Suppression of the invasive potential of highly malignant tumor cells by KIOM-C, a novel herbal medicine, via inhibition of NF-κB activation and MMP-9 expression

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Abstract. KIOM-C, a novel herbal formula, was recently reported to be effective for treating pigs suffering from porcine circovirus-associated disease (PCVAD). In addition, administration of KIOM-C promoted clearance of influenza virus via production of antiviral cytokines, such as TNF-α and IFN-γ. Since metastasis is the major cause of cancer-related death and the greatest challenge in cancer treatment, we investigated the effect of KIOM-C on the metastatic potential of HT1080 and B16F10 cells. We observed inhibitory properties of KIOM-C in colony-forming activity, migration and invasion. Matrix metalloproteinase-9 (MMP-9) activity in the resting and PMA-stimulated state in HT1080 cells was dose-dependently decreased by KIOM-C treatment via suppression of NF-κB activation. In addition, daily oral administration of KIOM-C at doses of 170 and 510 mg/kg, the corresponding human adult daily doses, efficiently blocked lung metastasis in C57BL/6J mice following injection of B16F10 cells in the tail veins. In particular, none of the mice administered KIOM-C during the experimental period exhibited systemic toxicity, such as body weight loss or liver and kidney dysfunction. Collectively, our results suggest that KIOM-C is a potential therapeutic formula useful as a safe herbal medicine for controlling metastatic cancer.

Introduction

Metastatic spread of tumor cells from the original site to distant organs via blood or lymph vessels results in a high mortality rate in cancer patients and remains a challenge in cancer treatment (1). Metastasis involves a complex multi-step process and various cytophysiological changes, including invasion by degradation of the environmental barriers surrounding cancer cells such as the extracellular matrix (ECM) and basement membranes; entrance into vasculature; migration to distant organs; adhesion to endothelial cells; extravasation leading to infiltration into the underlying tissue; and establishment of metastatic foci at the secondary site (2). Degradation of the ECM by cancer cells mediated through a variety of proteolytic enzymes, such as matrix metalloproteinases (MMPs), serine proteinase, cathepsins and plasminogen activators (PAs), plays a critical role in tumor invasion and metastasis. MMPs, a family of zinc-dependent endopeptidases, is composed of 4 groups according to their substrates, including collagenases, gelatinases, stromelysins and membrane-associated MMPs. Among them, MMP-2 and -9 are particularly elevated in malignant tumors and may be closely associated with the progression and metastasis due to their capacity to degrade type IV collagen, a major component of basement membranes. In addition, MMP-9 may be critical in the process of tumor angiogenesis by increasing the availability of vascular endothelial cell growth factor (VEGF) in malignant tumors (3-6). Thus, agents capable of modulating MMP-2 and -9 activity by targeting the upstream regulatory pathways may have therapeutic potential for controlling cancer metastasis (7-9).

Numerous studies have demonstrated that natural herbal medicines have the potential to treat a wide range of human diseases including cancer. A herbal cocktail, a multi-herb mixture in a single formula, may act in concert to amplify the therapeutic efficacies of each single herb, leading to maximal therapeutic efficacy with minimal adverse effects (10,11). Our group has developed a novel herbal medicine, KIOM-C, which is composed of herbal medicinal plants including Radix Scutellariae, Radix Glycyrrhizae, Radix Paeoniae Alba, Radix Angelicae Gigantis, *Platycodon grandiflorum*, *Zingiber officinale* and *Lonicera japonica* Thunb., among others. Recently, KIOM-C was shown to improve the overall growth performance and restore viability in pigs suffering from porcine circovirus-associated disease (PCVAD) by increasing body weight and stimulating immune responses (12). Furthermore, oral administration of KIOM-C was clearly shown to reduce the influenza virus titer in the lungs and protect mice from a lethal challenge with A/Korea/maCJ01/2009 virus (13).

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In the present study, we evaluated the effect of KIOM-C on the metastatic potential of the highly metastatic malignant tumor cells lines, human fibrosarcoma HT1080 and murine melanoma B16F10 in an *in vitro* system and investigated whether KIOM-C administration inhibits pulmonary metastasis of B16F10 melanoma after intravenous injection in mice. Furthermore, we investigated the detailed mechanism of the antimetastatic activity of KIOM-C.

Materials and methods

Mice and cell cultures. B16F10 murine melanoma cells, which are highly metastatic to the lungs of C57BL/6J mice, and HT1080 human fibrosarcoma cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza, Walkersville, MD, USA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin/100 μg/ml streptomycin (both from Gibco, Invitrogen, Carlsbad, CA, USA) at 37˚C in a humidified 5% CO_2 incubator. For animal experiments, specific pathogen-free female C57BL/6J mice were purchased from Taconic Farms lnc. (Samtako Bio Korea, O-San, Korea) and maintained in our animal facility for 1 week before use. Mice were housed under specific pathogen-free conditions at a temperature of 24±1˚C and humidity of 55±5% in a barrier facility with a 12-h light-dark cycle. Animal experimental procedures were approved by the Korea Institute of Oriental Medicine Care and Use Committee (reference nos. 12-094 and 12-111) and performed in accordance with the Korea Institute of Oriental Medicine Care Committee Guidelines.

Antibodies and chemicals. Anti-IκBα, anti-phospho-IκBα (Ser32/36), anti-NF-κB p65, anti-MMP-9 and anti-tubulin antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-TATA sequence-binding protein (TBP) was obtained from Lifespan Biosciences, Inc. (Seattle, WA, USA). A cytotoxicity detection lactate dehydrogenase (LDH) kit and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Sigma Chemical Co. (St. Louis, MO, USA), respectively.

Herbal materials and preparation of KIOM-C. The herbs for preparing KIOM-C, as mentioned above, were purchased from Korea Medicine Herbs Association (Yeongcheon, Korea). The identification of all herbs was confirmed by Professor Ki Hwan Bae of the College of Pharmacy, Chungnam National University (Daejeon, Korea), and all voucher specimens were deposited in the herb bank of the Korea Institute of Oriental Medicine (KIOM, Daejeon, Korea). A total of 2456.5 g KIOM-C formula was placed in 15 liters distilled water and then heat-extracted for 3 h at 115˚C in an extractor (Cosmos-600 Extractor; Gyeonseo Co., Inchon, Korea), filtered using standard testing sieves (150-μm; Retsch, Haan, Germany) and then concentrated to dryness in a lyophilizer. Freeze-dried KIOM-C powder (50 mg) was dissolved in 1 ml distilled water, filtered through a 0.22-μm disk filter, and stored at -20˚C until use.

