3 and HER4 [7]. They combine with each other for homo/hetero dimerization. HER2 homo/hetero dimers activates RTK pathway, such as MAPK and ATK signaling pathways, resulted in increased abnormal proliferation and differentiation of the cancer cells [7]. Two drugs are currently approved for HER2 positive breast cancer. First, trastuzumab (Herceptin) is a monoclonal antibody, which has specific antigenicity against HER2 receptors. Second, lapatinib (Tykerb) is a dual tyrosine kinase inhibitor HER2 and EGFR [8]. They are often used in combination for the treatment of HER2 positive breast cancer, with a significantly enhanced therapeutic efficacy [9]. However, the chemotherapies have negligible effects on TNBC, because ER, PgR and HER2 receptors were not overexpressed [10, 11]. Recent study has reported that breast carcinoma is more detailed, which was identified by microarray profiling [12]. However, TNBC has not found the target proteins abnormally expressed, and thus the effective therapies [13]. Alternatively, TNBC was treated with cytotoxic chemotherapeutic agents such as cisplatin, but the side effects were very severe [11].

Furthermore, TNBC clinical trials of single kinase inhibitors generally failed, because it was activation of other survival pathways [14]. Therefore, discovering the new molecular marker in TNBC was important for efficient therapies. Recently, the research was reported that in 230 breast cancer samples were observed using for immunohistochemistry. Of the 230 breast cancer samples, 30 cases (13%) were detected B-RAF V600E mutant, and of the 132 cases of TNBC samples, 14 (10.6%) cases were showed B-RAF V600E mutation [15]. Indicating that B-RAF mutation maybe a significant marker of TNBC.

The RAF family consist of A-RAF, B-RAF and C-RAF, which activate mitogen-activated protein kinase (MAPK) signaling pathway [16]. Activation of MAPK signaling induces cell survival and proliferation [17]. Therefore, the RAF-MEK-ERK signaling pathway cascade, which is relevance in tumor carcinogenesis, has been the focus of cancer therapy [18]. Among them, the B-RAF isoform is mutated at a high frequency in human cancer [19]. In addition, the mutation B-RAF V600E increases the abnormal phosphorylation of the MAPK proteins and inhibits apoptosis and increases cell proliferation, thereby increasing carcinogenesis [20]. Although, researchers developed RAF inhibitor, sorafenib, sorafenib did not specifically target to the mutant B-RAF V600E, and therapeutic response was insignificant [21]. Accordingly, a specific inhibitor of B-RAF V600E mutant, one of which is vemurafeinb, is needed.

Vemurafenib (PLX4032), which is specific targeting to mutant B-RAF V600E, has used to melanoma neoplasm [22]. It is competitive small-molecule serine-threonine kinase inhibitor that binds to the ATP-binding domain of mutant B-RAF V600E [22]. Vemurafenib significantly improved the survival of melanoma patients over 6 months [23]. However, the use of vemurafenib in patients 6-8 months later had a poverty effect despite clinical success [24]. The present study aimed to elucidate the mechanism of the anti-tumor effects of noble B-RAF V600E inhibitors, M7 and M8. Here, we demonstrate that M7 and M8 treatment inhibit MEK-ERK signaling pathway, and AP-1 and *c-fos* promoter activity, and thereby inhibiting the progression of triple negative breast cancer cells, MDA-MB231 cells. Also, M7 and M8 were inhibited EGF-induced normal epidermal cell transformation of JB6 C141 cells in soft agar matrix and tumor progression of 4T1 cells in BALB/c mice.

# Ⅱ. Materials & Methods

## 1. Materials

### 1.1 Regents and antibody

PLX4032(Vemurafenib) was kindly provided from korea institute of science and technology (KIST, seoul, south korea). The phospho-specific and total antibodies against MEK1/2, ERK1/2, c-Fos, caspase3 and P53 and the phospho-specific antibodies against phospho-P53 (ser 6) and phospho-MEK, phospho-ERK, and phospho-c-Fos and cleaved antibodies against cleaved PARP and caspase3 were acquired from cell signaling technology Inc. (Beverly, MA, USA); and total antibodies against PARP and mouse IgG were acquired from santa cruz biotechnology (Santa Cruz, CA, USA).

### 1.2 Cell culture

MDA-MB231 and 4T1 breast cancer cells were grown in eagle's minimal essential medium (MEM) and roswell park memorial Institute medium (RPMI) supplemented with 10% fetal bovine serum, respectively. All cell lines were cultured and maintained at 37 °C in humidified air containing 5% $CO_2$.

