



## Original article

Demethylwedelolactone derivatives inhibit invasive growth *in vitro* and lung metastasis of MDA-MB-231 breast cancer cells in nude miceYean-Jang Lee<sup>a</sup>, Wea-Lung Lin<sup>b,1</sup>, Nai-Fang Chen<sup>c</sup>, Shien-Kai Chuang<sup>c</sup>, Tsui-Hwa Tseng<sup>c,d,\*</sup><sup>a</sup> Department of Chemistry, National Changhua University of Education, No. 1, Jin-De Road, Changhua 500, Taiwan<sup>b</sup> Department of Pathology, Chung Shan Medical University Hospital, No. 110, Section 1, Chien-Kuo N. Road, Taichung 402, Taiwan<sup>c</sup> School of Applied Chemistry, Chung Shan Medical University, No. 110, Section 1, Chien-Kuo N. Road, Taichung 402, Taiwan<sup>d</sup> Department of Medical Education, Chung Shan Medical University Hospital, No. 110, Section 1, Chien-Kuo N. Road, Taichung 402, Taiwan

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## ABSTRACT

The anticancer properties of demethylwedelolactone (DWEL) and wedelolactone (WEL), which are naturally occurring coumestans, have not been well characterized. In this study, we investigated the anti-invasive effects of synthetic WEL and DWEL on human MDA-MB-231 breast cancer cells. We found that WEL and DWEL inhibited the anchorage-independent growth and also suppressed cell motility and cell invasion of MDA-MB-231 cells. In addition, WEL and DWEL reduced the activity and expression of matrix metalloproteinases (MMPs) involved in blocking the  $\text{I}\kappa\text{B}-\alpha/\text{NF}\kappa\text{B}$  and MEK/ERK signaling pathways in MDA-MB-231 cells. Furthermore, DWEL suppressed the metastasis and lung colonization of the tumor cells in the nude mice. Altogether, these data suggest that DWEL derivatives exert anti-invasive growth effect on breast cancer cells.

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## 1. Introduction

Breast cancer is one of the most common malignancies among Western populations. Although recent advances have been made in the diagnosis and treatment of tumors, the development of clinical metastasis remains a significant cause of mortality from this disease [1]. Recently, natural products have been the subject of many drug discovery efforts [2,3]. In Asian countries, herbal teas have been used as both food and medicine throughout recorded history and are one of the natural sources of biologically active compounds. As part of our recent research to develop new biologically active substances, we were attracted to the coumestans [4] and phenanthraquinones [5], which are common components of biologically important natural products and pharmaceutical agents [6].

The development of metastasis is characterized by the aggressive spread of malignant tumor cells from the primary tumor to distant organs and represents a major cause of death in cancer patients. Metastasis is proposed to be largely dependent upon the

ability of tumor cells to invade the barrier formed by the basement membrane and migrates to adjacent tissues. Tumor invasion involves aberrant alterations in cell motility, cell adhesion, and extracellular protease production [7,8]. Of these proteases, the matrix metalloproteinases (MMPs), a group of zinc-dependent extracellular matrix (ECM)-degrading enzymes which participate in the degradation of environmental barriers, are thought to play a critical role in tumor cell invasion. Therefore, MMP inhibitors are being developed as anti-cancer or chemoprevention agents. In addition, many reports 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 nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) and mitogen-activated protein kinases (MAPKs) signaling pathways, have been reported to be involved in the initiation of the invasion process of tumor cells [9–12]. Therefore, any agent that can block the signaling pathways involved in the invasion process may represent a powerful tool for the prevention and therapy of metastasis.

Demethylwedelolactone (DWEL) and wedelolactone (WEL) (Fig. 1) are naturally occurring coumestans from *Eclipta alba* and *Wedelia calendulacea* [13]. These plants are used as traditional Asian and South American medicine for the treatment of septic shock, liver diseases, viral infections, and snake bites [14,15]. WEL has been demonstrated to exhibit a wide range of biological effects, including the inhibition of IKK kinase,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity,

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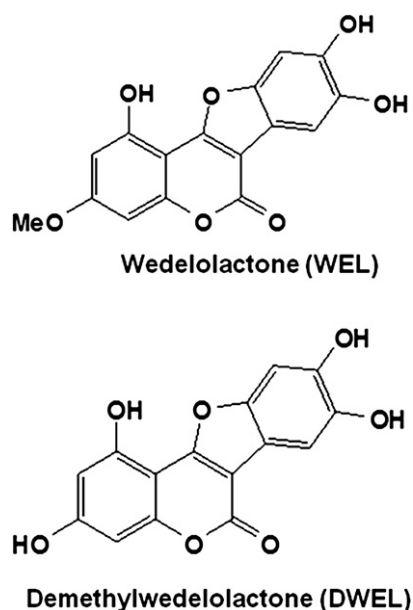


Fig. 1. The chemical structure of WEL and DWEL.

hepatitis virus C RNA-polymerase, and phospholipase A2 [14–17]. Recent research has highlighted the antitumor effects of WEL. Extracts of *Wedelia chinensis*, as well as combinations of its active components, including WEL, apigenin, and luteloin, were found to suppress the growth of prostate cancer cells *in vitro* and *in vivo* [18,19]. Furthermore, WEL was shown to inhibit the growth of pituitary adenoma cells and mammary carcinomas *in vitro* [20,21]. WEL was also found to suppress cellular growth and to induce apoptosis of androgen receptor-negative MDA-MB-231 breast cancer cells, an activity that was attributed to its inhibition of DNA topoisomerase II $\alpha$  and suppression of DNA synthesis [22]. However, the anti-invasive property of WEL in cancer cells is not well characterized, and our understanding of the biological activities of DWEL remains limited. In the present report, we investigated the mechanism(s) underlying the anti-invasive growth effects of synthetic WEL and DWEL on MDA-MB-231 breast cancer cells. Our investigation also demonstrated an inhibitory effect of DWEL on the lung metastasis and colonization of breast cancer cells in nude mice.

