Evodiamine induces caspase-dependent apoptosis and S phase arrest in human colon lovo cells

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Evodiamine, one of the major bioactive components derived from Wu-Chu-Yu, a long-standing Chinese herb, was reported to possess anticancer activity. In this study, we investigated the in-vitro and in-vivo anticancer effects of evodiamine on human colon lovo cells and their potential mechanisms. The 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay showed that the in-vitro proliferation of lovo cells was inhibited by evodiamine of various concentrations. Flow cytometry showed a time-dependent increase in the percentage of apoptotic cells and cells arrested in the S phase after treatment with 60 μmol/l evodiamine. Western blot indicated that evodiamine treatment decreased the expression of procaspase-8, procaspase-9, and procaspase-3 in lovo cells, accompanied by the activation of caspase-8, caspase-9, and caspase-3. However, the translocation of apoptosis-inducing factor and endonuclease G was not affected by evodiamine. Moreover, western blot assay also suggested that evodiamine-induced S phase arrest in lovo cells was associated with a marked decrease in the protein expression of cyclinA, cyclinA-dependent kinase 2, and cdc25c. In-vivo antineoplastic characteristics of evodiamine were examined in a human colon carcinoma lovo xenograft model and results showed that evodiamine increased the number of TUNEL-positive cells accompanied by the downregulated expression of procaspase-8, procaspase-9, and procaspase-3. In conclusion, these findings indicated that evodiamine could inhibit the in-vitro and in-vivo proliferation of human colon lovo cells by inducing caspase-dependent apoptosis and S phase arrest.
***Anti-Cancer Drugs* 21:766–776 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2010, **21**:766–776

Keywords: apoptosis, caspase, cell cycle, evodiamine, lovo cell

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Received 29 March 2010 **Revised form accepted** 13 June 2010

Introduction

Evodiamine is one of the major bioactive components derived from Wu-Chu-Yu, a long-standing Chinese herb. It has been reported that evodiamine has vasodilative [1], anti-inflammatory [2], nociceptive, and antinociceptive effects [3]. Furthermore, evodiamine has been shown to exert suppressive effects on the proliferation of a variety of cancer cells, including human myeloid leukemia KBM-5 cells through inducing nuclear factor-κB inactivation by inhibiting IκBα kinase activation [4], human breast MCF-7 cells by inhibiting covalent topoisomerase–DNA complex segregation [5], human prostate PC-3 cells by inducing cell cycle arrest in G2/M phase and subsequent caspase-dependent apoptosis [6], and human melanoma A375-S2 cells by inducing caspase-independent apoptosis [7].

Cell cycle checkpoints are the elaborate surveillance systems to monitor DNA damages and other forms of genotoxic stress. When damage occurs, the checkpoint uses a signal mechanism to either stall the cell cycle until repairs are made or, if repairs cannot be made, to target the cell for destruction through apoptosis [8,9]. Cell cycle checkpoints are driven by the activation of cyclin-dependent kinases (CDKs), which require a cyclin subunit for their activation. For instance, cyclinE/CDK2 plays a role in G1S transition and relates to the malignant transformation of cells. CyclinA associated with both CDK1 and CDK2 acts in both the S phase and mitosis, and cyclinB/CDK2 mediated by the cdc25 family of phosphatases regulates G2M checkpoint [10]. Apoptosis occurs normally during development and aging, and is a homeostatic mechanism in maintaining the balance of cell populations in tissues. So far, studies have indicated two main apoptotic pathways. One is the caspase-dependent pathway including the activation of caspase-8, caspase-9, and caspase-3 [11] and the other is the caspase-independent pathway in which apoptosis-inducing factor (AIF) and endonuclease G (endo G) releasing from the mitochondria into the nucleus can induce chromatin condensation and DNA fragmentation [12,13].

Colon cancer is the second leading cause of cancer death and remains a significant global health problem [14]. Widely accepted risk factors for colon cancer include age, dietary habit [15], metabolic syndrome [16], and genetic mutation [17]. Over the past years, fluorouracil-based chemotherapy has been the treatment of choice for colon cancer [18], which, however, may cause psychological and physiological side effects, thus limiting its wide application [19]. Therefore, it is imperative to identify and develop novel antineoplastic drugs against colon cancer. Evodiamine has been recognized as a compound having

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antimetastatic and antineoplastic characteristics in murine colon 26-L5 cells [20]. Our study aimed to investigate the effect of evodiamine on the proliferation of human colon lovo cells. Our results showed suppressive effects of evodiamine on the in-vivo and in-vitro proliferation of human colon lovo cells by inducing caspase-dependent apoptosis and S phase arrest.

Materials and methods

Materials

Evodiamine was purchased from Xi'an Guanyu Bio-tech Co. Ltd (Xi'an, China) with a purity of 98% determined by high-performance liquid chromatography. Evodiamine was dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI-1640 medium before use. The DMSO concentration in the cell culture medium was below 0.1%.

RPMI-1640 medium and fetal calf serum were purchased from Gibco BRL (Gaithersburg, Maryland, USA). DMSO and MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich (St Louis, Missouri, USA). An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was purchased from KeyGen Biotech (Nanjing, China) and caspase-8, caspase-9, and caspase-3 activity assay kits from Beyotime Institute of Biotechnology (Nantong, China). A TUNEL apoptosis detection kit was purchased from Roche Applied Science (Indianapolis, Indiana, USA). Athymic mice were from the Chinese Academy of Sciences (Shanghai, China). The following primary antibodies were used in this study: procaspase-8, procaspase-9, Bcl-2, Bax, AIF, endo G antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA), procaspase-3 (Thermo Fisher Scientific, Hudson, New Hampshire, USA), cyclinA, CDK2, cyclinB1, CDK1, and cdc25c antibodies (Millipore Corporation, Billerica, Massachusetts, USA).

Cell line and cell culture

Lovo human colon cancer cells were maintained in RPMI-1640 medium supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. Cells in the logarithmic growth phase were harvested for use.

MTT assay and cell morphological assay

A modified MTT assay was used to quantify the cell proliferation. Lovo cells were seeded at a density of 1×10^4/well in 96-well plates. After incubation with evodiamine of different concentrations for 24 h, 20 μl of 5 mg/ml MTT solution was supplemented to each well followed by incubation for 6 h. Then, 50 μl of 20% SDS solution was added into each well followed by incubation overnight. The optical density was determined with a microplate reader at 570 nm.

Lovo cells were seeded in six-well plates and maintained for 12 h. The medium was discarded and replaced with fresh medium or medium containing 60 μmol/l evodiamine for 12 h. Cell morphology was observed and representative photographs were captured under a light microscope.