## 2. Methods

### 2.1 Cell proliferation assay

MDA-MB231 cells were seeded (5000 cells per well) in 96-well plates in 100㎕ of 10% FBS-modified eagle`s medium. After 24h, the cells were treated with M7, M8 and PLX4032 (Vemurafenib) for 48h, labeled with 10㎕/well BrdU-labeling solution and then reincubated for additional 4h at 37°C in a 5% CO2 atmosphere. After sucked the media, FixDenat solution was added in each well, incubated at RT for 30 min and then removed. Anti-BrdU-POD-working solution was added in each well and incubated for further 90min at RT. The cells were then washed with washing solution for three times and 100 ㎕of

substrate solution was added in each well and incubated for 30min. Cell proliferations was estimated by measuring the absorbance at 370 nm.

## 2.2 Immunoblot assay

The cells were disrupted in RIPA lysis buffer [50mM Tris (pH7.5), 150mM NaCl, 0.5% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitors cocktail]. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked and hybridized with the appropriate primary antibody overnight at 4℃. The protein bands were visualized using a chemiluminescence detection kit (Amersham HRP ChemiluminescentSubstrates, Amersham Biosciences, Piscataway, NJ) after hybridization with HRP-conjugated secondary antibody from rabbits or mice. A LAS4000-mini(GE healthcare, little Chalfont, England) imaging system used.

## 2.3 Reporter gene assay

The roporter gene assay for firefly luciferase activity was performed using lysates from AP-1-luc or *c-fos*-luc transfected MDA-MB231 cells. In addition, the reporter gene vector pRL-TK-luciferase plasmid (Promega) was co-transfected into each cell line and the renilla luciferase activity generated by this vector was used to normalize the results for transfection efficiency. Cell lysates were prepared by first washing the transfected MDA-MB231 cells once with phosphate-buffered saline (PBS) at RT. After removing the PBS completely, passive lysis buffer (PLB, Promega) was added, and then cells were incubated at RT for 1h with gentle shaking. The supernatant fraction was used to measure firefly and renilla luciferase activities. Cell lysates (50㎕) were mixed with 50㎕ of luciferase assay Ⅱ reagent, and firefly luciferase light emission was measured by GloMax®-Multi detection system (Promega). Subsequently, 50㎕ of renilla luciferase substrate was added in order to normalize the firefly luciferase data. The *c-Fos*-luc promoter (pFos-WT GL3) constructs were kindly provided by Dr. Ron Prywes (Columbia

University, New York, NY). The AP-1 luciferase repoter plasmid (-73/+63 collagenase-luciferase) was kindly provided by Dr. Dong Zigang (Hormel Institute, University of Minnesota, Austin, MN).

### 2.4 Anchorage-independent cellular transformation assay

The effect of M7 and M8 in the transformation was investigated in JB6 C141 cells. Briefly, 8X103 cells were exposed to different doses of M7 or M8 in 1ml of 0.3% basal medium Eagle (BME) agar containing 10% FBS, 2mM L-glutamine, and 25ug/mL gentamicin. The cultures were maintained at 37℃ in a 5% CO2 incubator for 14-20 days, and cell colonies were scored using an Axiovert 200 M fluorescence microscope and Axio Vision software (Carl Zeiss Inc., Thornwood, NY).

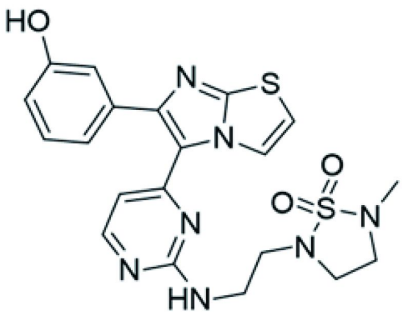
# Ⅲ. Results

## 1. Structure of M7 and M8

Vemurafeinb (PLX4032) is synthetic molecule which was potent inhibitor of oncogenic B-RAF kinase activity in melanoma [25]. Vemurafeinb only works in melanoma patients whose cancer has a V600E B-RAF mutation [26]. Thus, we had analyzed new synthetic compounds M7 and M8. M7 and M8 are derivatives of vemurafenib, which target to B-RAF V600E mutant (Figure 1A, 1B).

