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Artical

Praeruptorin A inhibits cervical cancer HeLa cell growth and invasion by suppressing MMP-2 expression and ERK1/2 signaling

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ABSTRACT

Praeruptorin A(PA), a naturally existing pyranocumarin, is isolated from the dried root of *Peucedanum praeruptorum* Dunn. So far the anti-cancer effect and molecular mechanism behind Praeruptorin A action in human cervical cancer HeLa cells remain unknown. In the present study, we find that PA reduces cell proliferation and colony formation of human cervical cancer HeLa cells through inducing cell cycle arrest at G0/G1 phase. PA-upregulated p21 and p27 proteins are observed, accompanied with inhibition of cyclin D1 and S-phase kinase-associated *protein* 2(Skp2) proteins expression. PA could significantly inhibit migration and invasion of human HeLa cells. Meanwhile, PA significantly reduces invasive protein expression of matrix metalloproteinase-2 (MMP-2), and increases protein expression of tissue inhibitor of metalloproteinase-2 (TIMP-2). PA is observed to possess the capacity in suppressing ERK1/2 activation. PD98059 (ERK specific inhibitor) significantly enhances PA-induced downregulation of MMP-2 expression, and upregulation of TIMP-2 expression. Moreover, we found that PA treatment notably inhibits 12-O-tetradecanoylphorbol-13-acetate(TPA)-upregulated ERK1/2 activation, MMP-2 expression, cellular migration and invasion in human HeLa cells. Taken together, these findings are the first to demonstrate the anti-cancer activity of PA, which may act as a promising therapeutic agent for the treatment of human cervical cancer.

Keywords: Praeruptorin A, human cervical cancer, ERK, Invasion, MMP-2, TIMP-2

1. Introduction

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Cervical cancer is the second most frequent type of gynecologic cancer worldwide with 530,000 new cases in 2012, and 300,000 deaths from cervical cancer in 2015. Cervical cancer patients mainly die from disease recurrence or progression despite the advanced chemotherapeutic treatments [1]. The high lethality of cervical cancer results from its resistance to available chemotherapy and rare adaptability to radiotherapy [1], which thus leaves cervical cancer with no effective therapy and the very poor prognosis. Metastasis of cervical cancer to other sites such as lymph nodes, lung, bone, liver, and bowel [2-6] are the critical factors, which leads to the high mortality in cervical cancer patients. So far surgical tumor resection represents the effective therapeutic method for cervical cancer patients due to unsuccessful chemotherapy. Therefore, the novel potential strategy for inhibiting cell growth and metastasis in cervical cancer needs to be explored.

Metastasis of cancer is the major cause leading to the poor clinical outcomes and the high mortality. Metastasis is a complex process that includes cell adhesion, migration, invasion and the proteolytic degradation of the extracellular matrix (ECM). ECM degradation by extracellular proteinases contributes to the progress of tumor cell invasion and metastasis [7]. Matrix metalloproteinases (MMPs) belongs to the proteolytic proteinase systems which are primarily responsible for ECM degradation *in vivo* [7-8]. MMPs are the zinc-containing enzymes that include interstitial collagenases, gelatinases, stromelysin, matrilysin, metalloelastase, and membrane-type MMPs [9,10]. MMP-2 and MMP-9 is shown to possess the important role in the malignant development of cervical cancer in animal models and patients[11-13]. MMP activity is closely regulated by the physiological inhibitors, tissue Inhibitor of metalloproteinase (TIMPs), through modification in the activation and stability of MMPs [14].

The compounds from plants are being examined for their efficacy for the clinical

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treatment of many cancers that are in the screening program for detection of early cancer lesions. Herbal medicine or natural food used to treat different types of cancers for cancer therapy are revealed to might possess the capacity of the synergistic effects in the treatment combining antitumor agents, or might replace the conventional chemotherapy [15]. Praeruptorin A(PA), a major bioactive pyranocoumarin, is isolated from the dried root of *Peucedanum praeruptorum* DUNN (*P. praeruptorum*). Many pharmacological studies reveal that the extract of *P. praeruptorum* might possess a wide variety of activities such as anti-inflammation, anti-microbiome, neuroprotection and ani-cancer [15]. PA is reported to possess the cytotoxicity activity against P-388 lymphocytic leukemia cells, the induction of differentiation of human HL-60 promyelocytic leukemia cells toward both myelocytic and monocytic lineages, the inducement of cell apoptosis inhuman multidrug resistant (MDR) KB-V1 cells [16]. However, so far the effects and molecular mechanisms of anti-cancer growth and anti-invasive motility behind PA-treated human cervical cancer cells remain unknown.

The potentially effective plant-based products against human cervical cancer cells need to be explored and identified. In the present study, we investigated whether PA inhibits cell growth, migration and invasion, and further and identified the precise molecular mechanisms in human cervical cancer HeLa cells. The results demonstrated that PA treatment inhibits cell growth, induces cell cycle arrest at G0/G1 phase through the increase in p21 and p27 levels and the inhibition of cyclin D1 and Skp2 expression. PA significantly suppressed TPA-induced invasive motility through deactivation of ERK1/2 signaling pathway, inhibition of MMP-2 expression, and upregulation of TIMP-2 level in human cervical cancer HeLa cells. This study suggested that PA possesses the anticancer properties through inhibiting cell growth and invasive potential of human cervical cancer cells.