## 2. Results and discussion

### 2.1. Cytotoxicity and anchorage-independent growth inhibition of WEL and DWEL in MDA-MB-231 cells

We evaluated the cytotoxicity of WEL and DWEL in human MDA-MB-231 breast cancer cells by MTT assay after treatment for 24 h. Treatment with either WEL or DWEL exhibited significant differences in cell viability compared to the control-treated cells at concentrations of 25–100  $\mu$ M (Fig. 2A). We further examined the anti-invasive effects of WEL and DWEL at concentrations that were sub-lethal or non-toxic for these cells. Soft agar growth assays 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 used soft agar assays to evaluate the anti-metastatic potential of WEL and DWEL. As shown in Fig. 2B, both WEL and DWEL inhibited the colony formation of MDA-MB-231 cells in a dose-dependent manner.

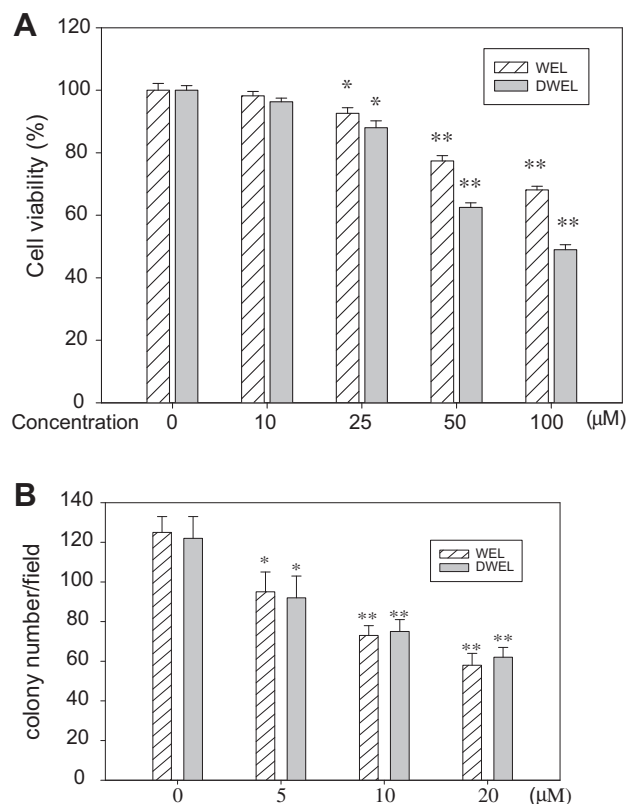
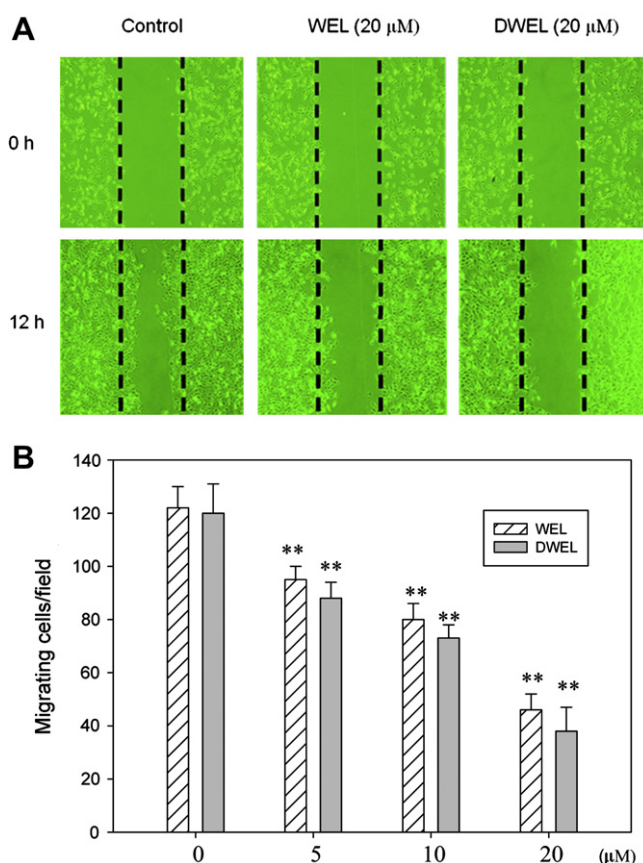


Fig. 2. The effects of WEL and DWEL on cell viability and anchorage-independent growth on MDA-MB-231 breast cancer cells. (A) Cells were treated with the indicated concentrations of WEL or DWEL (0–100  $\mu$ M) for 24 h. The viability of MDA-MB-231 cells was measured by MTT assay. \* $P < 0.05$  and \*\* $P < 0.001$  versus control (0.2% of DMSO). (B) After treatment with various concentrations of DWEL or WEL (0–20  $\mu$ M) for 24 h, the cells were seeded into 6-well plates in culture medium containing 0.33% low-melting agarose over a 0.5% agarose layer. After 3 weeks, the colonies were counted. Data are represented as the means  $\pm$  SD of 3 independent experiments. \* $P < 0.01$  and \*\* $P < 0.001$  versus control (0.2% of DMSO).