Cytotoxicity assay. Cytotoxicity was evaluated using MTT and LDH release assays. Briefly, cells seeded in 96-well culture plates at a density of $5x10^3$ cells/well were incubated with specific KIOM-C concentrations between 10 and 250 μg/ml. After a 48-h treatment, cells were incubated with 10 μl MTT solution (5 mg/ml in PBS) for an additional 4 h. Formazan precipitates were dissolved with dimethyl sulfoxide (DMSO), and then the absorbance was measured at 570 nm with the Infinite® M200 microplate reader (Tecan Group Ltd., Switzerland). In addition, LDH release into the culture supernatant from KIOM-C-treated cells was determined by a commercial cytotoxicity detection kit according to the manufacturer's instructions.

Soft agar colony formation assay. To determine anchorage-independent cell growth, cells ($1x10^4$) suspended in 3 ml of medium containing 0.3% agar and 10% FBS were applied to the solidified bottom agar containing 0.6% agar and 10% FBS. During 3 weeks of incubation, colonies on soft agar were observed under a phase-contrast microscope and photographed.

Colony formation assay. Two hundred cells were seeded in a 12-well culture plate in 1 ml 10% FBS/DMEM and incubated to allow attachment. After adding KIOM-C at the specified concentrations, cells were incubated for 10 days, and colonies were stained with 0.2% crystal violet/20% methanol (wt/vol) solution.

Wound healing assay. Cells were pre-incubated with 25 μg/ml mitomycin C (Sigma Chemical Co.) for 30 min, and injury lines were drawn on a confluent monolayer of cells. After washing with DMEM, cells were allowed to migrate in the presence of KIOM-C, and migration was observed under a phase-contrast microscope at specific time points.

Gelatin zymography. Cells were pre-incubated for 12 h in serum-free DMEM with KIOM-C at the specified concentrations and then stimulated with 5 nM PMA for an additional 24 h. The equivalent volumes of the conditioned medium were electrophoresed on an 8% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) containing 0.1% gelatin. Gels were washed thoroughly with washing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5% Triton X-100) and then incubated in activation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 1 μM $ZnCl_2$) at 37˚C. The gels were stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad Laboratories, Hercules, CA, USA) and destained (10% isopropanol, 10% acetic acid). MMPs were detected as clear bands against a dark blue background.

Transwell migration and Matrigel invasion assays. The *in vitro* migration and invasion assays were performed using a Transwell chamber with a 10-mm diameter and an 8-μm pore size polycarbonate membrane (Corning Costar, Cambridge, MA, USA). In brief, after filling the lower chamber with 600 μl 10% FBS/DMEM, cells ($1x10^5$/100 μl) in serum-free DMEM were added to each upper chamber and incubated for 12-36 h at 37˚C. Cells attached to the upper surface of the filters were removed by wiping with a cotton swab, and the filters were stained with 0.2% crystal violet/20% methanol

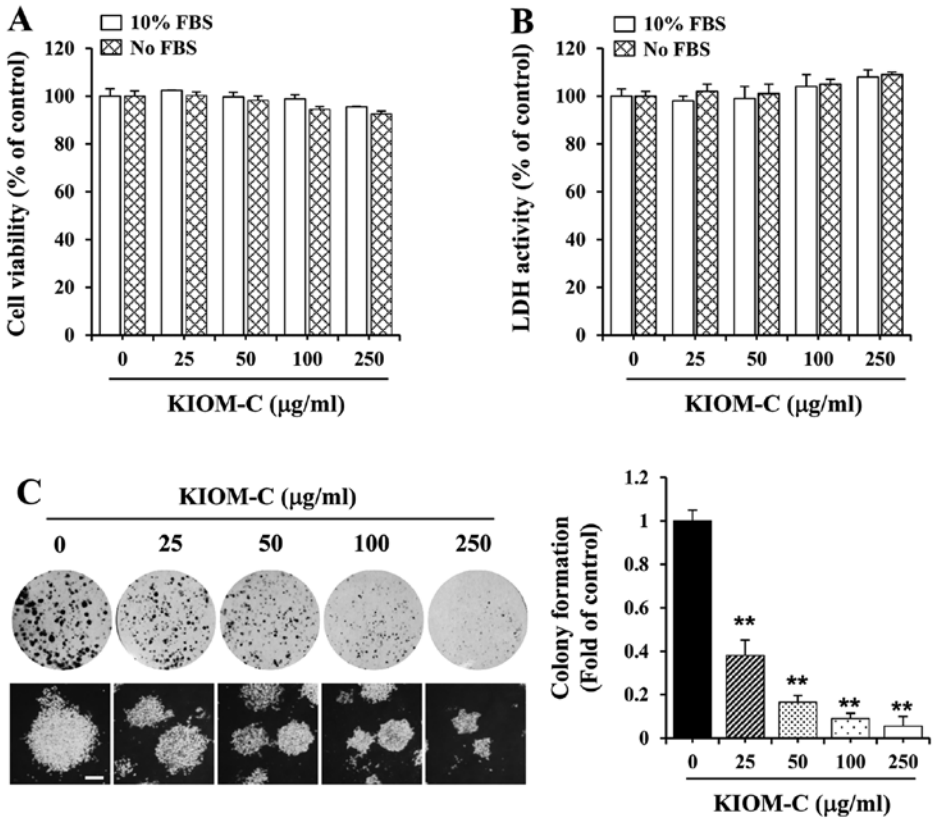


Figure 1. Effect of KIOM-C on the viability and colony-forming activity of B16F10 cells. Cells were seeded onto a 96-well culture plate and treated with the specified concentrations of KIOM-C with or without 10% FBS for 48 h. Cell cytotoxicity was estimated by (A) MTT and (B) LDH release assays. (C) Representative images of anchorage-dependent (upper) and -independent (lower) colony formation in the presence or absence of KIOM-C. At the end of incubation, cells were stained with crystal violet staining solution, the areas of 20 representative colonies were measured, and relative colony formation was quantitated by ImageJ. Each bar of the histogram represents the means ± SD. $^{**}p<0.01$ vs. untreated control. Magnification, x40; scale bar, 0.5 mm. FBS, fetal bovine serum; LDH, lactate dehydrogenase.