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2. Results

2.1. PA reduces cell viability and colony formation in human cervical cancer HeLa cells

We observed the effect of PA on cell viability and colony formation in human HeLa cells. HeLA cells were treated with various concentrations of PA (0, 10, 20, 30, 40 and 50 μM) for 24 h, and followed by MTT assay and colony formation assay (Figure 1A-C). The results showed that PA possesses the effect in the inhibition of cell growth in human cervical cancer HeLa cells.

2.2. PA induces cell cycle arrest at G0/G1 phase in human cervical cancer HeLa cells

Flow cytometry assay was performed to further confirm the effect of PA on regulation of cell cycle (Figure 2A). The results showed that PA showed the effect in the induction of cell cycle arrest at G0/G1 phase. The factors cyclin D1, p21, Skp2 and p27 involved in cell cycle regulation were measured in human HeLa cells. PA significantly inhibited the expression of cyclin D1 and Skp2 proteins, accompanied with the increase of p21 and p27 protein levels (Figure 2B).

2.3. PA inhibits cell migration/invasion and MMP-2 expression in human cervical cancer HeLa cells

To identify the effect of PA on cellular migration and invasion activity in human HeLa cells, we treated hum HeLa cells with various concentrations of PA (0, 10, 20 and 30 μM) for 24h. We found that PA significantly inhibited cellular migration and invasion activity in human HeLa cells. The inhibitory effect of PA presented a dose-dependent manner in the reduction of HeLa cell migration and invasion (Figure. 3A). MMP-2 and MMP-9 have been reported to play a critical role in cancer cell migration and invasion by contributing to the degradation of the ECM and cancer progression. To identify the effect of PA on gene and protein expression of MMP-2 and -9 in human

cervical cancer cells, we treated hum HeLA cells with various concentrations of PA (0, 10, 20 and 30 μM) for 24h, and subjected to immuniblotting assay and qRT-PCR. The results showed that protein and gene levels of MMP-2 in human HeLa cells were significantly reduced when exposed to PA (10 and 20 μM) for 24h. The significant reduction of MMP-2 in protein and gene by PA treatment presented a phenomenon of dose-dependent manner. Furthermore, we observed the expression of TIMP-1 and -2 in the levels of protein and gene. TIMP-2 was significantly upregulated in a dose-dependent manner after PA treatment (10, 20 and 30μM) (Figure 3B, 3C).

2.4. Role of ERK1/2 in PA-modified MMP-2 and TIMP-2 expression in human cervical cancer HeLa cells

To identify which signal transduction pathway(s) was involved in the mechanism behind PA-regulated activity of migration and invasion in human HeLA cells, we treated human HeLa with PA (10, 20 and 30 μM). HeLa cells then were harvested for immunoblotting assay to observe the activation of signaling pathways. We found that PA inhibits the activity of endogenous ERK1/2 signaling pathway with no influence on activation of JNK1/2 and p38 pathways (Figure 4A). To identify the role of ERK1/2 signaling pathway in PA-inhibited invasive motility, we applied PD98059 (specific MEK1/2-ERK1/2 pathway inhibitor) to specifically blocked the activation and expression ERK1/2. We found that PD98059 significantly enhanced PA-induced inhibitory effect on invasive motility (Figure 4B), downregulation of MMP-2 expression, and upregulation of TIMP-2 in human HeLa cells (Figure 4C, 4D). The results suggested that ERK1/2 signaling pathways was involved in PA-inhibited invasive motility in human HeLa cells.

2.5. PA inhibits TPA-induced ERK1/2 activation, MMP-2 expression and migration/invasion in human cervical cancer HeLa cells

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We further examined whether PA suppressed the effect of 12-O-tetradecanoylphorbol-13- acetate (TPA) on human HeLa cells. We observed that TPA(50 ng/ml) significantly induced the activation of ERK1/2, JNK1/2 and p38 proteins in a time-dependent manner (Figure 5A). Human HeLa cells were treated with various concentration of PA (0, 20 and 30 μM) in the presence of TPA (50 ng/ml). We found that PA significantly inhibited TPA increased cell invasive motility, activation of ERK1/2, and expression of MMP-2 at the protein and mRNA in human HeLa cells (Figure 5B-5D). These results provided that PA notably reversed the effect of TPA in human cervical cancer HeLa cells.

