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EXPERIMENTAL STUDY

Evodiamine induces extrinsic and intrinsic apoptosis of ovarian cancer cells *via* the mitogen-activated protein kinase/phosphatidylinositol-3-kinase/protein kinase B signaling pathways

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Abstract

OBJECTIVE: To explore the effects of evodiamine on ovarian cancer cells and the mechanisms underlying such effects.

METHODS: Human ovarian cancer cells HO-8910PM were treated with evodiamine at 0, 1.25, 2.5, and 5 μM for 1-4 d. 3-(4,5-Dimethiylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect the growth inhibition rate of evodiamine-treated HO-8910PM cells. The cell cycle was observed via propidium iodide (PI) staining. Apoptosis induction was assessed via Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) double staining assay. To verify the mechanism of apoptosis, caspase-dependent apoptotic pathway-related protein was detected by Western blot analysis. The expression levels of mitogen-activated protein kinase (MAPK) and/or phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway-related proteins were also investigated.

RESULTS: Evodiamine significantly inhibited the proliferation of HO-8910PM cells in a dose- and time-dependent manner. Evodiamine induced G2/M arrest with an increase of cyclin B1 level, and promoted cell apoptosis with a decrease of B cell lymphoma/lewkmia-2 (Bcl-2) and an increase of Bcl-2-associated X protein (Bax) level. In addition, evodiamine treatment led to the activation of caspase-8, caspase-9, and caspase-3 and the cleavage of poly (ADP-ribose)-polymerase (PARP). Evodiamine targeted the MAPK and/or PI3K/Akt pathways by reducing the expression and activity of PI3K, Akt, and extracellular signal-regulated kinase mitogen-activated protein kinase (ERK1/2 MAPK) and the activity of p38 MAPK.

CONCLUSION: Evodiamine can inhibit the growth of ovarian cancer cells by G2/M arrest and intrinsic and extrinsic apoptosis. In addition, evodiamine-induced PI3K/Akt, ERK1/2 MAPK, and p38 MAPK signaling may be involved in cell death.

Key words: Evodiamine; Ovarian neoplasms; Apoptosis; Caspases; Mitogen-activated protein kinase phosphatases; MAP kinase signaling system

INTRODUCTION

Ovarian cancer is one of the most common gynecologic cancers, next to cervical and endometrial cancers. However, the mortality rate of ovarian cancer is the

highest among the above-mentioned cancer types. The 5-year survival rate of ovarian cancer is poor despite the availability of chemotherapy and routine surgery. Tumor recurrence and metastasis are considered as the major reasons for poor clinical outcome and ovarian cancer-related deaths.[1] Therefore, developing effective measures against ovarian cancer is critical.

Evodiamine is a quinolone alkaloid isolated from a traditional Chinese medicinal plant, Wuzhuyu (*Fructus Evodiae Rutaecarpae*). Evodiamine has pharmacological properties, including anti-anoxic, antinociceptive and vasorelaxant properties. Recent studies show that evodiamine can inhibit various tumors, including human pancreatic tumor cell SW1990,[2] human thyroid cancer cell ARO,[3] human melanoma cell A375-S2,[4] and human cervical cancer cell HeLa[5] through cycle arrest and apoptosis. In addition, evodiamine has been reported to inhibit the invasion and metastasis of cancer cells including human breast cell MDA-MB-231[6] and colon 26-L5 cell.[7] Evodiamine shows no toxic effects on normal peripheral blood mononuclear cells and normal human mammary epithelial cell line H184B5H5/M10.[3,8] Therefore, evodiamine is a promising candidate for further development as an antitumor agent. However, the effect of evodiamine on ovarian cancer cell is still unclear. In the study, we examined the effect of evodiamine on the growth of human ovarian cancer cell line HO-8910PM in vivo.