### 2.2. Effects of WEL and DWEL on cell motility and invasion in MDA-MB-231 cells

To better understand the anti-invasive effects of WEL and DWEL, we first examined the motility and invasion of MDA-MB-231 cells with or without the treatment of WEL or DWEL. According to our results, WEL and DWEL showed cytotoxicity significantly in the concentration of 25–100  $\mu$ M and treatment for 24 h. To decrease the interference in the anti-invasive evaluation all the assay were performed in the concentration of 0–20  $\mu$ M and treatment for 12 h. By scratch motility assay, the MDA-MB-231 cells exhibited wound closure activity, whereas WEL- or DWEL-treated cells (12 h) decreased this activity significantly (Fig. 3). Because cytoskeletal proteins have been linked with cell motility [23,24], we investigated possible cytoskeletal alterations by fluorescence microscopy using TRITC-conjugated phalloidin staining of the actin cytoskeleton. As shown in Fig. 4A, MDA-MB-231 cells that were cultured in complete medium showed spindle-shaped with loose cell–cell contact. Treatment with WEL and DWEL resulted in actin reorganization and changed the invasive phenotype morphology of MDA-MB-231 cells that were cultured in complete medium (Fig. 4A). In addition, focal adhesion kinase (FAK) is an intracellular protein-tyrosine kinase that acts as a coordinator of cell motility-associated signaling events [25,26]. Thus, we assessed the effects of WEL and DWEL on the activation of FAK. WEL and DWEL inhibited the phosphorylation of FAK in a dose-dependent manner

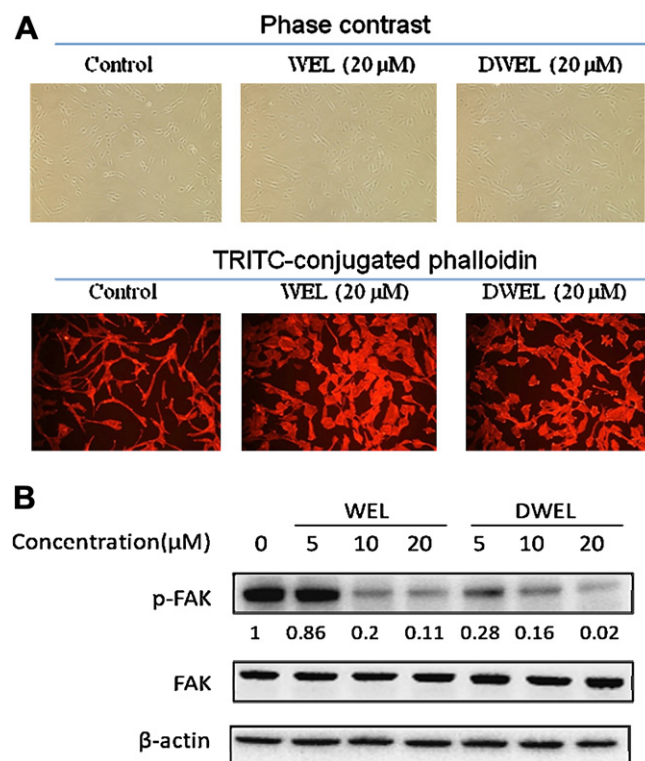


**Fig. 3.** The effects of WEL and DWEL on the cell motility of MDA-MB-231 cells. The serum-starved cells grown in monolayer were scratched with a pipette tip and then treated with or without various concentrations of WEL or DWEL in serum-containing media for 12 h. The cell motility was assessed by microscope. (A) Photographs of the administration representative were taken when the wound was created at 0 h and again at 12 h. (B) The histogram represents the number of cells that migrated into the wound at 12 h. The quantified results of the experiment were performed on 5 distinct fields along the wound in 3 independent experiments. \*\* $P < 0.001$  versus control.

(Fig. 4B). Next, we examined the anti-invasive effects of WEL and DWEL on MDA-MB-231 cells. After pretreatment with or without WEL and DWEL for 12 h, the cells were harvested for Boyden chamber assay. Our data indicated that WEL and DWEL pretreatment decreased the invasive capacity of MDA-MB-231 cells in a dose-dependent manner (Fig. 5).