3. Discussion

Plant-derived compounds are showed to be as the potential agents with many anti-cancer bioactivities. Prenylated coumarins, the ethanol extracts of *Peucedanum praeruptorum*, exhibits *in vitro* cytotoxic activity against human cancer cell lines including HL-60, A-549, SMMC-7721, MCF-7 and SW-480 cells [17]. Angular pyranocoumarin extracted from *Peucedanum praeruptoruon* inhibits the proliferation and induces the apoptosis in U266 cells through upregulating expression of caspase-8 and -3 proteins and downregulating expression of phospho-ERK, phospho-AKT proteins and the hTERT mRNA [18]. Pyranocoumarins from root extracts of *Peucedanum praeruptorum* Dun are showed to downregulate nitric oxide (NO) production, and inhibit the efflux of drugs by multidrug-resistance (MDR) proteins[19]. Praeruptorin A (PA) from *Peucedanum praeruptorum* Dun shows antiproliferative and cytotoxic effects on human gastric cancer SGC7901 cells. Moreover, PA enhances the inhibitory effects of doxorubincin (DOX) on human SGC7901 cells. Inhibition of cell growth is higher when co-treatment with PA and DOX than that with the chemotherapy agent applied

alone[20]. So far no study explores the effect of PA on the development/progress of human cervical cancer cells. Expression change of cell cycle-regulation factors and the subsequent dysregulation of cell cycle are critical steps in leading to the development and progress of tumor, which are the most events founded in human cancers. Overexpression of cyclin D1 and Skp2 are associated with the poor prognosis in various human cancers[13,21]. Cyclin D1 plays a key intracellular regulator involved in the progression of the cell cycle through G1, and contributes to a worse prognosis. Skp2 acts as a oncogene and is involved in the double negative feedback loops with both p21 and p27 proteins that control cell cycle entry and G1/S transition [22,23]. In this study, we investigated the properties of PA in the anti-proliferation and anti-metastasis, and identified the molecular mechanism of PA in human cervical cancer cells. We observed that PA shows the induction in cell growth inhibition and cell cycle arrest at G0/G1 phase in human cervical cancer HeLa cells. PA might significantly inhibits cell proliferation and colony formation through downregulation of cyclin D1 and Skp2 proteins, and upregulation of p21 and p27 proteins. The findings suggested that PA might exert the protective effects against progression of human cervical cancer by modulating cell cycle.

Extracellular matrix (ECM) remodeling contributes to migration and invasion of cancer cells during distant metastasis. The process in disrupting the interaction between cells and ECM leads to the development of malignant tumor. Upregulation in MMPs expression/activation is showed to be associated with ECM remodeling progress, tumor cell invasion and metastasis. MMP activation is closely is regulated by physiological inhibitor, tissue inhibitor of metalloproteinases(TIMPs). Four type of TIMP species have been identified as TIMP-1, TIMP-2, TIMP-3 and TIMP-4. TIMP binds to MMP in a 1:1 stoichiometric ratio. TIMPs not only directly inhibit MMPs but also form

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199 complexes with MMPs to control activation and stability of MMPs. Increased
200 expression of MMP-2 and MMP-9, and decreased expression of TIMP-1 and TIMP-2
201 might serve as markers of invasive and metastatic potential of the
202 squamous cervical carcinoma (SCC) tumor [24]. TIMP-2 gene methylation is reported
203 to be the characteristic in human cervical lesions [25]. Staurosporine might induce anti-
204 tumor response in the cervical tumor microenvironment, and
205 inhibits cancer progression and metastases through suppression of MMP-1 and MMP-
206 2 [26]. HPV16E6 and E7 oncoproteins cooperate and promote the cervical
207 cancerinvasiveness by specifically upregulating MMP-2 transcription [27]. Anti-viral
208 drugs Ribavirin and Indinavir are reported to act as therapy against HPV-18
209 induced cervical cancer by decreasing MMP-2 and MMP-9 secretion [28]. MMP-2 and
210 MMP-9 are associated with the progression of cervical cancer when exposed to low
211 concentrations of arsenic trioxide and humic acid [29]. Both MMP-2 and MMP-9 play
212 the important role in cancer progression and remodeling of the ectocervix. MMP-2 and
213 MMP-9 could be used diagnostic detection for cervical lesion and cancer[30]. In this
214 study, we found that PA dramatically inhibits cell migration and invasion through
215 downregulating MMP-2 expression and upregulating TIMP-2 expression in human
216 HeLa cells. The results indicate PA as potential antimetastasis agents against human
217 cervical cancer cells.

218 Mitogen-activated protein kinases (MAPKs) participate in many biological
219 functions and cellular responses such as cell survival, proliferation, invasion and
220 apoptosis, depending on the stimuli, intensity, and duration, as well as cell types. Some
221 studies show that ERK1/2 mediates fisetin-induced apoptosis by activating caspase-
222 8/caspase-3 dependent pathways in human cervical HeLa cells [31]. ERK1/2
223 contributes to Securinine-induced cell cycle arrest and apoptosis in human HeLa