(wt/vol) solution. The invasion assay was conducted using the Transwell chamber after coating with 20 μl of a 1:2 mixture of Matrigel:DMEM (Matrigel; BD Biosciences, Bedford, MA, USA) as the intervening invasive barrier.

Western blot analysis. Whole cell lysates and nuclear/cytosolic extracts were prepared using M-PER Mammalian Protein Extraction Reagent and NE-PER Nuclear and Cytosolic Extraction Reagent (Pierce Biotechnology, Inc., Rockford, IL, USA), respectively, according to the manufacturer's instructions. Protein concentration was determined using the bicinchoninic acid (BCA) assay. After immunoblotting, proteins were visualized using a Power Opti-ECL Western blotting detection reagent (Animal Genetics, Inc., Korea) and an ImageQuant LAS 4000 mini (GE Healthcare, Piscataway, NJ, USA). Band intensities were calculated using ImageJ software (National Institutes of Health, USA).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCT). Total RNA was extracted using PureHelix™ RNA extraction solution and reverse transcribed to cDNA using HelixCript™ 1st Strand cDNA Synthesis kit (NanoHelix Co., Daejeon, Korea). cDNA aliquots corresponding to 1 μg RNA were analyzed by semi-quantitative PCR using the following primers; hMMP-9, 5'-TCTTCCCTGGAG ACTGAGAA-3' and 5'-GGCAAGTCTTCCGAGTAGTTT-3'; GAPDH, 5'-TCATGACCACAGTCCATGCC-3' and 5'-TCCA CCACCCTGTTGCTGTA-3'.

In vivo experimental pulmonary metastasis assay. The female C57BL/6J mice were divided into 3 groups (n=5 for each group), and B16F10 cells ($3x10^5$ cells/0.2 ml) were injected via the tail veins. The amount of KIOM-C for human adults with an average body weight of 60 kg is ~50-150 g/day, and the yield of powdered extraction is ~20.7% (wt/wt). Therefore, KIOM-C at doses of 170 or 510 mg/day/kg of body weight and saline (controls) were orally administered to mice for 17 days. The mice were sacrificed, their lungs were fixed in Bouin's solution (Sigma) and the number of B16F10 colonies on the surface of the lung was determined by visual inspection.

Statistical analysis. Statistical significance of the difference between groups was analyzed using the Student's t-test with the SigmaPlot 8.0 software, and a p-value <0.05 was considered to indicate a significant result. Data are presented as the means ± standard deviation (SD), and all experiments were repeated at least 3 times.

Results

KIOM-C suppresses colony-forming activity and anchorage-independent growth of B16F10 melanoma cells. To determine

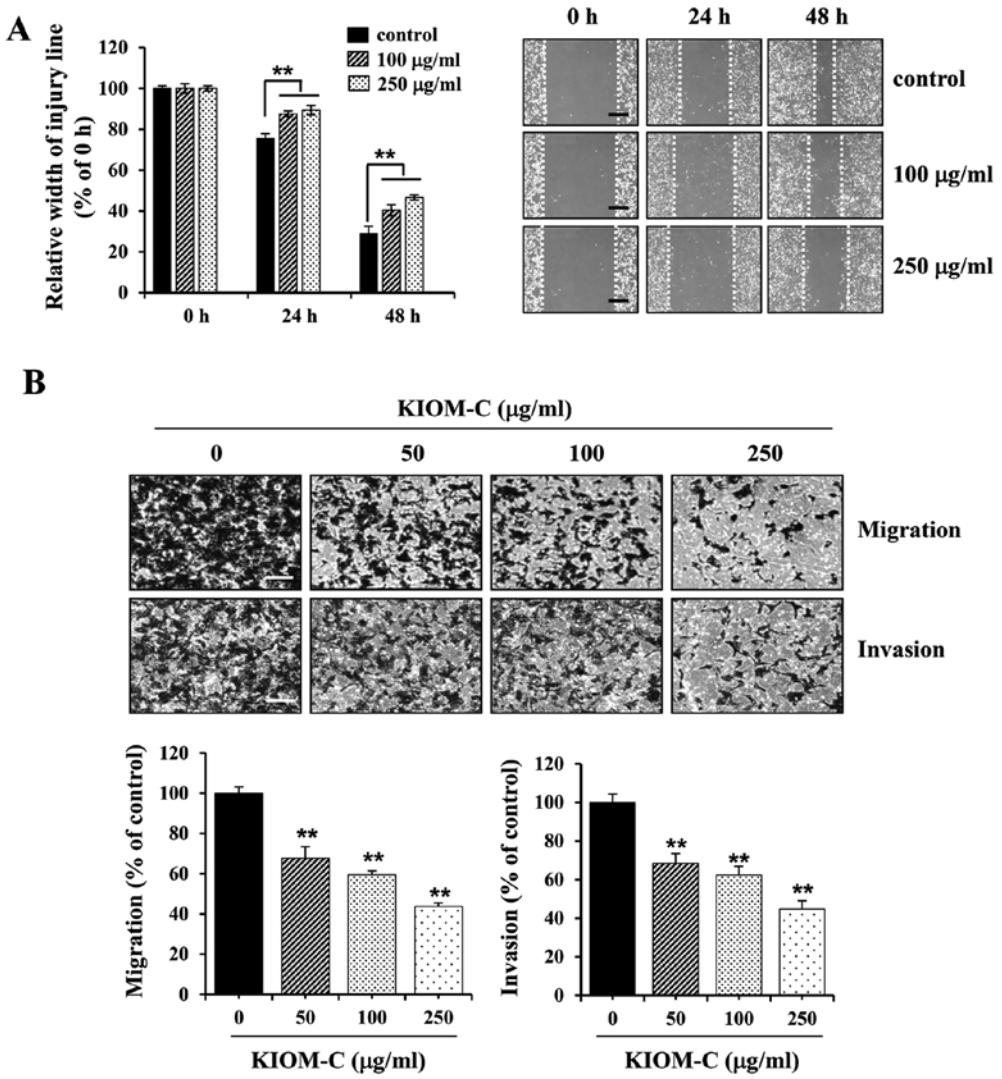
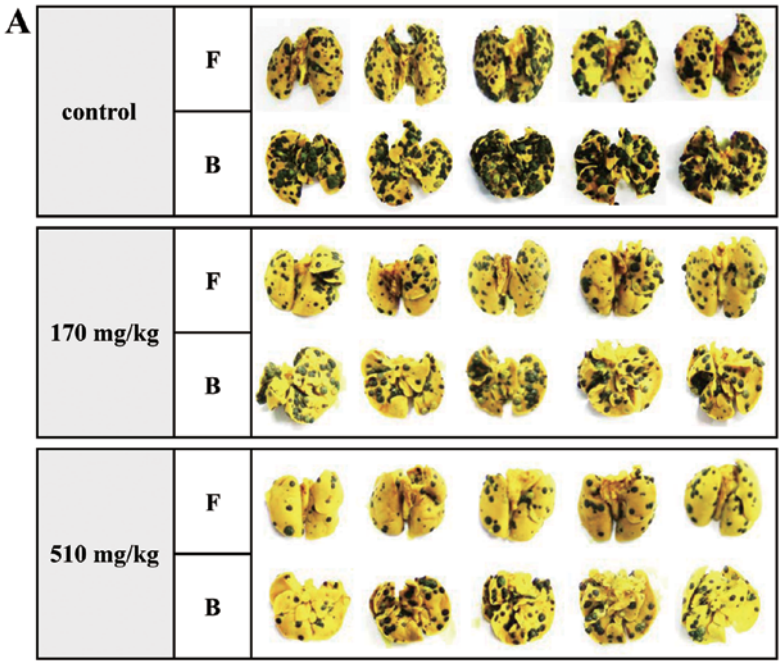