### 2.3. Effects of WEL and DWEL on the activity and expression of MMP-2 and MMP-9

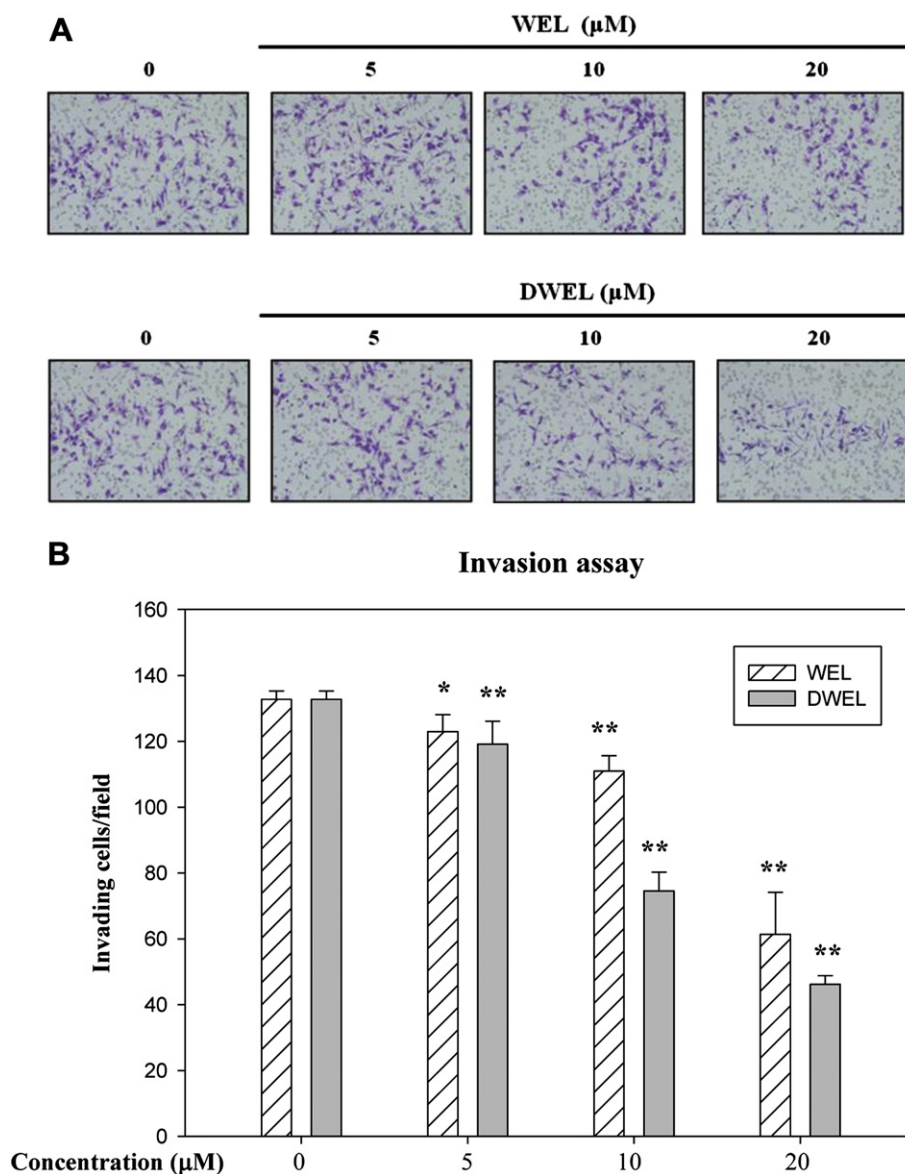
The secretion of extracellular proteases plays an important role in cancer metastasis. Of these proteases, the matrix metalloproteinases (MMPs), a group of zinc-dependent ECM-degrading enzymes, are thought to play a critical role in tumor cell invasion. The activity and expression of MMP-2 and MMP-9 have been found to correlate with the metastatic potential of tumor cells [27]. As shown in Fig. 6A, the activity of MMP-2 and MMP-9 is increased in MDA-MB-231 cells in a time-dependent manner. Treatment with DWEL inhibited MMP-2 and MMP-9 activity apparently. In addition, the individual activities of MMP-9 and MMP-2 can be inhibited by tissue inhibitor metalloproteinase-1 (TIMP-1) and tissue inhibitor metalloproteinase-2 (TIMP-2), respectively [28]. Thus, we evaluated the effects of WEL and DWEL on the protein expression of MMPs and TIMPs. We found that treatment with WEL and DWEL resulted in reduced expression of MMP-2 and MMP-9 and increased expression of TIMP-1 and TIMP-2 (Fig. 6B).



**Fig. 4.** The effects of WEL and DWEL on the morphology of and FAK phosphorylation in MDA-MB-231 cells. (A) Cells were cultured in serum-free medium for 24 h and then were treated with or without 20 μM of WEL or DWEL for 12 h in DMEM medium with 10% FBS. The F-actin was stained with TRITC-conjugated phalloidin, which exhibited a red fluorescence. (B) The MDA-MB-231 cells were treated with WEL or DWEL (0–20 μM) for 12 h. The cell lysates were subjected to immunoblotting analysis using phospho-specific antibodies against phospho-FAK and antibodies against total FAK and β-actin (loading control).

