Research Article

Camphorataimide B, a maleimide in mycelium of *Antrodia camphorate,* inhibits progression of human MDA-MB-231 breast cancer cells

Yean-Jang Lee[1], Chi-Chou Huang[2,3], Wea-Lung Lin[2,4,†], Chia-Hung Hung[5], Pei-Yun Huang[5], Tsui-Hwa Tseng[6,7, *]

1. Department of Chemistry, National Changhua University of Education, No. 1, Jin-De Road, Changhua 500, Taiwan.
2. School of Medicine, Chung Shan Medical University, No.110, Sec.1, Jianguo N.Rd.,Taichung City 402,Taiwan.
3. Division of Colorectal surgery Department of Surgery Chung Shan Medical University Hospital, No.110, Sec.1, Jianguo N. Rd.,Taichung City 402,Taiwan.
4. Department of Pathology, Chung Shan Medical University Hospital, No.110, Sec.1, Jianguo N.Rd.,Taichung City 402,Taiwan.
5. Institute of Biochemistry and Biotechnology, college of medicine, Chung Shan Medical University, No.110, Sec.1, Jianguo N.Rd.,Taichung City 402,Taiwan.
6. School of Medical Applied Chemistry, Chung Shan Medical University, No. 110, Section 1, No.110,Sec.1,Jianguo N.Rd.,Taichung City 402,Taiwan.
7. Department of Medical Education, Chung Shan Medical University Hospital, No.110, Sec.1, Jianguo N.Rd.,Taichung City 402,Taiwan.

† contribution as the first author

ABSTRACT

Breast cancer is one of the most common malignancies among women worldwide. The degree of metastasis negatively affects a breast cancer patient's prognosis and treatment benefits. In a previous investigation, camphorataimide B (Cam B), a maleimide derivative, was isolated from the mycelium of *Antrodia camphorate*, and inhibited cell cycle progression and tumor growth in MDA-MB-231 human breast cancer cells. However, it is not clear whether this compound exerts other anticancer effects. The results demonstrated that synthetic Cam B can inhibit the anchorage-independent growth of MDA-MB-231 breast cancer cells. In addition, Cam B decreased motility and invasion of MDA-MB-231 breast cancer cells. Moreover, Cam B reduced the expression of hypoxia-inducible factor-1α (HIF-1α) and its target gene product such as vimentin, cathepsin D and matrix metalloproteinase-2 (MMP-2), which play important roles in tumor progression. Additionally, Cam B reduced the phosphorylation of AKT and p65 NFκB, which associated the downregulation of HIF-1α. Furthermore, Cam B inhibited pulmonary colonization of MDA-MB-231 breast cancer cells in nude mice. By histological and gross examination of mouse lung, it showed that pretreated Cam B reduced the lung colonization of MDA-MB-231 breast cancer cells. The immunohistochemical data exhibited that pretreated Cam B decreased HIF-1α in lung section. These results demonstrate that Cam B reveals a novel role in inhibiting tumor progression.

Keywords: AKT, camphorataimide B, HIF-1α, p65 NF-κB.

Introduction

Breast cancer is one of the most common malignancies among women worldwide. Although recent advances have been made in the diagnosis and treatment of tumors, the patients continue to experience high morbidity and mortality from this disease. Accordingly, the essential characteristics of tumor progression are associated with an increased invasiveness and metastasis, and the degree of invasion and metastasis negatively affects a breast cancer patient's prognosis and treatment benefits (1). Consequently, there is great need to develop pharmacological compounds that could effectively block tumor progression. Furthermore, metastasis is the spread of cancer cells from a primary lesion to distant sites and is a major feature of tumor progression. In addition to cell motility, tumor invasion plays a crucial role in metastasis and involves a number of important steps including enzymatic digestion of the basement membrane by proteolytic enzymes followed by migration through the extracellular membrane with the subsequent growth and proliferation of cells at a new site. Many studies have demonstrated that the ability of a cancer cell to invade its surroundings generally results from aberrant cell signaling mechanisms. Several signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/AKT, nuclear factor-κB (NFκB) and mitogen-activated protein kinases (MAPKs) signaling pathways, have been reported to be involved in the tumor cell invasion process (2-5). Therefore, any agent that can block the signaling pathways involved in the invasion process of cancer cells may represent a potential for anti-tumor progression.

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor composed of an inducible subunit (HIF-1α), which is oxygen-sensitive, and a constitutively expressed subunit (HIF-1β). The HIF-1 transcriptional activity is determined by the regulated expression of the HIF-1α subunit. It has been found that HIF-1α is overexpressed in common human solid tumors, which is likely achieved through both intratumoral hypoxia and normoxic pathways including genetic alterations and extracellular stimuli (6). Overexpression of HIF-1α or HIF-1-dependent genes is associated with aggressive behavior in human cancers including breast cancer (7-8). Therefore, scientists approach to HIF-1α as an anti-cancer progression target.

Chang-Chih, specifically referred to as the fruiting body of *Antrodia camphorate* , is well known in Taiwan as an expensive folk medicine for hypertension and cancer. There is increasing evidence that both the fruiting bodies and the mycelium of *A. camphorate* possess an extensive range of biological activities, including anti-inflammatory, antioxidative, antihypertensive, antihepatotoxic, neuroprotective effects, and anticancer activities (9). The chemical components in *A. camphorate*, which include polysaccharides, triterpenoids, steroids, benzenoids, and maleic/succinic acid derivatives, have been identified (9). Both the fruiting bodies and the mycelium of *A. camphorate* exert potent anticancer activity against a variety of cancer cells, including breast, bladder, prostate, and lung cancer cells. Camphorataimide B (Cam B; Fig. 1), a maleimide derivative, has been isolated from mycelium of *A. camphorate* by Nakamura et al. and exhibits cytotoxic against LLC cells (ED_{50}=7.5 μg/mL) (10). Previously, we found Cam B exhibits cytotoxicity in MDA-MB-231 (IC_{50}= 11.4 μM) and MCF-7 (IC_{50}= 10.8 μM) breast cancer cells (11-12). Cam B also reduces tumor growth and inhibits cyclooxygenase-2 activity (COX-2) in MDA-MB-231 cells (12). Stasinopoulos et al. reported that silencing of COX-2 abolishes the metastatic potential of MDA-MB-231 cells *in vivo* (13). Recently, it has been reported that antrodin C, a maleimide, inhibits TGF-β1-induced migration and invasion of MCF-7 cells (14). Despite the emerging evidence of chemopreventive and chemotherapeutic importance of *A. camphorate* (14-16), the biological activities of the components of *A. camphorate* against cancer invasion are not well defined. In the present study, we investigated the inhibitory potential of synthetic Cam B against the motility and invasion of MDA-MB-231 breast cancer cells.