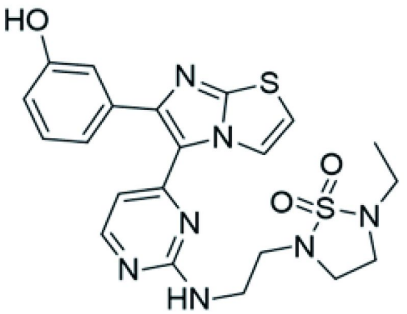
**Figure 1.**

**A**




**M7**

**B**




**M8**

**Figure 1. Structure of M7 and M8.**

(A) Chemical structure of M7 (B) Chemical structure of M8

## 2. M7 and M8 inhibits of EGF-induced MAPK signaling.

TNBC patient samples were observed mutation of B-RAF gene, whereas these were not find mutations of KRAS or EGFR [27]. First, we examined the anti-proliferation effect of tamoxifen, vemurafenib, and new B-RAF V600E mutant inhibitors, M7 and M8, in MDA-MA231 cells, using Brd-U assay. We showed that tamoxifen treatment didn`t decrease cell proliferation, whereas vemurafinb, a B-RAF V600E mutant inhibitor, reduced cell proliferation. Moreover, the newly synthesized B-RAF inhibitors, M7 and M8, were more effective in suppressing cell proliferation than vemurafenib. Thus, we hypothesized that the inhibition of mutant B-RAF ware effective for down-regulation of growth or proliferation in TNBC cells (Figure 2A). Next, we examined whether M7 and M8 regulated the phosphorylation of MAPK signaling cascade. The results showed that M7 and M8 dose- and time- dependently inhibited phosphorylation of MEK, ERK and c-Fos signaling pathway (Figure 2B, 2C). We next investigated the effect of M7 and M8 on the EGF-induced RAF down-stream signaling. The result showed that phosphorylations of MEK, ERK, and c-Fos induced by EGF were inhibited by M7 and M8 (Figure 2D).

**Figure 2.**

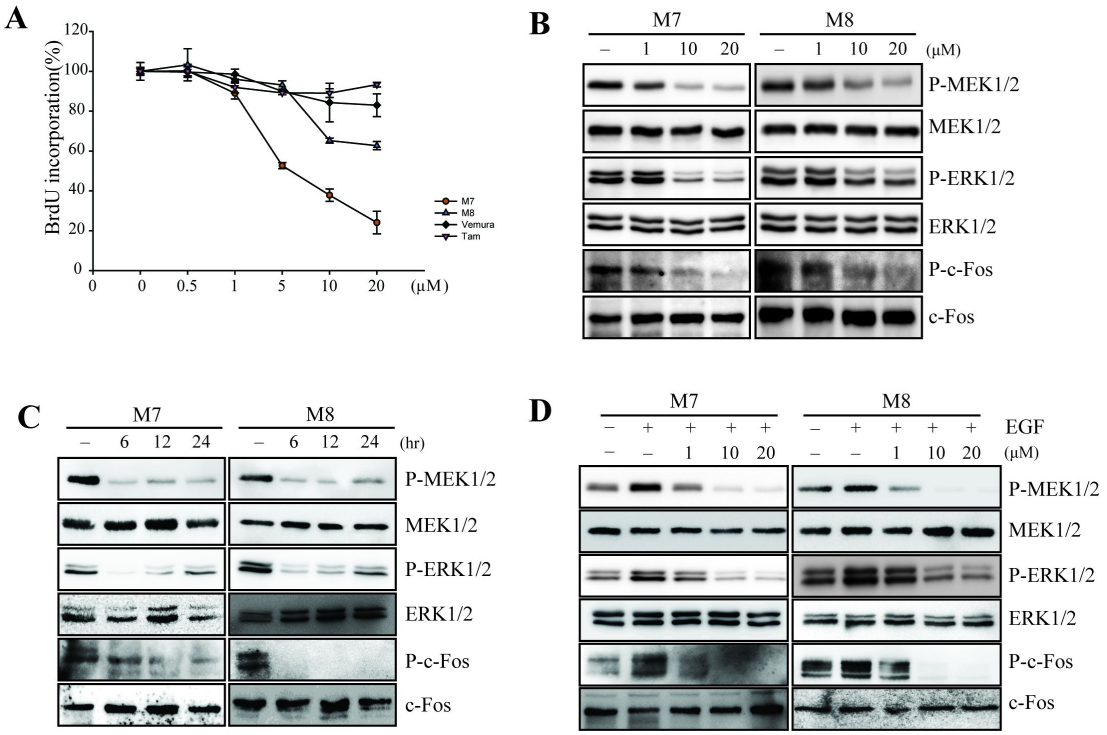


**Figure 2. Effects of M7 and M8 on cell proliferation and phosphorylation of MAPKs in MDA-MB231 cells.**

(A) The cells were treated with M7 and M8 and vemurafenib dose-dependently as indicated. Cell proliferation was measured by Brd-u assay, as described in Materials and Methods. (B and C) Cells were incubated 37 °C for 48 h, treated with M7 and M8 at the indicated concentrations for 24 h (B) or with 20 $\mu$M of M7 and M8 for indicated times (C). The levels of phosphorylated and total proteins related with MEK-ERK and c-Fos signaling cascades in whole cell lysates were determined by immunoblotting analysis using specific antibodies against the corresponding proteins, respectively. (D) The cells were treated with M7 and M8 at the indicated concentrations for 24 h with 10 ng/mL EGF and harvested. The levels of phosphorylated and total proteins related with MEK-ERK and c-Fos signaling cascades in whole cell lysates were determined by immunoblotting analysis using specific antibodies against the corresponding proteins, respectively.