Flow cytometry

The DNA content was analyzed by measuring the distribution of cells in different phases in the cell cycle. Lovo cells were harvested, washed with PBS, and fixed in 70% ethanol at 4°C overnight. After washing twice with PBS, these cells were then resuspended in a DNA staining solution containing PI (80 μg/ml), RNase A (100 μg/ml), and 0.1% Triton X-100 followed by incubation for 30 min at room temperature in the dark. Next, the cells were analyzed with a flow cytometer equipped with the CellQuest software (Becton Dickinson, Heidelberg, Germany).

Apoptosis was evaluated by measuring the phosphatidylserine exposed on cell membranes with an apoptosis detection kit. Lovo cells were harvested and then resuspended in a staining solution containing PI (50 μg/ml) and Annexin V-FITC (25 μg/ml) followed by incubation for 15 min at room temperature in the dark. The cells were then resuspended in the binding buffer and apoptotic cells were determined with a flow cytometer equipped with CellQuest software.

Western blot assay

Lovo cells were harvested and washed twice in ice-cold PBS. The cells were lysed with a lysis buffer. The tumors from mice were washed with cold PBS, and homogenized in the lysis buffer. The cell lysate and tumor homogenate were centrifuged at 13 000 g for 15 min at 4°C. The protein concentration was determined by a BCA protein assay kit (KangChen, Shanghai, China). Protein was loaded onto an SDS–PAGE gel and electrotransferred to polyvinylidene fluoride membranes. After being blocked with 5% non-fat milk, the membranes were incubated with specific primary antibodies followed by horseradish peroxidase conjugated secondary antibodies. Finally, the membranes were visualized with an enhanced chemiluminescence kit (KangChen, Shanghai, China). Signal intensity was quantified by densitometric analysis.

Activities of caspases

Lovo cells treated with evodiamine were harvested and washed twice with PBS. The cells were resuspended in lysis buffer and centrifuged at 13 000 g for 15 min at 4°C, and 100 μg of protein was incubated with enzyme-specific colorigenic substrates at 37°C overnight. The activities of caspase-8, caspase-9, and caspase-3 were determined by measuring absorbance at 405 nm.

Immunocytochemistry

Lovo cells were grown and treated with evodiamine. These cells were then fixed in 4% paraformaldehyde for 30 min, blocked with 5% bovine serum albumin, and stained with anti-AIF and anti-endo G antibodies (1 : 100

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dilution) overnight at 4°C. Subsequently, these cells were incubated with FITC-conjugated mouse anti-rabbit antibody (1 : 200) for 1 h. Cell nuclei were stained with 10 mg/ml 4′,6-diamidino-2-phenylindole and representative photographs were obtained under a laser confocal scanning microscope.

In-vivo antitumor effect

A total of 32 female athymic mice aged 4–6 weeks were given ad libitum access to sterilized food and water. Mice were subcutaneously administrated with 3×10^6 of lovo cells in 0.1 ml of PBS. Ten days later, when the tumor size reached approximately 100 mm^3, the mice ($n = 8$ per group) were treated with evodiamine, 5-fluorouracil (5-FU), or DMSO for 2 weeks (thrice weekly). Evodiamine (1 mg/kg) was dissolved in DMSO and intratumorally injected. 5-FU (30 mg/kg) dissolved in DMSO was intraperitoneally administered as a positive control. The mice undergoing intratumoral or intraperitoneal administration with DMSO served as controls. The tumor volume and body weight of these mice were measured during the experimental period, and tumor volume was determined by the following formula: $0.5 \times L \times W^2$ (L: length; W: width). The animals in the control and treatment groups were decapitated after 2 weeks of treatment and the tumors were removed followed by fixation in 4% paraformaldehyde for 3 days. Next, the tumors were cut into 5 μm sections and mounted on slides for further analysis.

Apoptosis in tumors

Tumor tissues were cut into sections followed by staining with hematoxylin and eosin (H&E). Apoptosis was measured with a TUNEL staining kit according to the manufacturer's instructions. The sections were deparaffinized in xylene and dehydrated in gradient ethanol. Next, these sections were permeabilized with 20 μg/ml protease for 30 min at 37°C followed by 50 μl of TUNEL reaction mixture for 60 min at 37°C. The sections were analyzed using a fluorescence microscope.

Statistical analysis

Data were presented as means ± standard deviation. Statistical analysis was carried out with a one-way analysis of variance or Student's *t*-test for independent variables. A *P* value of less than 0.05 was considered statistically significant.

Results

Evodiamine inhibits cell proliferation *in vitro*

To investigate the effect of evodiamine on cell proliferation, lovo cells were treated with evodiamine of different concentrations for 24 h, and cell viability was assessed by the MTT assay. As shown in Fig. 1a, the viability of lovo cells was decreased in a dose-dependent manner with 54.08% in the 60 μmol/l evodiamine-treated group. The following experiments were performed on lovo cells exposed to 60 μmol/l evodiamine.

The in-vitro morphological changes in the evodiamine-treated cells were examined. After treatment with 7.5, 15, 30, and 60 μmol/l evodiamine for 12 h, cells became small, round, and floating, in a concentration-dependent manner (Fig. 1b).

Evodiamine promotes cell apoptosis

To determine the effects of evodiamine on apoptosis, the cells were treated with evodiamine for 6, 12, and 24 h, and apoptotic cells were determined by flow cytometry. By Annexin V labeling to phosphatidylserine, the number of early apoptotic cells (Annexin V-FITC positive) was increased in a time-dependent manner, and that of late apoptotic or necrotic cells (Annexin V-FITC positive and PI positive) was not markedly changed (Fig. 2).

Caspase-dependent signaling pathway is involved in evodiamine-induced apoptosis

As evodiamine promoted the apoptosis of lovo cells, the underlying mechanism was further explored. Caspases exist as inactive zymogens in normal and survival cells, and proteolysis occurs after activation resulting in apoptosis. In this study, the expression of procaspase-8, procaspase-9, and procaspase-3 in the cells treated with different concentrations of evodiamine for 24 h were markedly downregulated in a dose-dependent manner. Meanwhile, administration with 60 μmol/l evodiamine for 6, 12, and 24 h caused a significant reduction in the expression of procaspase-8, procaspase-9, and procaspase-3 in a time-dependent manner (Fig. 3a).

As caspases are the critical enzymes responsible for the execution of apoptosis, caspase activities were directly determined with caspase activity kits in our study. Results showed that treatment with 60 μmol/l evodiamine for 24 h significantly increased the activities of caspase-8, caspase-9, and caspase-3 (240.06, 163.77, and 188%, respectively) when compared with those in the control group. These findings suggested that evodiamine could induce apoptosis by activating caspases (Fig. 3b).