Figure 2. Effect of KIOM-C on the *in vitro* migration and invasion capabilities of B16F10 cells. (A) Cells were pretreated with mitomycin C (25 μg/ml) for 1 h, and an injury line was drawn on the confluent monolayer. After washing cell debris completely, cells were incubated with or without 100 or 250 μg/ml KIOM-C. Cell migration was monitored with a phase-contrast microscope at the specified time-points. The relative width of the gap region was quantified by measuring four selected fields. Magnification, x40; scale bar, 0.5 mm. (B) An upper Transwell chamber was filled with cells suspended in serum-free DMEM ($1x10^5$ cells/100 μl), whereas the lower chamber was filled with 10% FBS/DMEM (600 μl) and coated with Matrigel for the invasion assay. After incubation for 24 h (migration) and 36 h (invasion) with or without KIOM-C, cells that migrated and invaded to the lower surface of the membrane were stained and observed with a phase-contrast microscope. Magnification, x200; scale bar, 0.5 mm. The relative degrees of migration and invasion were quantified by ImageJ. Data are expressed as means ± SD of 3 independent experiments. $^{**}p<0.01$ vs. untreated control.

the non-cytotoxic concentration of KIOM-C, B16F10 cells were treated with various KIOM-C concentrations for 48 h followed by MTT and LDH assays. Compared to the control cells, the viability and morphology did not significantly change in response to KIOM-C, suggesting that KIOM-C at a concentration ranging from 25 to 250 μg/ml was non-cytotoxic to B16F10 cells (Fig. 1A and B); thus we used this concentration range for KIOM-C in all subsequent experiments. We further investigated whether KIOM-C at non-cytotoxic doses influences the malignant phenotype in terms of anchorage-dependent colony formation at low density. As shown in Fig. 1C (upper panel), untreated control B16F10 cells displayed rapid proliferation and formed sizable colonies from one cell, whereas KIOM-C treatment suppressed colony-forming activity involving a lower number of sizable colonies and a reduced colony size in a dose-dependent manner. Anchorage-independent growth, the ability of cells to form colonies in a semi-solid medium, which is closely correlated with metastatic potential (14), was also significantly decreased in the KIOM-C-treated cells when compared to the untreated control cells by ~60-95% at concentrations of 25-250 μg/ml (Fig. 1C, lower panel). The inhibition of colony formation was not due to KIOM-C cytotoxicity, as shown in Fig. 1A and B.

KIOM-C prevents in vitro migration and invasion of B16F10 melanoma cells. To investigate the effect of KIOM-C on metastatic activity, we first assessed migration activity using a wound healing assay. Control B16F10 cells migrated across the wound area, leading to ~25 and 70% healing at 24 and 48 h, respectively, whereas KIOM-C treatment at 100 and 250 μg/ml significantly suppressed wound migration to 60 and 55% at 48 h, respectively (Fig. 2A). Next, we performed



B

Treatment	Number of colonies	% of inhibition
Control	542.3 ± 93.56	-
170 mg/kg	227.5 ± 20.10*	58.05*
510 mg/kg	154.7 ± 52.81*	71.47*

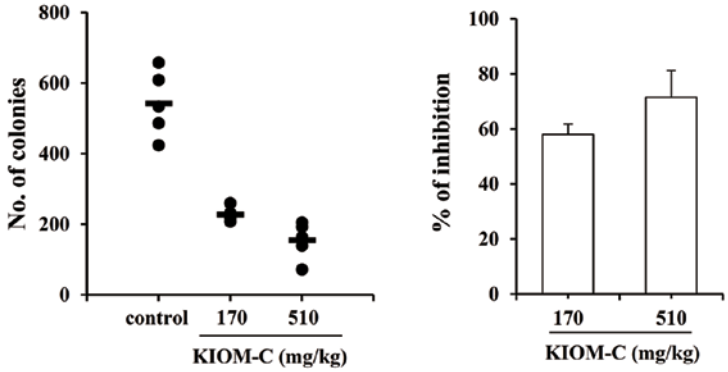


Figure 3. Effect of KIOM-C on pulmonary metastasis of B16F10 cells. Cells ($3x10^5$/200 μl PBS) were injected into the tail vein of C57BL/6J mice and were daily administered KIOM-C (170 or 510 mg/kg). Seventeen days later, mice were sacrificed, and the colonies on the lung surface were counted macroscopically. (A) Images of metastatic lung nodules at the front (F) and back (B) sides of the lungs are shown. (B) Colonies were counted, and the relative inhibition compared to the control group was calculated. Data are representative of 2 independent experiments (each group, n=5). *p<0.01 vs. control.