### 3. M7 and M8 inhibits EGF-induced *c-fos* and AP-1 transcriptional activity.

c-Fos protein is in part of transcription factor of AP-1, which was expressed by activation of MAPK signaling pathway [28]. And the increased activity of AP-1 induces invasive and proliferative properties in TNBC cells [29]. So, we investigated whether M7 and M8 regulated *c-fos* transcriptional activity. The results showed that M7 and M8 treatment inhibited *c-fos* promoter activity in MDA-MB231 cells (Figure 3A). Given that AP-1 promoter activity was regulated by fos family proteins, such as c-Fos, which was up-regulated by EGF-induced MAPK signaling pathway [14], we examined whether AP-1 transcriptional activity were inhibited by dose dependently treatment with M7 and M8. The results showed that AP-1 transcriptional activity was inhibited by M7 and M8 (Figure 3B). In addition, the treatment with M7 and M8 suppressed EGF-induced AP-1 or *c-fos* transcriptional activity (Figure 3C, 3D). The results suggested that the inhibition of the AP-1 and *c-fos* promoters by EGF is one of the mechanisms on the anti-proliferative effect of M7 and M8.

**Figure 3.**

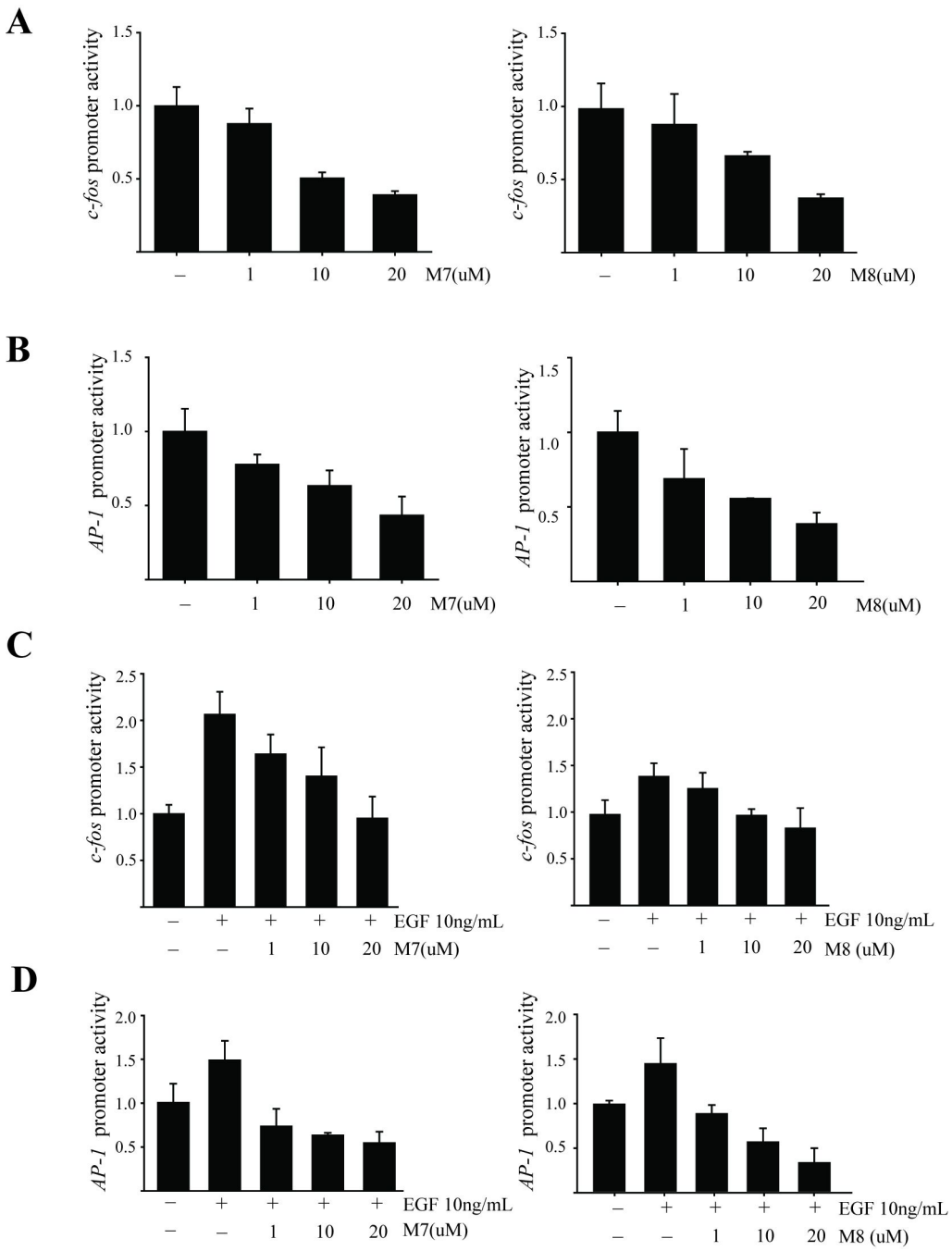


**Figure 3. Effects of M7 and M8 on transcriptional activity of *c-Fos* and AP-1 in MDA-MB231 Cells.**

(A and D) Cells were transfected with a plasmid mixture containing *c-fos-luc* (A and C) and AP-1-luc promoter gene (B and D) with the pRL-TK vector. At 24 h after transfection, cells were serum-starved for 12 h, and then treated with the indicated concentration of M7 and M8 and then either exposed (C and D) or not exposed (A and B) with 10 ng/mL EGF for 24h. In all of the promoter assays, the firefly luciferase activity was determined in cell lysates and normalized against renilla luciferase activity, and these luciferase activities are expressed relative to control cells, respectively.