Evodiamine induces a decline in Bcl-2/Bax ratio

Cells were treated with different concentrations of evodiamine for 24 h, and the expressions of Bcl-2 and Bax were determined. Results showed that Bax expression was enhanced in the 15–60 μmol/l evodiamine-treated cells ($P < 0.05$), but Bcl-2 expression was only slightly attenuated. In addition, in cells exposed to 60 μmol/l evodiamine for 6, 12, and 24 h, Bax expression was increased ($P < 0.05$), whereas Bcl-2 was only slightly decreased (Fig. 3a).

Caspase-independent apoptosis driven by apoptosis-inducing factor and endonuclease G is not caused by evodiamine

As AIF and endo G are involved in the caspase-independent pathway, the role of AIF and endo G in the evodiamine-induced apoptosis was further explored. Translocation of AIF and endo G was detected by observing

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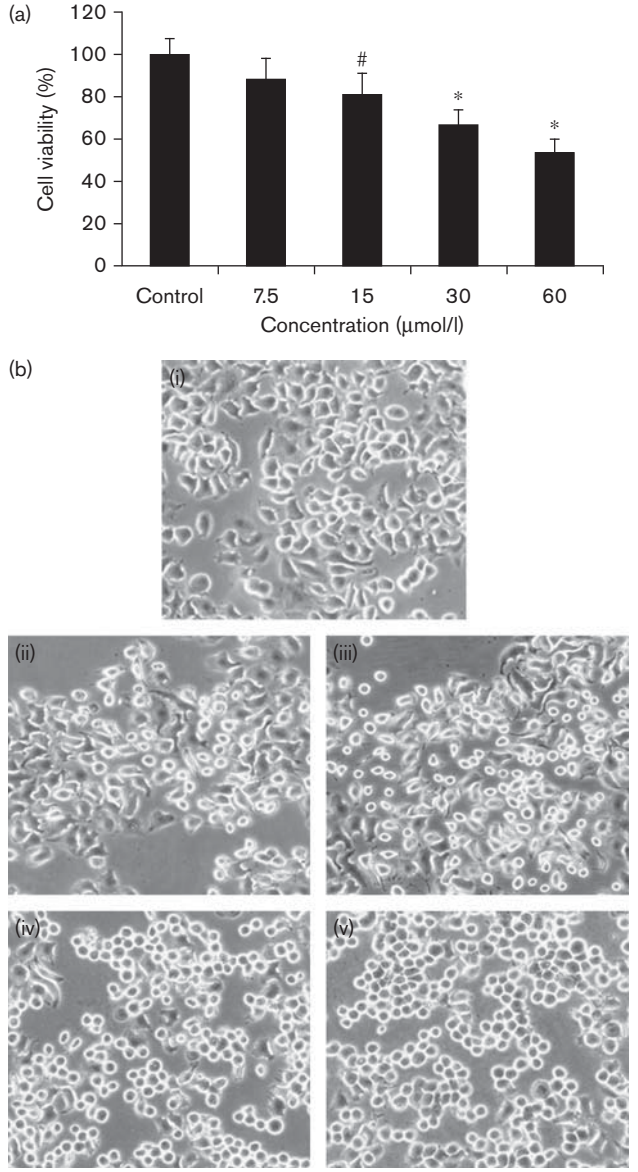
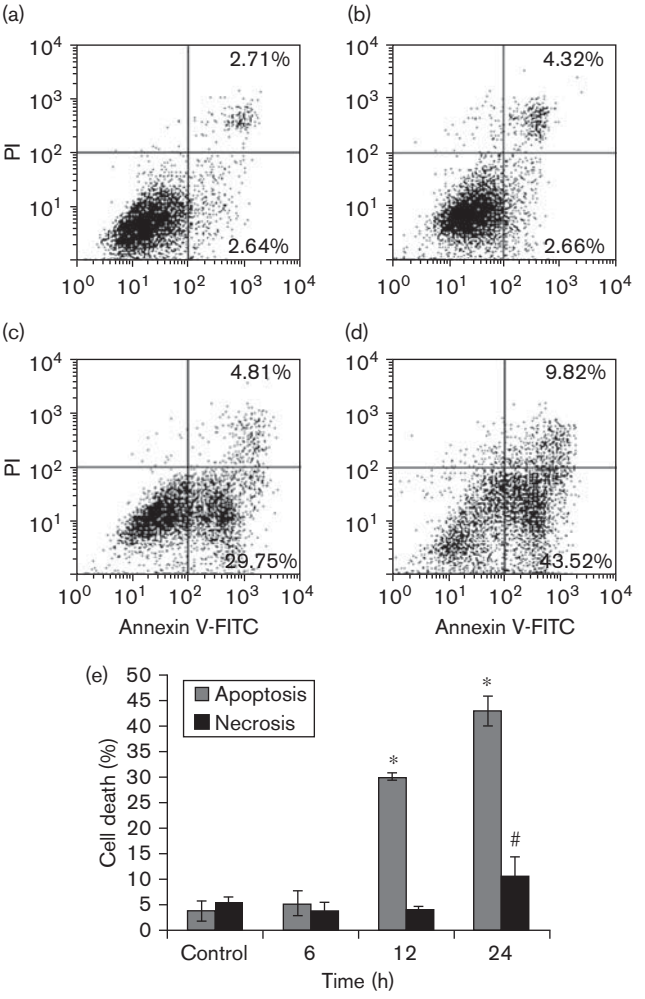
Fig. 1



Evodiamine inhibited cell proliferation *in vitro*. (a) Lovo cells were treated with evodiamine at different concentrations (7.5, 15, 30, and 60 μmol/l) for 24 h. Cell viability was determined using the MTT assay. Data were presented as the percentage by control, which was defined as 100% ($n=5$) and means ± SD. $^{*}P<0.01$ and $^{\#}P<0.05$ versus control group. (b) Lovo cells were treated with 7.5, 15, 30, and 60 μmol/l evodiamine for 12 h, and representative photographs were captured under a light microscope; (i) control, (ii) 7.5 μmol/l, (iii) 15 μmol/l, (iv) 30 μmol/l, and (v) 60 μmol/l (× 200).

Fig. 2



Evodiamine-induced cell apoptosis. Lovo cells were treated with 60 μmol/l evodiamine for 6, 12, and 24 h. Apoptosis was examined by flow cytometry ($n=3$). Representative photographs from three independent experiments were displayed; (a) control, (b) 6 h, (c) 12 h, and (d) 24 h. (e) The data of apoptosis and necrosis from three independent experiments were presented as means SD, $^{*}P<0.01$ and $^{\#}P<0.05$ versus control group. FITC, fluorescein isothiocyanate; PI, propidium iodide

their release from the mitochondria and translocation to the nucleus under a laser confocal microscope. Results displayed that both AIF and endo G were not translocated into the nucleus after evodiamine treatment (Fig. 4). Meanwhile, western blot showed that after 60 μmol/l evodiamine treatment for 2 h, no significant changes in the expressions of AIF and endo G were observed in the nucleus (data not shown).