Transwell migration and invasion assays. As shown in Fig. 2B, the migration activity of serum-induced B16F10 cells was significantly decreased following KIOM-C treatment in a dose-dependent manner. In addition, the invasiveness, which is determined by the ability of cells to invade a Matrigel barrier, was also considerably suppressed in KIOM-C-treated cells. Treatment with KIOM-C at 250 μg/ml prevented serum-induced migration and invasion by ~55% compared to the untreated control cells (Fig. 2B).

KIOM-C administration dramatically inhibits in vivo pulmonary metastasis of B16F10 cells. To confirm the inhibitory effect of KIOM-C on tumor metastasis *in vivo*, we evaluated the ability of B16F10 cells to colonize the lungs of C57BL/6J mice after intravenous injection. As shown in Fig. 3, the incidence of metastatic black colonies was significantly decreased by KIOM-C administration in a dose-dependent manner. In particular, administration with 510 mg/kg KIOM-C resulted in ~71.47% inhibition of lung metastases as compared to the control group. To evaluate whether repeated administration of KIOM-C elicits systemic toxicity, mice were medicated with saline only (control) and KIOM-C at doses of 170 and 510 mg/kg. During the 2-week experimental period, the administration of KIOM-C did not cause death or abnormal behavior and did not affect weight gain in mice (Table I). Organ weight changes were not significantly different between the KIOM-C-treated

Table I. Mean body weights of mice administered 170 or 510 mg/kg of KIOM-C.

	Body weight (g)				
	Days after treatment				
Treatment	0	3	7	10	14
Control	14.61±0.31	14.93±0.29	15.33±0.51	15.71±0.36	16.43±0.22
170 mg/kg	15.09±0.13	15.37±0.01	15.64±0.31	16.12±0.25	16.96±0.16
510 mg/kg	14.82±0.27	15.19±0.45	15.50±0.07	15.89±0.06	16.65±0.46

Data are presented as means ± SD. Each group of mice (n=3) was orally administered 170 or 510 mg/kg KIOM-C daily, and the body weight was determined on days 0, 3, 7, 10 and 14.

Table II. Mean organ weights of mice administered 170 or 510 mg/kg of KIOM-C.

	Weight of organs (g)					
Treatment	Liver	Heart	Lung	Spleen	Kidney (L)	Kidney (R)
Control	1.05±0.07	0.10±0.01	0.13±0.00	0.09±0.01	0.11±0.01	0.12±0.00
170 mg/kg	0.90±0.01	0.09±0.00	0.13±0.01	0.08±0.00	0.11±0.00	0.11±0.02
510 mg/kg	0.89±0.01	0.09±0.00	0.14±0.00	0.08±0.00	0.10±0.00	0.11±0.01

Data are presented as means ± SD. Each group of mice (n=3) was orally administered 170 or 510 mg/kg KIOM-C daily. Mice were sacrificed on day 14, and organ weights were determined.

Table III. Chemical analysis of serum sample obtained from mice administered 170 or 510 mg/kg of KIOM-C.

Treatment	GOT (IU/l)	GPT (IU/l)	ALP (IU/l)	BUN (mg/dl)	CRE (mg/dl)
Control	55.0±0.0	20.0±0.0	185.0±21.2	9.5±1.4	1.0±0.0
170 mg/kg	55.0±0.0	22.5±3.5	212.5±3.54	10.5±1.4	1.0±0.0
510 mg/kg	55.0±0.0	20.0±0.0	202.5±3.54	11.5±4.2	1.0±0.0

Data are presented as means ± SD. Each group of mice (n=3) was orally administered 170 or 510 mg/kg KIOM-C daily. Mice were sacrificed at day 14, and the levels of GOT, GPT, ALP, BUN and CRE were determined. GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CRE, creatinine.

groups and the controls (Table II). In addition, no critical differences in the serological parameters between KIOM-C-treated and control groups were observed (Table III). The ratios of GOT/GPT and BUN/CRE were not significantly altered in the KIOM-C-treated group when compared to levels in the control, suggesting that KIOM-C administration did not cause hepatic and renal damage. The hematological parameters of KIOM-C-treated mice were also similar to the control mice (Table IV). The numbers of RBCs and the Hb level, an indicator of RBC count balance and anemia, were not altered by KIOM-C. The numbers of WBCs and other parameters were within the normal ranges. These data indicate that KIOM-C administration efficiently suppressed pulmonary metastasis of melanoma cells when compared to the metastasis noted in the controls, but without any adverse effect during treatment based on serological and hematological findings.

KIOM-C suppresses in vitro PMA-induced migration and invasion of HT1080 fibrosarcoma cells. We first examined the cytotoxicity of KIOM-C on HT1080 cells by MTT and determined that the non-cytotoxic concentrations ranged from 25 to 250 μg/ml which were used for subsequent experiments (Fig. 4A). Incubation with specified concentrations of KIOM-C substantially inhibited colony formation (Fig. 4B)

Table IV. Hematological analysis of blood obtained from mice administered 170 or 510 mg/kg of KIOM-C.

Parameter	Control	170 mg/kg	510 mg/kg
WBCP (x10^3 cells/μl)	2.38±0.41	2.11±0.27	2.33±0.39
WBCB (x10^3 cells/μl)	2.62±0.63	2.36±0.25	2.38±0.41
RBC (x10^6 cells/μl)	9.43±0.69	9.17±0.09	9.47±0.04
Mean HGB (g/dl)	13.9±1.13	13.4±0.07	13.9±0.07
HCT (%)	50.8±2.55	50.0±1.20	50.9±0.92
MCV (fl)	53.9±1.27	54.6±0.78	53.8±0.78
MCH (pg)	14.8 ±0.14	14.6±0.00	14.6±0.00
MCHC (g/dl)	27.4±0.85	26.8±0.42	27.2±0.42
PLT (x104 cells/μl)	34.3±1.54	29.8±1.04	31.9±1.88
% NEUT	7.11±0.99	8.40±0.00	8.42±0.37
% LYM	88.0±3.54	85.7±0.35	84.9±5.94
% MONO	0.50±0.14	0.57±0.09	0.65±0.15

Data are presented as means ± SD. Each group of mice (n=3) was orally administered 170 or 510 mg/kg KIOM-C daily. Mice were sacrificed at day 14, and hematologic parameters were analyzed. CBC, complete blood cell count; WBCP, white blood cell count (peroxidase method); WBCB, white blood cell count (basophil method); RBC, red blood cell count; HGB, hemoglobin, HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; NEUT, neutrophils; LYM, lymphocytes; MONO, monocytes.

and wound migration (Fig. 4C) in a dose-dependent manner. In particular, KIOM-C at 250 μg/ml almost completely suppressed colony formation and wound migration. As shown in Fig. 4D, the ability of HT1080 cells to migrate and invade across the Transwell was markedly increased following PMA stimulation to ~3.5- and 4.5-fold, respectively. In contrast, treatment with KIOM-C attenuated migration and invasion by ~50% when compared to the control cells under the PMA-stimulated conditions.