### 4. M7 and M8 suppressed tumor progression of MDA-MB231 cells in vitro.

DNA damage is an important cause of cancer cells and damaged cells try to recover DNA in cell cycle arrest by P53 protein [30]. Thus, activation of P53 indicates the cell cycle arrest at G1 in DNA damaged cancer cells. Therefore, we analyzed whether M7 and M8 affected on the phosphorylation of P53. The result showed that the phosphorylation of P53 was increased by M7 and M8 in dose-dependent manner (Figure 4A). Also, we examined whether M7 and M8 induced cell cycle G1 arrest in MDA-MB231 cells. We observed that M7 and M8 significantly induced cell cycle arrest during G1 in dose-dependent manner (Figure 4C). Recently, it was reported that the inhibition of mutant B-RAF increased apoptotic activity and cell cycle arrest, resulted in increased nuclear PARP cleavage and caspase activation [17]. Therefore, we investigated the effects of M7 and M8 on apoptosis and cell cycle regulation. The results showed that M7 and M8 increased cleaved caspase-3 and PARP dose-dependently manners (Figure 4B). Next, the TUNEL assay was performed to examine the apoptotic signal in MDA-MB231 cells. The result showed that the treatment of M7 and M8 significantly increased the apoptosis signal in the MDA-MB231 cells (Figure 4D). The results suggest that M7 and M8 may have critical effects on the apoptosis or cell cycle phases in MDA-MB231 cells.