### 2.4. Effects of WEL and DWEL on the signaling pathway involving invasion

The ability of a cancer cell to invade its surroundings is an important hallmark of malignant tumors and results from aberrant cell signaling mechanisms. The signal transduction that leads to tumor invasion is composed of a complex network. NFκB and MAPKs (such as ERK1/2, JNK 1/2, and p38) have been reported to be important mediators of pro-invasive signaling [9–12]. NF-κB is a heterodimeric complex of Rel family proteins, and its activation transduces the signal from upstream stimuli, such as cytokines, mitogens, growth factors, and bacterial and viral gene products [29,30]. Accordingly, elevated levels of NF-κB are frequently detected in breast cancer cells, and NF-κB is a downstream mediator of growth signaling in estrogen receptor-negative cancers that exhibit invasive phenotypes [31]. Because WEL has been reported to be an IKK inhibitor, we examined the effects of WEL and DWEL on the phosphorylation of IκB-α in MDA-MB-231 cells. We found that after 3–24 h of treatment, DWEL inhibited the phosphorylation of IκB-α, which might initiate activation of the NFκB signal pathway. Similarly, WEL significantly inhibited phosphorylation of IκB-α after treatment for 12–24 h (Fig. 7A). Furthermore, WEL and DWEL exhibited dose-dependent inhibitory effects on IκB-α phosphorylation, significantly inhibiting phosphorylation after treatment for 12 h (Fig. 7B). Furthermore, both WEL and DWEL inhibited the phosphorylation of ERK (Fig. 7B). We next examined the effects of signaling inhibitors including an NFκB inhibitor (pyrrolidine dithiocarbamate, PDTC) and a MEK/ERK inhibitor (PD98059) on the expression of MMP-2 and MMP-9 in MDA-MB-231 cells. We found



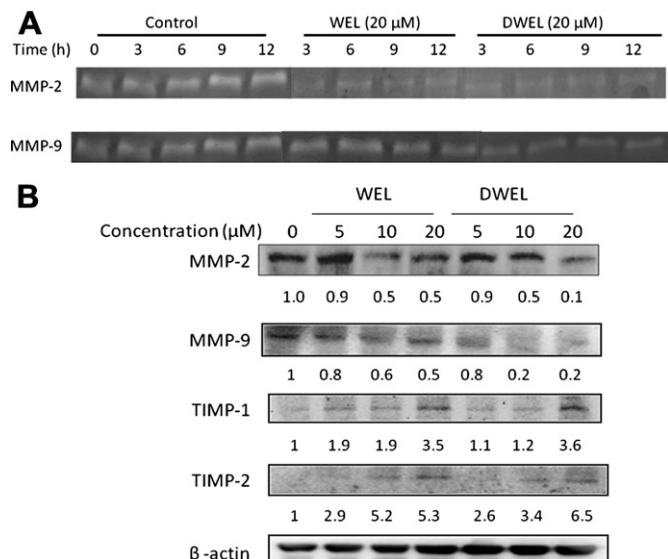
**Fig. 5.** The effects of WEL and DWEL on the invasiveness of MDA-MB-231 breast cancer cells. The invasion assay was performed using the Boyden chamber assay as described in the text. (A) A representative photograph of MDA-MB-231 breast cancer cells after invasion. (B) Cells that had invaded the lower surface of the membranes were counted in 5 randomly selected fields per well ( $n = 4$ ) using a light microscope. \* $P < 0.01$  and \*\* $P < 0.001$ , compared with the solvent control.

that both inhibitors suppressed the expression of MMP-2 and MMP-9 (Fig. 7C). Notably, WEL has been demonstrated by Benes et al. to suppress growth and induce apoptosis in MDA-MB-231 breast cancer cells. However, the activity of NF- $\kappa$ B was not affected by treatment with 30  $\mu$ M WEL for 8 h [22], suggesting that the growth-suppressing effects of WEL are likely a result of its ability to inhibit topoisomerase II $\alpha$  and DNA synthesis. Our results demonstrated that WEL significantly inhibited phosphorylation of I $\kappa$ B- $\alpha$  after treatment for 12 h and that DWEL also exhibited potent inhibitory effects (Fig. 7A). Concentrations of WEL and DWEL between 5 and 20  $\mu$ M inhibited the phosphorylation of I $\kappa$ B- $\alpha$  in MDA-MB-231 breast cancer cells treated for 12 h (Fig. 7B), which might have suppressed the activation of NF- $\kappa$ B, resulting in decreased expression of MMPs (Fig. 7C). In addition, FAK was reported to mediate cancer cell invasion by regulating focal adhesion and signaling connections [25]. FAK has been proposed to enhance the activation of ERK, which promotes transcriptional upregulation of proteases [26]. Taken together, our data indicate for the first time

that WEL and DWEL can inhibit the invasiveness of MDA-MB-231 human breast cancer cells by inhibiting the FAK, ERK, and NF $\kappa$ B signaling pathways, thereby inhibiting MMP-2 and MMP-9.