KIOM-C suppresses PMA-induced MMP-9 expression and activity through blockage of NF-κB activation. Since MMP-2 and -9 play essential roles in facilitating cancer metastasis by degrading the surrounding ECM (2,6), we examined whether KIOM-C modulates the expression and activity of these MMPs in order to elucidate the inhibitory mechanism of KIOM-C on migration and invasion. As shown in Fig. 5A, mRNA expression of MMP-9 was significantly decreased by KIOM-C treatment in HT1080 cells both under a resting and PMA-stimulated condition. In addition, KIOM-C treatment significantly decreased secreted MMP-9 expression and gelatinolytic activity in the resting state. PMA, a potent inducer for MMP-9 activation (15), increased MMP-9 expression and activity 15.3- and 26.1-fold, respectively, in the control cells. In the KIOM-C-treated cells, the increase in MMP-9 expression and activity in response to PMA was significantly lower than these values in the control cells (Fig. 5B and C). PMA stimulation also increased the secretion of active MMP-2 in the control cells, which was confirmed by western blotting and gelatin zymography (15); however, KIOM-C did not prevent the PMA-induced conversion of latent pro-MMP-2 into its active form (data not shown). In addition, in B16F10 cells, KIOM-C treatment also significantly decreased MMP-9 activity and MMP-9 expression (Fig. 5D). Previous reports have demonstrated that transcription factors such as NF-κB and activator protein-1 (AP-1) are significantly involved in the regulation of the MMP-9 gene expression and enhanced tumor invasion in various types of cells (15-17). To elucidate whether the inhibitory effect of KIOM-C on MMP-9 expression and invasion is linked to NF-κB activity, we examined the levels of IκBα and phospho-IκBα, along with p65 nuclear translocation. As shown in Fig. 6A, PMA stimulation in control HT1080 cells immediately increased the level of IκBα phosphorylation, accompanied by IκBα degradation. In addition, the p65 subunit was rapidly translocated from the cytosol to the nucleus following PMA stimulation (Fig. 6B). However, in the KIOM-C-treated HT1080 cells, the increase in IκBα phosphorylation and p65 nuclear translocation in response to PMA stimulation was insignificant when compared to the control cells, collectively suggesting that KIOM-C inhibits migration and invasion by reducing MMP-9 activity via suppression of NF-κB activation.

Discussion

Early-stage primary tumors can generally be controlled by conventional treatment such as surgery, radiation and chemotherapy. However, tumors retaining invasive and metastatic potential are relatively resistant to current chemotherapeutic agents, accounting for poor prognoses and high mortality rates (18). Although many anticancer drugs that target cell proliferation and/or induce apoptosis are believed to block or hinder the progression of cancer cells, these agents are also associated with undesirable and severe side-effects due to the non-selective killing of proliferating cells, which limit clinical applications (1,19,20). Thus, identification of agents with little