cells[32]. p38 MAPK mediates fisetin-suppressed urokinase plasminagen activator (uPA) expression and invasion in human cervical cancer cells [33]. α-Mangostin could inhibit the tumor growth of cervical cancer cells through enhancing ROS amounts to activate ASK1/p38 signaling pathway and damage the integrity of mitochondria and thereby induction of apoptosis in cervical cancer cells [12]. p38 MAPK kinase mediates DEPTOR-regulated survival and apoptosis in human squamous cervical carcinoma (SCC) tumor[34]. Activation of p38 MAPK kinase mediates Neferine-enhanced antitumor effect of mitomycin-c in human HeLa cells[35]. Goniothalamin possesses a cytotoxic effect against human HeLa cells via the induction of mitochondria-mediated apoptosis, associated with ER stress-induced activation of JNK1/2 [36]. In this study, we attempted to identify the role of these factors including ERK1/2, p38 and JNK1/2 in PA-regulated expression MMPs expression, TIMPs expression, migration and invasion in human HeLa cells. PA significantly suppresses endogenous activity of ERK1/2 signaling pathway, but not JNK1/2 and p38 pathways. We found that PD98059 (specific ERK1/2 inhibitor) significantly enhanced PA-inhibited migration, invasion and MMP-2 gene/protein expression, and PA-increased TIMP-2 gene/protein expression in human HeLa cells. Furthermore, we observed that PA notably suppresses TPA-upregulated ERK1/2 activity, MMP-2 gene/protein expression and invasive motility in human HeLa cells. These findings suggested that PA exerts the strong anti-cancer bioactivity against human cervical cancer cells.

The present results suggested PA might possess the anti-cancer capacity in suppressing cell growth and invasive motility through deactivating ERK1/2 signaling pathway and MMP-2 expression, and increasing TIMP-2 level. Moreover, PA significantly inhibited TPA-induced invasive motility, ERK1/2 pathway activation and MMP-2expression in human cervical cancer HeLa cells. To our knowledge, this study

249 first demonstrates the effects and molecular mechanisms underlying the anti-cancer
250 potential properties of PA against human cervical cancer cells.

251 **4. Materials and Methods**

252 *4.1. Reagents*

253 A stock solution of paeruptorin A (PA) was made at a concentration of 100 mM in
254 ethanol and stored at -20 °C. Antibodies against cyclin D1, p21, Skp2, p27, MMP-2,
255 MMP-9, TIMP-1, TIMP-2, p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, p-p38, p38 and β-
256 actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The MEK1/2
257 inhibitor, PD98059 was purchased from Calbiochem (San Diego, CA). Horseradish
258 peroxidaselabeled anti-mouse and anti-rabbit secondary antibodies were obtained from
259 Promega (Madison, WI). MTT was purchased from Sigma (St. Louis, MO). All stock
260 solutions were wrapped in foil and kept at -20 °C.

261 *4.2. Cell Culture*

262 Human cervical cancer cell line, HeLa (BCRC No 60005) was obtained from the
263 Bioresources Collection and Research Center, Food Industry Research and
264 Development Institute (Hsinchu, Taiwan). HeLa cells were maintained in Dulbecco's
265 modified Eagle's medium (DMEM, GibcoInvitrogen Corporation, CA), cells were
266 supplemented with 10 % fetal bovine serum (FBS, Gibco-Invitrogen Corporation, CA)
267 and 1 % antibiotics (10,000 units/mL penicillin, 10 μg/mL streptomycin (Invitrogen
268 Life Technologies, Carlsbad, CA) in a humidified atmosphere of 5 % CO_2 at 37 °C.

269 *4.3. Immunoblotting*

270 To isolate total proteins, cells were washed with cold PBS and resuspended in lysis
271 buffer (50 mM Tris , pH 7.5, 0.5M NaCl, 1.0 mM EDTA ,pH 7.5, 10% glycerol, 1mM
272 BME, 1% NP40) plus proteinase inhibitor cocktail and phosphatase inhibitor cocktail

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(Roche Molecular Biochemicals). After incubation for 30 min on ice, the supernatant was collected by centrifugation at 12,000 g for 15 min at 4 °C, and the protein concentration was determined by the Bradford method. Sample containing equal proteins (40μg) were loaded and analyzed by immunoblotting. Briefly, proteins were separated by 12% SDS-PAGE and transferred onto PVDF membrane (Life Technologies, Carlsbad, CA, USA). Membrane were blocked with blocking buffer (5% non-fat dry milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for at least 1 h at room temperature. Membranes were incubated with primary antibodies in the above solution on an orbit shaker at 4 °C overnight. Following primary antibody incubations, membranes were incubated with horseradish peroxidase-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG). Antibody-bound protein bands were detected using high sensitive Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA), and photographed with Bio-Rad Chemiluminescence Imaging System (Bio-Rad Laboratories, Inc. Hercules, CA, USA)

4.4. Cell Viability Assay

To determine the effect of PA on human cervical cancer HeLa cell viability, cells were treated with PA and were subjected to MTT assay. The absorbance of blue formazan crystals was measured at 570 nm using an enzyme-linked immunosorbent assay plate reader. The quantity of the formosan product was directly proportional to the number of viable cells in the culture medium. The cell viability of cells was determined according to the absorbance corrected to a background reading.