Materials and methods

Reagents. Cam B was synthesized from commercially available succinic anhydride (Fig. 1)(11). Anti-phospho-p65 NFκB, anti-phospho-AKT, anti-phospho-ERK1/2 (Thr[202]/Tyr[204]; p-ERK1/2), anti-Snail and anti-
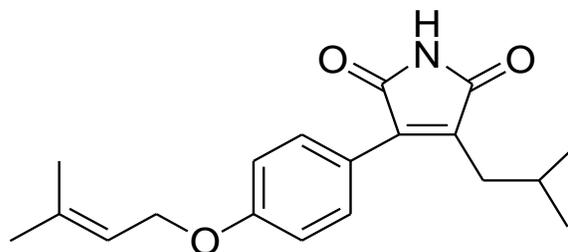
Figure 1. Structure of camphorataimide B (Cam B).

E-cadherin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The other antibodies, such as anti-ERK1/2, AKT, p65 NFκB, MMP-2, HIF-1α, cathepsin D, vimentin and β-actin antibodies, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Matrigel was provided from Collaborative Research (Bedford, MA, USA), and the other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. The estrogen-independent breast cancer cell lines MDA-MB-231 (ER⁻, HER2/neu⁻) and MDA-MB-453 (ER⁻, HER2/neu⁺) are metastatic breast cancer cell lines. Both cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (FBS; Invitrogen-Gibco), 1% penicillin/streptomycin/neomycin, and 1% non-essential amino acids. Cells were maintained at 37 °C in an incubator with 5% CO_2 and 95% air.

Cell viability assay. MDA-MB-231 and MDA-MB-453 cells were seeded in 24-well plates (5×10^4 cells/well), and cultured in complete grown medium for 24 h. Cells were treated with or without of Cam B (0- 5 μM) for 24 h. The medium was subsequently changed and incubated in the presence of 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye solution (Promega, Southampton, UK) for 4 h. The number of viable cells was directly proportional to the production of formazan, which was then dissolved with DMSO and measured using a spectrophotometer at 570 nm by Emax Microplate reader (Molecular Devices, Sunnyvale, CA).

Anchorage-independent cell growth assay. The MDA-MB-231 cells were treated with Cam B (0-2.5 μM) for 24 h. The cells ($5x10^3$) were suspended in 1 ml of 0.33% basal Eagle medium (BEM) agar containing 10% FBS and then overlaid with 3.5 ml of 0.5% BME agar containing 10% FBS. The cells were cultured at 37 °C in 95% air and 5% CO_2. After 21 days, the colonies that contained more than eight cells were photographed under an inverted microscope at 100x magnification and counted.

Scratch assay. A total of 4×10^5 MDA-MB-231 cells were plated into 6-well plates and cultured overnight in serum-containing media, and then cells were serum-starved for 24 h. The monolayer was scratched with a pipette tip, washed with PBS to remove the floating cells and photographed (0 h). Then, various concentrations of Cam B in serum-containing media were then applied for 24 h. The number of cells that migrated into the scratched area were photographed, and counted in five randomly selected fields (100x magnification) in three independent experiments. The mean value per field was calculated.

In vitro cell invasion assay. Matrigel (BD Transduction Laboratories) was diluted 1:25 with serum-free DMEM. Ten microliters of the diluted Matrigel was used to coat the top of each Boyden chamber and allowed to gel for 4 h at room temperature. The MDA-MB-231 or MDA-MB-453 cells were pretreated with or without the indicated concentration of Cam B for 24 h. The cells (2×10^5 cells/ml) were suspended in serum-free media, and 50 μl of the suspended cells was placed into the upper chamber. The complete growth medium (10% FBS) was placed in the lower chamber. After incubation for 24 h, the cells on the upper surface of the filter were wiped with a cotton swab. The cells on the lower surface of the filters were fixed for 10 min with methanol and stained with Giemsa for 1 h, and the cells that had invaded the lower surface of the filter were then counted by light microscopy. For each replicate (n=4), the cell in five randomly selected fields were determined, and the counts were averaged.

RNA isolation and reverse transcription-PCR analysis. Total RNA was isolated from individual samples using TRIzol reagent (Invitrogen, Carlsbad, CA. USA). The purified RNA was used as a template to generate first-strand cDNA synthesis using a GoScript reverse transcription kit (Promega, Madison, WI, USA). PCR was amplified with the primer pairs for a 441-bp fragment of HIF-1α (forward primer 5'-TGCACTCAATCAAGAAGTTG-3' and reverse primer 5'-GTGGCATTAGCAGTAGGTTC-3') and a 366-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer 5'-ATCCCATCACCATCTTCCAG-3' and reverse primer 5'-GAGTCCTTCCACGATACCAA-3'). RT-PCR experiments were performed in triplicates for each sample. GAPDH was used as a control. The correct size of the PCR products was confirmed by agarose gel electrophoresis.

Preparation of total cell extracts and immunoblot analysis. The cells were washed with PBS plus zinc ion (1 mM) and lysed in radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-buffer, 5 mM EDTA, 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinin, 170 μg/ml leupeptin, and 100 μg/ml PMSF; pH 7.5). After mixing for 30 min at 4 °C, the mixtures were centrifuged ($10000\times g$) for 10 min, and the supernatants were
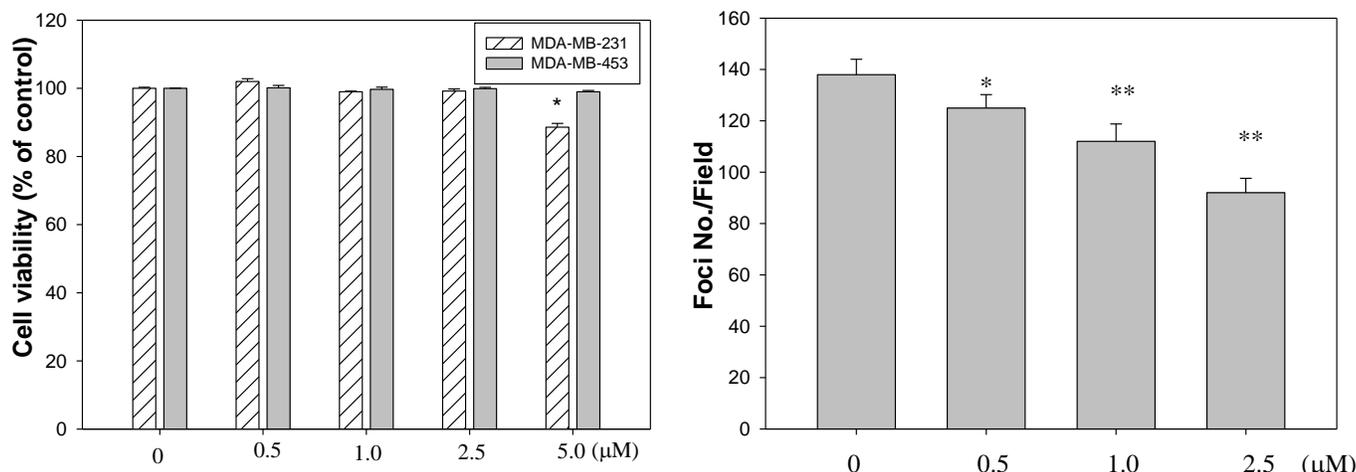
Figure 2. Effect of camphorataimide B on the cell viability and anchorage-independent growth of MDA-MB-231 breast cancer cells. (A) MDA-MB-231 and MDA-MB-453 cells were treated with various concentration of Cam B (0 - 5 μM) for 24 h. The viability of cells was measured by MTT assay. (B) MDA-MB-231 cells after treatment with various concentration of Cam B (0 - 2.5 μM) were seeded onto 6-well plate in culture medium containing 0.33% low-melting agarose over a 0.5% agarose layer. After 3 weeks, the colonies were counted. Each data represents the mean ± SD of three independent experiments. *$P<0.05$ and **$P<0.01$ versus control.

collected as the whole-cell extracts. The protein content was determined with the Bio-Rad protein assay reagent using bovine serum albumin as a standard. The samples containing 50 μg of proteins were boiled in Laemmli sample buffer, separated on SDS polyacrylamide gel, electrophoretically transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, USA), and blotted with the indicated primary antibodies. The proteins were visualized with the horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratory, Inc., South San Francisco, CA, USA), followed by chemiluminescence detection (ECL-Plus; Santa Cruz Biotechnology). The relative photographic density was quantitatedby densitometry.