MATERIALS AND METHODS

Chemicals and reagents

Evodiamine was purchased from Sigma (Louis, MO, USA), and its purity was determined to be approximately 99% by high performance liquid chromatography. Evodiamine was dissolved in dimethylsulfoxide (DMSO) to create a stock solution and was diluted in RPMI-1640 medium before the experiments. The DMSO concentration in the cell culture medium was below 0.1%. The RPMI-1640 medium and penicillin/streptomycin were purchased from Gibco (Shanghai, China), whereas the fetal bovine serum (FBS) was purchased from Hyclone (Beijing, China). Trypsin-ethylenediaminetetraacetic acid, phosphate buffer solution (PBS) and bicinchoninic acid protein assay kit were purchased from KeyGen Biotech (Nanjing, China), whereas DMSO and 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphemyltetrazolium bromide (MTT) were from Sigma (Shanghai, China). The cell cycle detection kit was purchased from Multi Biotech (Hangzhou, China). Annexin V/PI apoptosis detection kit was purchased from BD Biosciences (San Diego, CA, USA). The B cell lymphoma/lewkmia-2 (Bcl-2), Bcl-2-associated X protein (Bax), caspase 3, caspase 8, caspase 9, cleaved caspase-9, poly (ADP-ribose)-polymerase (PARP), phosphatidylinositol 3-kinase (PI3K), phospho-PI3K (p-PI3K), phospho-protein kinase B (p-Akt), extracellular signal-regulated protein kinases (ERK1/2), p38, phospho-p38 (p-p38), stress-activated protein kinase/c-Jun N-terminal kinase (SPAK/JNK), and phospho-SPAK/JNK (p-SPAK/JNK) antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Cyclin B1, phospho-cell division cycle 2 (p-Cdc2) (Tyr15) and Akt were from Abcam (Cambridge, UK). Cdc2 and phospho-ERK1/2 (p-ERK1/2) antibody were from Antibody Revolution, Inc. (San Diego, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse IgG and rabbit IgG were from Merck Millipore (Billerica, MA, USA). Enhanced chemiluminescent kits were obtained from Fude Biotech (Hangzhou, China).

Cell culture

The human ovarian epithelial cancer cell line HO-8910PM which was purchased from Keygen Biotech (Nanjing, China), was cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humid atmosphere incubator containing 95% room air and 5% CO_2 at 37 ℃. The cells were harvested for the experiment under logarithmic growth conditions.

Cell viability assay

The cell viability was measured using the MTT assay, as previously described.[9] The HO-8910PM cells were seeded in 96-well plates (Nest Biotech Co., Ltd., Shanghai, China) at a density of 5000 cells/well. After 12 h, the cells were treated with different concentrations of evodiamine for the indicated time. 10 μL MTT (5 mg/mL) was supplemented into each well and incubated at 37 ℃ for 4 h. Subsequently, the MTT solution was removed and replaced by 150 μL DMSO with the absorbance at 490 nm. The wavelength (A490) was measured by a microplate reader from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The rate of cell growth inhibition was calculated as follows: Inhibitory rate = [A490 (control) - A490]/ A490 (control) × 100%.

Flow cytometry analysis of the cell cycle distribution

After treatment with or without evodiamine for 24 h, the cells were harvested, washed, and fixed with 70% cold ethanol for 24 h at 4 ℃. The cells were washed twice with cold PBS and stained with 500 μL propidium iodide (PI) solution in the dark for 30 min at room temperature. The cells were analyzed using fluorescence-activated cell sorter (FACSCalibur) flow cytometer from Becton, Dickinson and Company (East Rutherford, NJ, USA).

Flow cytometry detection of apoptotic cells

After treatment with or without evodiamine for 48 h, the adherent and suspended cells were collected and washed with cold PBS. Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) apoptosis detection kit was used according to the manufac-

turer's protocol. 5 μL Annexin V-FITC and 5 μL PI were added to the cells, which were incubated at room temperature for 20 min in the dark prior to analysis.

Western blot assay

Western blot assay was performed to analyze the levels of protein expression. After treatment with or without evodiamine for 24 h, the all cells were collected and lysed in RIPA buffer containing a protease inhibitor cocktail. Cellular proteins (60 μg) from each sample were separated by 8% to 12% SDS-polyacrylamide gel electrophoresis. The separated fragments were transferred to 0.22 μm polyvinylidene fluoride membranes, which were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h at room temperature. The membranes were incubated and allowed to react with primary antibodies overnight at 4 ℃. After washing with TBS-T buffer thrice (for 3 min to 10 min each time), the membranes were incubated with HRP-conjugated secondary antibodies and diluted in 5 mL 0.5% blocking buffer for 1 h. After washing twice with TBS-T buffer for 10 min, the bound antibodies were detected using enhanced chemiluminescence reagents. Chemiluminescent signals were captured using the FluorChem FC2 System (Alpha Inootech Corporation, San Leandro, CA, USA). The expression level of each protein was quantified using Alphaview software with glyceraldehyde-3-phosphate dehydrogenase (GADPH) as the loading control.

Statistical analysis

Triplicate experiments with triplicate samples were performed. The SPSS 16.0 (SPSS Inc., Chicago, IL, USA) software package was used for statistical analyses. The data were expressed as the mean ± standard error of mean (SEM). Comparisons were made between the groups using Student's *t*-test or one-way analysis of variance. P values of < 0.05 were accepted as significant.