4.5. Colony formation assay

Human cervical cancer HeLa cells were seeded into 6-well plates for 2 weeks. Colonies composing more than 50 cells were stained with 0.5% crystal violet for 30 min at room

297 temperature. Triplicate independent experiments were performed.

298 *4.6. Flow Cytometric Analysis*

299 Cells were centrifuged at 800 rpm at 4 °C for 5 min, washed with ice-cold PBS
300 and stained with propidium iodide (PI) buffer (4 μg/ml PI, 1% Triton X-100, 0.5 mg/ml
301 RNase A in PBS). The cells then were analyzed using a FACS Vantage flow cytometer
302 that uses the Cellquest acquisition and analysis program (Becton Dickinson FACS
303 Calibur, San Joes, CA, USA). Cells were gated to exclude cell debris, doublets, and
304 clumps. The apoptotic cells with hypodiploid DNA content were detected in the sub-
305 G1 region.

306 *4.7. Migration and Invasion Assay*

307 Cell migration and invasion assays were performed using the 24-well modified Boyden
308 chambers containing membrane filter inserts with 8-μm pores (Corning Incorporated
309 Life Sciences, Tewksbury, MA, USA). Membrane filter inserts were pre-coated with
310 Matrigel Matrix (Cat. No.354230; Corning Incorporated Life Sciences, Tewksbury, MA,
311 USA) for invasion assay. The lower compartment was filled with DMEM containing
312 10% FCS. Cells were placed in the upper part of the Boyden chamber containing serum-
313 free medium and incubated for 16~24 h. The migratory and invasive phenotypes were
314 determined by counting the cells that migrated to the lower side of the filter with
315 microscopy at 100x and 200x magnification, respectively. The fourth fields were
316 counted for each filter, and each sample was assayed in triplicate.

317 *4.8. Reverse Transcription and Real-Time PCR Assay*

318 Total RNA was isolated from cultured cells. The cells were homogenized in RNA
319 lysis/binding buffer. The High Pure RNA Tissue Kit (Roche Applied Science,
320 Mannheim, Germany) was used for RNA extraction. The standards reverse

transcription and real-time PCR protocol was used in this study. For reverse transcription, the samples were incubated at 25 °C for 10 min, real-time PCR was initiated with a hot start (10 min at 95°C, 1 cycle), the samples were then subjected to 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Data was analysed by StepOne real-time PCR system (Applied Biosystems, Foster City, California, USA). Primers were as follows: human MMP-2 forward primer 5'TGGCAAGTACGGCTTCTGTC-3', reverse primer 5'TTCTTGTCGCGGTCGTA GTC-3'; human MMP-9 forward primer 5′-CCTGCCAGTTTCC ATTCATC-3′; reverse primer 5′-GCCATTCACGTCGTCCTTAT-3′; human TIMP-1 forward primer 5′-CAGTAGAATGGGAGAGTC-3′, human TIMP-1 reverse primer 5′-GGTGATG AAGCAGCCCAG-3′; human TIMP-2 forward primer 5′-GGCGTTTTGCAATGCA GATGTAG-3′, human TIMP-2 reverse primer 5′-CACAGGAGCCGTCACTTCTCT TG-3′; human β-actin forward primer 5′-GCACTCTTCCAGCCTTCCTTCC-3′ reverse primer 5′-TCACCTTCACCGTTCC AGTTTTT-3′ (MISSION BIOTECH, Taipei, Taiwan). Relative gene expression was obtained after normalization with endogenous β-actin and determination of the difference in threshold cycle (Ct) between treated and untreated cells using $2^{-\Delta\Delta C}$t method.

4.9. Statistical Analysis

Each experiment was repeated at least three times. Results were presented as the mean ± SE, and statistical comparisons were made using the Student's *t* test. Significance was defined at the $p<0.05$ or 0.01 levels.

CONFLICT OF INTEREST

The authors do not have any conflict of interest about this paper.

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Author Contributions: Min-Hua Wu, Yi-Hsien Hsieh and Chung-Jung Liu designed the experiments and research project. Chia-Liang Lin and Shun-Fa Yang performed the experiments and analyzed the data. Hui-Ling Chiou participated in the discussion. Yi-Hsien Hsieh and Chung-Jung Liu wrote the paper.

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Figure Legend

Fig. 1. The effect of PA on cellular growth in human cervical cancer HeLa cells (A) Structure of Praeruptorin A. **(B)** Cell viability of HeLa cells treated with various concentrations of PA (0, 10, 20, 30, 40 and 50 μM) for 24h were measured by MTT

assay. **(C)** Colony formation of HeLa cells treated with various concentrations of PA (0, 10, 20 and 30 μM) was measured. *, $p < 0.05$ *versus* control (line 1); **, $p < 0.01$ *versus* control, (mean ± SE, n = 3).