#### 2.5. Inhibitory effects of DWEL on lung colonization of cancer cells in nude mice

Our previous *in vitro* data indicated that DWEL exhibits more potent anti-invasive and kinase inhibitory effects than WEL. We assessed whether DWEL would decrease the ability of MDA-MB-231 cells to colonize the lungs of nude mice. Four weeks after intravenous (i.v.) injection of the MDA-MB-231 cells, all mice including cell-free vehicle-injected group (normal), cancer cell-injected group (positive control group), and cancer cell-injected with DWEL-pretreated groups were euthanized and pulmonary colonization of cancer cells was examined for the development of lung metastasis. Both the size and total weight of the lungs were significantly increased in the positive control group compared with



**Fig. 6.** The effects of WEL and DWEL on MMP-2 and MMP-9 in MDA-MB-231 cells. (A) The activities of MMP-2 and MMP-9 were evaluated by zymography. Media from MDA-MB-231 cells treated with or without WEL or DWEL for the indicated times were subjected to electrophoresis as described in the text. (B) MDA-MB-231 cells were treated with WEL and DWEL for 12 h. The total cell lysates were prepared and subjected to immunoblot analysis against MMP-9, MMP-2, TIMP1, TIMP2 and β-actin (loading control).

the normal group. Moreover, mice injected with DWEL exhibited decreases in the size and total weight of the lungs (Fig. 8A and B). Furthermore, histological examination of the lungs revealed that the positive control group had significantly more massive tumor cells, which exhibited a solid histological appearance. The groups that were pretreated with DWEL exhibited reduced metastasis (Fig. 8C). These data demonstrate that DWEL derivatives exhibit anti-invasive and anti-growth effects on cancer cells.

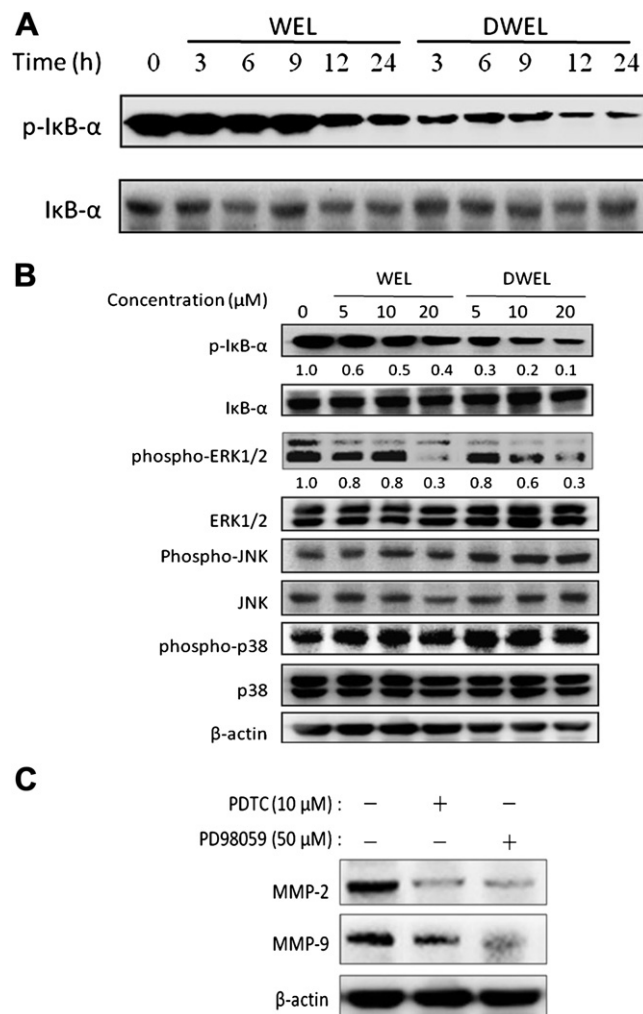
### 3. Conclusion

In conclusion, DWEL and WEL inhibited cancer cell motility and altered multiple signaling pathways, suppressing the invasion and growth of MDA-MB-231 cells. Both DWEL and WEL reduced the phosphorylation of FAK, ERK and IκB-α, thereby mediating the downregulation of MMPs. Lastly, DWEL treatment suppressed the lung metastasis and colonization of these cancer cells in nude mice. In addition, both WEL and DWEL exhibited anti-invasive effects on other invasive cancer cell-types, such as SK-Hep1 hepatoma cells (data not shown). Our data clearly indicate that DWEL derivatives hold significant potential as anti-tumor progression agents. Our *in vitro* data indicated that DWEL is more effective than WEL. Further investigation is required to clarify the structure–activity relationship of DWEL derivatives and their applications in cancer treatment.

### 4. Experimental protocols

#### 4.1. Materials

Anti-phospho-FAK (p-FAK), -phospho-IκB-α (p-IκB-α), -phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>, p-ERK1/2), -phospho-JNK/SAPK (Thr<sup>183</sup>/Tyr<sup>185</sup>, p-JNK), and -phospho-p38 (Thr<sup>180</sup>/Tyr<sup>182</sup>, p-p38) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Other antibodies, including anti-ERK1/2, -JNK/SAPK, -p38, -FAK, -MMP-2, -MMP-9, -TIMP-1, -TIMP-2 and -β-actin antibodies, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Matrigel is