RESULTS

Evodiamine inhibited the growth of ovarian cancer cells and induced cell cycle arrest at G2/M phase.

The cell proliferation was measured by MTT assay. After incubation with evodiamine for 24 h, the growth inhibition was not obvious ($P > 0.05$). The significant inhibitory effect of evodiamine on HO-8910PM cell growth was observed at 48 h and persisted for 96 h ($P < 0.05$) (Figure 1). The IC_{50} values in 48, 72, and 96 h were 3.94, 2.16, and 1.66 μM, respectively.

To gain insight into the mechanism of evodiamine cell growth inhibition, the cell cycle distribution was assessed by PI staining after evodiamine treatment for 24 h. The number of cells during each phase was significantly different ($P < 0.0001$) between the control and experimental groups. As shown in Figure 2A and 2B, the G2/M percentage was dramatically increased from 19.7% in the control group to 33.9%, 56.0%, and 66.0% in the groups treated with evodiamine at 1.25, 2.5, and 5 μM, respectively. The G0/G1 percentage was markedly reduced from 65.3% in the control group to 43.0%, 19.9%, and 6.4% in the groups treated with evodiamine at 1.25, 2.5, and 5 μM, respectively. To explore the G2/M arrest mechanism caused by evodiamine, Western blot analysis was performed to measure the expressions of cell cycle-regulating molecules. Compared with the control group, the expression of cyclin B1 was significantly increased (Figure 2C) in the evodiamine-treated groups.

Evodiamine induced apoptosis via extrinsic and intrinsic apoptosis pathways in HO-8910PM cells

To determine the effects of evodiamine on apoptosis, the cells were treated with evodiamine for 48 h. Annexin V-FITC/PI double staining assay was used to determine apoptotic cells. Figures 3A and 3B showed that the HO-8910PM cells treated with evodiamine displayed much higher apoptosis rates than the cells in the control group, and the increase in apoptosis rate was dose dependent ($P < 0.05$). The underlying mechanism was subsequently explored. After evodiamine treatment for 24 h, caspase-3 activation and caspase substrate PARP cleavage were detected by Western blot analysis (Figure 3E). The activation of upstream proteins was further examined. The results showed that the activation of the extrinsic pathway-related protein caspase-8, the activation of the intrinsic pathway through the decrease of Bcl-2, the increase of Bax, and the activation of caspase-9 were initiated after the evodiamine treatment (Figures 3C and 3D).

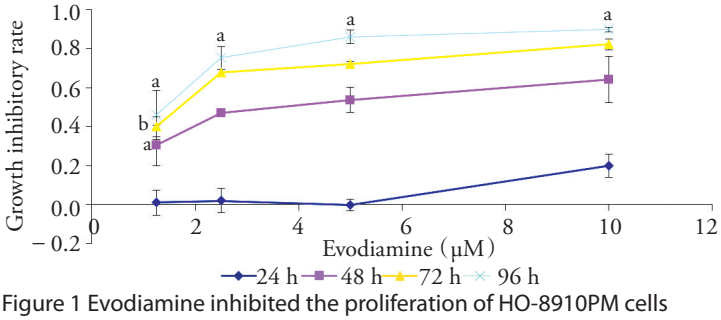


Figure 1 Evodiamine inhibited the proliferation of HO-8910PM cells

Cells were treated with various concentrations of evodiamine (0 μM to 10 μM) for 1 day to 4 day. The cell viability was measured using MTT assay. MTT: 3-(4,5-Dimethiylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Compared with the control group, [a]$P < 0.05$, [b]$P < 0.001$.