Fig. 2. The effect of PA on cell cycle regulation in human HeLa cells **(A)** Cell cycle of HeLa cells treated with various concentrations of PA (0, 10, 20 and 30 μM) was measured by flow cytometry. (mean ± SE, n = 3). **(B)** The related proteins of cell cycle regulation were measured in HeLa cells treated with various concentrations of PA (0, 10, 20 and 30 μM) for 24h. *, $p < 0.05$ *versus* control; **, $p < 0.01$ *versus* control, (mean ± SE, n = 3).

Fig. 3. The effect of PA on cell migration / invasion, and protein expression of MMPs and TIMPs in human cervical cancer HeLa cells (A) HeLa cells were treated with various concentrations of PA (0, 10, 20 and 30μM) for 24h, then followed by measuring the capacity of cell migration and invasion. **(B)** HeLa cells were treated with various concentrations of PA (0, 10, 20 and 30 μM) for 24h. Cells were then harvested for detection of protein and gene expression by western blotting and **(C)** qRT-PCR. *, $p < 0.05$ *versus* control (line 1); **, $p < 0.01$ *versus* control, (mean ± SE, n = 3).

Fig. 4. The role ERK1/2 in PA-regulated MMP-2 and TIMP-2 expression in human HeLa cells (A) HeLa cells were treated with various concentrations of PA (0, 10, 20 and 30 μM) for 1 hr. Cells were harvested and lysed at the indicated dose points. Target protein level was measured by western blotting with specific antibodies. **(B)** HeLa cells were treated with/without PA in the presence of PD98059 (ERK1/2 specific inhibitor) for 24h. Migration and invasion in human HeLa cells were measured. **(C, D)** Effect of PA (20 μM) and PD98059 (20 μM) on protein and gene expression in MMP-2 and TIMP-2 were measured by immunoblotting assay and qRT-PCR. **, $p < 0.01$ *versus* control; #, $p < 0.05$ *versus* only PA treatment (mean ± SE, n = 3).

Fig. 5. The inhibitory effect of PA on TPA-induced ERK1/2 activation, MMP-2 expression and migration/invasion in human cervical cancer HeLa cells (A) HeLa cells were treated with/without PA (20 μM) in the presence of TPA (50ng/ml) for 30, 60, 90 and 120 min. Cells were harvested and lysed at the indicated time points. Target protein level was measured by western blotting with specific antibodies. **(B)** HeLa cells were treated with/without various concentrations of PA (20 μM) in the presence of TPA (50ng/ml) for 24hrs. Migration and invasion of human HeLa cells then were measured. **(C, D)** HeLa cells were treated with/without various concentrations of PA (0, 10, 20 and 30 μM) in the presence of TPA(50 ng/ml) for 1 hr to detect phospho-ERK1/2 protein, and for 24 hrs to detect MMP-2 protein and gene levels. **, $p < 0.01$ *versus* control (line1); #, $p < 0.05$ *versus* only TPA treatment (mean ± SE, n = 3).