Evodiamine induces cell cycle arrest in S phase

The cell cycle was examined by flow cytometry. The cells treated with 60 μmol/l evodiamine were mostly arrested in the S phase, which was in a time-dependent manner. As early as 6 h after evodiamine treatment, the cells arrested in S phase were observed. Furthermore, evodiamine treatment for 24 h increased the percentage of cells in the S phase from 28.5 to 95.78%, with a parallel reduction in the percentage of cells in the G0/G1 phase from 64.49 to 1.94% (Fig. 5).

To evaluate the roles of cell cycle-regulating proteins in the effects of evodiamine on the cell cycle, proteins were extracted from the evodiamine-treated cells at designed time points for western blot analysis. When compared with

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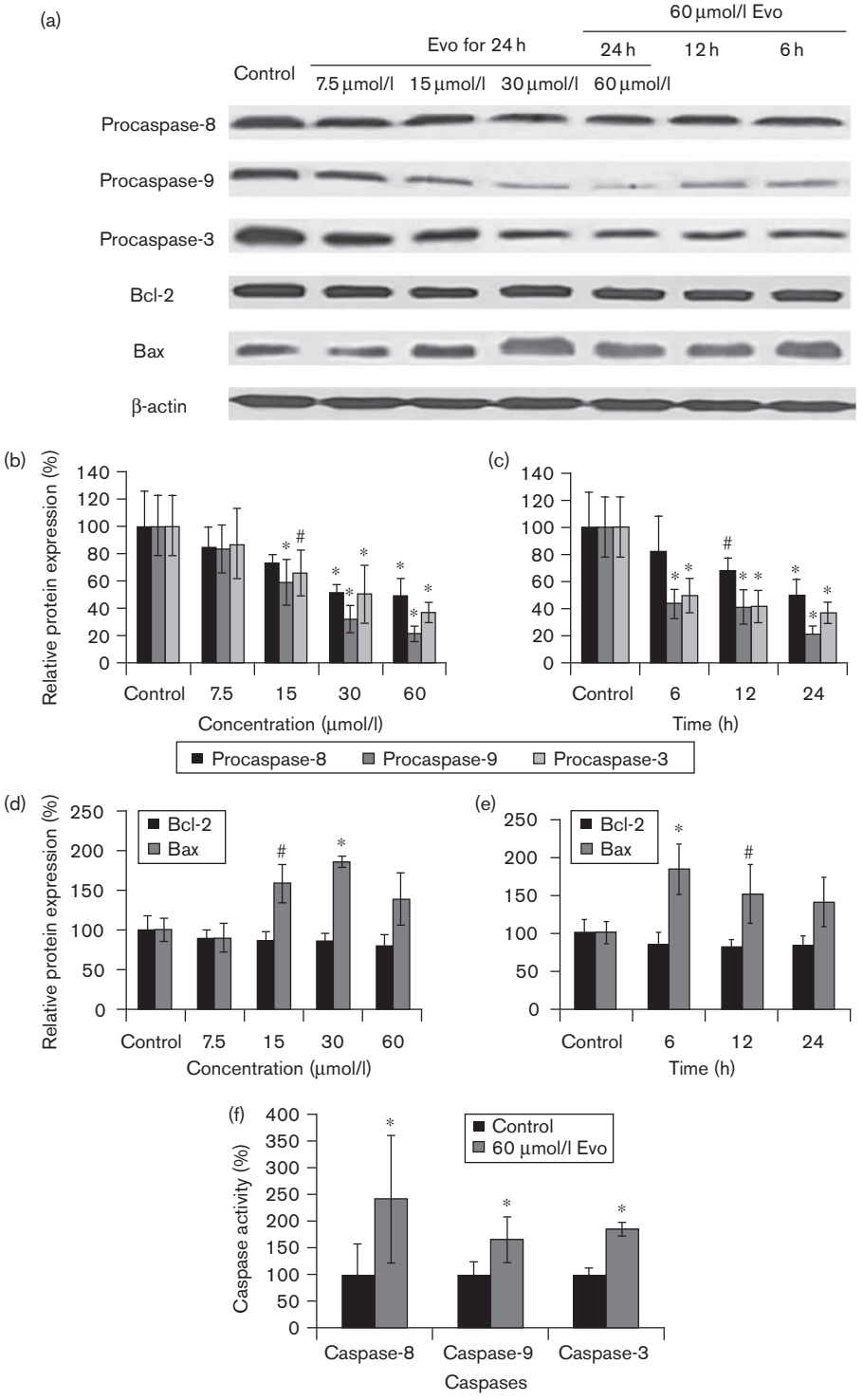
Fig. 3



The caspase-dependent signaling pathway was involved in evodiamine-induced apoptosis. (a) Lovo cells were treated with 7.5, 15, 30, and 60 μmol/l evodiamine for 24 h, or with 60 μmol/l evodiamine for 12 and 6 h. The expressions of procaspase-8, procaspase-9, procaspase-3, Bcl-2, and Bax were measured by western blot analysis. The amount of β-actin was measured as an internal reference. Representative photographs from three independent experiments were displayed (b), (c), (d), (e) The expression levels of proteins were presented as means ± SD, $*P<0.01$ and $*P<0.05$ versus control group. (f) Lovo cells were treated with 60 μmol/l evodiamine for 24 h. The activities of caspase-8, caspase-9, and caspase-3 were determined with caspase activity kits. Data were expressed as percentage by control, which was defined as 100% ($n=3$). Data were presented as means ± SD, $*P<0.01$ versus control group. Evo, evodiamine.