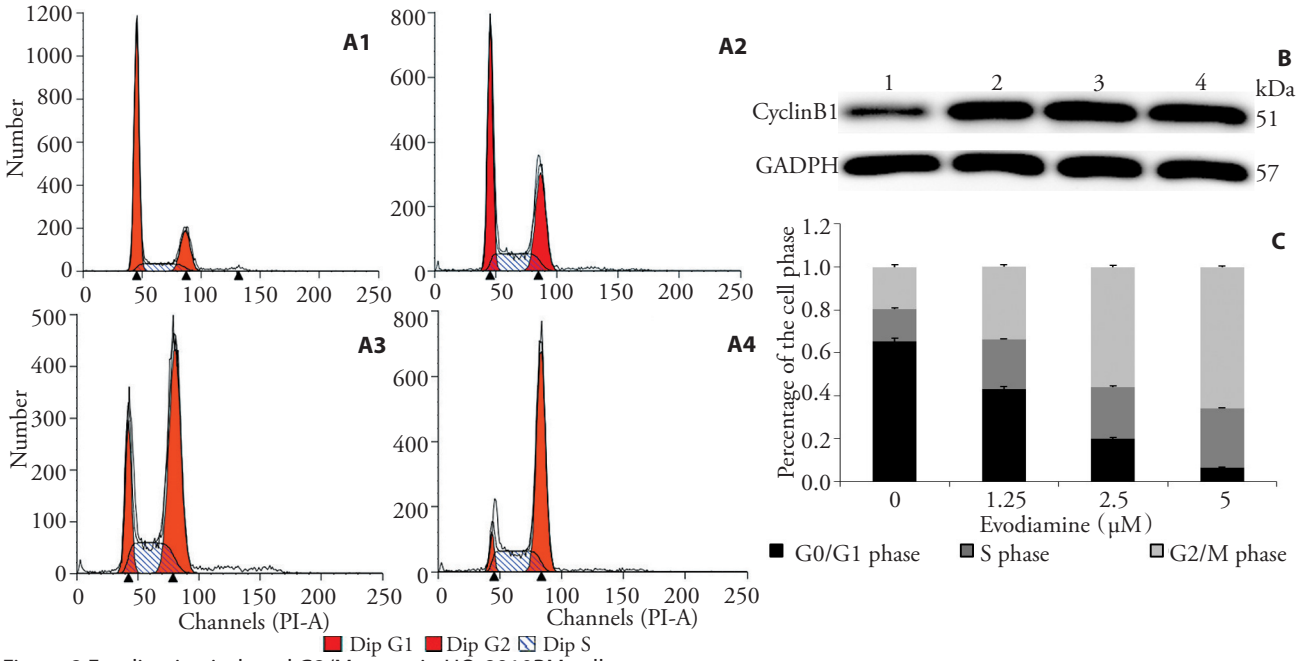
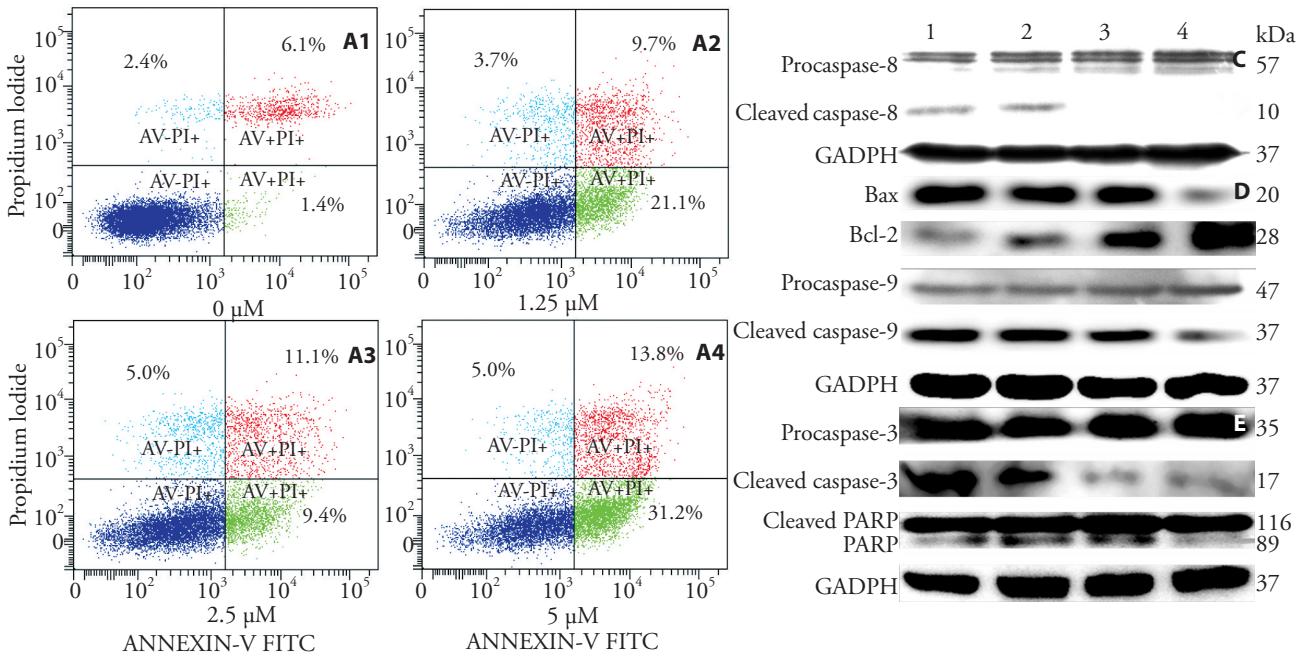