# Figure 4.

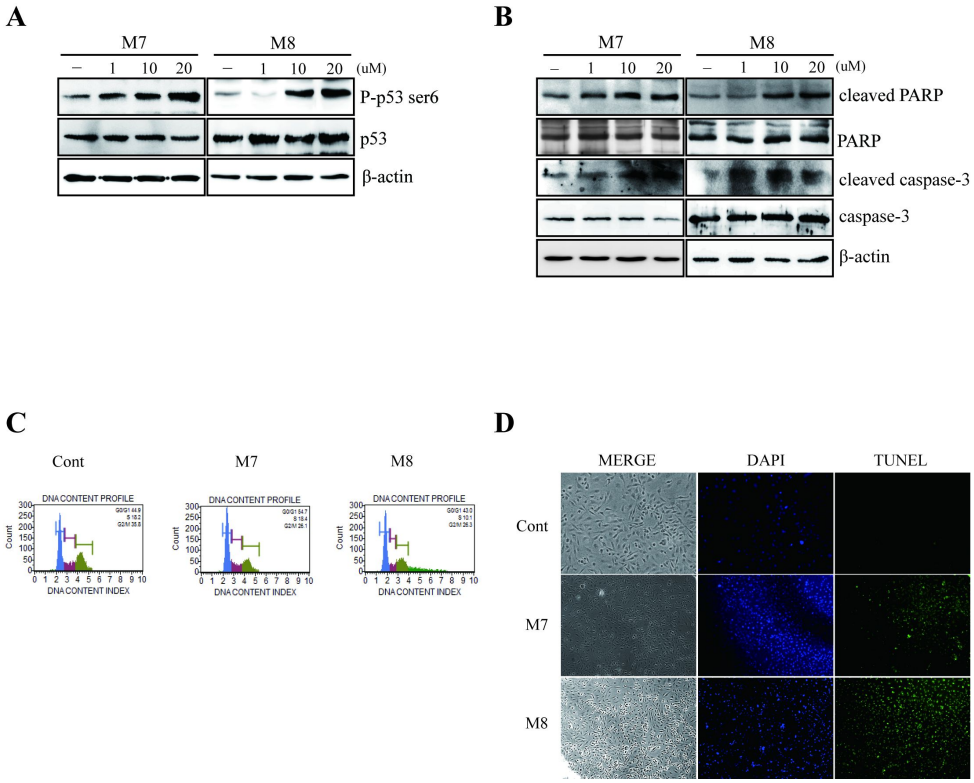


**Figure 4 Effects of apoptosis and cell cycle arrest in MDA-MB231cells.**

(A and B) Cells were incubated 37 ℃ for 48 h, treated with M7 and M8 at the indicated concentrations for 24 h, and harvested. (A) The levels of phosphorylated and total proteins related with p-P53 (Ser6), P53 and β-actin cascades or (B) cleaved and total proteins related with cleaved PARP, PARP, cleaved caspase-3, caspase-3 in whole cell lysates were determined by immunoblotting analysis using specific antibodies against the corresponding proteins, respectively. (C) MDA-MB231 cells were treated with 20 μM M7 and M8, and was measured by Muse cell cycle assay, as described in Materials and Methods. (D) MDA-MB231 cells were treated with 20 μM M7 and M8 or nothing for 24h and use to cell death kit (tunnel assay).

### 5. M7 and M8 suppresses epithelial cells transform and carcinogenesis in vitro or in vivo.

The c-Fos/AP-1 complex and MAPK pathway induced cell transformation and proliferation [31]. Therefore, we investigated whether M7 and M8 can prevent normal epithelial cell transformation and in vivo carcinogenesis. MDA-MB231 cells were treated with M7 and M8 followed by incubation at 37°C in a 5% $CO_2$incubatorfor48hours, and the cell proliferation assay was performed. The results showed that M7 and M8 can significantly suppress the cell proliferation induced by EGF (Figure 5A). Next, we observed whether M7 and M8 prevented abnormal transformations by EGF using a soft agar matrix. Our results showed that M7 and M8 significantly inhibited the EGF-induced cell transformation of JB6 cells in a dose-dependent manner (Figure 5B, 5C). These data strongly suggest that M7 and M8 inhibits the neoplastic cell transformation in epidermal mouse skin cell stimulated with EGF. Furthermore, we demonstrated that whether M7 and M8 can suppress tumor development in vivo. The effect of M7 and M8 on tumor suppress in vivo were studied in a syngeneic mouse 4T1 breast tumor metastasis model. M7 and M8 treatment significantly inhibited 4T1 cells in a dose- dependent manner. And, representative tumor images demonstrated that M7 and M8 significantly suppress mammary gland tumor development (Figure 5D). The results, therefore, suggest that M7 and M8 significantly inhibits EGF-induced cell proliferation and suppresses normal epithelial cell metastasis.