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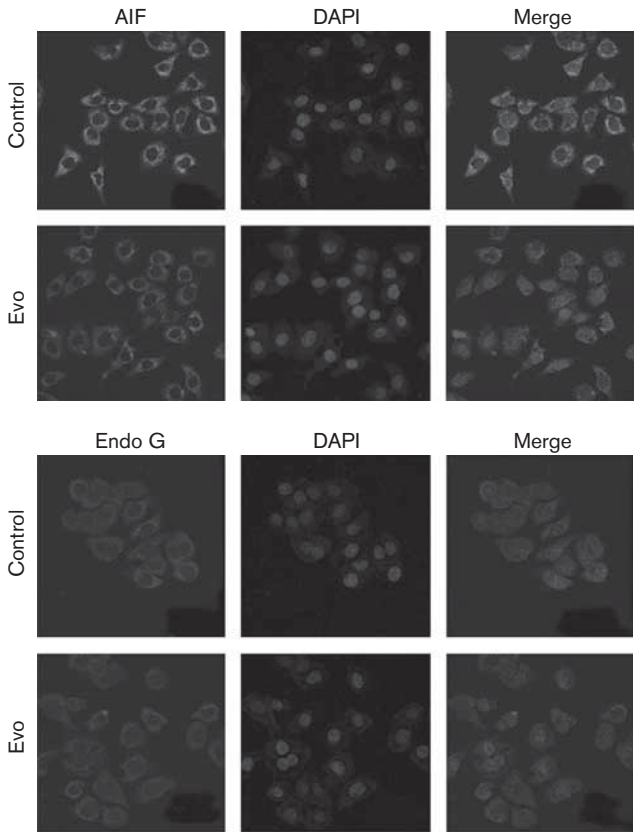
Fig. 4



Caspase-independent apoptosis driven by apoptosis-inducing factor and endonuclease G was not caused by evodiamine. Lovo cells were treated with 60 μmol/l evodiamine for 2 h and then incubated with specific antibodies overnight at 4°C followed by fluorescein isothiocyanate-conjugated secondary antibodies. After 4′,6-diamidino-2-phenylindole staining, images were captured under a laser confocal scanning microscope. Representative photographs from three independent experiments were displayed; Evo, Evodiamine; AIF, apoptosis-inducing factor; Endo G, endonuclease G; DAPI, 4′,6-diamidino-2-phenylindole (× 400).

the control group, the expressions of cyclinA, CDK2, and cdc25c were significantly decreased, which was in a time-dependent manner, whereas the expressions of cyclinB1 and CDK1 were increased at 6 and 12 h, and decreased at 24 h after evodiamine treatment (Fig. 6).

Evodiamine suppresses lovo xenograft tumor growth through caspase-dependent apoptosis

To investigate the in-vivo effects of evodiamine on tumor growth, athymic mice were inoculated with human colon cancer lovo cells and tumor volume was determined. Results indicated that the tumor size in the control group was gradually increasing. Evodiamine and 5-FU were found to slow the tumor growth compared with the control group. Furthermore, the tumor growth rate was lowest in the evodiamine-treated group. Moreover, death because of evodiamine toxicity was not observed in these mice and body weight was not significantly affected (Fig. 7). In addition, H&E staining showed that the cell nucleus had shrinkage, pyknosis, and karyorrhexis in the evodiamine-treated group accompanied by significantly increased number of TUNEL-positive cells (Fig. 8a). Furthermore, the expressions of procaspase-8, procaspase-9, and procaspase-3 were lowered in the tumors of evodiamine-treated mice when compared with the controls, which was consistent with the results in the in-vitro study (Fig. 8b).

Discussion

As colon cancer is the second leading cause of cancer deaths, effective treatments are needed to be developed against colon cancer. Evodiamine is one of the major bioactive components derived from a long-standing Chinese herb named Wu-Chu-Yu. Earlier studies have indicated that evodiamine can affect many physiological functions and might be a promising medicine in the future [21–23]. In this study, the in-vitro and in-vivo effects of evodiamine on the proliferation of human colon cancer lovo cells, and the potential mechanisms were explored.

It has been shown that evodiamine at concentrations ranging from 10^{-6} to 10^{-4} mol/l significantly decreased the proliferation of LNCaP cells [24], and from 2.5 to 80 μmol/l resulted in a significant increase in the loss of Hela cell line [25]. Tumor growth of mice with human breast NCI/ADR-RES xenografts orally treated with evodiamine (10 mg/kg) twice daily was slow compared with carboxymethyl cellulose administration [26]. In this study, the results showed that administration of 7.5 μmol/l evodiamine inhibited the growth of lovo cells and a higher dose of evodiamine caused lower viability of lovo cells. In addition, in our earlier study, the in-vivo antineoplastic characteristics of evodiamine of different concentrations were evaluated, and results showed that 1 mg/kg was an optimal dose with the lowest toxicity (data not shown). At the same time, evodiamine (1 mg/kg) significantly inhibited tumor growth when compared with the control group. Moreover, the inhibition of cell proliferation by evodiamine was more profound than that by 5-FU (30 mg/kg), which is a well-known anti-cancer drug for colon cancer. Therefore, we postulated that evodiamine could inhibit the in-vitro and in-vivo proliferation of lovo cells.

Apoptosis, a major control mechanism by which cells die when DNA damage is not repaired, has provided the basis for novel targeted therapies that can induce death of cancer cells or sensitize them to established cytotoxic agents [27]. The morphological features of apoptosis can represent cell viability to a certain extent. At the early stage of apoptosis, cell shrinkage and pyknosis are visible under light microscope. Extensive plasma membrane blebbing occurs followed by karyorrhexis, and cell fragments formed into apoptotic bodies, which are subsequently phagocytosed by other cells and degraded within phagolysosomes [11,28,29]. Our study indicated that the lovo cells treated with 7.5, 15, 30 and 60 μmol/l evodiamine for 12 h

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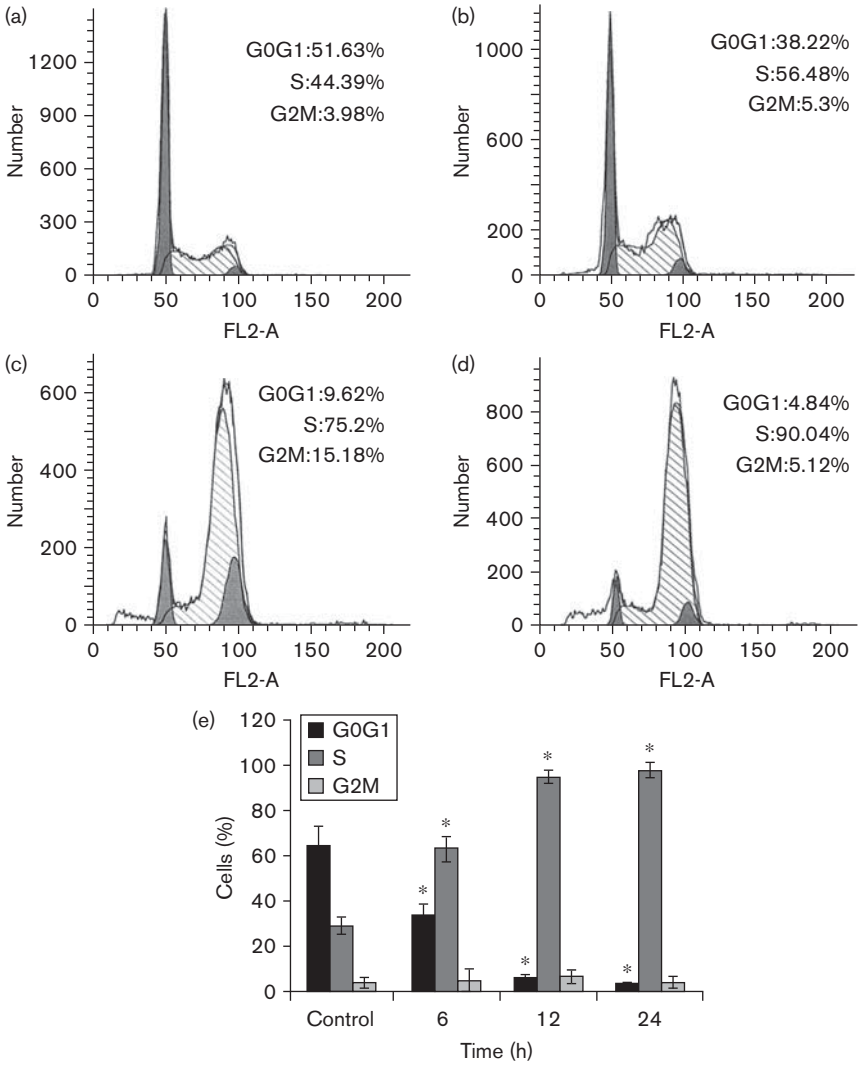
Fig. 5