Figure 2 Evodiamine induced G2/M arrest in HO-8910PM cells

A: after exposure to evodiamine (at 0 μM to 5 μM) for 24 h, cell cycle analysis was performed by flow cytometry. A1: 0 μM evodiamine; A2: 1.25 μM evodiamine; A3: 2.5 μM evodiamine; A4: 5 μM evodiamine. G1 was the DNA pre-synthetic phase; S was the DNA synthesis stage; and G2 was the DNA post-synthetic phase. B: After evodiamine treatment (0 μM to 5 μM) for 24 h, the lysates were analyzed for the level of cyclin B1 by Western blot assay. C: The data represent the percentage of cells in each phase of the cell cycle. 1: 0 μM evodiamine; 2: 1.25 μM evodiamine; 3: 2.5 μM evodiamine; 4: 5 μM evodiamine; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.



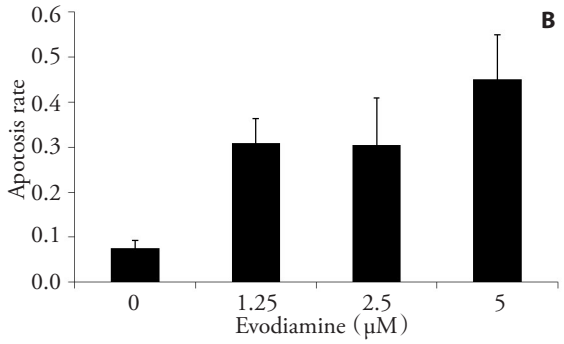


Figure 3 Evodiamine induced apoptosis in HO-8910PM cells through the extrinsic and intrinsic apoptosis pathways

A: measurements of apoptosis. A1: 0 μM evodiamine; A2: 1.25 μM evodiamine; A3: 2.5 μM evodiamine; A4: 5 μM evodiamine. After exposure to evodiamine (at 0 μM to 5 μM) for 48 h, apoptosis was detected using the Annexin V/PI double staining assay. Lower left: normal cells; upper left: necrotic cells; lower right: early apoptotic cells; upper right: late apoptotic cells. B: the proportion of apoptotic cells after evodiamine treatment were quantified and illustrated; Compared with the group, the significance levels in the treated groups were $^{a}P < 0.05$ and $^{b}P < 0.01$. C-E: cells were treated with evodiamine (at 0 μM to 5 μM) for 24 h. The cell lysates were analyzed for the expression of caspase-3, caspase-8, caspase-9, PRAP, Bax, and Bcl-2 by Western blot assay. 1: 5 μM evodiamine; 2: 2.5 μM evodiamine; 3: 1.25 μM evodiamine; 4: 0 μM evodiamine; Bcl-2: B cell lymphoma/lewkmia-2; Bax: Bcl-2-associated X protein; PRAP: poly (ADP-ribose) polymerase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Evodiamine induced the inactivation of PI3K/Akt pathway

The PI3K/Akt pathway is important for cell growth, proliferation, and survival.[10] To further establish the effect of evodiamine on PI3K/Akt signaling, the levels of PI3K, p-PI3K, Akt, and p-Akt proteins in HO-8910PM cells exposed to the indicated concentrations of evodiamine for 24 h were evaluated via Western blot analysis. The low levels of PI3K, p-PI3K, Akt, and p-Akt were observed as compared with the control group ($P < 0.05$, Figures 4A-4C).