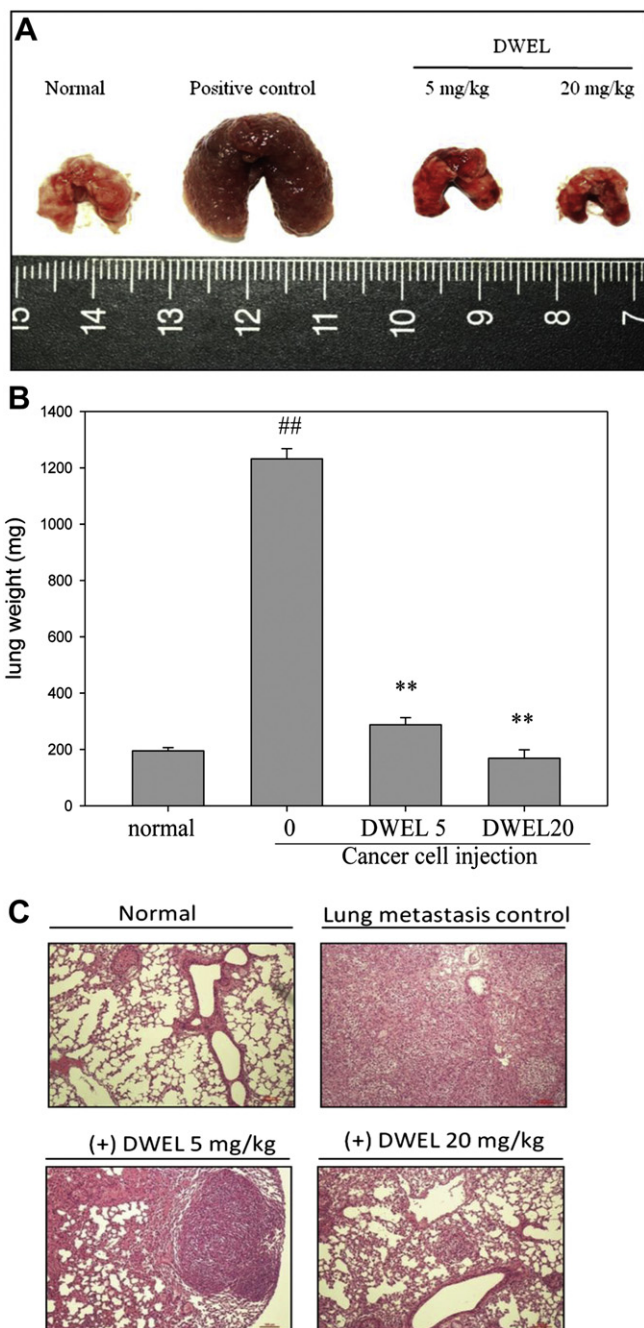


**Fig. 7.** The effects of WEL and DWEL on IκB-α and MAPKs and their association with MMPs in MDA-MB-231 cells. (A) The MDA-MB-231 cells were treated with 20 μM of WEL or DWEL for the indicated times. The cell lysates were subjected to immunoblot analysis using phospho-specific antibodies against p-IκB-α and IκB-α. (B) The MDA-MB-231 cells were treated with 5, 10, 20 μM of WEL or DWEL for 12 h. The cell lysates were subjected to immunoblot analysis using phospho-specific antibodies against p-IκB-α, p-ERK, p-JNK/SAPK, and p-p38, which react with the active forms of the respective kinases. The blots were also probed with antibodies against total IκB-α, ERK, JNK/SAPK, p38, and β-actin antibodies. (C) The effects of the signaling inhibitors on the expression of MMP-2 and MMP-9 were evaluated by immunoblot analysis using antibodies against MMP-2, MMP-9, and β-actin (loading control).

a solubilized basement membrane preparation extracted from murine sarcomas, containing laminin, type IV collagen, proteoglycans, and growth factors (Collaborative Research, Bedford, MA). Other chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO). WEL and DWEL were synthesized from commercially available phloroglucinol [4].

#### 4.2. Cell culture

The estrogen-independent, invasive human breast cancer cells, MDA-MB-231, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin–streptomycin–neomycin. Cells were maintained at 37 °C in an incubator with 5% CO<sub>2</sub> and 95% air.



**Fig. 8.** The effect of DWEL on the lung colonization by MDA-MB-231 cells in nude mice. The female mice were divided into 4 groups ( $n = 6$ ). DWEL was administered (i.p.) 3 times/week for 5 weeks. After 1 week of DWEL administration, MDA-MB-231 cells ( $1 \times 10^6$ ) were suspended in 100  $\mu$ l of DMEM and injected into the tail veins of the positive control group and the DWEL-treated group. Mice were sacrificed 4 weeks after injection and the lungs were harvested for assessment. (A) The representative pictures of lung size were photographed. (B) The whole lung weight was measured. Data are presented as the means  $\pm$  SD ( $n = 6$ ). \*\* $P < 0.001$ , compared with the positive control group. ## $P < 0.001$ , compared with the normal group. (C) A representative histological view of the lung sections (H & E stain, 100 $\times$ ) was photographed. Scale bar = 100  $\mu$ m.

#### 4.3. Cytotoxicity assay

Cancer cells were seeded into 24-well plates ( $5 \times 10^4$  cells/well) and cultured in complete medium for 24 h. Cells were treated with or without WEL and DWEL (0–100  $\mu$ M) for 24 h. The medium was

subsequently changed and incubated in the presence of 10  $\mu$ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye solution 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.

#### 4.4. Anchorage-independent growth assay

MDA-MB-231 cells were treated with WEL or DWEL (0–20  $\mu$ M) for 24 h. The cells ( $5 \times 10^3$ ) were suspended in 1 ml of 0.33% basal Eagle medium (BEM) agarose containing 10% FBS and then overlaid with 3.5 ml of 0.5% BME agarose containing 10% FBS. The cells were cultured at 37  $^{\circ}$ C in 5% CO<sub>2</sub> and 95% air. After 21 days, the colonies were counted under an inverted microscope at 40 $\times$  magnification.