Evodiamine induced cell cycle arrest in S phase. Lovo cells were treated with 60 μmol/l evodiamine for 6, 12, and 24 h. Cell cycle was detected by flow cytometry. Approximately 1×10^5 cells were included in each group and the cell numbers in different phases were expressed as percent of the total cell number ($n=3$). Representative photographs from three independent experiments were displayed; (a) control; (b) 6 h; (c) 12 h; (d) 24 h. (e) Data were presented as means ± SD. *$P<0.01$ versus control group.

became small, round, and floating. H&E staining showed nucleus pyknosis and karyorrhexis in the evodiamine-treated tumors. Subsequently, the apoptosis of evodiamine-treated lovo cells was determined by Annexin V-FITC/PI double staining. Results showed that 60 μmol/l evodiamine caused time-dependent early apoptosis but not late apoptosis (necrosis). Meanwhile, TUNEL assay showed that the number of TUNEL-positive cells in the evodiamine-treated group was significantly increased when compared with the control group.

It is well known that caspases play critical roles in the caspase-dependent apoptotic pathway and are widely expressed as inactive proenzymes in most cell types. Once activated, they can activate other procaspases [30,31]. Caspase-dependent apoptosis occurs through two main pathways. The first is the extrinsic pathway that is triggered through the Fas death receptor and forms the death-inducing signaling complex, leading to cleavage and activation of procaspase-8. The second pathway is the intrinsic pathway in which the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome-c, and subsequent cleavage and activation of procaspase-9. Both pathways converge to a final common pathway involving the activation of procaspase-3, which cleaves the inhibitor of caspase-activated deoxyribonuclease, leading to the activation of caspase-activated deoxyribonuclease, and subsequent apoptosis [32]. Our results showed that evodiamine markedly down-regulated the expressions of procaspase-8, procaspase-9,

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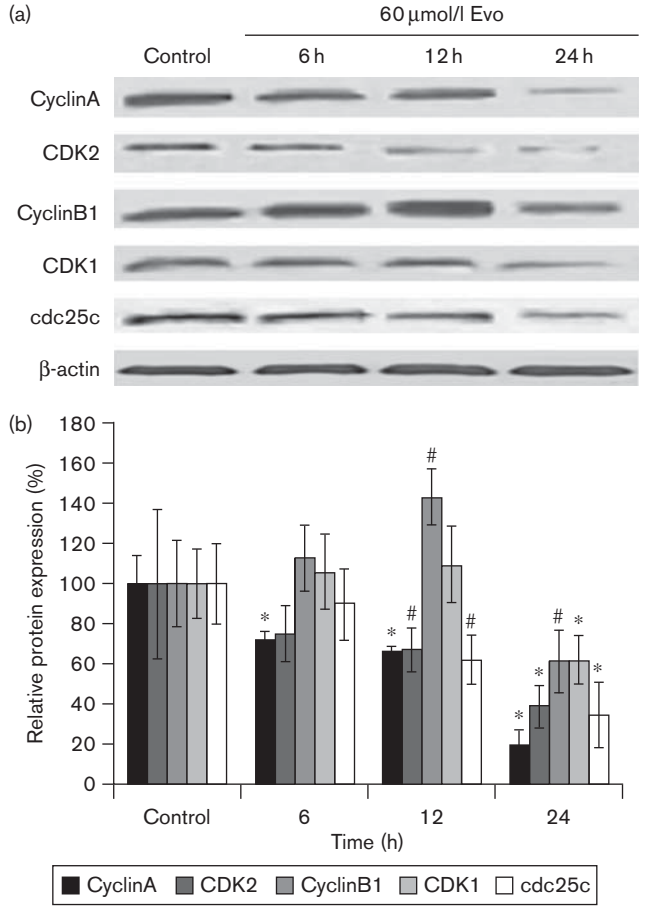
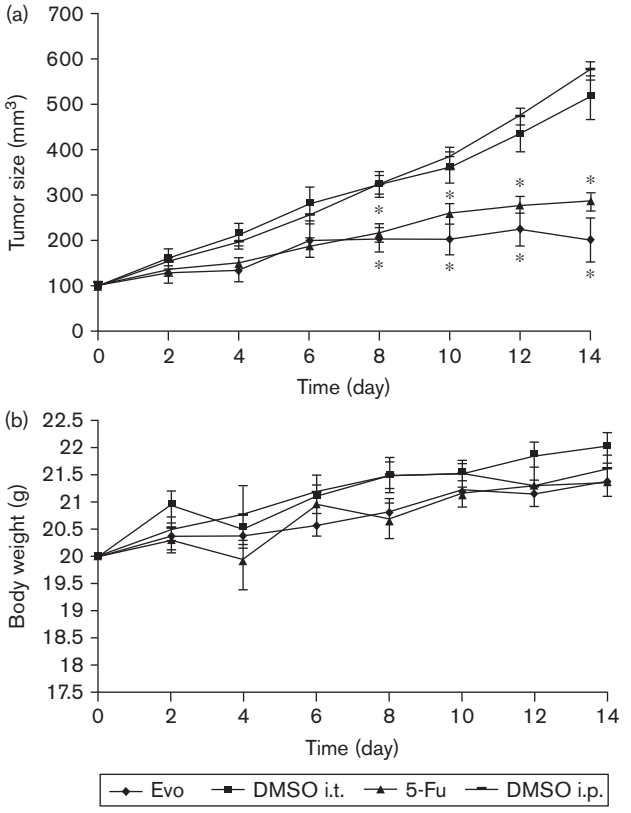
Fig. 6



Evodiamine altered the expressions of cell cycle-related proteins.(a) Lovo cells were treated with 60 μmol/l evodiamine for 6, 12, and 24 h. After treatment, the expressions of cyclinA, cyclin dependent kinase 2 (CDK2), cyclinB1, CDK1, and cdc25c were measured by western blot analysis. The amount of β-actin was measured as an internal reference. Representative photographs from three independent experiments were displayed. (b) Data were presented as means ± SD. *$P<0.01$ and $^{\#}P<0.05$ versus control group. Evo, evodiamine.