**Figure 5.**

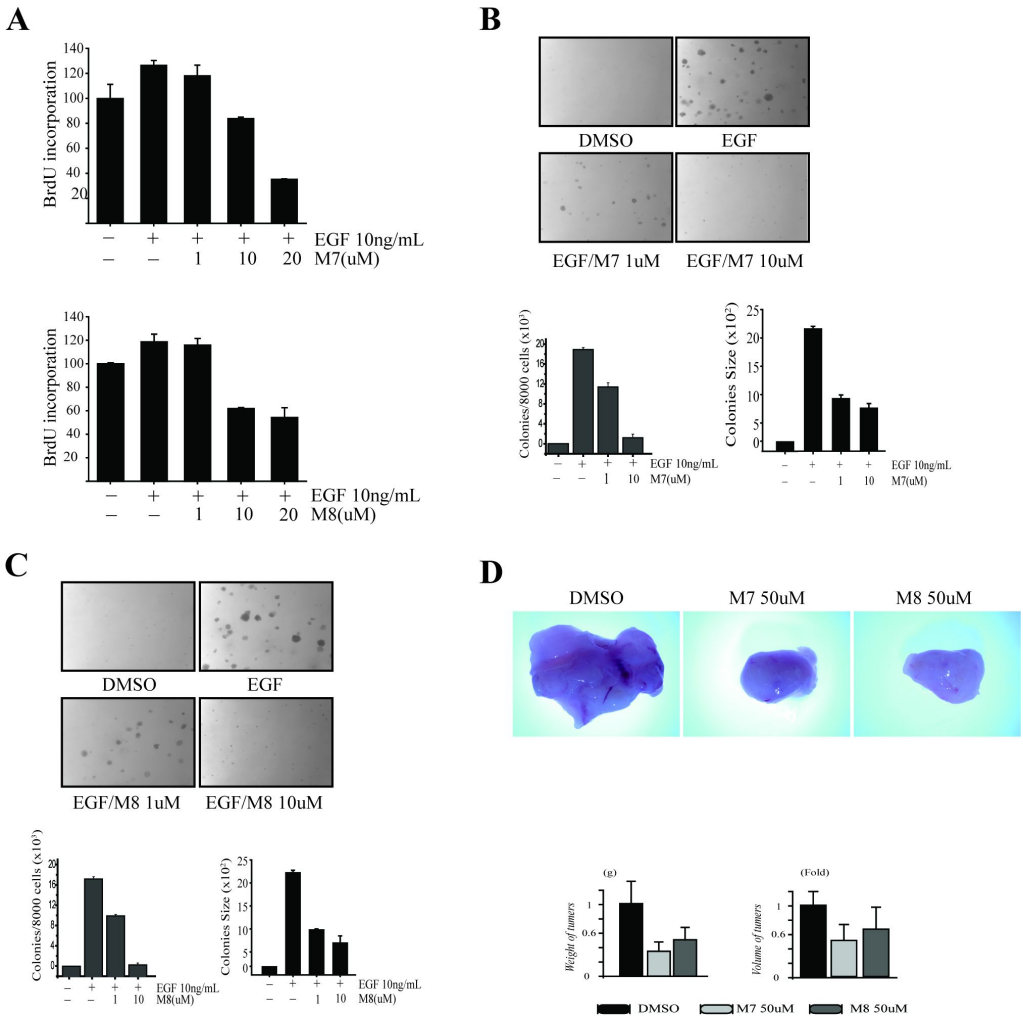


**Figure 5. In vitro and in vivo effect of M7 and M8 on tumorigenicity of JB6 and 4T1 cells.**

(A) The cells were treated with M7 and M8 dose-dependently with/without EGF 10ng/mL as indicated. Cell proliferation was measured by Brd-u assay, as described in Materials and Methods. (B and C) MDA-MB231 cells were treated with 20μM M7 and M8 with/without treatment of 10ng/mL EGF as indicated in soft agar matrix, and incubated at 37°C in a 5% CO2 atmosphere for 14 days. (D) 4T1 cells were injected into the mammary gland of BALB/c mice in the presence or absence of M7 and M8 50μM and allowed to grow until tumors formed (14 days).

# Ⅳ. Discussion

There is a significant unmet medical need for the high efficacious cure of chemotherapy on TNBC. A clinically relevant subtype classification is generally determined by immunohistochemistry analysis of the tumor expression, such as ER, PgR and HER2 positive subtype [32]. Triple negative breast cancers, which didn`t express estrogen receptor, progesterone receptor and ErBB2 (Her2) proteins, show bad prognosis even when treated or medicines are consumed [33]. TNBC accounts for about 15% of all breast cancer diagnosis, which showed high proliferation rate, high metastasis and necrosis [34, 35]. Microarray analysis has confirmed the type of breast cancer in more detail : normal breast like, luminal A, luminal B, HER2 and basal like [12]. There is overlap between TNBC and basal like tumors, but they have differences of status, which is oncogene expression such as BRCA1 [36]. BRCA responds to DNA damage with poly (ADP-ribose) polymerase (PARP), which repairs single strand DNA break [37]. In cancer, the lacking of functional BRCA1 perpetuated cellular proliferation in the setting of potential oncogene activation [38]. PARP target treatment on BRCA-defective TNBC was demonstrated by the ability of BRCA deficiency to sensitize tumor cells to PARP inhibition [39, 40]. Through the reports, we thought that the finding of new character in TNBC was important for TNBC treatment.

Recently studies reported that triple negative breast cancer tissue samples have mutant B-RAF V600E positive [15]. And, the inhibition of mutation of B-RAF V600E had a good clinical effect in B-RAF mutant cancers [25, 41]. Thus, we predicted that B-RAF is the key protein, when targeted therapy is performed on TNBC. Thus, we tried to treatment B-RAF inhibitor, such as vemurafenib, in MDA-MB231 cells, which are TNBC cell lines. However, the results showed that vemurafenib had just a little effect on EGF-induced phosphorylation inhibition of proliferation of MDA-MB231 cells (Figure 2A). It is speculated that vemurafenib is too specific for human melanoma cancers [42]. In particular, when a B-RAF mutation melanoma patient took vemurafenib, the previously treated breast cancer recurred [43]. These results are indicated that B-RAF mutation inhibitor, M7 and M8, may be the possible candidates for TNBC therapy as an alternative to vemurafenib.