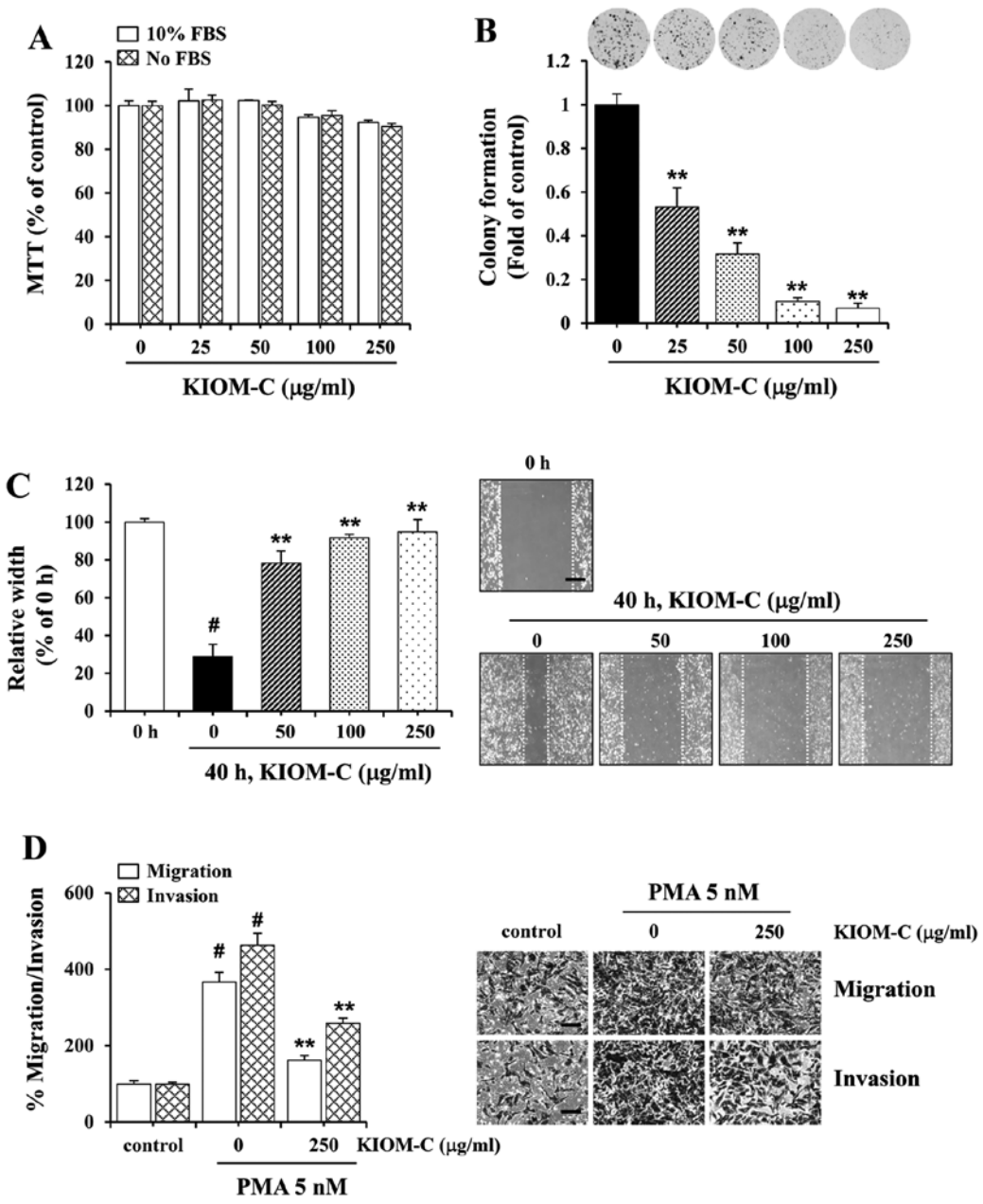


Figure 4. Effect of KIOM-C on colony formation, migration and invasion of HT1080 cells. (A) The non-cytotoxic concentration of KIOM-C in the HT1080 cells was determined by MTT assay. (B) Colony formation and (C) wound migration (magnification, x40; scale bar, 0.5 mm) of HT1080 cells in the presence or absence of KIOM-C at the specified concentrations were examined. $^{\#}p<0.01$ vs. 0 h, $^{**}p<0.01$ vs. control at 40 h. (D) PMA-induced migration and invasion were measured using the Transwell system. Cells were pretreated with or without 250 μg/ml KIOM-C for 12 h, harvested, counted and plated on the Transwell as described in Fig. 2B. After incubation with PMA (5 nM) as stimulator for 12 h (migration) and 24 h (invasion), cells that migrated and invaded were stained and images were captured. Magnification, x200; scale bar, 0.5 mm. Relative migration and invasion were calculated using ImageJ. $^{\#}p<0.01$ vs. no PMA control, $^{**}p<0.01$ vs. PMA + no KIOM-C.

or no toxicity to normal cells that can interrupt one or more steps of metastasis and limit the spread of cancer cells to a new site may be a valid strategy by which to improve the efficacy of cancer treatment and to enhance the survival of cancer patients. The metastatic cascade occurs in a sequential order including cell adhesion, invasion, proliferation and vessel formation. In particular, degradation of the basement membrane and stromal ECM is essential for invasion and metastasis of malignant cancer cells, and type IV collagen-degrading enzymes, mainly MMP-2 and -9, participate in these initial steps. Recent studies have revealed that invasive cells have higher expression levels of MMP-9 and that its expression is closely correlated with vascular invasion and aggressive malignant phenotypes (4,6). In addition, MMP-2 has been found to be associated with adverse prognosis and relapse in breast cancer patients (21-23). Recently, natural plant products have received considerable attention for their potential use in the treatment of malignant invasive progression, and several herbal medicines are frequently used as a supplemental therapy for many chronic diseases including cancer due to the possible beneficial effect of flavonoids (24-30).

In the present study, we demonstrated that KIOM-C, a novel herbal medicine, abrogates the metastatic potential of malignant HT1080 cells by reducing MMP activity via suppression

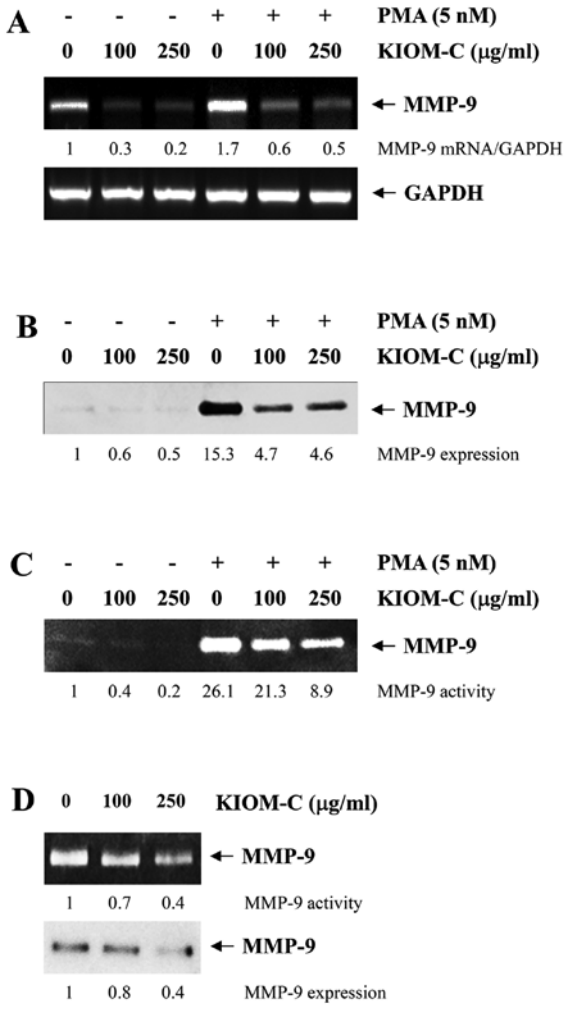


Figure 5. Effect of KIOM-C on PMA-induced MMP-9 expression and gelatinolytic activity. (A) HT1080 cells were pretreated with KIOM-C for 12 h and then further incubated in serum-free media for 24 h with or without 5 nM PMA. Expression of MMP-9 mRNA was measured by RT-PCR. (B and C) Each conditioned medium was collected and analyzed for the expression and enzyme activity of MMP-9 by western blotting and gelatin zymography, respectively. (D) B16F10 cells were incubated in serum-free media for 48 h with or without the indicated concentrations of KIOM-C. Each conditioned medium was collected, concentrated using a Centricon (Amicon Ultra centrifugal filter, 30K; Millipore Co., Billerica, MA, USA), and analyzed for MMP-9 expression and activity by western blotting and gelatin zymography, respectively. Band intensity was quantified using ImageJ software.