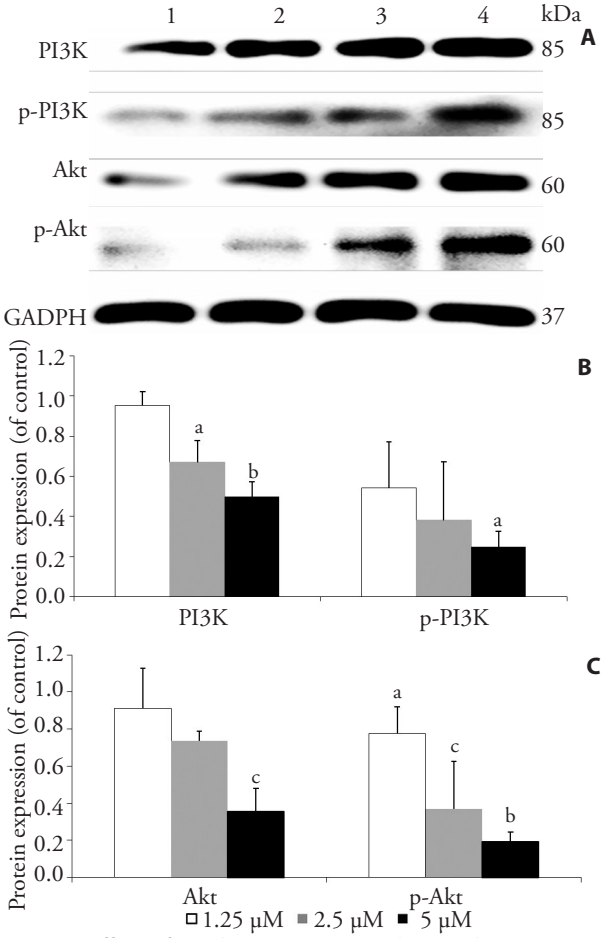


Figure 4 Effect of evodiamine on PI3K/Akt signaling

A: after treatment with evodiamine (at 0 μM to 5 μM) for 24 h, Western blot assay was used to detect the expression of PI3K, Akt, and their phosphorylation in HO-8910PM cells. B: the data represent the percentage of PI3K and phospho-PI3K. C: the data represent the percentage of Akt and phospho-Akt. 1: 0 μM evodiamine; 2: 1.25 μM evodiamine; 3: 2.5 μM evodiamine; 4: 5 μM evodiamine; PI3K: phosphatidylinositol 3-kinase; p-PI3K: phospho-PI3K. Akt: protein kinase B; p-Akt: phospho-Akt; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Compared with the control group, the significance levels in the treated groups were $^{a}P < 0.05$, $^{b}P < 0.001$, and $^{c}P < 0.01$.

MAPK pathways were involved in the pharmacological action of evodiamine

Under environmental stress, the MAPK family members are crucial for cell maintenance.[11] To investigate whether MAPKs were involved in the pharmacological action of evodiamine, the expressions of ERK1/2 MAPK, p38 MAPK, and SAPK/JNK signaling kinases were examined. The result demonstrated that the evodiamine treatment reduced the expression of ERK1/2 MAPK ($P < 0.05$, Figures 5A and 5B), instead of p38 MAPK and SAPK/JNK ($P > 0.05$, Figure 5A and 5C). Phosphorylation was directly linked to kinase activity. Thus, we also analyzed the expression of the phosphorylated forms of these MAPKs. We found a significant decrease in the expression of p-ERK1/2 and p-p38 after evodiamine treatment ($P < 0.05$, Figures 5A to 5C), but p-SAPK/JNK was less sensitive to the alkaloid than the other kinases, and thus, the data was not listed.