Mutant B-RAF V600E can enhance the phosphorylation to MAPK cascade that increase proliferation and decrease apoptosis [44]. The B-RAF inhibitor vemurafenib inhibits MAPK signaling and tumor cell proliferation rate [45]. Therefore, we anticipated that M7 and M8 are an alternative inhibitor of vemurafenib, that inhibits TNBC through MAPK signaling phosphorylation. Our data showed that M7 and M8 inhibited phosphorylation of MAPK downstream signaling and also inhibited EGF-induced MAPK phosphorylation. This result can be expected that M7 and M8 regulated the progression of TNBC through inhibition of MAPK signaling in mutant B-RAF. Furthermore, MDA-MB231 have gene mutation of B-RAF G464V and KRAS. In future studies, it will be important that TNBC could be induced by amino acid substitution mutation of B-RAF and M7 and M8 show a therapeutic effect by specifically acting on B-RAF mutations, or simply by blocking RAS downstream signaling.

AP-1 was discovered in human cells, which binds to selective enhancer elements in cis control region of the SV40 virus [46]. AP-1 is dimeric transcription factors consist of Jun, Fos or activating transcription factor (ATF) subunits that bind to the AP-1 binding site, a common DNA region [47] [48]. Fos and jun enhance the activity of both AP-1 complex and DNA-binding site to regulate transcriptional regulation [49]. The AP-1 protein modulates the expression and function of cell cycle-regulating proteins such as cyclin D1, p53 and p21 to regulate cell death [50]. In addition, AP-1 is mainly regulated by MAPK signaling, and then AP-1 regulates cell growth or proliferation rate [51]. Expletively, estrogen induce to c-Fos expression in human breast cancer cells [52]. ER acts at the AP-1 active site, since activation of ER is required for binding to AP-1 and Jun or Fos [53]. In this study, M7 and M8 inhibited AP-1 or *c-fos* transcriptional activity in MDA-MB231 cells, and this inhibition of the AP-1 and *c-fos* promoter activity led to the suppression of cell cycle of G1 phase. Moreover, arrested G1 induced phosphorylation P53 or activation of apoptosis signaling. These results indicated that the inhibition of mutant B-RAF V600E by M7 and M8 might be responsible for M7 and M8`s strong inhibition of AP-1 or *c-fos* promoter activity in TNBC through inhibition of MAPK phosphorylation. In summary, M7 and M8 inhibited EGF-induced cell proliferation and neoplastic transformation of JB6 C141 through its inhibiting with B-RAF V600E mutation. This inhibition was increased mainly

through the suppressing of the MAPK signaling pathway, including the AP-1 and *c-fos* promoter activity, and increased apoptosis and cell cycle arrest at G1 phase via increase phosphorylation P53 and cleaved caspase3 and PARP. Furthermore, M7 and M8 significantly inhibited the tumor progress in mice. Collectively, these data suggeseted that B-RAF V600E mutation is the most potent molecular target of M7 and M8 for treatment TNBC.

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# ABSTRACT

## The mechanistic study of noble compounds on the inhibition of epithelial cell transformation and tumorigenesis in triple negative breast cancer

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As triple negative breast cancer (TNBC) does not have a marker for treatment, general chemotherapy has little effect on patient treatment. Therefore, it is very important to find the new target for therapy. Recent studies have reported that B-RAF is a notable protein for targeting TNBC. In this study, we demonstrated that the proliferation and progression of MDA-MB231 cells induced by endothelial growth factor (EGF) were inhibited by noble B-RAF mutant inhibitors, M7 and M8. Moreover, the M7 and M8 inhibited mitogen-activated protein kinase/extracellular signal-regulated kinase kinases (MEK)1/2 induced by EGF in MDA-MB231 cells. In addition, the M7 and M8 inhibited associated activator protein-1 (AP-1) as well as *c-fos* promoter activity, and thus colony formation in MDA-MB231 cells. Consistent with these results, *in vivo* tumorigenicity assay showed that the M7 and M8 suppressed tumor growth in BALB/c mice. Also, the M7 and M8 inhibited the neoplastic cell formation of JB6 cells in soft agar and induced a cell cycle arrest at G1 phase in MDA MB231 cells. In conclusion, these results shows that M7 and M8 might produce chemotherapeutic effects through the inhibition of MAPK signaling pathway, and inducing the arrest of cell cycle at G1 phase, suggesting that B-RAF V600E mutant may be a key molecular marker of TNBC for therapy.