Lung colonization of breast cancer cells in nude mice. Female athymic nude mice (BALB/*c-nu*) were purchased from GlycoNex Inc. (Taiwan) and maintained in cages in a specifically designed pathogen-free isolation facility with a 12/12h light/dark cycle. Rodent chow and water were provided *ad libitum.* All experiments were conducted in accordance with the guidelines of the Chung Shan Medical University Animal Ethics Research Board. Six-week-old female nude mice were used in the experimental metasatsis assay. To generate metastasis in nude mice, MDA-MB-231 cells (1×10^6) with or without treatment of Cam B (1.0 and 2.5 μM)were resuspended in 100 μl of PBS

and injected into the tail vein on day 0 (n=6). The mice were euthanized four weeks after the injection of the cancer cells. The disseminated tumors formed principally in the lung. The size and weight of the lungs were assessed. Furthermore, lung sections were fixed in formaldehyde and embedded in paraffin blocks. The slides were stained with hematoxylin and eosin, and mounted for microscopic examination. For immunohistochemical analysis, 5 μm thick sections from each subcutaneous lung specimen were fixed and incubated with monoclonal anti-HIF1-α antibodies (Santa Cruz Biotechnology) and then with 1:100 diluted biotinylated horse anti-mouse IgG for 1 h. After washing with PBS, they were reacted with 1:100 diluted avidin-biotin peroxidase mixtures for 30 min. The sections were washed thoroughly in PBS and a substrate (3-amino-9-ethylcarbazole) solution was added, and slides were counterstained with hematoxylin. Finally, slides were washed, dehydrated, and mounted for microscopic examination. The immunoreative cells presented brown color.

Data analysis. The statistical significances were analyzed through one-way analysis of variance (ANOVA) and post hoc Dunnett's test. Differences with *P* values less than 0.05 were considered statistically significant.
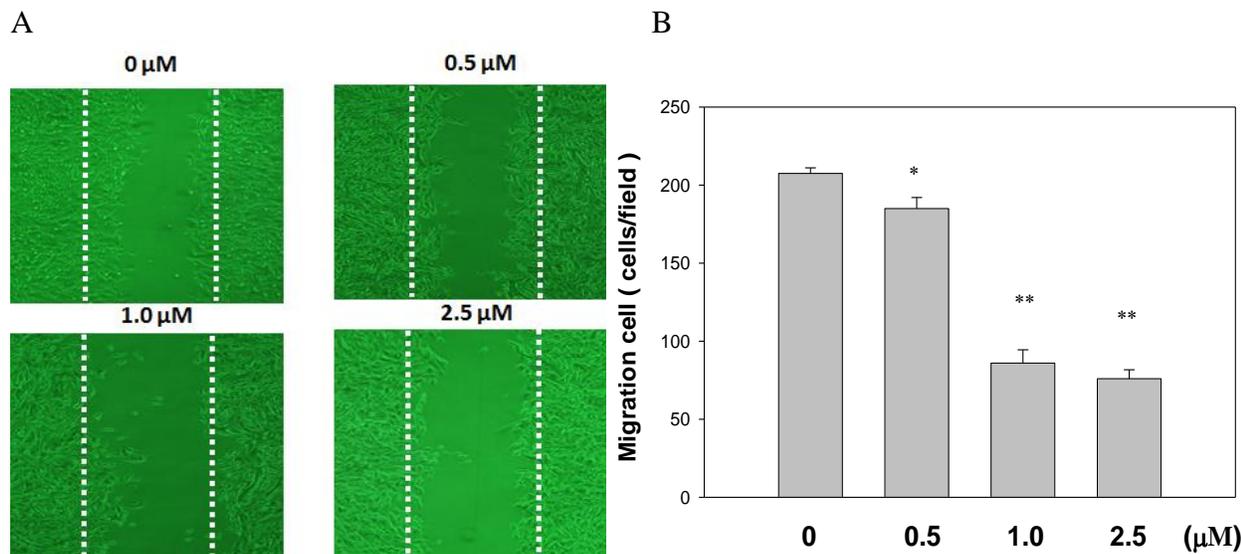
Figure 3. Effect of camphorataimide B on cell motility of MDA-MB-231 cells. **(A)** The serum-starved cells grown in monolayer were scratched with a pipette tip and then treated with or without various concentrations of Cam B in serum-containing media for 24 h. Then, the phenomenon of cell motility was observed by microscope. (A) Representative photographs were taken. (B) The number of cells migrated that into the wound after 24 h were counted in five distinct fields along the wound. Each data represents the mean ± SD of three independent experiments. *P<0.01 and **P<0.001, compared with the control.

Results

Inhibitory effect of Cam B on the anchorage-independent growth of tumor cells. Our previous study showed that Cam B triggered the apoptosis in MDA-MB-231 cells above the concentration of 10 μM (12), but not in Chang liver cells which is a normal cell line. In the Fig. 2A, it showed Cam B was noncytotoxic at concentrations of 0-2.5 μM in MDA-MB-231 and MDA-MB-453 breast cancer cells, and further used in the following study. Soft agar growth assay can provide a system in which to mimic the molecular events involved in tumor metastatic dissemination, because tumor cells reside, proliferate, and invade to form colonies in soft agar. Thus, we performed soft agar assay to determine the anti-metastasis potential of Cam B. As shown in Figure 2B, Cam B inhibited the colony formation of MDA-MB-231 cells in a dose-dependent manner.

Inhibitory effect of Cam B on cell motility and invasion of MDA-MB-231 cells. Cell motility is one of the critical steps in the metastasis of cancer cells. As shown through a scratch motility assay, treatment with Cam B for 24 h significantly decreased the migrating growth of MDA-MB-231 cells as compared to control (Fig. 3). In addition, the Boyden chamber assay was carried out to determine the ability of cells to invade through biological matrices *in vitro*. As shown in Fig. 4A, MDA-MB-231 cells with the pretreatment of Cam B (0.5, 1, and 2.5 μM) decreased the invasion of MDA-MB-231cell significantly. 2.5 μM Cam B leaded to about 50% reduction of MDA-MB-231 cell invasion compared with the untreated cells. As shown in Fig. 4B, Cam B also inhibited the invasion effect of MDA-MB-453 breast cancer cells.