Fig.1

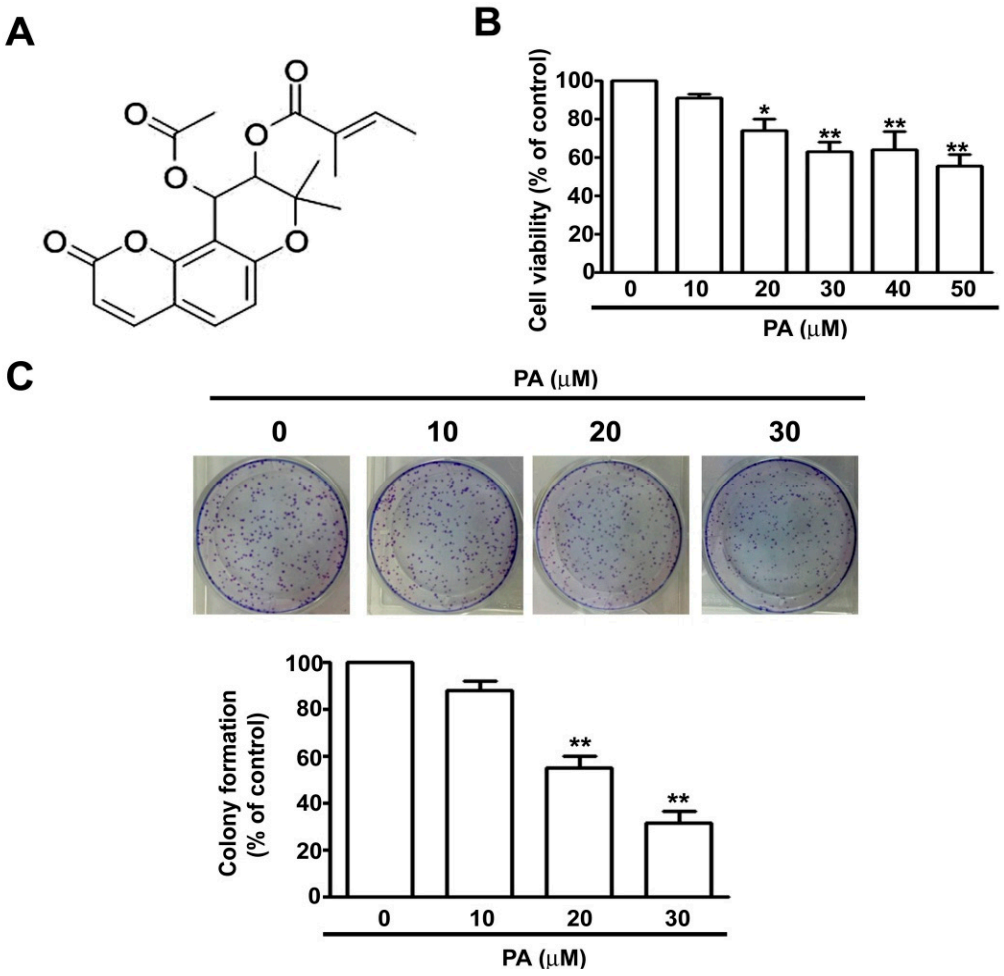

Fig. 2

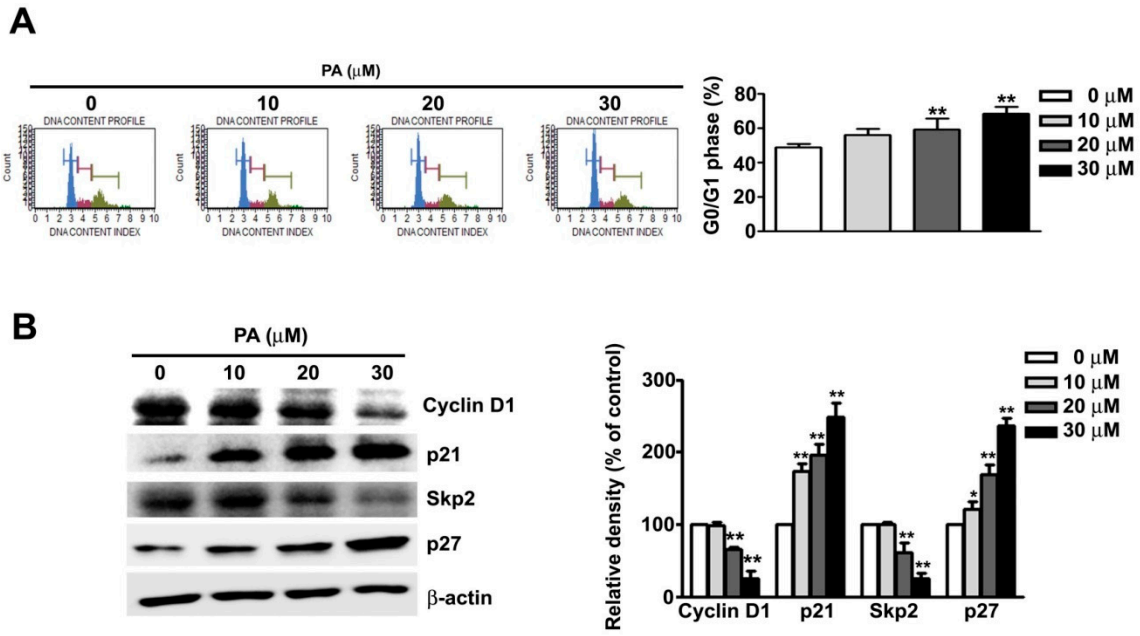

Fig. 3

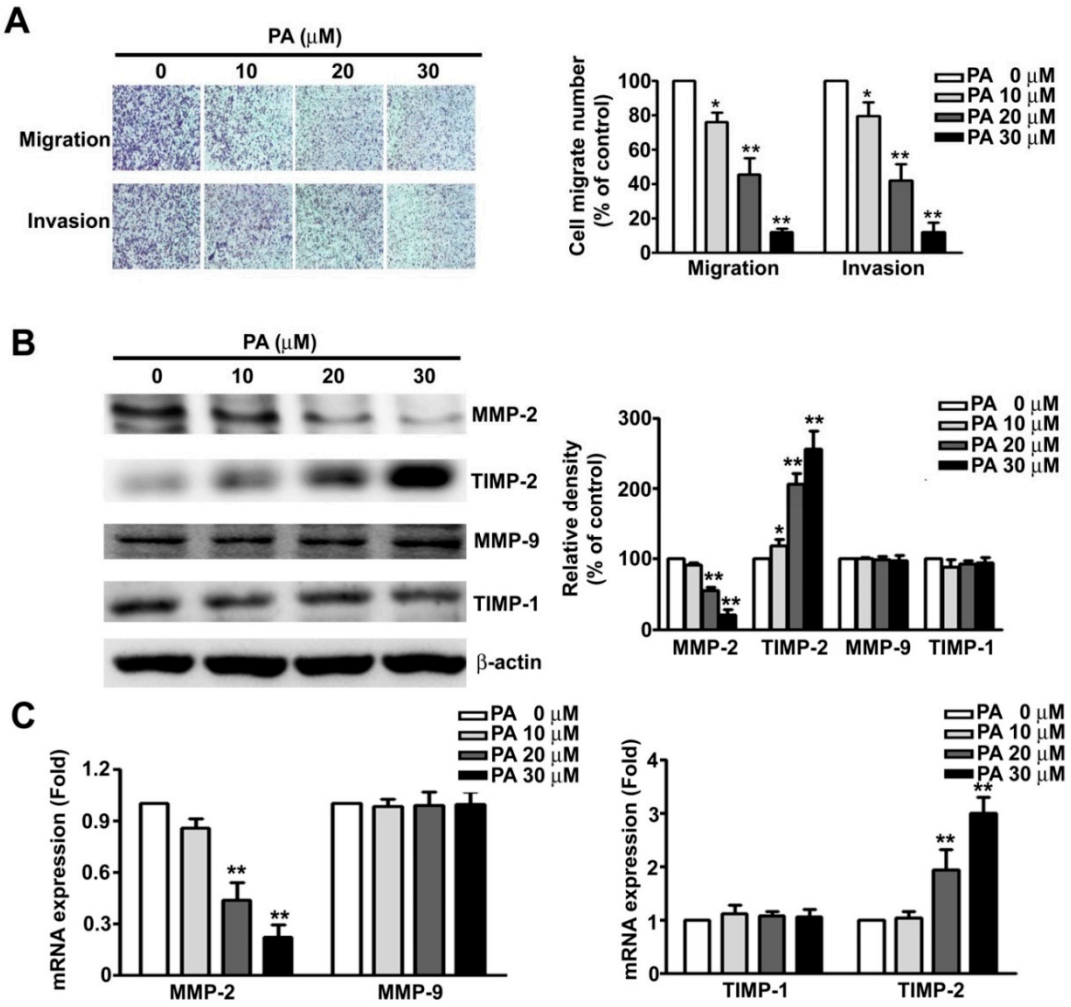