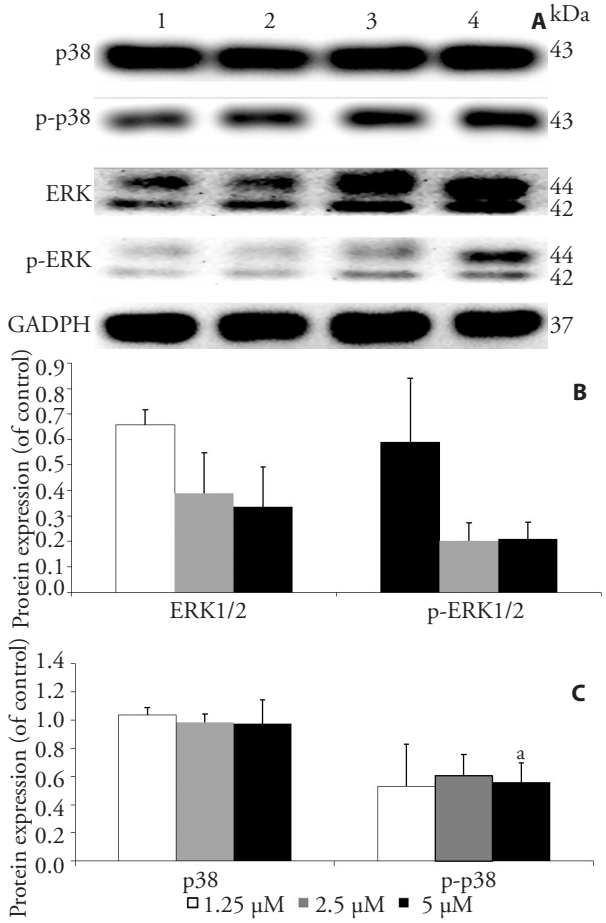


Figure 5 Effect of evodiamine on MAPK signaling

A: after treatment with evodiamine (at 0 μM to 5 μM) for 24 h, the levels of ERK1/2, phospho-ERK1/2, p38 and phospho-p38 were examined via Western blot analysis. B: the data represent the percentage of ERK1/2 and phospho-ERK1/2 in HO-8910PM cells. C: the data represent the percentage of p38 and phospho-p38 in HO-8910PM cells. 1: 0μM evodiamine; 2: 1.25μM evodiamine; 3: 2.5μM evodiamine; 4: 5μM evodiamine. ERK1/2: extracellular signal-regulated protein kinases; p-ERK1/2: phospho-ERK1/2; p-p38: phospho-p38; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Significance levels of $^{a}P < 0.05$ and $^{b}P < 0.01$ were obtained in the treatment groups compared with the control group.

DISCUSSION

Ovarian cancer is one of the most common gynecologic cancers. Its 5-year survival rate is poor, despite the availability of routine surgery and chemotherapy because of tumor recurrence and metastasis. Thus, the de-

velopment of effective measures against ovarian cancer is necessary. Evodiamine is a quinolone alkaloid isolated from a traditional Chinese medicinal plant. Previous studies have demonstrated that evodiamine can inhibit the growth of various tumors. In the present study, evodiamine exhibited significant antitumor activity against ovarian cancer cells, and the potential mechanisms underlying such activity were explored.

The cell cycle comprises a series of events leading to cell division and replication to produce two daughter cells. The cell cycle progression is regulated by cyclins, cyclin-dependent kinases, and other regulatory proteins.[12] The activation of cyclin B1/cdc2 kinase complex is required for the cell to enter the M phase from the G2 phase.[13] Cdc2 is activated *via* phosphorylation on Thr161 and dephosphorylation on Thr 14 and Tyr15 in the late G2 phase, thereby resulting in the activation of the cdc2/cyclin B1 complex.[14] In this report, evodiamine induced the accumulation of cells in the G2/M phase. Western blot analysis showed that the evodiamine treatment increased the level of cyclin B1, but the decreased phosphorylation of cdc2 on Tyr15 was not observed. HO-8910PM cells were arrested in the G2/M phase after evodiamine treatment.

Apoptosis is a programmed cell death process that occurs in abnormal or damaged cells. To examine whether evodiamine induces apoptosis, the Annexin V-FITC/PI double staining assay was used. The results showed that evodiamine caused apoptosis in a dose-dependent manner. Two key signaling pathways, namely the intrinsic and extrinsic pathways, are involved in the induction of apoptosis. The extrinsic pathway is activated by a death receptor from outside the cell, which indirectly induces the activation of caspase-8.[15] The intrinsic pathway is activated by the members of the Bcl-2 family proteins, including anti-apoptotic proteins, such as Bcl-2, pro-apoptotic proteins (Bax), and downstream mitochondrial signals (functional caspase-9).[15] Evodiamine treatment leads to the cleavage of caspase-8 and caspase-9, the down-regulation of Bcl-2, and the up-regulation of Bax. The both pathways stimulate the activation of caspase-3 and the downstream cleavage of PARP. We observed that evodiamine activated caspase-3 and induced the degradation of PARP. Therefore, the extrinsic and intrinsic apoptosis pathways might be implicated in evodiamine-induced apoptosis.

MAPK pathways are known to play a role in tumorigenesis. There are three different MAPK cascades, namely, ERK1/2 MAPK, p38 MAPK, and SAPK/JNK. The ERK1/2 MAPK signaling pathway regulates cell growth, differentiation, proliferation, apoptosis, and migration functions, and this pathway is often up-regulated in human tumors.[16] Therefore, the specific blockage of the ERK1/2 MAPK pathway is expected to result in an anti-proliferative, anti-metastatic, and anti-angiogenic effects in tumor cells. Our study demonstrated that evodiamine could down-regulate the expression and activity of ERK1/2 MAPK, as expected. The p38 MAPK signaling pathways are involved in a variety of cellular responses, and the outcomes of cellular response are varied and complicated. The p38 MAPK signaling promotes cell death,[17,18] but some reports suggest that p38 MAPK can promote cancer cell growth and survival.[19,20] Our study confirmed that evodiamine could reduce the activity of p38 MAPK. SAPK/JNK is also activated in response to a variety of cellular and environmental stresses.[11] However, evodiamine failed to affect the activity of SAPK/JNK in HO-8910PM cells. The PI3K/Akt pathway plays an important role in regulating critical cellular functions, including cell growth and metabolism.[10] The study showed that evodiamine treatment could attenuate the PI3K/Akt signaling, as expected.