Fig. 7



Evodiamine suppressed lovo xenograft tumor growth. Mice were injected subcutaneously with 3×10^6 of lovo cells in 0.1 ml of PBS. Ten days later, the tumor volume reached 100 mm^3. Mice ($n=8$ per group) were treated with evodiamine, 5-fluorouracil (5-FU) or vehicle. Evodiamine (1 mg/kg) dissolved in dimethyl sulfoxide (DMSO) was administered intratumorally thrice weekly. 5-FU (30 mg/kg) dissolved in DMSO was given intraperitoneally thrice weekly. The mice undergoing intratumoral or intraperitoneal administration with DMSO served as controls. The length and width of the tumors and the body weight of these mice were measured during the experimental period, and tumor volume was determined using the following formula $0.5 \times L \times W^2$ ($n=8$); (a) tumor size, (b) body weight. Data were presented as means ± SD. *$P<0.01$ versus control group. Evo, evodiamine; i.p., intraperitoneally; i.t., intratumorally.

and procaspase-3 in a time-dependent and dose-dependent manner *in vitro*. Meanwhile, when compared with the control group, significantly downregulated expressions of procaspase-8, procaspase-9, and procaspase-3 were found in the evodiamine-treated mice with lovo xenografts. We also found that 60 μmol/l evodiamine could markedly enhance the activities of caspase-8, caspase-9, and caspase-3. The Bcl-2 family proteins are key regulators of apoptosis, including the anti-apoptotic proteins (Bcl-2, Bcl-X_L, and Mcl-1) and proapoptotic proteins (Bax, Bad, and Bid). Anti-apoptotic proteins act as repressors by blocking the release of cytochrome-c, whereas proapoptotic members act as promoters. These effects are dependent on the Bcl-2/Bax ratio [32,33] (Fig. 9). Our results showed that evodiamine decreased Bcl-2 expression and significantly increased Bax expression. These findings are similar to our earlier study in which evodiamine could activate caspase-8, caspase-9, and caspase-3. Therefore, our findings suggested that evodiamine could induce caspase-dependent apoptosis accompanied by a decline in the Bcl-2/Bax ratio.

Caspase family members were once thought to be indispensable for the occurrence of cell apoptosis. However, some proapoptotic proteins, such as AIF and endo G, can also induce apoptosis in a caspase-independent manner [34]. AIF and endo G exist in the mitochondrial intermembrane space, and can be translocated into the nucleus after apoptosis induction causing chromatin condensation and DNA fragmentation [12,13] (Fig. 9). It was shown earlier that evodiamine could induce apoptosis in human leukemic U937 cells through caspase-dependent and caspase-independent pathways

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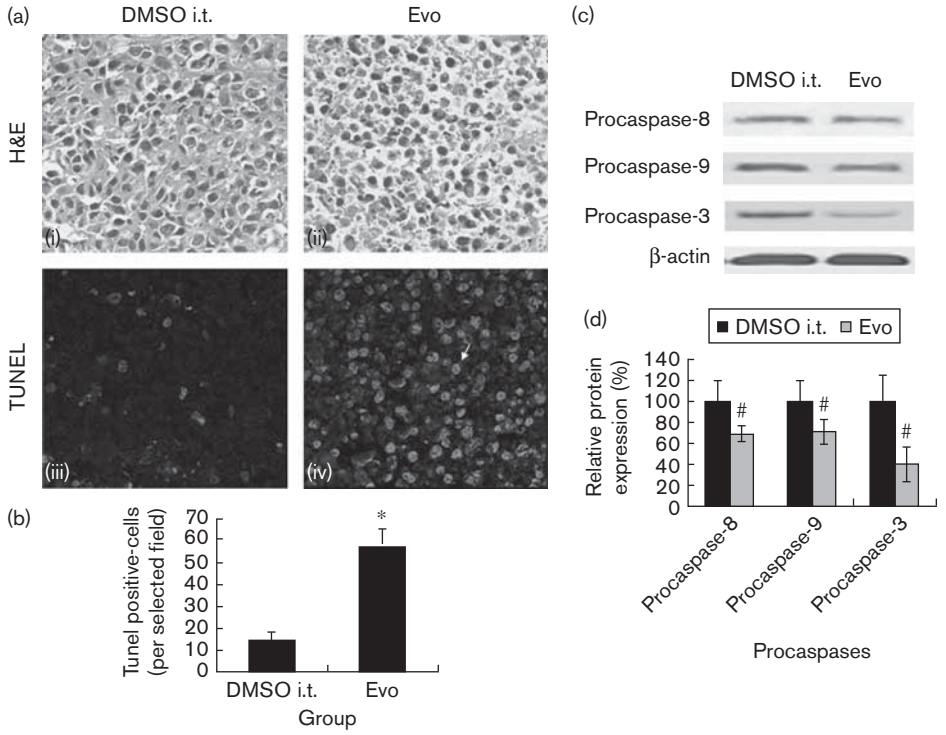
Fig. 8



Evodiamine suppressed lovo xenograft tumor growth through caspase-dependent apoptosis. (a) Sections from the dimethyl sulfoxide (DMSO) intratumoral (i.t.) group and evodiamine-treated group were processed for hematoxylin and eosin (H&E) staining. (i) DMSO i.t group; (ii) evodiamine-treated group ($\times$ 200). TUNEL staining was also performed in these sections. TUNEL-positive cells in 10 randomly selected fields at high magnification were counted and representative images were captured. The white arrow indicated TUNEL-positive cells. (iii) DMSO i.t. group; (iv) evodiamine-treated group ($\times$ 200) ($n=8$). Representative photographs from three independent experiments were displayed. (b) The data of TUNEL positive cells were presented as means $\pm$ SD. $^{*}P<0.01$ versus control group. (c) Tumors were lysed and the proteins were extracted and the expressions of procaspase-8, procaspase-9, and procaspase-3 were measured by western blot analysis. The amount of β-actin was measured as an internal reference. Representative photographs from three independent experiments were displayed. (d) The expression levels of proteins were presented as means $\pm$ SD. $^{\#}P<0.05$ versus control group. Evo, evodiamine.