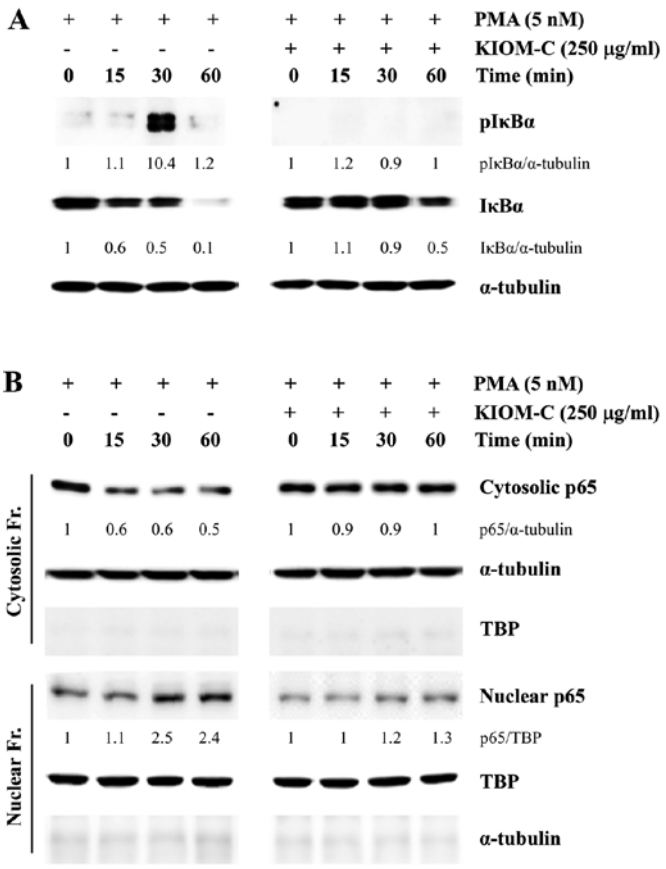


Figure 6. Effect of KIOM-C on PMA-induced NF-κB activation. (A) Control and KIOM-C-pretreated HT1080 cells were stimulated with 5 nM PMA for the specified times, and cell lysates were subjected to western blot analysis for phosphorylation and degradation of IκBα. (B) To examine the nuclear translocation of the NF-κB p65 subunit, cell lysates were fractionated into cytosolic and nuclear compartments. Relative band intensities normalized to α-tubulin or TBP are shown. Results are from a single analysis, representative of 2 independent experiments.

of NF-κB activation, and inhibits *in vivo* pulmonary metastasis of B16F10 melanoma (Figs. 1-6). Long-term intake of KIOM-C showed a dose-dependent reduction in the number of pulmonary metastatic colonies, and intake of the most effective dose of 510 mg/kg, which corresponds to the human daily dose, did not result in systemic toxicity throughout the experimental period (Table I-IV). Furthermore, oral administration of a single dose of KIOM-C of up to 2,000 mg/kg in Sprague-Dawley rats did not show acute toxicity or genotoxicity (12). These results suggest that KIOM-C can potentially be used as an antimetastatic remedy.

An herbal cocktail may target multiple cellular pathways in multifactorial diseases such as cancer, and may show synergy and reciprocal action among the myriad of phytochemicals present. The aqueous extract of *Platycodon grandiflorum*, one of the constituents of KIOM-C, has been reported to strongly suppress *in vivo* experimentally induced lung cancer, and to prolong survival by possibly inhibiting the adhesion of B16F10 melanoma cells to the basement membrane and by activating NK cells (31). Extracts of *Lonicera japonica* Thunb. inhibit HepG2 cell motility by suppressing MMP-2 activity and inducing G2/M cell cycle arrest via ERK activation (32). In addition, baicalein, baicalin, and woogonin isolated from Scutellariae Radix exhibited strong antitumor activity by interruption of cell proliferation, migration and invasion (33-35). In HPLC analysis, baicalin was recovered as a dominant component (~15.03-15.12%) in KIOM-C (13). In previous studies, baicalin was demonstrated to inhibit basic fibroblast growth factor (bFGF)-induced neovascularization in a chicken chorioallantoic membrane (CAM) assay by reducing cell-associated MMP-2 activity and inhibiting migration and proliferation (34), indicating that it plays a pivotal role in mediating anticancer activity. In addition, baicalin was shown to inhibit migration and invasion of MDA-MB-231 cells *in vitro* and suppress tumor growth and pulmonary metastasis in a xenograft model of MDA-MB-231 cells *in vivo* with no change

in body weight or liver or kidney function, which was explained by a decrease in MMP-2, MMP-9, uPA and uPAR expression via the p38 mitogen-activated protein kinase (MAPK) pathway (36). Decursin was shown to inhibit the growth of cancer cells via apoptosis, cell cycle arrest in the G_1 phase and ERK activation (37). Furthermore, it reduced the expression as well as the activity of MMP-9 in CT-26 murine colon carcinoma via suppression of ERK and JNK phosphorylation, and significantly reduced the formation of tumor nodules in the lung and increased lung weight caused by CT-26 metastasis (38). In addition, 6-gingerol reduced MMP-2/MMP-9 activities and inhibited the metastatic potential of MDA-MB-231 human breast cancer cells (39). These results suggest that KIOM-C may have potential antimetastatic effects via these active components.

PMA reportedly stimulates MAPKs, including p38, ERK and JNK, which leads to the activation of AP-1 and NF-κB transcription factors. Activation of NF-κB and AP-1 downstream of MAPKs or PI3K-Akt pathways is involved in many pathological processes, such as inflammation, cancer cell adhesion, invasion, metastasis and angiogenesis (2,40). In particular, enhanced activation of MMP-9 by treatment with PMA in HT1080 cells is mediated through the activation of NF-κB (15). In accordance with previous results, KIOM-C suppressed PMA-induced MMP-9 expression through inhibition of NF-κB activation in HT1080 cells (Figs. 5 and 6).

In summary, our results clearly demonstrated the antimetastatic activity of KIOM-C via suppression of NF-κB activation in highly malignant cancer cells. Moreover, oral administration of KIOM-C considerably prevented pulmonary metastasis of intravenously injected B16F10 melanoma cells with no systemic toxicity, possibly through suppression of migration and invasion. Collectively, these results suggest that KIOM-C may be a safe herbal medicine for controlling metastatic cancer.

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