Reduction in HIF-1α, vimentin, cathepsin D, and MMP-2 expression by Cam B. It has been reported that HIF-1α is highly expressed in metastatic human cancers, such as MDA-MB-231 cells (7). Therefore, we investigated whether Cam B affects the expression of HIF-1α in MDA-MB-231 cells. As shown in Fig. 5A, treatment with Cam B for 12 and 24 h decreased the expression of HIF-1α significantly. Cam B also reduced the mRNA and the protein level of HIF-1αin a dose dependent manner (Fig. 5B and 5C). Furthermore, we assessed the effect of Cam B on the expression of HIF-1α regulated protein. It showed that Cam B reduced the protein level of vimentin, cathepsin D, MMP-2 and Snail, and increased the expression of E-cadherin after treatment for 24 h (Fig. 5D).

Suppression of PI3K/AKT and NFκB signaling

A

B
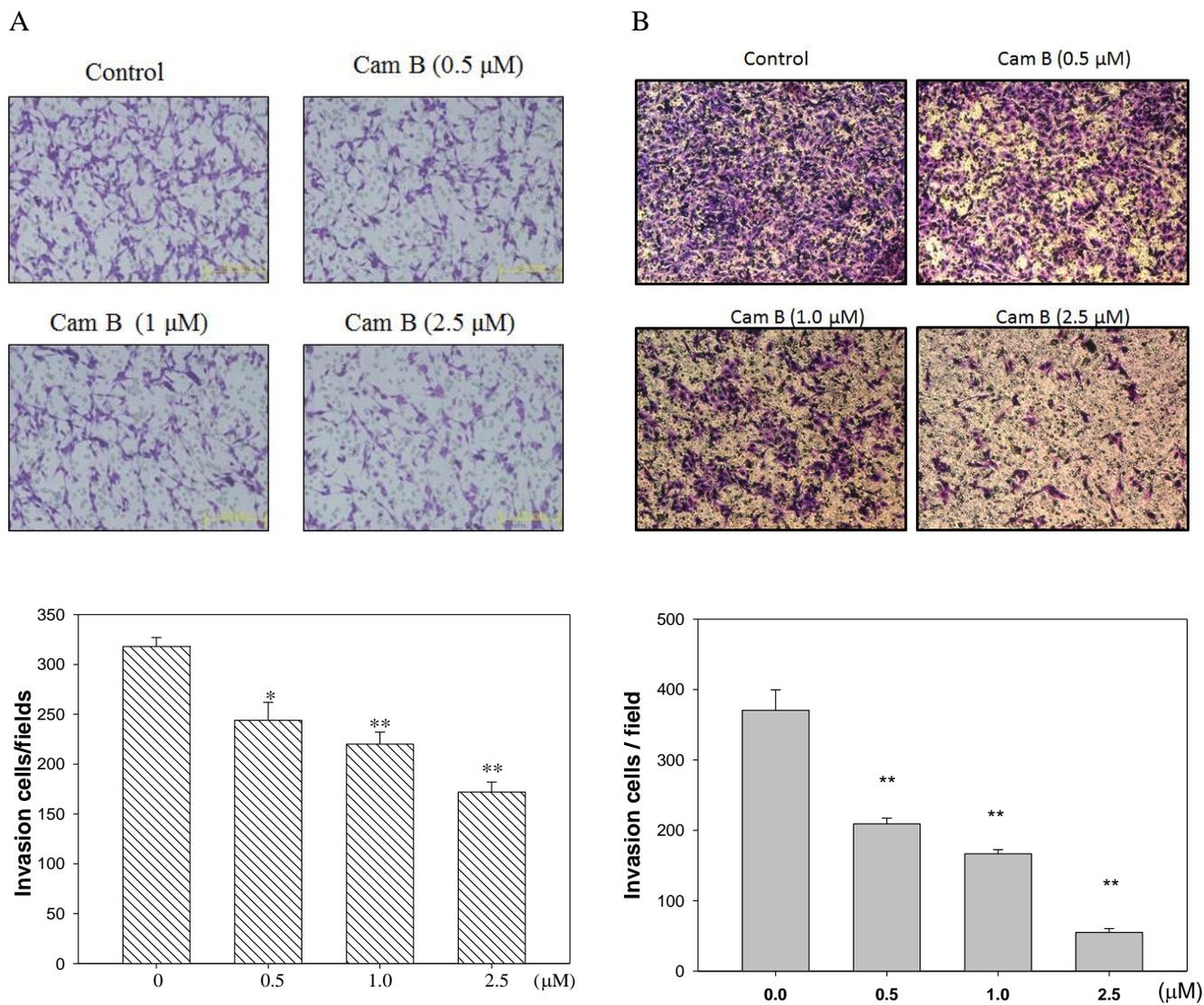
Figure 4. Effect of camphorataimide B on cell invasion of MDA-MB-231 breast cancer cells. The invasion assay was performed using the Boyden chamber assay as described in the text. (A) MDA-MB-231 and (B) MDA-MB-453 breast cancer cells that had invaded the lower surface of the membranes were taken photograph (upper) and counted in 5 randomly selected fields per well (n = 4) using a light microscope. Each data represents the mean ± SD. *P < 0.0*1 and **P < 0.00*1, compared with the control.

_____

pathways by Cam B. The effect of Cam B on the phosphorylation of ERK, AKT, and p65 NFκB was determined through an immunoblotting analysis. It showed the phosphorylation of AKT and p65 NFκB was decreased significantly after treatment with Cam B for 1-6 h. (Fig. 6A and 6B). The results implied Cam B inactivated the AKT and p65 NFκB signaling pathways. We further examined the effect of signaling inhibitors, including the NFκB inhibitor (pyrrolidine

dithiocarbamate, PDTC) and the PI3K/AKT inhibitor (wortmannin), on the expression of HIF-1α in MDA-MB-231 cells. It showed that both inhibitors reduced the expression of HIF-1α (Fig. 6C).