In conclusion, evodiamine can inhibit the growth of ovarian cancer through G2/M arrest and intrinsic and extrinsic apoptosis. In addition, PI3K/Akt, ERK1/2 MAPK, and p38 MAPK pathways are involved in evodiamine-induced cell death. Therefore, evodiamine shows therapeutic potential for ovarian cancer treatment. However, further animal studies are required to validate these findings.

REFERENCES

1 **Chaffer CL**, Weinberg RA. A perspective on cancer cell metastasis. Science 2011; 331(6024): 1559-1564.

2 **Wei WT**, Chen H, Wang ZH, et al. Enhanced antitumor efficacy of gemcitabine by evodiamine on pancreatic cancer *via* regulating PI3K/Akt pathway. Int J Biol Sci 2012; 8 (1): 1-14.

3 **Chen MC**, Yu CH, Wang SW, et al. Anti-proliferative effects of evodiamine on human thyroid cancer cell line ARO. J Cell Biochem 2010; 110(6): 1495-1503.

4 **Wang C**, Li S, Wang MW. Evodiamine-induced human melanoma A375-S2 cell death was mediated by PI3K/Akt/caspase and Fas-L/NF-kappa B signaling pathways and augmented by ubiquitin-proteasome inhibition. Toxicol *In Vitro* 2010; 24(3): 898-904.

5 **Yang J**, Wu LJ, Tashino SI, Onodera S, Ikejima T. Protein tyrosine kinase pathway-derived ROS/NO productions contribute to G2/M cell cycle arrest in evodiamine-treated human cervix carcinoma HeLa cells. Free Radical Res 2010; 44(7): 792-802.

6 **Du J**, Wang XF, Zhou QM, et al. Evodiamine induces apoptosis and inhibits metastasis in MDA-MB-231 human breast cancer cells *in vitro* and *in vivo*. Oncol Rep 2013; 30(2): 685-694.

7 **Ogasawara M**, Matsubara T, Suzuki H. Inhibitory effects of evodiamine on *in vitro* invasion and experimental lung metastasis of murine colon cancer cells. Biol Pharm Bull 2001; 24(8): 917-920.

8 **Fei XF**, Wang BX, Li TJ, et al. Evodiamine, a constituent of Evodiae Fructus, induces anti-proliferating effects in tumor cells. Cancer Sci 2003; 94(1): 92-98.

9 **Mosmann T**. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity

assays. J Immunol Methods 1983; 65(1-2): 55-63.

10 **Vivanco I**, Sawyers CL. The phosphatidylinositol 3-kinase-AKT pathway in human cancer. Nature reviews. Cancer 2002; 2(7): 489-501.

11 **Wada T**, Penninger JM. Mitogen-activated protein kinases in apoptosis regulation. Oncogene 2004; 23(16): 2838-2849.

12 **Draetta G**. Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. Trends Biochem Sci 1990; 15(10): 378-383.

13 **Taylor WR**, Stark GR. Regulation of the G2/M transition by p53. Oncogene 2001; 20(15): 1803-1815.

14 **Devault A**, Martinez AM, Fesquet D, et al. MAT1 ('menage a trois') a new RING finger protein subunit stabilizing cyclin H-cdk7 complexes in starfish and Xenopus CAK. Embo J 1995; 14(20): 5027-5036.

15 **Debatin KM**. Apoptosis pathways in cancer and cancer therapy. Cancer Immunol Immun 2004; 53(3): 153-159.

16 **Kohno M**, Pouyssegur J. Targeting the ERK signaling pathway in cancer therapy. Ann Med 2006; 38(3): 200-211.

17 **Gil Araujo B**, Toledo Lobo MV, Gutierrez Salmeron M, et al. Dual specificity phosphatase 1 expression inversely correlates with NF-kappa B activity and expression in prostate cancer and promotes apoptosis through a p38 MAPK dependent mechanism. Mol Oncol 2014; 8(1): 27-38.

18 **Rudolf E**, Kralova V, Rudolf K, John S. The role of p38 in irinotecan-induced DNA damage and apoptosis of colon cancer cells. Mutat Res-fund Mol M 2013; 741: 27-34.

19 **Porras A**, Zuluaga S, Black E, et al. P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. Mol Biol Cell 2004; 15(2): 922-933.

20 **Serini S**, Trombino S, Oliva F, et al. Docosahexaenoic acid induces apoptosis in lung cancer cells by increasing MKP-1. Apoptosis 2008; 13(9): 1172-1183.