[35]. However, translocation of AIF and endo G into the nucleus was not observed in the human lovo cells after evodiamine treatment. Our results indicated that evodiamine could induce apoptosis of lovo cells through the caspase-dependent pathway, but not in an AIF and endo G-mediated caspase-independent manner.

In eukaryotes, mitosis is dependent on the completion of DNA synthesis. Highly conserved DNA-repair and cell-cycle checkpoint pathways allow cells to deal with both endogenous and exogenous DNA damage [8,36]. So far, studies have shown that several therapeutic agents, such as cisplatin, paclitaxel, hydroxyuracil, and 5-FU, can disrupt cell cycle regulation and impair checkpoint controls ultimately inducing growth arrest and apoptosis in cancer cells. It is a new finding that evodiamine could induce S phase arrest in lovo cells with a parallel reduction in the number of cells in the G0/G1 phase. The cell cycle progression is regulated by different classes of cyclins, CDKs, and other regulatory proteins [37]. CyclinA accumulates at the G1/S phase transition and can be observed throughout the S phase and mitosis. CDK2 is required for the entry into the S phase, and completion of the S phase [38,39]. The critical target of the G2 checkpoint is the mitosis-promoting activity of cyclinB/CDK1, whose activation is regulated by cdc25c [40] (Fig. 10). In this study, we found that evodiamine treatment caused S phase arrest and a decrease in the protein expression of cyclinA, CDK2, and cdc25c in a time-dependent manner. However, the expressions of cyclinB1 and CDK1 were increased at the early stage after evodiamine treatment and decreased at the late stage. These findings indicated that evodiamine treatment could downregulate the expression of the cyclinA/CDK2 complex and subsequently induce the S phase arrest in lovo cells. The S phase arrest could induce a positive feedback resulting in an increase in the cyclinB1/CDK1 expression at an early stage and downregulation of cdc25c induced a decrease in their expressions at the late stage.

In conclusion, this study suggested that evodiamine could exert suppressive effects on the proliferation of human colon cancer lovo cells and induce apoptosis both *in vitro*

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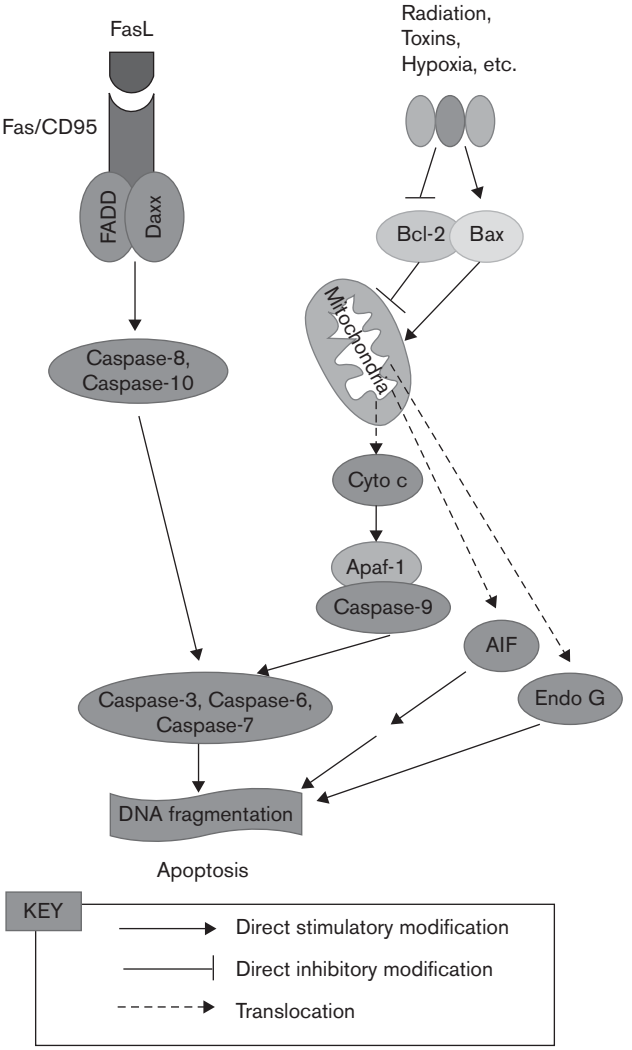
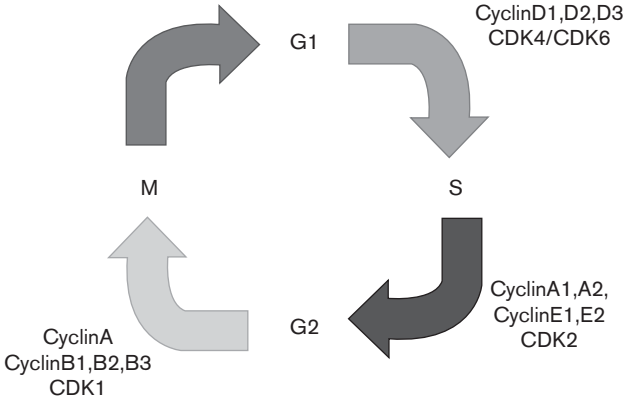
Fig. 9



Overview of the pathway for evodiamine-induced apoptosis in human colon lovo cells. AIF, apoptosis induced factor; Cyto c, cytochrome c; endo G, endonuclease G.

Fig. 10



A schema of cell cycle and approximate time of activity for different combinations of cyclins and cyclin-dependent kinases (CDKs).

and *in vivo*. Evodiamine-induced apoptosis was achieved through the activation of caspase-8, caspase-9, and caspase-3 accompanied by a decline in the Bcl-2/Bax ratio. However, the caspase-independent pathway driven by AIF and endo G was not involved in the evodiamine-induced apoptosis. In addition, evodiamine could also arrest lovo cells in the S phase by reducing cyclinA/CDK2 expression. Taken together, our findings showed that the in-vitro and in-vivo antineoplastic effects of evodiamine on human colon cancer lovo cells were mediated by inducing apoptosis, which was in a caspase-dependent manner and related with S phase arrest.

Acknowledgements

This study was supported by Grants from the National High Technology Research and Development Program ('863'Program) of China (No. 2008AA02Z440), the National Natural Science Foundation of China (No. 30672414; No. 30973431), and Project of State Key Laboratory of Trauma, Burns, and Combined Injury (No. SKLZZ200802). The authors also appreciate Qianglin Duan from Tongji Hospital for English usage and paper revision.

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