Inhibitory effect of Cam B on the lung colonization of cancer cells in nude mice. We assessed whether Cam B would decrease the ability of MDA-MB-231 cells to colonize the lung of nude mice. First, nude mice were injected with MDA-MB-231 cells that were either
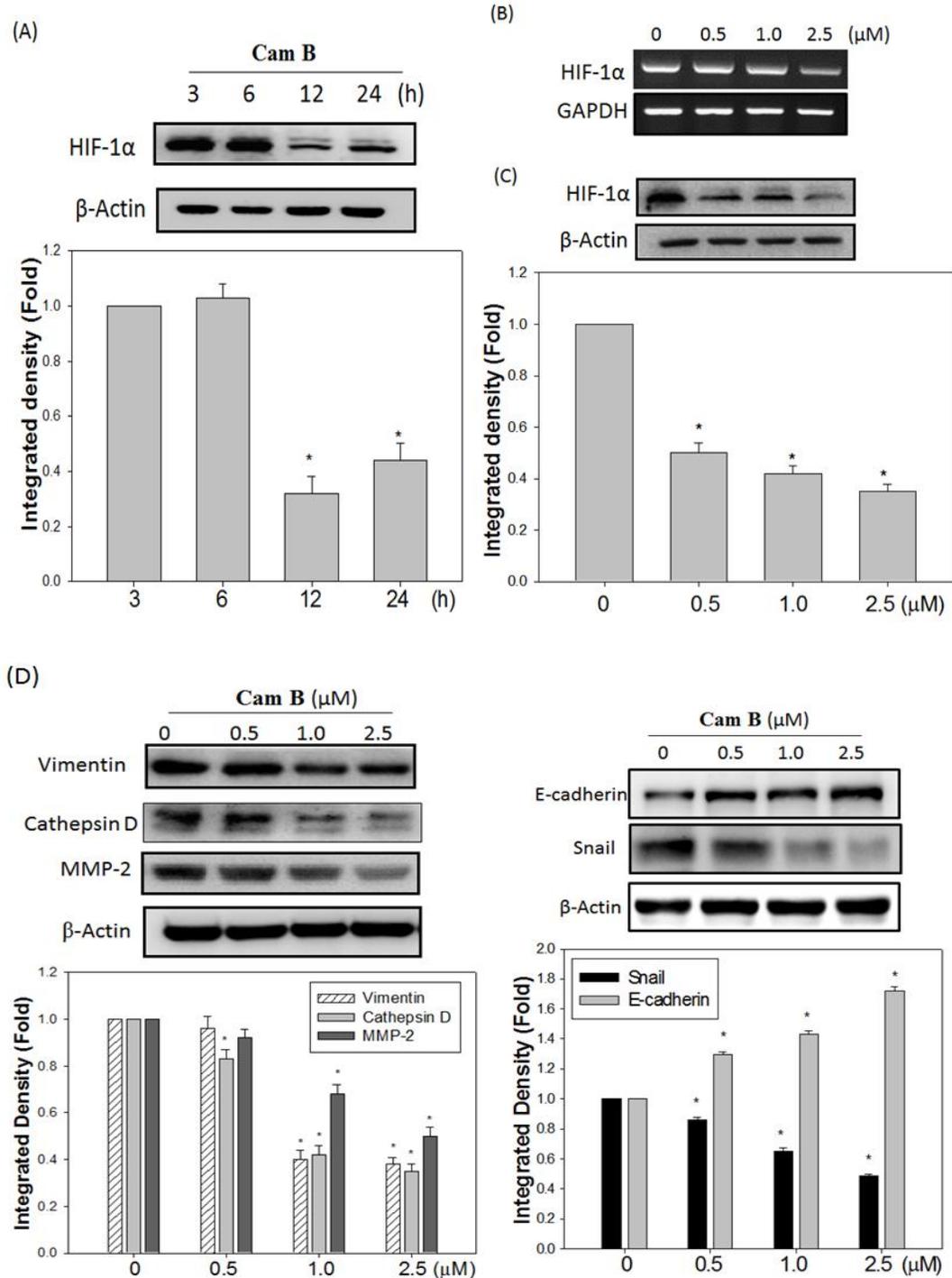
Figure 5. Effect of camphorataimide B on the protein expression of HIF-1α and its target genes in MDA-MB-231 cells. (A) Cells were treated with Cam B for indicated times. The level of HIF-1α was analyzed by immunoblotting analysis against anti-HIF-1α and anti-actin antibody was used as an internal control. In addition, the cells were treated with various concentration of Cam B for 24 h. (B) The mRNA level of HIF-1α was analyzed by RT-PCR as described in the text. (C) The protein level of HIF-1α and (D) the indicated protein were analyzed by immunoblotting analysis against anti-HIF-1α, -vimentin, -cathepsin D, -MMP-2, -E-cadherin, -Snail and anti-actin antibody was used as an internal control. After blotting onto NC paper, the bands were visualized using the ECL reagent. The result shown is one data set representative of three independent experiments. The average densitometric value was shown as the mean±SD.*$P < 0.05$, compared with the lane 1.

pretreated or not pretreated with Cam B for 24 h. Four weeks later, the mice were euthanized, and the pulmonary colonization of the cancer cells was examined to assess the development of lung metastasis. Both the total weight and the size of the lungs were increased in the positive control group compared with the normal group. Moreover, the total weight, size and nodule number of the lung of the mice injected with the cells pretreated with Cam B were significantly decreased as compared to positive control group (Fig. 7A, 7B and 7C). Furthermore, histological examination of the lung revealed that the positive control group had significantly more massive tumor cells, which exhibited a solid histological appearance. The groups that were pretreated with Cam B exhibited reduced lung colonization of the breast cancer cells (Fig. 7D). In addition, immunohistochemical analysis against HIF-1α antibody showed that pretreated with Cam B decreased HIF-1α in the lung section (Fig. 7E).

Discussion
Natural products have been the subject of many drug discovery efforts. There is an increasing body of evidence that shows *A. camphorate* exhibit antitumor activity. It has been reported that the triterpenoids in *A. camphorate* contribute to the activity (15). In addition, Hsu et al. reported that ethyl acetate extractions from the fruiting body of *A. camphorate* exert an anti-invasive effect in hepatoma cells (16). Yang et al. demonstrated that the fermented culture broth of *A. camphorate* from submerged cultures can significantly inhibit the metastatic ability of MDA-MB-231 breast cancer and B16F10 melanoma cells (17). Senthil Kumar and his co-workers found antrodin C inhibits epithelial-to-mesenchymal transition and metastasis of breast cancer cells (14). Our previous study showed that Cam Bsignificantly inhibits the xenograft tumor growth of MDA-MB-231 cells at dose of 3 mg/kg (i.p.; thrice a week for 4 weeks) in nude mice (12). It showed administration of 10 mg/kg of Cam B (i.p.; thrice a week for 4 weeks) in mouse did not display any signs of toxicity by examination of individual organs such as liver, lung and kidney. The present study provides the demonstration that Cam B, a maleimide derivative, exerts anti-tumor progression potential in estrogen-negative breast cancer cells. According to our nude mice study, Cam B inhibited pulmonary colonization of MDA-MB-231 breast cancer cells in nude mice. By histological and gross examination of mouse lung, it showed that pretreated Cam B reduced the lung colonization of MDA-MB-231 breast cancer cells (Fig.

7). However, the *in vivo* study should be done in the future in order to define anti-metastatic effect of Cam B.

There is growing evidence supporting that the inflammatory activity plays a role in the development of cancer. For instance, inappropriate up-regulation of COX-2 prolongs the survival of malignant or transformed cells and leads to phenotypic changes associated with metastatic potential (18). It has been suggested that the inhibition of COX-2 can decrease breast cancer cell motility, invasion, and matrix metalloproteinase expression (13, 19). In accordance with our results, Cam B, a COX-2 inhibitor (12), demonstrates anti-migration and anti-invasion in MDA-MB-231 cells.