#### 4.5. Scratch assay

Forty thousand MDA-MB-231 cells were plated into 6-well plates and cultured overnight. The cell monolayers were scratched with a pipette tip, washed with PBS to remove the floating cells and photographed before (0 h) and at 12 and 24 h after the application of 5, 10, or 20  $\mu$ M WEL and DWEL. The number of cells that migrated into the scratched area was photographed and counted in 5 randomly selected fields (100 $\times$  magnification) in 3 independent experiments and the mean value per field was calculated.

#### 4.6. Microscopic examination

Cells were seeded into a 6-well culture dish. After 24 h, they were starved of serum (0.1%) overnight and were then treated with indicated concentrations of WEL or DWEL for 12 h. Cells were fixed in 4% paraformaldehyde for 10 min and then permeabilized in 0.1% triton X-100 in PBS for 5 min. The morphology was assessed by inverted microscopy. Cytoskeletal changes were analyzed using fluorescence microscopy by staining with TRITC-conjugated phalloidin F-actin (500 ng/ml) for 1 h. Images were acquired on a fluorescence microscope (Nikon Microscope SE, Nippon Kogaku KK, Tokyo, Japan) at 100 $\times$  magnification.

#### 4.7. In vitro cell invasion assay

Matrigel (BD Transduction Laboratories) was diluted 1:25 with serum-free DMEM. Ten microliters of diluted Matrigel was used to coat the top of each Boyden chamber and allowed to gel for 4 h at room temperature. MDA-MB-231 cells were pretreated with or without the indicated concentrations of WEL or DWEL for 12 h. The cells ( $2 \times 10^5$  cells/ml) were suspended in serum-free media, and 50  $\mu$ l of suspended cells was placed into the upper chamber. The complete growth medium (10% FBS) was placed in the lower chamber. After incubation for 12 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 (200 $\times$ ). For each replicate ( $n = 4$ ), the cells in 5 randomly selected fields were determined, and the counts were averaged.

#### 4.8. Western blotting 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% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81  $\mu$ g/ml aprotinin, 170  $\mu$ g/ml

leupeptin, and 100 µg/ml PMSF; pH 7.5). After mixing for 30 min at 4 °C, the mixtures were centrifuged (10,000 × g) for 10 min, and the supernatants were collected as 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 50–100 µg of proteins were boiled in Laemmli sample buffer, separated on SDS polyacrylamide gel, electrophoretically transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL) and blotted with the indicated primary antibodies. The proteins were visualized with the horseradish peroxidase (HRP)-conjugated secondary antibodies (Zymed Laboratory, Inc., South San Francisco, CA), followed by chemiluminescence detection (ECL-Plus; Santa Cruz Biotechnology). The relative photographic density was quantitated by densitometry.

#### 4.9. Zymography assay of MMP-2 and MMP-9

The activities of MMP-2 and MMP-9 from conditioned media were measured by gelatin-zymogram protease assays. First, cells were seeded into 6 cm<sup>2</sup> dishes at a density of 4 × 10<sup>5</sup> cells/ml overnight and treated with 20 µM of WEL or DWEL for 0–12 h. The media were subjected to electrophoresis with 8% SDS-polyacrylamide gel containing 0.1% gelatin. Electrophoresis was performed at 150 V for 3 h. After the electrophoresis, the gels were washed three times with 100 ml distilled water (pH 7.4) containing 50 mM Tris–HCl and 2% Triton X-100 on a gyratory shaker for 30 min at room temperature to remove SDS. Then, the gel was incubated in reaction solution (10 mM CaCl<sub>2</sub>, 0.01% NaN<sub>3</sub>, 40 mM Tris–HCl; pH 8.0) for 16 h at 37 °C, stained with 0.125% Coomassie blue R-250 (in 50% methanol and 10% acetic acid) for 1 h with shaking at room temperature, and then destained with methanol/acetic acid/water (50/75/875, v/v/v).

#### 4.10. Experimental metastasis assay in nude mice

Six-week-old female athymic nude mice (BALB/*c-nu*) were purchased from GlycoNex Inc. (Taiwan) and maintained in cage housing in a specifically designed pathogen-free isolation facility with a 12/12 h 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. The mice were intraperitoneally (i.p.) administered either 100 µl of phosphate-buffered saline (PBS) or 100 µl of vehicle containing DWEL (5 mg/kg and 20 mg/kg) 3 times/week (*n* = 6). After 1 week, MDA-MB-231 cells (1 × 10<sup>6</sup>) were resuspended in 100 µl of PBS and injected into the tail vein to generate metastasis in nude mice. The mice were euthanized 4 weeks after the injection of cancer cells. The size and weight of the lungs were assessed. Furthermore, the hematoxylin-stained lung sections were histologically evaluated for the presence or absence of disseminated cancer cells within the lung. Other organs including the liver and kidney were immediately frozen, fixed in 10% neutral-buffered formalin, and embedded in paraffin. To monitor drug toxicity, the body weight of each animal was measured weekly, and a pathologist examined the mice organs.

#### 4.11. Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) with the post hoc Dunnett's test. *P* values less than 0.05 were considered statistically significant.

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