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Fig. 4

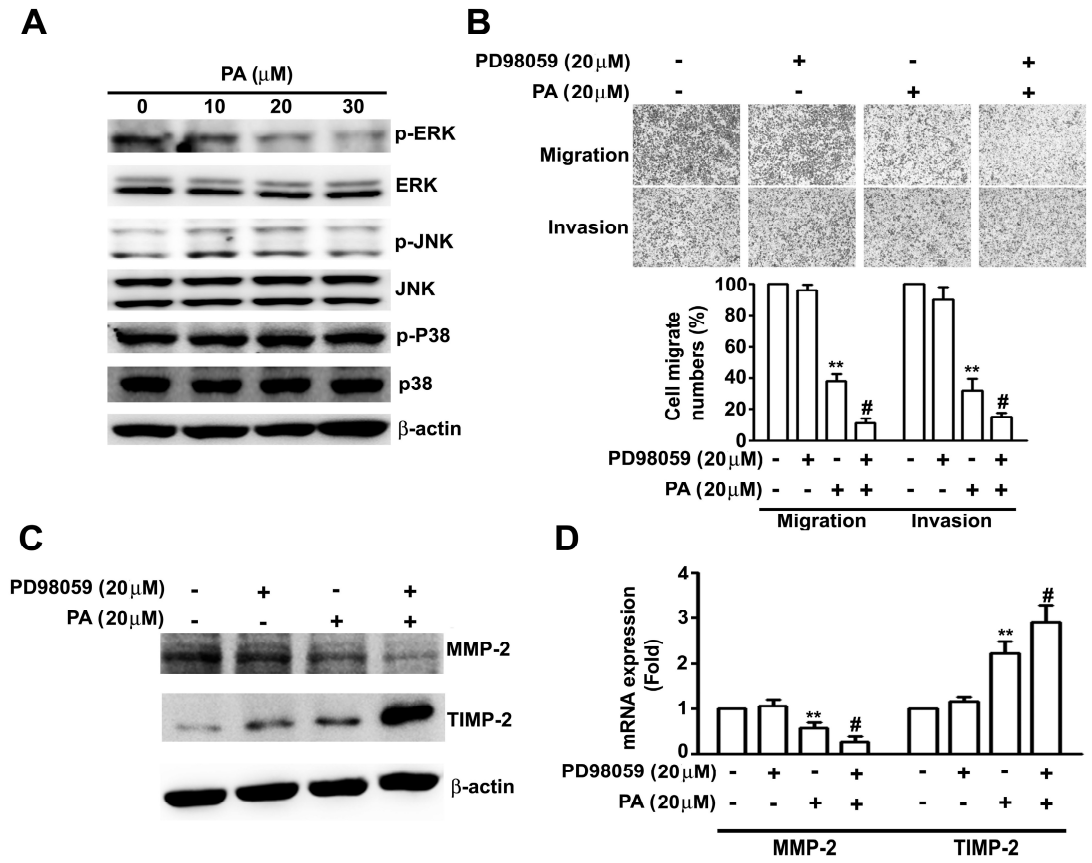

Fig. 5