HIF-1α regulated many gene expression involving tumor invasion and metastasis such as vimentin, cathepsin D, MMP2 and Snail (6, 20). Vimentin is a type of III intermediate filament protein that is expressed frequently in epithelial carcinoma correlating with cell motility and invasion (21-22). Cathepsin D is a lysosomal protease known to play tumor-promoting function in cancer progression (23-24). In addition to lysosomal protease, tumor cells acquire ability to surmount extracellular matrix barriers by expressing MMPs (25). Snial affects tumor progression in diverse tumors through induction of epithelial mesenchymal transition (20). Moreover, HIF-1α plays a central role in tumor progression by activating oncogenes and inactivating tumor suppressor genes. Cancer cell invasion involves aberrant alteration in extracellular protease production. Of these proteases, the matrix metalloproteinases (MMPs), which are a group of zinc-dependent extracellular matrix (ECM)-degrading enzymes that participate in the degradation of environmental barriers are thought to play a critical role in tumor cell invasion (26). In addition, cathepsin D is one aspartic protease involved in cell invasion and metastasis, particularly in breast cancer (27). Downregulation of cathepsin D by anti-sense gene transfer inhibited tumor growth and experimental metastasis of human breast cancer (28). Cathepsin D may be a good potential therapy target for estrogen-negative breast cancer (29). In the present study, Cam B decreased the expression of MMP-2 and cathepsin D, which are target gene products of HIF-1 in MDA-MB-231 cells (Fig. 5). The data showed that anti-invasion effect of Cam B is associated with downregulation of HIF-1α and its target gene products in MD-MB-231 cells.

The development of metastasis is the major cause of
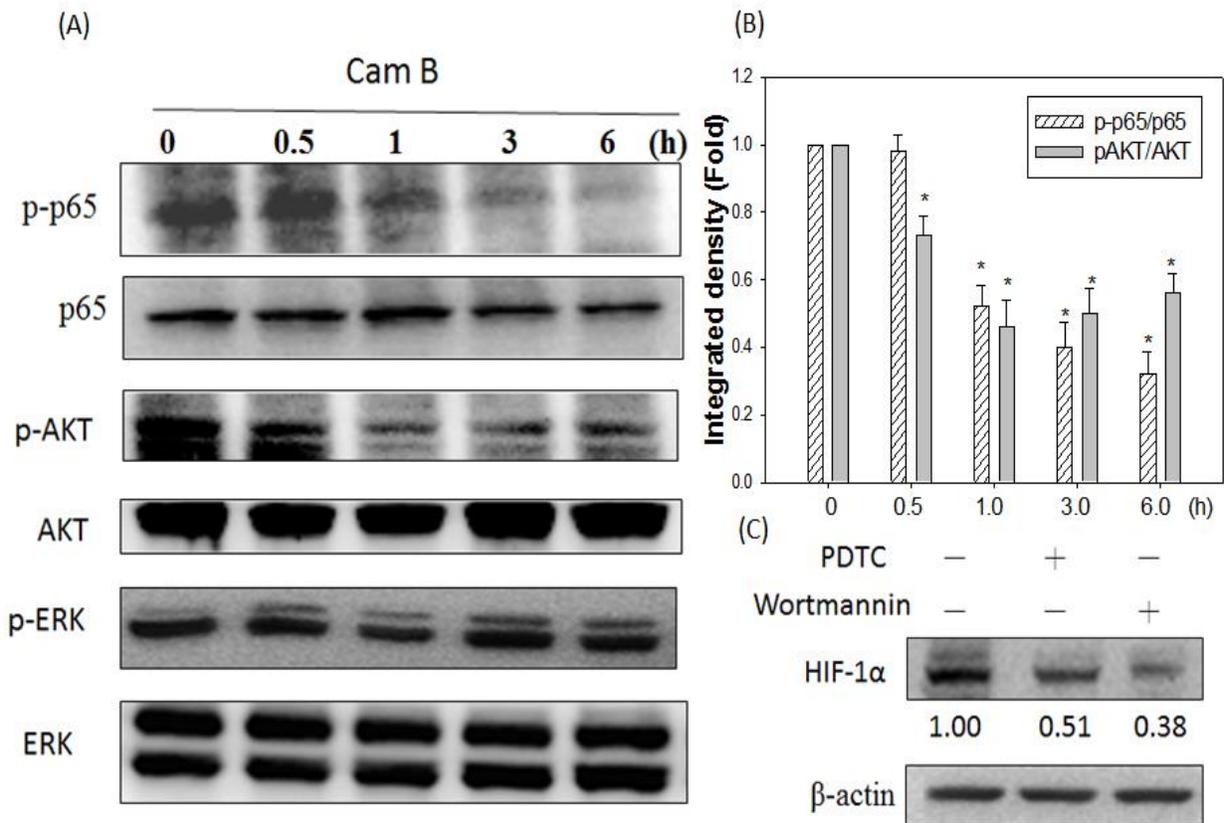
Figure 6. Effect of camphorataimide B on the p65 NFκB, AKT, and ERK signaling pathways in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with Cam B for the indicated times. The cell lysate from each sample was subjected to immunoblot analysis using phosphor-specific antibodies against p-p65 NFκB, p-AKT and p-ERK, which react with the active forms of the respective kinase. The blots were also probed with antibodies against total p65 NFκB, AKT, and ERK. The result shown is one data set representative of three independent experiments. After blotting onto NC paper, the bands were visualized using the ECL reagent. (B) The average densitometric value was shown as the mean±SD.*$P < 0.05$, compared with the lane 1. (C) The effect of signaling inhibitors, PDTC and wortmannin, on the expression of HIF-1α, were evaluated by immunoblot analysis. After blotting onto NC paper, the bands were visualized using the ECL reagent.

treatment failure in breast cancer patients. Metastasis is a complex multistep processes triggered by genetic and epigenetic alterations that result changes in the activity of signaling pathways. The signal transduction that leads to tumor invasion comprises a complex network. ERK, PI3K/AKT and NFκB have been reported to be important mediators of pro-invasive signaling (2-5). In a previous study, we demonstrated that ERK and NFκB inhibitor can decrease the activity of MMPs (30). In addition, our previous study found that the PI3K/AKT pathway involved in cell motility (31). Recently, a growing body of evidence demonstrates that the uncontrolled activation of the NFκB and PI3K/AKT pathway contribute to the development and progression

of human cancers (32). It has been found that HIF-1α is a target gene of NFκB under normoxia. NFκB may act in concert with translation and stabilization of HIF-1α (33). In addition to hypoxia, PI3K/AKT pathway plays a key role in the control of HIF-1α translation and synthesis in certain types of cancer cells (34). In the present study, Cam B inhibits the phosphorylation of p65 NFκB and AKT, which are associated the expression of HIF1-α. Consequently, our data provide the first demonstration that Cam B can inhibit PI3K/AKT and NFκB signaling pathways, which is associated with downregulation of HIF-1α in MDA-MB-231 cells (Fig. 6). These results further imply anti-tumor progression potential of Cam B by blocking the
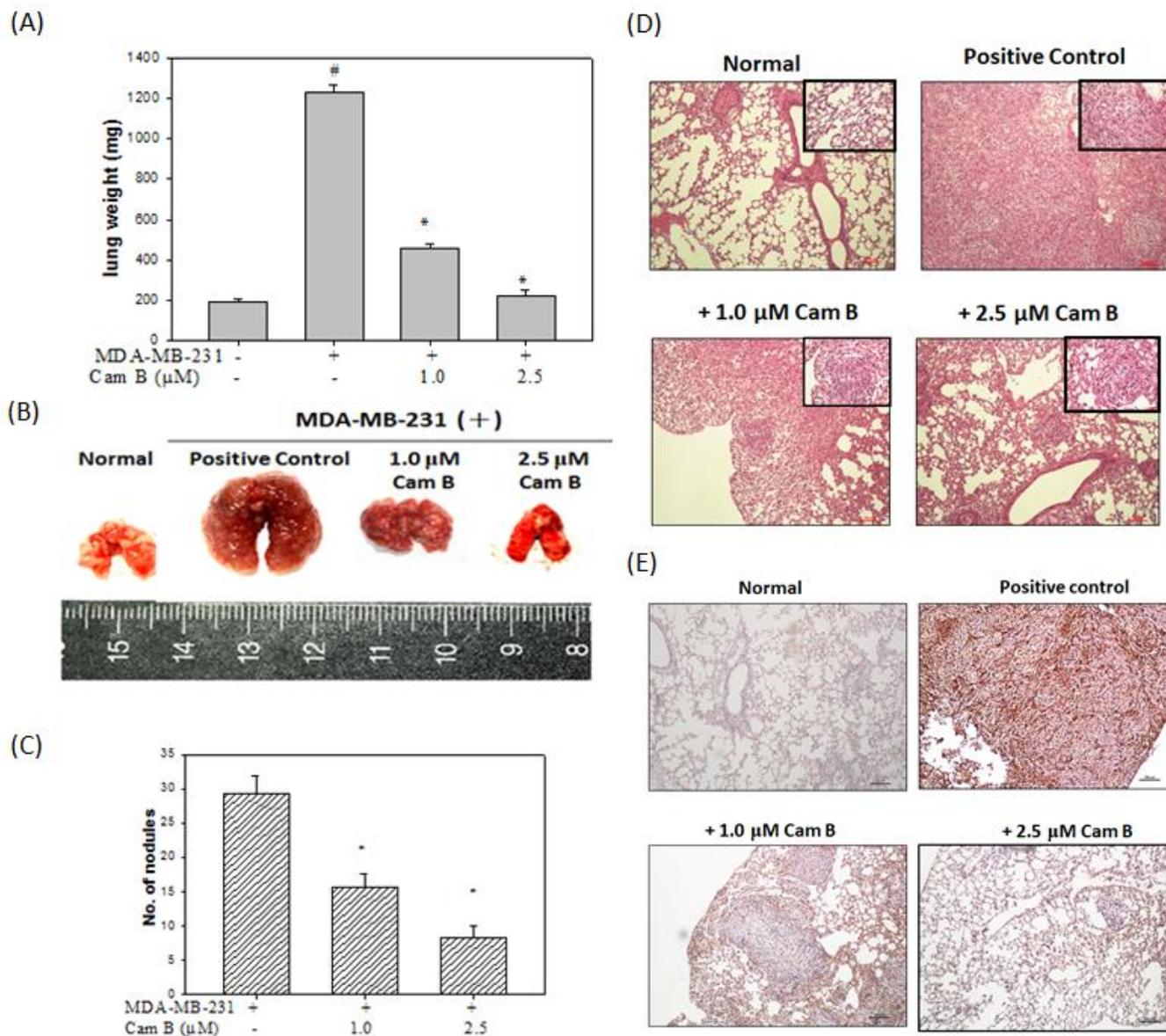
Figure 7. Effect of camphorataimide B on lung colonization of MDA-MB-231 in nude mice. MDA-MB-231 cells were pretreated with 0.5 or 1.0 µM Cam B for 24 h. The cells were then injected into the lateral tail vein of six-week-old female nude mice (n=6), and the mice were sacrificed four weeks after injection. (A) The weight of the lungs was measured. (B) The gross view of representative lungs was photographed. (C) The nodules of the lung were counted. (D) A representative set of histological views of the lung sections was photographed (H & E, 100X). Scale bar = 100 µm. (E) The effect of Cam B on HIF-1α expression in lung section by immunohistochemical analysis. The lung sections were stained for anti-HIF-1α using Catalyzed signal Amplification system, which is based on streptavidin-biotin-horseradish peroxidase complex formation. Scale bar = 100 µm.

NFκB and PI3K/AKT signaling pathways.

Conclusion
Our previous and the present studies demonstrate that Cam B inhibits the proliferation and invasion of estrogen receptor-negative breast cancer cells. Cam B, a component in *A. camphorate,* reduced the expression of HIF1-α by blocking NFκB and PI3K/AKT signaling pathways in MDA-MB-231 cells. These results demonstrated that Cam B is a candidate compound for

the prevention and treatment of breast cancer.

Abbreviations:
Cam B,   camphorataimide B;
COX-2,   cyclooxygenase-2;
GAPDH,   glyceraldehyde-3-phosphate dehydrogenase;
HIF-1α,   hypoxia-inducible factor 1;
MAPKs,   mitogen-activated protein kinases;
MMP-2,   matrix metalloproteinase-2;
NFκB,   nuclear factor-κB;
PI3K,   phosphatidylinositol 3-kinase.

References
1. Chiang AC, Massagué J. Molecular basis of metastasis. N Engl J Med. 2008 Dec; 359:2814-2823. DOI: 10.1056/NEJMra0805239.
2. Rosen LS, Ashurst HL, Chap L. Targeting signal transduction pathways in metastatic breast cancer: a comprehensive review. Oncologist. 2010 Mar; 15 (3):216-235. DOI: 10.1634/theoncologist.2009-0145.
3. Dolcet X, Llobet D, Pallares J, Matias-Guiu X. NF-κB in development and progression of human cancer. Virchows Arch. 2005; 446:475-482. DOI: 10.1007/s00428-005-1264-9.
4. Reddy KB, Nabha SM, Atanaskova N. Role of MAP kinase in tumor progression and invasion. Cancer Metastasis Rev. 2003 Dec; 22 (4):395-403.
5. Garcia-Echeverria C, Sellers WR. Drug discovery approaches targeting the PI3K/AKT pathway in cancer. Oncogene. 2008 Sep; 27 (41):5511-5526. DOI: 10.1038/onc.2008.246.
6. Nagy MA. HIF-1 is the commander of gateways to cancer. J Cancer Sci Ther. 2011; 3 (2):35-40. DOI: 10.4172/1948-5956.1000054.
7. Kimbro KS, Simons JW. Hypoxia-inducible factor-1 in human breast and prostate. Endocr Relat Cancer. 2006 Sep; 13 (3):739-749. DOI: 10.1677/erc.1.00728.
8. Patiar S, Harris AL. Role of hypoxia-inducible factor-1α as a cancer therapy target. Endocr Relat Cancer. 2006 Dec; 13 (Suppl 1):S61-S75. DOI: 10.1677/erc.1.01290.
9. Geethangili M, Tzeng YM. Review of pharmacological effects of *Antrodia camphorate* and its bioactive compounds. Evid Based Complement Alternat Med. 2011 Jan; 2011:212641. DOI: 10.1093/ecam/nep108.
10. Nakamura N, Hirakawa A, Gao JJ, Kakuda H, Shiro M, Komatsu Y, Sheu CC, Hattori M. Five new maleic and succinic acid derivatives from the Mycelium of Antrodia camphorate and their cytotoxic effects on LLC tumor cell line. J Nat Prod. 2004; 67:46-48.
11. Cheng CF, Lai ZC, Lee YJ. Total synthesis of (±)-camphorataimides and (±)-himanimides by NaBH$_4$/Ni(OAc)$_2$ or Zn/AcOH stereoselective reduction. Tetrahedron. 2008 May; 64 (19):4347-4353. DOI: 10.1016/j.tet.2008.02.077.
12. Lin WL, Lee YJ, Wang SM, Huang PY, Tseng TH. Inhibition of cell cycle progression, tumor growth and cyclooxygenase-2 activity in MDA-MB-231 breast cancer cells by camphorataimide B. Eur J Pharmacol. 2012 Apr; 680 (1-3):8-15. DOI: 10.1016/j.ejphar.2012.01.032.
13. Stasinopoulos I, O'BBrien DR, Wildes F, Glunder K, Bhujwalla ZW, Silencing of cyclooxygenase-2 inhibits metastasis and delays tumor onset of poorly differentiated metastatic breast cancer cells. Mol Cancer Res. 2007; 5:435-442.
14. Senthl Kumar KJ, Gokila Vani M, Chueh PJ, Mau JL, Wang SY. Antrodin C inhibits epithelial-to-mesenchymal transition and metastasis of breast cancer cells via suppression of Smad2/3 and β-catenin signaling pathways. PLOS ONE 2015 Feb;10(2):e0117111. DOI:10.1371/journal.pone.0117111.
15. Lee YP, Tsai WC, Ko CJ, Rao YK, Yang CR, Chen DR, Yang MH, Yang CC, Tzeng YM. Anticancer effects of eleven triterpenoids derived from Antrodia camphorate. Anticancer Res. 2012 Jul; 32 (7):2727-2734.
16. Yang HL, Kuo YH, Tsai CT, Huang YT, Chen SC, Chang HW, Lin E, Lin WH, Hseu YC. Anti-metastatic activities of Antrodia camphorata against human breast cancer cells mediated through suppression of the MAPK signaling pathway. Food Chem Toxicol. 2011 Jan; 49 (1):290-298. DOI: 10.1016/j.fct.2010.10.031.
17. Hseu YC, Tsou HT, Kumar KJS, Lin KY, Yang HL. The antitumor activity of Antrodia camphorate in melanoma

cells: modulation of Wnt/β-catenin signaling pathways. Evid Based Complementary Altern Med. 2012; 2012:197309. DOI: 10.1155/2012/197309.

18. Singh B, Berryl JA, Shoher A, Ramakarishnan V, Lucci A. COX-2 overexpression increases motility and invasion of breast cancer cells. Int. J. Oncol. 2005; 26: 1393-1399.

19. Larkins T, Nowell M, Singh S, Sanford G. Inhibition of cyclooxygenase-2 decreases breast cancer cell motility, invasion and matrix metalloproteinase expression. BMC Cancer 2006; 6: 181-192.

20. Luo D, Wang J, Li J, Post M. Mouse Snail is a target gene for HIF. Mol Cancer Res. 2011; 9(2):234-245.

21. Mendez MG, Kojima SI, Goldman RD. Vimentin induces changes in cell shape, motility and adhesion during the epithelia to mesenchymal transition. FASEB J. 2010 Jun; 24 (6):1838-1851. DOI: 10.1096/fj.09-151639.

22. Hendrix MJC, Seftor EA, Seftor REB, Trevpr KT. Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasion behavior. Am J Pathol. 1997 Feb; 150 (2):483-495.

23. Rochefort H, Liaudet-Coopman E. Cathepsin D in cancer metastasis: a protease and a ligand. APMIS. 1999 Mar; 107 (1-6):86-95. DOI: 10.1111/j.1699-0463.1999.tb01530.x.

24. Hu L, Roth JM, Brooks P, Luty J, Karpatkin S. Thrombin up-regulates cathepsin D which enhances angiogenesis, growth, and metastasis. Cancer Res. 2008 Jun; 68(12): 4666-4673. DOI: 10.1158/0008-5472.CAN-07-6276.

25. DeClerck YA, Perez N, Shimada H, Boone TC, Langley KE, Taylor SM. Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res. 1992 Feb; 52:701-708.

26. Stallings-Mann M, Radisky D. Matrix metalloproteinase-induced malignancy in mammary epithelial cells. Cells Tissues Organs. 2007; 185 (1-3):104-110. DOI:10.1159/000101310.

27. Benses P, Vetvicka V, Fusek M. Cathepsin D-Many functions of one aspartic protease. Crit Rev Oncol Hemat**.** 2008 Oct; 68 (1):12-28. DOI: 10.1016/j.critrevonc.2008.02.008.

28. Glondu M, Liaudet-Coopman E, Derocq D, Platet N, Rochefort H, Garcia M. Down-regulation of cathepsin-D expression by antisense gene transfer inhibits tumor growth and experimental lung metastasis of human breast cancer cells. Oncogene. 2002 Aug; 21 (33):5127-5134. DOI:10.1038/sj.onc.1205657.

29. Rochefort H, Glondu M, Sahla ME, Platet N, Garcia M. How to target estrogen receptor-negative breast cancer? Endocr Relat Cancer. 2003 Jun; 10 (2):261-266.

30. Lee YJ, Lin WL, Chen NF, Chuang SK, Tseng TH. Demethylwedelolactone derivatives inhibit invasive growth in vitro and lung metastasis of MDA-MB-231 breast cancer cells in nude mice. Eur J Med Chem. 2012 Oct; 56:361-367. DOI: 10.1016/j.ejmech.2012.07.041.

31. Lee WJ, Chen WK, Wang CJ, Lin WL, Tseng TH. Apigenin inhibits HGF-promoted invasive growth and metastasis involving blocking PI3K/Akt pathway and □4 integrin function in MDA-MB-231 breast cancer cells. Toxicol Appl Pharmacol. 2008 Jan; 226 (2):178-191.

32. Levitzki A, Klein S. Signal transduction therapy of cancer. Mol Aspects Med. 2010 Aug; 31 (4):287-329. DOI: 10.1016/j.mam.2010.04.001.

33. Prasad S, Ravomdram J, Aggarwal BB. NF-κB and cancer: how intimate is this relationship. Mol Cell Biochem. 2010 Mar; 336 (1-2):25-37. DOI: 10.1007/s11010-009-0267-2.

34. Li J, Zhang C, Jiang H, Cheng J. Andrographolide inhibits hypoxia-inducible factor-1 through phosphatidylinositol 3-kinase/AKT pathway and suppresses breast cancer growth. Onco Targets Ther. 2015 Feb; 8:427-435. DOI: 10.2147/OTT.S76116.